Heterozygous mis-sense mutations in *Prkcb* as a critical determinant of anti-polysaccharide antibody formation

Running title: Heterozygous mutations in *Prkcb* affect T independent antibody formation

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Abstract

To identify rate-limiting steps in T cell-independent type 2 (TI-2) antibody production against polysaccharide antigens, we performed a genome-wide screen by immunizing several hundred pedigrees of C57BL/6 mice segregating ENU-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 Beta (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 PKCβI whereas the amount of alternatively spliced PKCβII protein was not markedly reduced. Circulating B cell subsets were normal and acute responses to BCR-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.

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. Firstly, antigens have been classified as eliciting either T cell-dependent or T cell-independent (TI) responses based on the ability to induce antibodies to a given antigen in athymic *nude* mice[1]. Antigens that elicit T-independent antibody responses have been further sub-divided 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 receptors (BCR). *Xid* mice are capable of making antibodies against TI-1 antigens, such as bacterial lipopolysaccharide or flagellin, because these signal B cell proliferation through Toll-like receptors[2]. 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[1, 3, 4]. TI-2 responses are mediated primarily by the secretion of short-lived IgM isotype antibodies by marginal zone (MZ) B cells in the spleen[5] and the B-1 cells in the serous cavities[6, 7].

In a T-independent antibody response, the stimulation of the B cell receptor (BCR) leads to the phosphorylation of immunoreceptor tyrosine-based activation motifs in the cytoplasmic tails of the signal transducing molecules CD79a (Igα) and CD79b (Igβ). This allows the phosphorylation of multiple signalling kinases such as Bruton's tyrosine kinase (Btk) and the phospholipase c gamma-2 (PLCγ2)-mediated elevation of intracellular calcium. These kinases activate protein kinase c beta (PKCb) which starts a phosphorylation cascade activating the Card11/Malt1/Bcl-10 complex. This results in the recruitment of the nuclear factor kappa B 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 influenza* type b, *Neisseria meningitides* and *Streptococcus pneumoniae*[8]. These infections severely impact 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[9-11] 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 immunisation 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 increase in the frequency of heterozygous deleterious mutations[12-16] with each person carrying on average 100 deleterious mutations in the genome with approximately 20 being complete loss of function variants[17]. Each of these mutations are individually too recently arising and rare in the population at large to have yet been removed by purifying selection, yet cumulatively they amount to a large genetic burden. Rare variants account for 95% of the protein-altering mutations predicted to be deleterious in the current generation of humans, and account for ~25% of the deleterious mutations in any individual's genome. Based on the *de-novo* mutation rate in human reproduction (1.4×10^{-8}/bp), the current generation of humans is now large enough (6×10^{9} individuals) to saturate every nucleotide of the genome with *de-novo* mutations[12]. Among this burden of recent mutations will be many unique heterozygous mutations in the large network of genes that control antibody production. Heterozygous mutations that affect

rate-limiting steps in antibody production will account for a substantial amount of inherited variability in antibody formation, but no systematic efforts have yet been made to identify these rate-limiting steps.

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-Ficoll[18]. Two semi-dominant mutations were isolated causing selective deficits in TI-2 antibody response, and both were due substitutions in conserved residues in the catalytic domain of Protein kinase c beta (*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 T-cell independent antibody production. First generation offspring (G_1) 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 G_2 progeny to generate a G_3 generation. Twelve-week-old G_3 offspring from 130 pedigrees were screened by immunization with a panel of antigens that differentially elicit T-cell independent and T-dependent antibody responses. T cell-dependent antibody response were measured by immunizing each G_3 animal with alum-precipitated chicken gamma globulin coupled to hapten arsonate (ABA-CGG) and heat killed *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[18, 19]. Three of five mice tested in the *Tilcara* pedigree exhibited lower TI-2 responses compared to littermate counterparts (Figure 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 F_2 intercross was performed between offspring from an affected C57BL/6 (B6) G_3 mouse and a wild-type C57BL/10 (B10) mouse. Immunization of the F_2 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 was tested using a panel of single nucleotide polymorphisms (SNPs) between the B6 and B10 strains, linking the *Tilcara* trait to chromosome 7 distal to SNP 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)F_1 animals with an intermediate response and wild-type B6 mice. Genotyping of intermediate responders and unaffected animals localised 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 base pair 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 PKCβ 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 beta sheet 5 near the conserved gatekeeper residue (Met420) that controls access to the ATP binding pocket (Figure 1f, g and h). In *Tilcara*, Ser552 in the middle of alpha helix G on the C-lobe of the kinase domain, on the lower lip of the substrate binding cleft (Figure 1f, g and h), is mutated to a proline that would be predicted to break the helical structure. The mutated S552 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 protein kinase C beta, PKCβI and PKCβII are produced in mice, humans and other mammals by alternative mRNA splicing of exons encoding PKCβI residues 622-671 (622-673 in PKCβII). 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 PKCβI isoform in purified B cell lysates from *Prkcb*$^{til/til}$ mice compared to wild-type controls, while *Tilcara* heterozygous mice had intermediate protein expression (Figure 2a). By contrast, there was no consistent decrease in the PKCβII isoform (Figure 2a).

Following translation, both PKCβI and PKCβII undergo sequential phosphorylation at threonine and serine residues to become catalytically active, and these modifications can be revealed indirectly by slower mobility in SDS-PAGE and directly by phospho-specific antibodies[21]. When the mutant or wild-type PKCβI isoform was expressed in 293T cells, the *Tilcara* S552P mutation caused almost complete absence of the slower migrating PKCβI form although the more rapidly 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 PKCβII 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 PKCβI and II, involving Thr500 phosphorylation in the kinase domain activation loop by phosphoinositide-dependent kinase-1 (PDK-1)[22] 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 *Prkcb*$^{til/til}$ B cell lysates. This activation loop phosphorylation in turn allows PKCβI to auto-phosphorylate T642 in the turn motif and S660 in the hydrophobic motif of the alternatively spliced C-terminal tail, releasing the fully active enzyme into the cytosol[23]. Western blotting with phospho-T642 PKCβI antibody revealed a profound decrease in autophophorylated protein in B cells from homozygous *Prkcb*$^{til/til}$ mutant mice and an intermediate deficit in heterozygotes (Figure 2d). Thus, the S552P mutation almost completely eliminates the fully active species of PKCβI in B cells, establishing that the *Prkcb*$^{til/til}$ mutation diminishes PKCβ function both in situ in primary B cells and when the mutant protein is expressed on its own in a heterologous cell line.

Diminished basal serum IgM and IgG3 and T-independent antibody responses in *Tilcara* mouse

The semi-dominant biochemical effects of the *Tilcara* S552P PKCβ 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 seven days post immunization, *Tilcara* heterozygous mice made significantly less antibody than wild-type controls while *Prkcb*$^{til/til}$ homozygotes made lower responses than heterozygotes. Likewise, in unimmunised 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 to 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 alum precipitated chicken gamma globulin coupled to hapten arsonate (ABA-CGG) and heat killed *Bordetella pertussis* (BP), which depend on T_H2 and T_H1 cells to elicit CGG-specific IgG1 and BP-specific IgG2c response[24]. 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 T-independent 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 – marginal zone and B-1 cells. In the bone marrow, no significant differences in percentages of early pro-B/pre-B ($B220^{lo}IgM^{lo}$), immature ($B220^{lo}IgM^{hi}$) and mature ($B220^{hi}IgM^{hi}$) B cells could be detected between wild-type, *Prkcb*$^{wt/til}$, *Prkcb*$^{til/ti}$ mice (Figure 4a). Similarly, there was no discernable effect of the mutation on transitional 1 ($CD93^{hi}IgM^{lo}CD23^{lo}$), transitional 2 ($CD93^{hi}IgM^{hi}CD23^{hi}$), transitional 3 ($CD93^{hi}IgM^{lo}CD23^{hi}$), follicular ($CD93^{lo}CD23^{hi}CD21^{int}$) and marginal zone ($CD93^{lo}CD23^{lo}CD21^{int}$) B cell subpopulations in the spleen (Figure 4b). Cell surface IgM was downregulated normally during maturation of follicular B cells in *Tilcara* homozygotes: a result that stands in contrast to mutations in BTK[25] or PLCγ2[26] immediately upstream of PKCβ or with mutations in CARD11[27] 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 $CD11b^{hi}IgM^{hi}$ B-1 cells were markedly decreased in frequency selectively in *Prkcb*$^{til/til}$ homozygous mice compared with wild-type and *Prkcb*$^{wt/til}$ heterozygotes (Figure 4c). Sub-division of the B-1 cells into $CD5^{+}$ B-1a and $CD5^{-}$ 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*$^{til/til}$ donors. Analysis of the reconstituted chimeric animals showed that the *Prkcb*$^{til/til}$ 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 marginal zone cells were preferentially derived from the wild-type lymphocytes and not from those harbouring the *Prkcb*$^{til/til}$ 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 analysed the consequences of the S552P *Prkcb*^{til} 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*$^{til/til}$ 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*$^{til/til}$ cells. Thus, the early steps in BCR signalling leading to Plcγ activation, which lies immediately upstream of PKCβ, appeared unaffected by the *Prkcb*^{til} 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[27], a known immediate substrate of PKCβ[28, 29]. Flow cytometric analysis showed that CD25 was induced by anti-IgM stimulation of wild-type B cells but poorly

induced in homozygous mutant *Prkcb*$^{til/til}$ B cells stimulated in the same culture (Figure 5b and c). By contrast, CD25 was induced equivalently in mutant and wild-type B cells stimulated with 1 μg/ml IgM + 10μg/ml 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 (Figure 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*$^{til/til}$ 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 (Figure 6a-d). Heterozygous *Prkcb*$^{til/+}$ 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*$^{til/til}$ cells (Figure 6e). Measurement of B cell division by dilution of the intracellular dye, 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, while the percentage of divided *Tilcara* heterozygous B cells was significantly lower than in wild-type controls (Figure 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, while 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 to 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 mis-sense mutations. Two independent, semi-dominant mutations were revealed, *Tilcara* and *Untied,* both changing individual amino acids within the catalytic domain of PKCβ. Semi-dominant loss of TI-2 antibody was also reported in *Prkcb* knockout mice[20]. 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 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 PKCβI and PKCβII isoforms. The active, autophosphorylated PKCβI species was preferentially diminished and the overall amount of PKCβI protein was greatly decreased in homozygotes and markedly decreased in heterozygotes, whereas the overall amount of PKCβII protein was not markedly reduced. More complete loss of autophosphorylated PKCβI than PKCβII was also observed when tagged versions of the proteins were transiently expressed in 293 cells. Preferential loss of autophosphorylated PKCβI 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, EDEDELFQS^{552}, that interact with basic Arg or Lys residues in the pseudosubstrate inhibitory domain that lies at the PKCβ 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 inhibitory domain and to kinase substrates. Decreased substrate binding may diminish autophosphorylation to generate the fully active species, while decreased binding to the pseudosubstrate domain may accelerate dephosphorylation and degradation of the kinase. When the pseudosubstrate domain is allosterically dislodged from Helix G upon PKCβ binding to membrane lipids, this opening up is rapidly followed by dephosphorylation of the kinase and, in the case of PKCβI, rapid protein degradation[33]. By contrast, PKCβII degradation after dephosphorylation occurs more slowly, and this may explain the selective loss of PKCβI in *Tilcara* B cells.

The resulting semi-dominant immunological effects of the *Tilcara* mutation favour the view that catalytically active PKCβI is the critical enzyme isoform for progressive B cell division in response to BCR-crosslinking and for TI-2 antibody formation. This genetic inference is supported by biochemical evidence that PKCβI preferentially associates with other essential proteins for BCR-induced NFκB signalling and proliferation, in particular BTK and CARD11[29, 34].

The immune phenotype of *Tilcara* homozygotes had a number of significant differences compared to knockout mice that completely lack both PKCβ isoforms. PKCβ-null mice had defects in both TI-2 and TD antibody responses[20], whereas *Tilcara* homozygous mice had low TI-2 antibody responses but normal TD antibody responses. PKCβ-null mice also had fewer mature $IgM^{lo}IgD^{hi}$ follicular B cells in the spleen[35] and fewer mature follicular B cells that had recirculated to the bone marrow[36], whereas the numbers of both subsets were normal in *Tilcara* homozygotes. There are two plausible explanations for the different phenotypes. Firstly, the $Prkcb^{til/til}$ mutation may result in the expression of small amounts of catalytically competent PKCβI kinase with residual activity. This activity may be insufficient for the stimulation of TI-2 antibody responses but sufficient for TD responses and mature B cell survival. TI-2 antibody responses are highly dependent on BCR cross-linking and stimulation via the BCR receptor[8]. In contrast, the TD antibody response

involves other mitogenic signals such as CD40 ligand[37], that may activate complementary signalling pathways and kinases that counteract the partial defect in PKCβI kinase 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 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, where the PKCβI isoform is involved in TI-2 antibody response, whereas the PKCβII 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 play 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[12-16]. Whereas offspring of ENU-mutagenized mice inherit ~40 novel mis-sense variants, a "normal" current human genome inherits more than 5 000 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 amongst 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 2 440 individuals[16] annotates 21 independent mis-sense mutations in *PRKCB* including many in the kinase domain (http://evs.gs.washington.edu/EVS/). It will important to test if *PRKCB* is equally gene-dosage sensitive in human B cells. Extrapolating from the mouse experiments here, and from evidence that ~20% of missense mutations are profoundly crippling[39], of the 1% of people carrying unique *PRKCB* variants at least 20% of these individuals (0.2% of the population) would be expected to make less antibody to polysaccharide antigens of

enveloped bacteria but exhibit normal circulating B cell subsets, serum IgG, TD antibody to tetanus toxoid and MMR vaccination. One could readily envisage additive or epistatic interactions between heterozygous mutations in *PRKCB* and in other rate-limiting genes for BCR-induced proliferation, as we have shown occurs between heterozygous mutations in *Lyn*, *Cd22* and *Ptpn6* encoding interacting negative regulators of BCR signalling[40]. These experimental results, coupled with human exome sequencing, thus open up a new way to analyse variability in humoral immunity in the wider population, among individuals that would not be defined as having a primary immunodeficiency.

Materials and methods

ENU-treatment of mice. 8-15 week old male C57BL/6 mice were treated with three doses of 100 mg/kg *N*-ethyl-*N*-nitrosurea (Sigma) prepared in 10% ethanol, citrate buffer (pH5.0), one week apart, as previously described [41]. The mice were housed in specific pathogen free conditions at the Australian National University Bioscience Facility or the Scripps Research Institute vivarium. All animal procedures were approved by the Australian National University Animal Ethics and Experimentation Committee or the Institutional Animal Care and Use Committee of the Scripps Research Institute.

Immunization and enzyme-linked immunosorbent assay. Mice were immunized by intraperitoneal injections. For TI-2 immune response, the mice received 25 μg NP-Ficoll (Biosearch Technology) in sterile PBS. For TD responses, mice received 50 μg CGG with hapten arsonate (Biosearch Technology) and 5×10^8 whole killed *Bordetella pertussis* (Lee Labs, Becton Dickinson). Total and antigen specific antibodies in serially diluted sera were detected by enzyme-linked immunosorbent assay on Maxisord plates coated with CGG, sonicated *B. pertussis* or NP-Ficoll. Bound antibodies were detected with alkaline phosphate antibodies to specific mouse isotypes using the phosphatase substrate system. Plates were read at 405 to 650 nm using a

Thermomax microplate reader. The titres for serum samples were calculated as the difference of serum concentrations required to achieve 50% maximum optical density compared to a standard control ran on each plate.

Typing of mutation. Candidate mRNAs were amplified in 300-800 bp fragments using polymerase chain reactions from spleen cDNA and resequenced by the Biomolecular Resource Facility (JCSMR, ANU) using the ABI 3730 capillary sequencer and analysed using the Sequencher 4.2 software. The primers for *Prkbc* were:

*Prkcb*A forward primer: 5' CGCGCAAGATGGCTGACC 3'
*Prkcb*A reverse primer: 5' CGATGTGGGCCTGGATATAG 3'
*Prkcb*B forward primer: 5' AAGCGCTGCGTGATGAAC 3'
*Prkcb*B reverse primer: 5' TGGCCAATCTTGGCTCTC 3'
*Prkcb*C forward primer: 5' TGTGGATGGCTGGTTCAA 3'
*Prkcb*C reverse primer: 5' AATACAGCATGGGGCTCCT 3'
*Prkcb*D forward primer: 5' GCTCCATTCCTGCTTCCA 3'
*Prkcb*D reverse primer: 5' CTTGCCTGGGTGTTTGGT 3'
*Prkcb*E forward primer: 5' CCGTTTGAAGGGGAGGAT 3'
*Prkcb*E reverse primer: 5' CATGGCCACTCTAGCCTCA 3'

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Cell isolation and flow cytometry. Spleens and thymi were harvested from mice and single cells suspensions were prepared by sieving and gentle pipeting through a 70 μm nylon mesh filter. Bone marrow cells were flushed out from tibias 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 straining, samples were stained with the appropriate primary and secondary antibodies. A BD LSRII Benchtop Flow Cytometer with FACS Diva was used for acquisition of flow cytometric data, and Flowjo (Treestar, Inc.) was used for analysis.

Antibodies. Antibodies used for flow cytometry were from BD unless other wise indicated: 7-Amino-actinomycin D (Calbiochem), anti-mouse CD11b-APC/FITC (eBioscience), anti-mouse CD16/CD32 (Stemcell Technologies), anti-mouse CD1d-PE, anti-mouse CD21/CD35-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 Flour 700 (BioLegend), anti-mouse CD45.2-Biotin/PE, anti-mouse CD45.2-Pecific blue (BioLegend), anti-mouse CD45R-Alexa Fluor 405/APC-Cy7/FITC/PE-Cy7/PerCP, anti-mouse CD5-PE, anti-mouse CD62L-PE, anti-mouse CD69-FITC/PE Cy7, anti-mouse CD8- Alexa Fluor 700, PerCP Cy5.5 (eBioscience), anti-mouse CD86-APC/PE, anti-mouse CD93-APC (eBioscience), anti-mouse IgM-APC/FITC/PE Cy5.5, anti-mouse TCR beta- APC-Alexa Fluor 750 (eBioscience), streptavidin-PE/ PerCP/APC and streptavidin-Qdot 605 (Invitrogen). Antibodies used for ELISA: goat anti-mouse IgG/IgG1/IgG3/IgM-alkaline phosphate (Southern Biotechnology), goat anti-mouse IgG2a^{b}-biotin (Southern Biotechnology) and strepavidin-alkaline phosphate (Vector).

Bone marrow chimeras. All recipients were sub-lethally irradiated with two doses of 450 rads and reconstituted via intravenous injections with 2×10^6 bone marrow cells.

***In vitro* cell stimulation assays.** Goat anti-IgM F(ab')2 (Jackson Immunoresearch Laboratories), anti-CD40 (Clone 1C10), anti-TCR (Clone H57-597), bacterial DNA (CpG) (Geneworks) and lipopolysaccharide, from *E.coli* (Sigma) were used as agonist for stimulation. All cell stimulations were done in RPMI supplemented with 10% FCS, 50 units/mL penicillin/streptomycin, 10mM HEPES, 10mM sodium pyruvate, 10mM non-essential MEM and 55 μM β-mercaptoethanol. For DNA synthesis assay, 2×10^6 cells were cultured for 48 hours in 96-well flat bottom plates, before pulsing with 1 μCi [^{3}H]thymidine for 8 hours. Intracellular calcium was measured on an LSR flow cytometer following labelling with Indo-1 (Molecular Probes). For labelling with CFSE, cells were diluted to a concentration of 20×10^6 cells/mL in PBS and stained with a final concentration of 2.5 μM CFSE at 37°C for 10 minutes. Following two washed with cold RPMI, 2×10^6 cells were cultured for 48 hours 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 strepavidin MACS bead separation (Miltenyi Biotech). Cell lysates were subjected to SDS-PAGE with a 12.5% gel using the BioRad Mini-Protean II system. Nitrocellulose membranes (Trans-blot BioRad) and the BioRad Mini-Protean II system was used for transfer according to the manufacturers instructions. Western blotting using the following antibodies: donkey anti rabbit IgG F(ab')2-peroxidase (GE Healthcare), goat anti mouse IgG F(ab')2-peroxidase (GE Healthcare), mouse anti beta-actin monoclonal antibody (Sigma), rabbit anti PKC beta 1 polyclonal antibody (Sapphire Biosciences and Cell Signalling), rabbit anti PKC beta 2 phosphorylated at T642 polyclonal antibody (Sapphire Biosciences) and rabbit anti PKC beta 2 polyclonal antibody (Sapphire Biosciences and Cell Signalling). Blots were developed using the Western Lightning Western Blot Chemiluminescence Reagent (Perkin Elmer).

Bioinformatics. The following bioinformatic tools were used: Boxshade 3.2.1 (http://www.ch.embnet.org:80/software/BOX_form.html), ClustalW (http://www.ebi.ac.uk/clustalw/), Cn3D (http://0-www.ncbi.nlm.nih.gov.ilsprod.lib.neu.edu/Structure/CN3D/cn3d.shtml), ENSEMBL Genome Browser (http://www.ensembl.org/index.html), Mouse Genome Informatics (http://www.informatics.jax.org/) and Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

Statistical analysis. Data were analysed using the One-way ANOVA and Bonfferonni post-test to compare pairs of columns unless otherwise stated, using the GraphPad prism software. Results were considered significant if $P<0.05$.

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Conflict of interest

The authors declare no conflict of interest.

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Figure legends

Figure 1. (**a**) *Tilcara* strain pedigree and TI-2 antibody response. Open and black-filled shapes in pedigree denote third generation (G_3) 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 G_3 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$^{til/+}$)F$_1$×B6$^{+/+}$ 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 cDNA from mice of the indicated genotypes, centred on nucleotide 1654. (**e**) Semi-dominant low TI-2 antibody following immunisation with 2,4,6-trinitrophenyl (TNP)-Ficoll in G_3 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 & C1B, diacylgycerol and phospholipid binding domains; C2, calcium binding domain. The green box represents the region that differs in PKCβI PKCβII. (**i**) Alignment of amino acid sequences of PKCβ from the indicated species (top) and with other murine PKC family members (bottom).

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Figure 2. (**a**) Western blot for PKCβI, PKCβII in unstimulated purified B cell lysates from wild-type (*Prkcb*$^{wt/wt}$), *Tilcara* heterozygous (*Prkcb*$^{wt/til}$) and *Tilcara* homozygous (*Prkcb*$^{tl/til}$) mice. (**b**) Western blot of total NP-40 cell lysates and the pellet or supernatant fractions from 293 cells transfected with expression

vectors encoding V5-tagged wild-type or S552P mutant PKCβI or PKCβII. (**c,d**) Western blot of lysates from unstimulated B cells of the indicated genotypes, probed with (**c**) antibody to phosphorylated threonine 500 or (**d**) phophorylated threonine 642 in PKCβI. Blots in a, c and d were reprobed with β-actin to ensure equal loading. The results are representative of two independent experiments analyzing 3-4 mice of each genotype.

Figure 3. Semi-dominant defects in TI-2 antibody but normal TD antibody responses in *Tilcara*.

(**a**) NP-binding IgM and IgG3 antibody titres measured 6 days after immunization with the TI-2 antigen, NP-Ficoll. (**b**) Relative concentration of basal serum antibodies of the IgM, IgG, IgG1 and IgG3 isotypes. (**c**) IgG2a^{b} and IgG1 isotypes TD antibody titres measured 15 days after primary immunization with *Bordetella pertussis* (BP) and alum precipitated chicken gamma globulin coupled to hapten arsonate (ABA-CGG), respectively. For **a-c**, relative antibody titres are shown, compared to the mean value in C57BL/6 control sera set at an arbitrary value of 100. Each dot represents one mouse, the bars represent the means for groups of *Tilcara* mice of the indicated genotypes, and error bars represent the standard deviations. *, ** and *** indicates a significant difference of P<0.05, P<0.01, P<0.001, respectively, calculated by The One-way ANOVA statistical test and Bonferroni post-test. The results are representative of three independent experiments.

Figure 4. A representation of flow cytometric plots and percentages from *Prkcb*$^{wt/wt}$, *Prkcb*$^{wt/til}$and *Prkcb*$^{til/til}$ mice for (**a**) Pro-B/pre-B (B220^{int}IgM^{int}), immature (B220^{int}IgM^{hi}) and mature (B220^{hi}IgM^{hi}) B cell populations from total lymphocytes for bone marrow cell suspensions. (**b**) Transitional 1 (B220^{hi}CD93^{hi}IgM^{lo}CD23^{lo}), transitional 2 (B220^{hi}CD93^{hi}IgM^{hi}CD23^{hi}), transitional 3 (B220^{hi}CD93^{hi}IgM^{lo}CD23^{hi}), follicular (B220^{hi}CD93^{lo}CD23^{hi}CD21^{int}) and marginal zone (B220^{hi}CD93^{lo}CD23^{lo}CD21^{int}) 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<0.05,

P<0.01, P<0.001, respectively, calculated by The One-way ANOVA statistical test and Bonferroni post-test. Results from **a-c** representative of three independent experiments.

Figure 5. Cell autonomous effects of the *Tilcara* mutation on acute B cell response to BCR crosslinking. (**a**) Intracellular calcium response in mixtures of B cells from *Prkcb*$^{wt/wt}$ CD45.1 and *Prkcb*$^{til/til}$ CD45.2 mice in response to optimal (50 μg/mL) and sub-optimal (5 and 0.5 μg/mL) concentrations of anti-IgM antibody introduced at t=30 s. The vertical axis shows the geometric mean fluorescence ratio of calcium-bound Indo-1 (395 nm, FL5) over un-bound Indo-1 (495 nm, FL4). Paired responses of CD45.1 and CD45.2 cells are overlaid for each anti-IgM dose. The results are representative of three independent experiments. (**b and c**) Induction of CD25 on homozygous or heterozygous CD45.2$^+$B220$^+$ cells (open histograms) co-cultured with CD45.1 wild-type B cells (shaded histograms) for 16 hrs in the presence of the indicated stimuli. The mean % positive cells in each culture are shown. The results are representative of two independent experiments with n = 5 per group.

Figure 6. Effects of *Tilcara* mutation on induction of B cell proliferation. (**a-e**) Mean (± s.d.) ^{3}H-thymidine incorporation in triplicate cultures of spleen cells from five individual mice of the indicated genotypes, 48 hrs 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*$^{til/til}$ 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 for *Prkcb*$^{wt/til}$ (n = 5) and *Prkcb*$^{til/til}$ (n = 5) respectively; for 10μg/mL anti-IgM: 66.6±10.5% and 29.4±15.0%; and for 1μg/mL anti-IgM+10 μg/mL CD40L 89.6±3.7% and 65.9±8.7%.

Supplementary Table 1. At day 0, wild-type (WT) sub-lethally irradiated recipients (CD45.1) were reconstituted with bone marrow from wild-type (+/+), heterozygous (+/*til*) or homozygous (*til*/*til*) *Tilcara* donor mice in the following combinations - 50% wild-type and 50% wild-type bone marrow, 50% wild-type and 50% +/*til* bone marrow or 50% wild-type and 50% *til/til* bone marrow. Each combination was introduced into five recipient mice (n=5 per group). After 12 weeks, the mixed hematopoietic chimeras were sacrificed and tested for reconstitution of different subpopulations in the bone marrow, spleen and peritoneal cavity. For this statistical analysis, the lymphocytes population was used as a reference population (the percentages of CD45.1 and CD45.2 lymphocytes were taken to be the “expected” frequency). The expected frequencies were compared to the observe CD45.1 and CD45.2 allotype percentages for other subpopulations in the same organ (the “observed” frequencies). The P values for a chi-squared test for independence are seen in the table. The values were taken to be significant when $P<0.05$ only in the +/+:*til*/*til* chimeras, and are denoted by shaded backgrounds.

Organ	Subpopulation	Allotype	P-values of chi-squared test from the different chimeras		
			+/+:+/+	+/+:+/*til*	+/+:*til*/*til*
Preimmune blood	$CD45R^+$ B cells	CD45.1	0.3534	0.0726	0.8054
		CD45.2	0.6776	0.2496	0.6906
	$CD4^+$ non-B cells	CD45.1	0.0005	<0.0001	0.0672
		CD45.2	0.0283	<0.0001	0.0127
Bone marrow	Pro-B/Pre-B	CD45.1	0.0007	0.0712	0.0015
		CD45.2	0.1171	0.5317	0.0361
	Immature	CD45.1	0.2930	0.8242	0.5686
		CD45.2	0.6678	0.9842	0.5827
	Mature	CD45.1	0.4696	0.1042	0.0002
		CD45.2	0.8972	0.7091	0.0017
Spleen	$CD45R^+$ B cells	CD45.1	0.0512	0.0012	0.8686
		CD45.2	0.3501	0.0119	0.6143
	T1	CD45.1	<0.0001	<0.0001	<0.0001
		CD45.2	<0.0001	<0.0001	<0.0001
	T2	CD45.1	<0.0001	<0.0001	<0.0001
		CD45.2	0.0006	<0.0001	<0.0001
	T3	CD45.1	<0.0001	<0.0001	0.0135
		CD45.2	0.0011	0.0001	<0.0001
	B-2	CD45.1	0.1196	0.0009	0.7497
		CD45.2	0.4797	0.0088	0.5308
	MZ	CD45.1	0.6444	0.6728	<0.0001
		CD45.2	0.8761	0.7615	<0.0001
	$TCR\beta^+$ cells	CD45.1	0.0022	<0.0001	0.7887
		CD45.2	0.0975	<0.0001	0.4137
	$CD4^+$	CD45.1	0.0059	<0.0001	0.7968
		CD45.2	0.1447	<0.0001	0.4393
	$CD8^+$	CD45.1	<0.0001	<0.0001	0.3180
		CD45.2	0.0013	<0.0001	0.0411
Peritoneal cavity	$CD45R^+$ B cells	CD45.1	0.8300	0.4166	0.9987
		CD45.2	0.9390	0.5685	0.9900
	$CD5^+$ IgM^- cells	CD45.1	<0.0001	<0.0001	0.9267
		CD45.2	0.0005	<0.0001	0.6339
	B-1 cells	CD45.1	0.4547	0.9226	<0.0001
		CD45.2	0.7074	0.9586	<0.0001
	B-1a cells	CD45.1	0.1865	0.4777	<0.0001
		CD45.2	0.4669	0.6400	<0.0001
	B-1b cells	CD45.1	0.6481	0.0533	<0.0001
		CD45.2	0.8411	0.1359	<0.0001