To identify rate-limiting steps in T cell-independent type 2 antibody production against polysaccharide antigens, we performed a genome-wide screen by immunizing several hundred pedigrees of C57BL/6 mice segregating N-ethyl-N-nitrosourea-induced mis-sense mutations. Two independent mutations, Tilcara and Untied, were isolated that semi-dominantly diminished antibody against polysaccharide but not protein antigens. Both mutations resulted from single-amino-acid substitutions within the kinase domain of protein kinase C-β (PKCβ). In Tilcara, a Ser552→Pro mutation occurred in helix G, in close proximity to a docking site for the inhibitory N-terminal pseudosubstrate domain of the enzyme, resulting in almost complete loss of active, autophosphorylated PKCβII, whereas the amount of alternatively spliced PKCβIII protein was not markedly reduced. Circulating B cell subsets were normal and acute responses to B-cell receptor stimulation such as CD25 induction and initiation of DNA synthesis were only measurably diminished in Tilcara homozygotes, whereas the fraction of cells that had divided multiple times was decreased to an intermediate degree in heterozygotes. These results, coupled with evidence of numerous mis-sense PRKCB mutations in the human genome, identify Prkcb as a genetically sensitive step likely to contribute substantially to population variability in anti-polysaccharide antibody levels.

*Keywords:* protein kinase C-β; T-independent antibody response; mis-sense mutation

INTRODUCTION

A hallmark of the adaptive immune system is the production of antibodies by B cells targeting varying types of antigens. Genetic studies in mice have distinguished different routes for antibody production depending on the nature of the antigen. First, antigens have been classified as eliciting either T cell-dependent or T cell-independent (TI) responses based on their ability to stimulate antibodies to a given antigen in athymic nude mice.  

Antigens that elicit TI antibody responses have been further subdivided into TI-1 or TI-2 antigens based on their ability to stimulate responses in the X-linked immunodeficiency (Xid) mouse strain, which has a crippling Btk mutation that affects some intracellular signals elicited by antigen binding to the B-cell antigen receptor (BCRs). Xid mice are capable of making antibodies against TI-1 antigens, such as bacterial lipopolysaccharide (LPS) or flagellin, because these signal B-cell proliferation through Toll-like receptors (TLRs).  

By contrast, Xid mice fail to make antibodies to TI-2 antigens such as the polysaccharide envelopes of some bacteria or synthetic polysaccharides such as Ficoll, because these antigens signal B-cell proliferation by extensively cross-linking the BCR into large aggregates that persist on the cell surface.  

TI-2 responses are mediated primarily by the secretion of short-lived IgM isotype antibodies by marginal zone (MZ) B cells in the spleen and the B-1 cells in the serous cavities.  

In a TI antibody response, the stimulation of the BCR leads to the phosphorylation of immunoreceptor tyrosine-based activation motifs in the cytoplasmic tails of the signal transducing molecules CD79a (Igs) and CD79b (Igs). This allows the phosphorylation of multiple signalling kinases such as Bruton's tyrosine kinase (BTK) and the phospholipase c γ-2 (PLCγ2)-mediated elevation of intracellular calcium. These kinases activate protein kinase C-β (PKCβ), which starts a phosphorylation cascade activating the Card11/Malt1/Bcl-10 complex. This results in the recruitment of the nuclear factor-kB transcription factor to inducible genes that stimulate B-cell proliferation and TI-2 antibody production.  

Defects in TI-2 antibody responses result in increased susceptibility to encapsulated bacterial infections such as *Haemophilus influenzae* type b, *Neisseria meningitides* and *Streptococcus pneumoniae*. These infections severely impact on young children particularly in the developing world. Chronic infection and poor disease management can lead to mortality. Lower antibody production against these antigens is correlated with recurrent infection but the underlying basis for population variation is not known. Further understanding of the underlying genetic alterations that make individuals more susceptible to these diseases are important for the identification of therapeutic targets for the disease and to enhance vaccination and immunization strategies.  

A unique feature of modern humans is the massive degree of population expansion that has occurred in the last several hundred generations. Deep sequencing efforts have recently revealed that this growth has resulted in an equally massive
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In this study, we have taken an experimental approach in mice to evaluate the impact of increasing the genome-wide burden of rare variants on TI-2 antibody formation against polysaccharide antigens. We used N-ethyl-N-nitrosurea (ENU) mutagenesis of inbred C57BL/6 mice to increase the frequency of single-nucleotide variants, coupled with systematic screening for variability in the antibody response following immunization with a haptenated polysaccharide, NP (4-hydroxy-3-nitrophenylacetyl)-Ficoll. Two semi-dominant mutations were isolated causing selective deficits in TI-2 antibody response, and both were due to substitutions in conserved residues in the catalytic domain of PKCβ (Prkcb). These results identify PRKCB as a rate-limiting point in the network of genes controlling TI-2 antibody, and provide novel animal models to analyze the biochemistry of the two PKCβ enzyme isoforms.

RESULTS

Generation of the Tilcara and Untied mutant mice and identification of the causative mutation in Prkcb

A genetic screen was performed to identify genes that are critical for TI antibody production. First generation offspring (G1) from ENU-treated inbred C57BL/6 male mice were used as founders for separate inbreeding pedigrees by breeding with wild-type females, and subsequently intercrossing their G2 progeny to generate a G3 generation. Twelve-week-old G3 offspring from 130 pedigrees were screened by immunization with a panel of antibodies that differentially elicit TI and T-dependent antibody responses. T cell-dependent antibody response were measured by immunizing each G3 animal with alum-precipitated chicken Bordetella pertussis (BP). After 5 weeks, the mice were re-immunized with ABA-CGG to test T cell-dependent memory and affinity maturation, and immunized at the same time with NP-Ficoll, which elicits a T cell-independent type 2 (TI-2) antibody response. Three of five mice tested in the Tilcara pedigree exhibited lower TI-2 responses compared with littermate counterparts (Figures 1a and b), but had normal T-dependent responses indicating that these mice have no general problem in the generation of specific antibodies.

To link the mutation responsible for low TI-2 antibody to a single chromosome, an F2 intercross was performed between offspring from an affected C57BL/6 (B6) G3 mouse and a wild-type C57BL/10 (B10) mouse. Immunization of the F2 offspring with NP-Ficoll identified three groups of animals. The first group had a very low TI-2 antibody response (‘affected’), a second group had normal levels of antibodies (‘unaffected’) and a third group showed an intermediate response. Pooled DNA samples from affected and unaffected mice were tested using a panel of single-nucleotide polymorphisms between the B6 and B10 strains, linking the Tilcara trait to chromosome 7 distal to single-nucleotide polymorphism rs1347954 at 109 Mb. Because of the semi-dominant inheritance the location of the mutation was further refined by immunizing offspring from a cross between (C57BL/6 × CBA)F1 animals with an intermediate response and wild-type B6 mice. Genotyping of intermediate responders and unaffected animals localized the mutation to an 8.3 Mb interval (Figure 1c) containing 199 predicted genes, including 16 genes with known immunological function. Three of these, Cd19, Cd11b and Prkcb, were selected as candidate genes for sequencing of splenic mRNA, revealing a single T-to-C point coding sequence mutation in Prkcb at basepair 1654 (Figure 1d).

A second screen of another cohort of ENU mutagenized mice revealed another mouse strain, Untied, that also displayed a semi-dominant defect in IgM antibody selectively against TI-2 antigens (Figure 1e). Sequencing of Prkcb revealed an independent mutation in this strain: in this case a T to C transition at position 1469 of the Prkcb transcript. The Tilcara and Untied mutations both result in single-amino-acid substitutions of highly conserved residues in the PKCβ1 kinase domain. Untied results from a tyrosine to histidine substitution at amino acid 417. This alters a residue that is absolutely conserved from mammals to invertebrates, in the middle of β-sheet 5 near the conserved gatekeeper residue (Met420) that controls access to the ATP-binding pocket (Figure 1f-h). In Tilcara, Ser552 in the middle of the α-helix G on the C-lobe of the kinase domain, on the lower lip of the substrate-binding cleft (Figure 1f-h), is mutated to a proline that would be predicted to break the helical structure. The mutated Ser552 is absolutely conserved from mammals to fish and highly conserved between different murine PKC family members (Figure 1i). The genetic evidence that these are causal is thus supported by three independent mutations, since a comparable semi-dominant loss of TI2 antibody responses was also observed in Prkcb knockout mice.20

Two isoforms of PKCβ2, PKCβII and PKCβIII, are produced in mice, humans and other mammals by alternative mRNA splicing of exons encoding PKCβ1 residues 622–673 (622–673 in PKCβIII). Hence, the S552P Tilcara mutation affects the part of the protein that is common to both isoforms. Western blotting with antibodies specific for one or other isoform revealed a consistent reduction in the PKCβII isoform in purified B-cell lysates from Prkcbmut mice compared with wild-type controls, whereas Tilcara heterozygous mice had intermediate protein expression (Figure 2a). By contrast, there was no consistent decrease in the PKCβIII isoform (Figure 2a).

Following translation, both PKCβII and PKCβIII undergo sequential phosphorylation at threonine and serine residues to become catalytically active, and these modifications can be regulated indirectly by slower mobility in SDS-polyacrylamide gel electrophoresis and directly by phospho-specific antibodies. When the mutant or wild-type PKCβIII isoform was expressed in 293T cells, the Tilcara S552P mutation caused almost complete absence of the tyrosine-phosphorylated form, whereas the wild-type migrating species was present at comparable levels in soluble cytoplasmic component of the NP-40 lysed-transfected with mutant or wild-type vectors (Figure 2b). The S552P mutation had a similar effect when the PKCβIII isoform was expressed in transfected 293T cells, although a small amount of slowly migrating protein was present in this case. The first post-translational modification step in the maturation of PKCβII and II, involving Thr500 phosphorylation in the kinase domain activation loop by phosphoinositide-dependent kinase-1,23 was tested by western blotting B-cell lysates with an antibody to phospho-T500 (Figure 2c). Comparable amounts of phospho-T500 PKCβ were present in wild-type, heterozygous and Prkcbmut B cell lysates. This activation loop phosphorylation in turn allows PKCβII to auto-
Figure 1. (a) Tilcara strain pedigree and TI-2 antibody response. Open and black-filled shapes in pedigree denote third generation (G3) mice with normal or low TI-2 responses, respectively, based on ELISA measurement shown on right. NP-specific IgM antibodies were measured in 1:150 diluted serum from individual G3 mice in the Tilcara pedigree and other ENU6 pedigrees screened in parallel, 6 days after immunization with NP-Ficoll. (b) Semi-dominant low TI-2 antibody following immunization in G3 offspring from the Tilcara pedigree. (c) Informative recombinant Ch7 haplotypes in (CBA × B6)F1 mice with low (affected) or normal antibody response to NP-Ficoll. Black, homozygous B6 genotype; white, heterozygous CBA/B6 genotype. Maximum non-recombinant interval is shown. Map positions refer to Build GRCm38 of the mouse genome assembly (http://mouse.ensembl.org). (d) Sequencing chromatograms from amplified Prkcb splenic complementary DNA from mice of the indicated genotypes, centered on nucleotide 1654. (e) Semi-dominant low TI-2 antibody following immunization with 2,4,6-trinitrophenyl-Ficoll in G3 offspring from the Untied pedigree. (f) Location of Tilcara and Untied mutations within the human PKCβII kinase domain crystal structure (PDB ID: 210E). (g) Location of the S552 residue, Y417 residue and the series of acidic residues in helix G that interact with the N-terminal pseudosubstrate domain. The ATP-binding pocket within the catalytic cleft is also shown. (h) Location of Tilcara and Untied mutations with respect to PKCβ protein domains: ps, pseudosubstrate inhibitory domain; C1A and C1B, diacylglycerol- and phospholipid-binding domains; C2, calcium-binding domain. The green box represents the region that differs in PKCβII PKCβIII. (i) Alignment of amino-acid sequences of PKCβ from the indicated species (top) and with other murine PKC family members (bottom).
Diminished basal serum IgM and IgG3 and T1 antibody responses in *Tilcara* mouse

The semi-dominant biochemical effects of the *Tilcara* S552P PKCβ1 mutation defined above were accompanied by semi-dominant effects on antibody production to the TI-2 antigen, NP-Ficoll (Figure 3a). When NP-specific IgM and IgG3 were measured in a large cohort of animals 7 days post immunization, *Tilcara* heterozygous mice made significantly less antibody than wild-type controls, whereas *Prkcb*<sup_absolute>til/til</sup> homozygotes made lower responses than heterozygotes. Likewise, in unimmunized age-matched mice there was a semi-dominant decrease in natural serum IgM antibody (Figure 3b), which decreased ~50% and ~75% in *Tilcara* heterozygous and homozygous mice, respectively, compared with wild-type controls. Basal serum IgG3 was also decreased ~75% in *Tilcara* heterozygous and homozygous mice.

Serum IgG1 and total IgG were nevertheless comparable between mutant and wild-type animals (Figure 3b). Likewise, there were normal T cell-dependent antibody responses following immunization with ABA-CCG and heat killed BP, which depend on T<sub>ind</sub>2 and T<sub>ind</sub>1 cells to elicit CCG-specific IgG1 and BP-specific IgG2c response. There were no significant differences in these antibody responses between wild-type mice and *Tilcara* heterozygous or homozygous animals (Figure 3c). The S552P mutation in *Prkcb* thus causes a selective defect in T1 antibody responses.

Lymphocyte development and decreased B-1a cells in *Tilcara* mouse

B lymphocyte development and peripheral subsets were assessed in *Tilcara* mice by flow cytometry, with particular focus on B cells that are responsible for TI-2 antibody responses—MZ and B-1 cells. In the bone marrow, no significant differences in percentages of early pro-B/pre-B (*B220<sup>lo</sup>IgM<sup>lo</sup>), immature (*B220<sup>hi</sup>IgM<sup>hi</sup>) and mature (*B220<sup>hi</sup>IgM<sup>lo</sup>) B cells could be detected between wild-type, *Prkcb*<sup>wt/wt</sup>, *Prkcb*<sup>til/ti</sup> mice (Figure 4a). Similarly, there was no discernable effect of the mutation on transitional 1 (CD93<sup>lo</sup>IgM<sup>hi</sup>CD23<sup>lo</sup>), transitional 2 (CD93<sup>hi</sup>IgM<sup>hi</sup>CD23<sup>lo</sup>), transitional 3 (CD93<sup>hi</sup>IgM<sup>hi</sup>CD23<sup>hi</sup>), follicular (CD93<sup>hi</sup>IgM<sup>hi</sup>CD23<sup>hi</sup>) and MZ (CD93<sup>hi</sup>IgM<sup>lo</sup>CD21<sup>lo</sup>) B-cell subpopulations in the spleen (Figure 4b). Cell surface IgM decreased normally during maturation of follicular B cells in *Tilcara* homozygotes; a result that stands in contrast to mutations in BTK<sup>25,26</sup> or PLC<sub>δ1</sub><sup>27,28</sup> immediately downstream, which cause surface IgM to remain high on follicular B cells. T-cell subsets in the thymus, lymph nodes and spleen were also comparable (data not shown). The only striking abnormality in lymphocyte development was in the peritoneal cavity, where CD11b<sup>lo</sup>IgM<sup>lo</sup> B-1 cells were markedly decreased in frequency selectively in *Prkcb*<sup>til/ti</sup> homozygous mice compared with wild-type and *Prkcb*<sup>wt/wt</sup> heterozygotes (Figure 4c). Subdivision of the B-1 cells into CD5<sup>-</sup> B-1a and CD5<sup>+</sup> B-1b subpopulation revealed that this difference was attributed largely to the near absence of the B-1a population in homozygous mutant animals (Figure 4c).

Competitive mixed bone marrow chimeras were produced by transplanting irradiated mice with equal amounts of bone marrow from CD45.1 wild-type and CD45.2 *Prkcb*<sup>wt/wt</sup> donors. Analysis of the reconstituted chimeric animals showed that the *Prkcb*<sup>til/ti</sup> mutation did not affect the numbers of B- and T-cell subsets in central and peripheral lymphoid tissue, which were drawn equally from mutant and wild-type lymphocytes. By contrast, peritoneal B-1a cells and to a lesser extend MZ cells were preferentially derived from the wild-type lymphocytes and not from those harboring the *Prkcb*<sup>til/ti</sup> mutation, demonstrating an intrinsic, cell-autonomous requirement for fully active PKCβ selectively in the formation of B1 B cells (Supplementary Table1 and Supplementary Figure 1).
Recessive deficits in the acute BCR response

We next analyzed the consequences of the S552P Prkcb<sup>b<sup>til</sup></sup> mutation on B-cell responses to receptor stimulation in vitro. Increased intracellular calcium after BCR stimulation was monitored using the calcium-sensing dye, Indo-1, in co-cultured CD45.1 wild-type and CD45.2 Prkcb<sup>b<sup>til</sup></sup> cells stimulated with sub-optimal and optimal concentrations of anti-IgM antibody (Figure 5a). By overlaying the paired responses from mutant and wild-type cells stimulated together, there was no discernable difference in the intracellular calcium response between wild-type and Prkcb<sup>b<sup>til</sup></sup> cells. Thus, the early steps in BCR signalling leading to PLCγ activation, which lies immediately upstream of PKCζ, appeared unaffected by the Prkcb<sup>b<sup>til</sup></sup> mutation.

BCR, CD40 or TLR signalling induces an early wave of gene transcription in B cells that includes the cell surface protein CD25. CD25 induction by the BCR but not CD40 or TLR4 is compromised in B cells with mutations in Card11, a known immediate substrate of PKCζ. Flow cytometric analysis showed that CD25 was induced by anti-IgM stimulation of wild-type B cells but poorly induced in homozygous mutant Prkcb<sup>b<sup>til</sup></sup> B cells stimulated in the same culture (Figures 5b and c). By contrast, CD25 was induced equivalently in mutant and wild-type B cells stimulated with 1 μg ml<sup>−1</sup> IgM + 10 μg ml<sup>−1</sup> CD40L or the TLR4 agonist, LPS. Unlike the homozygous mutant B cells, Tilcara heterozygous B cells exhibited no discernable decrease in CD25 induction following anti-IgM stimulation (Figures 5b and c).

Entry of B cells into cell cycle was measured by incorporation of radioactive thymidine after 2 days in culture. Spleen cells from individual homozygous Prkcb<sup>b<sup>til</sup></sup> mice had markedly diminished thymidine incorporation in response to BCR stimulation, whereas their response to LPS (TLR4), CpG (TLR9) or antibody to CD3 was normal (Figures 6a–d). Heterozygous Prkcb<sup>b<sup>til</sup></sup> B cells did not have significantly reduced DNA incorporation upon anti-IgM stimulation. The combination of anti-IgM and anti-CD40 stimulation partially restored the initiation of proliferation in Prkcb<sup>b<sup>til</sup></sup> cells (Figure 6e). Measurement of B-cell division by dilution of the intracellular dye carboxyfluorescein-succinimidyl-ester (CFSE) yielded similar results and enabled cell autonomous effects of the Prkcb mutation to be confirmed by comparing CFSE dilution in co-cultured CD45.1 normal B cells 5 days post stimulation. Few homozygous mutant B cells had divided multiple times in response to anti-IgM, whereas the percentage of divided Tilcara heterozygous B cells was significantly lower than in wild-type controls (Figures 6f and g). Stimulation with anti-IgM and CD40 greatly enhanced the fraction of wild-type B cells that had divided multiple times, but only modestly increased the response of homozygous mutant B cells, whereas the response of heterozygous mutant B cells was clearly intermediate. Collectively, these results indicate that the semi-dominant effects of Prkcb mutation on BCR-induced proliferation are difficult to discern during the entry into cycle but become more apparent when measured after several cell divisions. This may reflect either less re-initiation of subsequent cell divisions in heterozygous mutants or diminished survival of their divided progeny compared with the wild-type B cells in the same culture.

**DISCUSSION**

The results above represent a genome-wide experimental approach to identify rate-limiting steps in Ti-2 antibody production that are particularly sensitive to heterozygous miss-sense mutations. Two independent, semi-dominant mutations were revealed, Tilcara and Untied, both changing individual amino acids within the catalytic domain of PKCζ. Semi-dominant loss of Ti-2 antibody was also reported in Prkcb knockout mice. The findings identify Prkcb as a key genetic node with a gene-dosage sensitive role selectively in BCR-induced cell division and Ti-2 antibody responses. In contrast with these semi-dominant effects on Ti-2 antibody responses, formation of B1a B cells in the peritoneal cavity and basal production of serum IgM and IgG3 were only diminished in Tilcara homozygous mutants. Likewise, early responses to BCR stimulation such as CD25 induction and initiation of DNA synthesis were only measurably diminished in Heterozygous mutations in Prkcb affect T-independent antibody formation

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homozygotes, whereas the fraction of cells that had divided multiple times was decreased to an intermediate degree in heterozygotes.

In Tilcara, the Ser^{552}>Pro mutation differential affected the alternatively spliced PKC\(\beta\)I and PKC\(\beta\)II isoforms. The active, autophosphorylated PKC\(\beta\)I species were preferentially diminished and the overall amount of PKC\(\beta\)I protein was greatly decreased in homozygotes and markedly decreased in heterozygotes, whereas the overall amount of PKC\(\beta\)II protein was not markedly reduced. More complete loss of autophosphorylated PKC\(\beta\)I than PKC\(\beta\)II was also observed when tagged versions of the proteins were transiently expressed in 293 cells. Preferential loss of autophosphorylated PKC\(\beta\)I has several possible explanations. Ser^{552} lies in helix G of the catalytic domain, which forms a lower lip on the catalytic site and contains a series of acidic residues, EDE-DELFQS^{552}, that interact with basic Arg or Lys residues in the pseudosubstrate inhibitory domain that lies at the PKC\(\beta\)N-terminus.\(^{30-32}\) Serine^{552} is absolutely conserved in vertebrates, and its substitution by proline would be predicted to break the \(\alpha\)-helical structure and diminish binding to the pseudosubstrate.

Figure 4. A representation of flow cytometric plots and percentages from Prkcb\(\text{wt/wt}\), Prkcb\(\text{wt/til}\) and Prkcb\(\text{til/til}\) mice for (a) Pro-B/pre-B (B220\(^{-}\)IgM\(^{-}\)), immature (B220\(^{-}\)IgM\(^{hi}\)) and mature (B220\(^{-}\)IgM\(^{hi}\)) B-cell populations from total lymphocytes for bone marrow cell suspensions. (b) Transitional 1 (T1; B220\(^{hi}\)CD93\(^{lo}\)IgM\(^{lo}\)CD23\(^{lo}\)), transitional 2 (T2; B220\(^{hi}\)CD93\(^{lo}\)IgM\(^{hi}\)CD23\(^{hi}\)), transitional 3 (T3; B220\(^{hi}\)CD93\(^{hi}\)IgM\(^{hi}\)CD23\(^{hi}\)), follicular (FO; B220\(^{hi}\)CD93\(^{lo}\)IgM\(^{hi}\)CD21\(^{hi}\)) and marginal zone (MZ; B220\(^{hi}\)CD93\(^{lo}\)IgM\(^{lo}\)CD21\(^{hi}\)) splenocytes. (c) B-1 cell (IgM\(^{hi}\)CD11b\(^{hi}\)) population in the peritoneal cavity. The histograms show the B-1a (CD5\(^{hi}\)) and B-1b (CD5\(^{lo}\)) cell populations. The graphs show the percentages of cells from total lymphocytes. ** and *** indicates a significant difference of \(P<0.01\), \(P<0.001\), respectively, calculated by the one-way analysis of variance statistical test and Bonferroni post-test. Results from a–c representative of three independent experiments.
The immune phenotype of Tilcara homozygotes had a number of significant differences compared with knockout mice that completely lack both PKCβ isoforms. PKCβ-null mice had defects in both TI-2 and T-dependent (TD) antibody responses, whereas Tilcara homozygous mice had low TI-2 antibody responses but normal TD antibody responses. PKCβ-null mice also had fewer mature IgMloIgDhi follicular B cells in the spleen and fewer mature follicular B cells that had recirculated to the bone marrow; whereas the numbers of both subsets were normal in Tilcara homozygotes. There are two plausible explanations for the different phenotypes. First, the Prkcb<sup>til/til</sup> mutation may result in the partial defect in PKCβ activity. This hypothesis is also supported by the observation that addition of CD40L to the anti-IgM stimulation was able to normalize upregulation of CD25 and
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helped at least partially to overcome the defective proliferation in response to anti-IgM alone. Alternatively, there may be a division of labor between the PKCβI and PKCβII isoforms, whereas the PKCβIII isoform is involved in TI-2 antibody response, whereas the PKCβIV isoform that was less compromised in *Tilcara* is involved in TD antibody production and mature B-cell survival. Support for this possibility comes from the observation that PKCβI and PKCβII have different roles in the insulin receptor signalling pathway.38 It would be interesting in future studies to engineer mice that selectively lack PKCβI protein entirely while retaining normal PKCβII.

Heterozygous mis-sense mutations, like those in *Tilcara* and *Untied*, have accumulated in the current human population at a massive scale, because of exponential population growth and outbreeding.2–12 Whereas offspring of ENU-mutagenized mice inherit ~40 novel mis-sense variants, a ‘normal’ current human genome inherits more than 5000 mis-sense variants including 300–600 that are predicted to be deleterious to protein function and 75–150 that represent rare, recent deleterious mutations yet to be subject to purifying selection.16 Hence, the finding of two heterozygous missense *Prkcb* mutations that reduce TI-2 antibody responses among several hundred ENU mouse pedigrees can be extrapolated to predict at least a similar ~1% frequency in human families. Indeed, recent exome sequence data from 2440 individuals16 annotates 21 independent mis-sense mutations in *PRKCB* including many in the kinase domain (http://evs.gs.washington.edu/EVS/). It will be important to test if ENU treatment of mice would not be defined as having a primary immunodeficiency.

Cell isolation and flow cytometry

Spleens and thymus were harvested from mice and single-cell suspensions were prepared by sieving and gentle pipetting through a 70-μm nylon mesh filter. Bone marrow cells were flushed out from tibia and femurs, which were aseptically cut at both ends. Peritoneal cells were collected by abdominal lavage. Blood was collected by retro-orbital bleeds from live mice. For surface staining, samples were stained with the appropriate antibodies and in secondary antibodies. A BD LSRII Flow Cytometry with FACS Diva (BD Biosciences, San Jose, CA, USA) was used for acquisition of flow cytometric data, and Flowjo (Treestar, Inc., Ashland, OR, USA) was used for analysis.

Antibodies

Antibodies used for flow cytometry were from BD (San Jose, CA, USA) unless otherwise indicated: 7-aminobutyrophenone D (Calbiochem, San Diego, CA, USA), anti-mouse CD11b-APC/FITC (eBioscience, San Diego, CA, USA), anti-mouse CD16/CD32 (Stemcell Technologies, Tullamarine, Vic, Australia), anti-mouse CD14-PE, anti-mouse CD21/CD235-FITC/PE, anti-mouse CD23-PE Cy7 (eBioscience), anti-mouse CD25-APC, anti-mouse CD4-FITC/PE Cy7, anti-mouse CD43-PE, anti-mouse CD45-1-biotin/FITC, anti-mouse CD45.1-Alexa Fluor 700 (BioLegend, San Diego, CA, USA), anti-mouse CD45.2-PE/Cy7, anti-mouse CD45.2-Biotin/PE, anti-mouse CD45.2-Pacific blue (BioLegend), anti-mouse CD45SR-Alexa Fluor 405/APC/CF70/C1/FITC/PE/Cy7/PerCP, anti-mouse CD5-PE, anti-mouse CD62L-PE, anti-mouse CD69-FITC/Cy7, anti-mouse CD86-APC/PE, anti-mouse CD93-APC (eBioscience), anti-mouse IgM-AF488/FITC/PE/Cy5.5, anti-mouse TCR beta-APC-Alexa Fluor 750 (eBioscience), streptavidin-Pert/PerCP/APC and streptavidin-Qdot 605 (Invitrogen, Carlsbad, CA, USA). Antibodies used for ELISA: goat anti-mouse IgG/IgG1/IgG3/ IgM-alkaline phosphate (Southern Biotechnology, Birmingham, AL, USA), goat anti-mouse IgG2a-biotin (Southern Biotechnology) and streptavidin-PE/PerCP/APC and streptavidin-Qdot 605 (Invitrogen, Carlsbad, CA, USA).

Bone marrow chimeras

All recipients were sub-lethally irradiated with two doses of 450 rads and reconstituted via intravenous injections with 2 × 106 bone marrow cells.

Figure 6. Effects of *Tilcara* mutation on induction of B-cell proliferation. (a–e) Mean (± s.d.) 3H-thymidine incorporation in triplicate cultures of spleen cells from five individual mice of the indicated genotypes, 48 h after stimulation with the indicated agonists. Representative of two independent experiments. (f) Cell division measured by dilution of CFSE in wild-type, heterozygous and homozygous CD45.2 *Prkcb*wt/wt or CD45.2 *Prkcb*wt/til B cells (open histograms) co-cultured with CD45.1 wild-type B cells (shaded histograms) for 5 days with the indicated stimuli. Histograms are gated on B220+CD45.2 or CD45.1 cells. (g) The mean percentage of divided CD45.2 cells. The mean ± s.d. for the percentage of all B cells that were CD45.2+ in unstimulated cultures are: 70.8 ± 7.7% and 41.8 ± 17.2% for *Prkcb*wt/til (n = 5) and *Prkcb*wt/til (n = 5), respectively; for 10 μg ml−1 anti-IgM 66.6 ± 10.5% and 29.4 ± 15.0%; and for 1 μg ml−1 anti-IgM + 10 μg ml−1 CD40 89.6 ± 3.7% and 65.9 ± 8.7%, c.p.m., count per minute.
In vitro cell stimulation assays
Goat anti-IgM Fab’12 (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), anti-IgD (Clone 1C10), anti-TCR (Clone H57-597), bacterial DNA (CpG; Geneworks, Thebarton, SA, Australia) and LPS, from Escherichia coli (Sigma) were used as agonist for stimulation. All cell stimulations were done in RPMI supplemented with 10% fetal calf serum, 50 U ml⁻¹ penicillin/streptomycin, 10 mM HEPES, 10 mM sodium pyruvate, 10 mM non-essential minimum essential medium and 55 μM β-mercapto-ethanol. For DNA synthesis assay, 2 x 10⁶ cells were cultured for 48 h in 96-well flat bottom plates, before pulsing with 1 μCi [³²P]thymidine for 8 h. Intracellular calcium was measured on an LSR flow cytometer (BD Biosciences) following labelling with Indo-1 (Molecular Probes, Eugene, OR, USA). For labelling with CFSE, cells were diluted to a concentration of 20 x 10⁶ cells per ml in phosphate-buffered saline and stained with a final concentration of 2.5 μM CFSE at 37°C for 10 min. Following two wash with cold RPMI, 2 x 10⁶ cells were cultured for 48 h in 96-well flat bottom plates with appropriate agonist for 5 days.

Western blotting
For B-cell purifications, red-cell-depleted splenocytes were incubated with biotinylated antibodies to CD43 (BD) and B cells were negatively selected using streptavidin MACS bead separation (Miltenyi Biotech, Macquarie Park, NSW, Australia). Cell lysates were subjected to SDS-polyacrylamide gel electrophoresis with a 12.5% gel using the Bio-Rad Mini-Protean II system (Bio-Rad, Hercules, CA, USA). Nitrocellulose membranes (Trans-blot Bio-Rad) and the Bio-Rad Mini-Protein II system was used for transfer according to the manufacturer’s instructions. Western blotting using the following antibodies: donkey anti-rabbit IgG Fab’12-peroxidase (GE Healthcare, Copenhagen, Denmark), goat anti-mouse IgG Fab’12-peroxidase (GE Healthcare), mouse anti-β-actin monoclonal antibody (Sigma), rabbit anti-PKC-β 1 polyclonal antibody (Sapphire Biosciences, Waterloo, NSW, Australia and Cell Signalling, Genesearch PTY Ltd, Arundel, QLD, Australia), rabbit anti-PKC-β 2 phosphorylated at T642 polyclonal antibody (Sapphire Biosciences) and rabbit anti-β-actin antibody (Sigma). For B-cell purifications, Western Lightning Western Blot Chemiluminescence Reagent (Perkin Elmer, Waltham, MA, USA).

Bioinformatics
The following bioinformatic tools were used: Boxshade 3.2.1 (http://www.ch.embnet.org/3.2.1/software/BOX_form.html), ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/), Cn3D (http://www.ncbi.nlm.nih.gov/3dstructure/), ENSEMBL Genome Browser (http://www.ensembl.org/index.html), Mouse Genome Informatics (http://www.informatics.jax.org/) and Primer3 (http://www.frodo.wi.mit.edu).

Statistical analysis
Data were analyzed using the one-way analysis of variance and Bonferroni post-test to compare pairs of columns unless otherwise stated, using the GraphPad prism software. Results were considered significant if P < 0.05.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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