PNAS
www.pnas.org

Supplementary Information for

B cell receptor ligation induces display of V-region peptides on MHC class II molecules to T cells

Peter Csaba Huszthy, Ramakrishna Prabhu Gopalakrishnan, Johanne Tracey Jacobsen, Ole Audun Werner Haabeth, Geir Åge Løset, Ranveig Braathen, Karl Schenck, Anders Aune Tveita, Ludvig Andre Munthe and Bjarne Bogen

Peter Csaba Huszthy
Email: peter.csaba.huszthy@rr-research.no

This PDF file includes:

Supplementary text
Figs. S1 to S22
Table S1
Extended Materials and Methods

www.pnas.org/cgi/doi/10.1073/pnas.1902836116

A

```
        1
        <------------------------------------------  FR1 - IMGT
         D   V   Q   L   Q   E   S   G   P       G   L   V   K   P
VH 315 gat gta cag ctt cag gag tca gga cct ... ggc ctc gtg aaa cct
VH GL  --- --- --- --- --- --- --- --- --- ... --- --- --- --- ---

        ------------------------------------------> ________________

         S   Q   S   L   S   L   T   C   S   V   T   G   Y   S   I
VH 315 tct cag tct ctg tct ctc acc tgc tct gtc act ggc tac tcc atc

VH GL  --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---

        __  CDR1 - IMGT  ____________ 34 <--------------------------

         T               S   G   Y   F   W   N   W   I   R   Q   F
VH 315 acc ... ... ... agt ggg tat ttc tgg aac tgg ata cgg cag ttt
                                   Y
VH GL  --- ... ... ... --- --t --- -a- --- --- --- --c --- --- ---

                                  49      51      53
        FR2 - IMGT  ------------------------> _____________  CDR2
         P   G   N   K   L   E   W   L   G   F   I   K   Y   D
VH 315 cca gga aac aaa ctg gaa tgg ttg ggc ttc ata aag tac gac ...
                                   M       Y       S
VH GL  --- --- --- --- --- --- --- a-- --- -a- --- -gc --- --- ...

                 59
        - IMGT  _____ <---------------------------------------
                 G   S   N   G   Y   N   P   S   L   K       N   R
VH 315 ... ... ggt agc aat ggc tac aat cca tct ctc aaa ... aat cga
                         N
VH GL  ... ... --- --- --- aa- --- --c --- --- --- --- ... --- ---

        68                              76
        -------------------------------  FR3 - IMGT  ---------------
         V   S   I   T   R   D   T   S   E   N   Q   F   F   L   K
VH 315 gtt tcc atc act cgt gac aca tct gag aac cag ttt ttc ctg aag
         I                              K
VH GL  a-c --- --- --- --- --- --- --- a-- --- --- --- --- --- ---

        ----------------------------------------------------------> ____
         L   N   S   V   T   T   E   D   T   A   T   Y   Y   C   A
VH 315 ttg aat tct gtg act act gag gac aca gct aca tat tac tgt gcc

VH GL  --- --- --- --- --- --- --- --- --- --- --- --- --- --- --a

        98 ____________  CDR3 - IMGT  _____________

         G   D   N   D   H   L   Y   Y   F   D   Y   W   G   Q   G
VH 315 gga gat aat gat cac ctc tac tac ttt gac tac tgg ggc caa ggc
         R
VH GL  a-- --


         T   T   L   T   V   S   S
VH 315 acc act ctc aca gtc tcc tca
```

B

```
3'V-REGION   P  N1      D-REGION       N2   P        5'J-REGION
tgtgccggaga  t  a  ......atgatcac...  ctc  t  actactttgactactgg
                      IGHD2-4*01               IGHJ2*01
```

C

```
        C   A   G   D   N   D   H   L   Y   Y   F   D   Y   W
VH 315 tgt gcc gga gat aat gat cac ctc tac tac ttt gac tac tgg
               R               Y
 VH GL--- --a a-- --- --- --- t-- --- --- --- --- --- --- ---
```

Fig.S1. Alignment of the MOPC315 V_H nucleotide sequence used to generate VDJ_H^{315} mice and the most similar germline sequence. (A) Alignment of MOPC315 V_H and the germline sequence IGHV3-6*02 (1). Nucleotides encoding the leader sequence (amino acids 1 to 18) are not shown. A dash (-) indicates a position where the V_H^{315} sequence is identical to IGHV3-6*02. Positions of the complementarity determining regions are indicated using unbroken lines, whereas the framework regions are shown with broken lines, as annotated by IMGT (http://www.imgt.org/IMGT_vquest/vquest). The V_H^{315} nucleotide sequence was obtained by sequencing the MOPC315.4 line in our laboratory (2) and (3). The amino acid sequence is identical to the annotated V_H^{315} sequence in UniProt (P01822-HVM46_MOUSE). The DNA sequence differs with one nucleotide at the last codon of a.a. 38 from the annotated V_H^{315} sequence (EMBL European Nucleotide Archive, http://www.ebi.ac.uk/ena/data/view/M27638), as published (4). The BALB/c germline sequence was obtained by sequencing genomic DNA, and was identical to IGHV3-6*02 (1). (B) Junction analysis; D and J alignment and N-region nucleotide additions are indicated, as predicted by IMGT. (C) Translation of the junction. Amino acid changes from GL are found at position 98 of the V region (R→G) and at position 102 in the D-region (Y→H).

Materials and Methods

A sequence definition of the BALB/cJ IGHV3-6 region was performed. Ear samples from adult mice were digested in lysis buffer (100 mM Tris pH 8.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCl) added 5 µl proteinase K (10 mg/ml, P6556, Sigma-Aldrich) at 55° C over night. Thereafter, the supernatants were treated with RNase A (VWR, A3832) at 100 µg/ml for 30 minutes at room temperature, which after the DNA was precipitated with pure isopropanol, washed in 70% ethanol and finally re-suspended in Tris-EDTA buffer. PCR was run using primers flanking the V_H regions using forward primer 5'-gatgtacagcttcaggagtcaggacct-3' and a reverse primer 5'-tctcttgcacagtaatatgta-3'. The PCR products were TOPO-cloned using Zero blunt TOPO PCR cloning kit (Thermo Fisher Scientific). Colonies containing inserts were picked on Kanamycin LB plates. DNA was prepared from overnight cultures of single colonies using SV Miniprep kit (Promega). Small-scale DNA preps were sent for sequencing and analyzed.

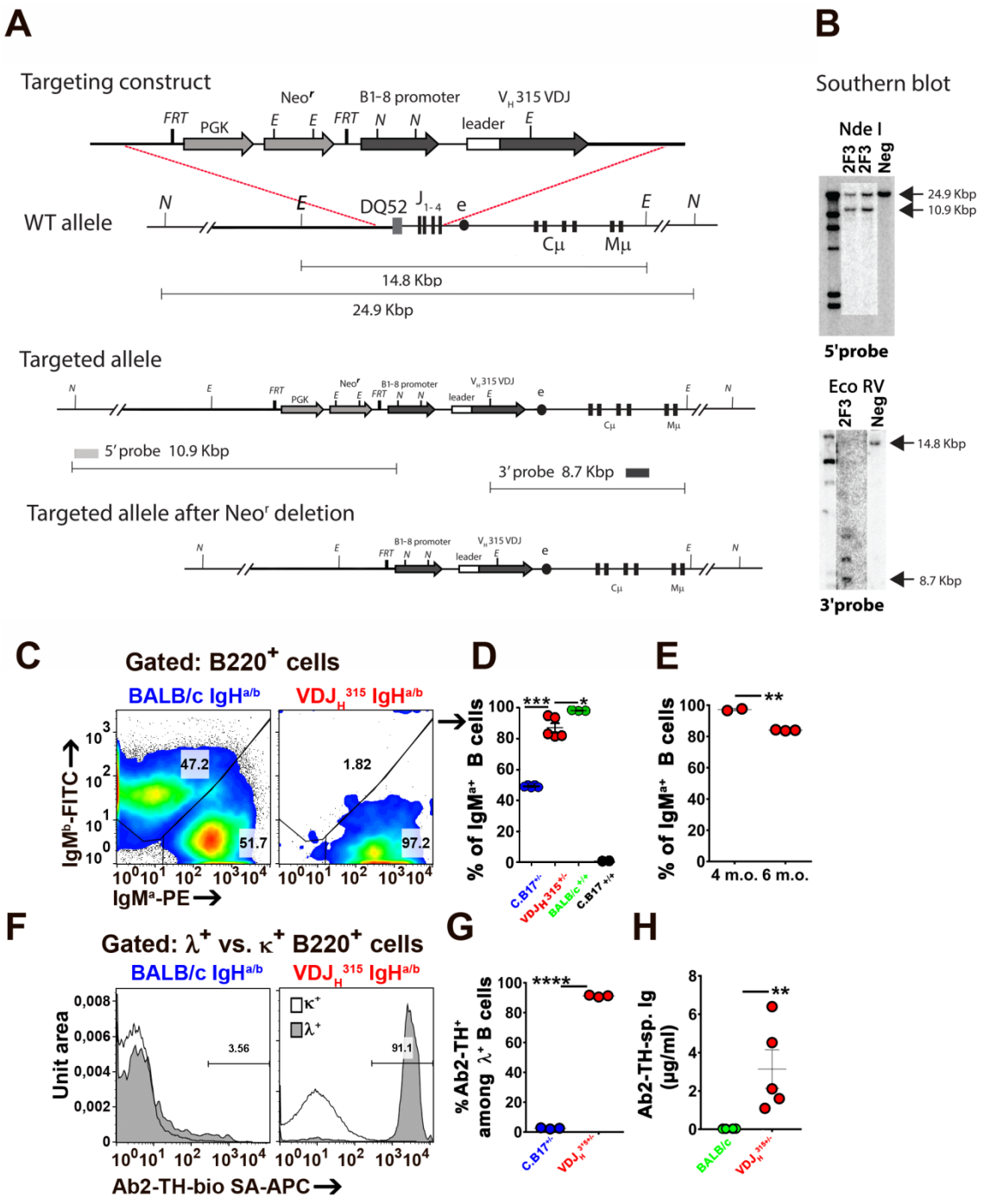

Fig.S2. VDJ_H^{315} mice show complete allelic exclusion. (A) Genomic targeting to generate VDJ_H^{315} mice. From top to bottom: targeting construct (exclusive of homology arms), WT allele, targeted allele and the targeted allele after removal of the Neomycin selection cassette. FRT: flippase recombination target sites, PGK: mouse phosphoglycerate kinase 1 promoter, Neo^r: neomycin resistance gene, $J_{1\text{-}4}$: joining genes 1-4. Insertion was targeted to replace IGHD-Q52*01-J_4. Restriction fragment sizes are indicated for WT and targeted loci. The 5' probes used to verify homologous recombination events at the 5' and 3' regions of the J_H locus are indicated. E=EcoRV

and N=Nde I restriction enzyme sites. (B) Southern blot analysis of WT (Neg) and VDJ_H^{315} targeted BALB/c ES cells (2F3). For analysis with the 5' probe, genomic DNA from ES cells was digested with Nde I, top. For Southern blot analysis using 3' probe, DNA was digested with EcoRV, bottom. (C-E) Allelic exclusion in VDJ_H^{315} mice. VDJ_H^{315} mice on a BALB/c (IgH^a) background were crossed with IgH^b congenic mice. Splenic B cells of the offspring were analyzed for cell surface expression of IgM^a vs. IgM^b allotypes. BALB/c WT x C.B17 F1 mice ($C.B17^{+/-}$) served as a control. (C) Representative FACS plots. (D) Scatter plot summary of data (n=5 for $C.B17^{+/-}$ and $VDJ_H^{315+/-}$, n=3 $C.B\text{-}17^{+/+}$, n=2 BALB/c). (E). Allelic exclusion as a function of age (4 vs. 6 months old VDJ_H^{315} mice, n=2 and n=3). (F) Expression of VDJ_H^{315} on peripheral B cells detected by the anti-Id mAb Ab2-TH, which binds VDJ_H^{315} expressed together with any λ L chain (λ1, λ2, λ3) but not with κ L chains (5). In VDJ_H^{315} mice, virtually all λ^+ B cells are Ab2-TH^+ while κ^+ B cells are not. (G) Summary of results (n=3). (H) Serum levels of Ab2-TH^+ Ig in $VDJ_H^{315+/+}$ (n=5) versus BALB/c (n=7) mice. Statistical comparisons: Tukey's multiple comparisons test (D), unpaired T tests (E, G), Mann-Whitney U test (H).

Materials and Methods

Generation of targeted VDJ_H^{315} knock-in mice

Mice that express the V-region of the M315 myeloma protein heavy chain, produced by the MOPC315 cell line (6) were generated by inserting the rearranged VDJ_H^{315} sequence into the D-J1-J4 region of the IgH locus through homologous recombination (see Fig. S2A above). First, the VDJ_H^{315} sequence was inserted next to the B1-8 promoter (derived from the pB1-8-LE vector, kindly supplied by Prof. K. Rajewsky) and fused to a Neo^r-encoding selection cassette, flanked by FRT sites for removal. Homology arms for positioning were the same as for the VDJ_H^{ald} mouse (7). Genomic targeting of BALB/c BL1 ES cells was performed at Ozgene Ltd. (Perth, Australia) as previously described (7). Offspring were obtained by standard procedures and germline transmission was confirmed by Southern blot analysis (see Fig. S2B above). VDJ_H^{315} mice were bred and maintained homozygous. Transgene expression was verified by PCR with primers annealing to the 5' Cre and the B1-8 promoter regions. Wild-type loci were detected using primers specific for the D region.

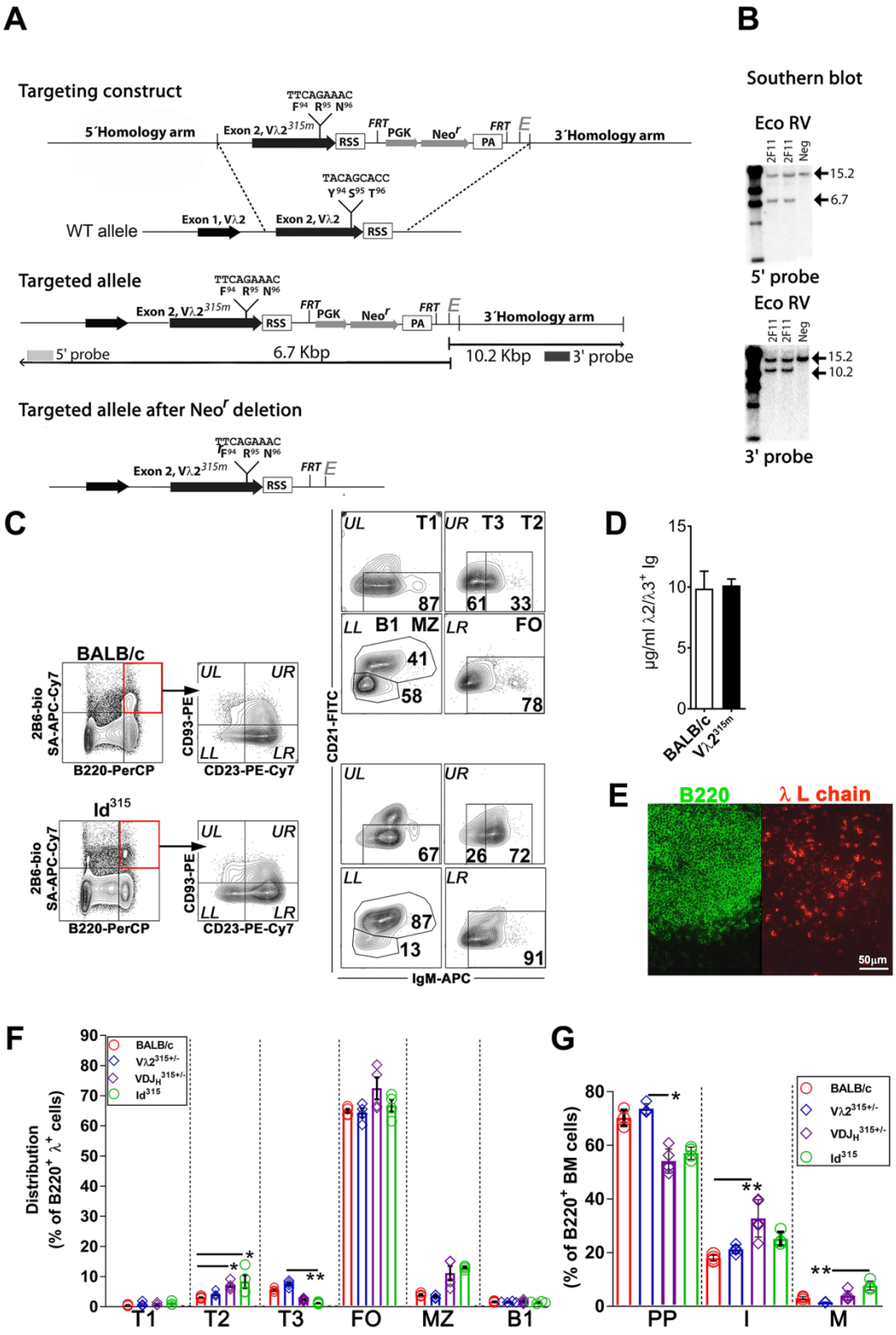

Fig.S3. Vλ2^{315m} mice and Id^{315} mice have a close-to physiological λ2^{+} B cell compartment. (A) The Vλ2^{315} construct (top). Targeted replacement of nine nucleotides in Vλ2 exon 2, resulting in an exchange of the germline sequence YST (residues 94-96) to FRN. The selection

cassette with the Neomycin resistance gene (Neo^r) and its PGK promoter is shown as grey arrows, flanked by *FRT* (Flippase recognition target) sites. An EcoRV restriction site (E) was introduced next to the 3′ FRT site to generate fragments for Southern blot analysis. EcoRV generates a 15.2 kB fragment in WT mice (endogenous sites not shown) whereas in the targeted genome, fragments of 6.7 kb (5' probe) and 10.2 kb (3' probe) are generated. (B) Southern blot analysis of targeted ES cells (2F11) and non-targeted ES cells (Neg) using the 5' and 3' probe. (C) FACS gating strategy for splenic B cell lineages. The B220$^+$ λ2/λ3 BCR L chain$^+$ population (red square, detected by the 2B6 mAb) was selected for analysis. Values given in the quadrants represent the mean frequency of the given population within the parent population (for both, n=4). (D) Serum levels of λ2/λ3$^+$ Ig in Vλ2^{315m} mice versus BALB/c WT mice (n=4). (E) Spleen section showing a follicle from a Vλ2^{315m} mouse co-stained for B cells (B220 FITC, left) and λ L chains (goat anti-mouse λ-TRITC, right) show the relative scarcity of B cells with λ$^+$ BCRs. (F) Distributions of B cell subsets among λ2/λ3$^+$ B220$^+$ splenic B cells in the indicated mouse strains [analyzed as in (C), n=4/strain]. T1-T3: Transitional cells, FO: Follicular B cells, MZ: Marginal zone B cells, B1: B1 cells. (G) Bone marrow subsets among B220$^+$ cells in the four strains of mice (n=4/strain). PP: Pre-B cells and Pro-B cells, I: Immature B cells, M: Mature B cells. Statistical comparisons: Dunn's multiple comparisons tests (F, G).

Materials and Methods

Generation of Vλ2^{315m} mice

In Vλ2^{315m} mice, the germline Ig Vλ2 exon 2 was replaced with a synthetic Vλ2^{315} exon 2. The codon TACAGCACC (Y^{94} S^{95} T^{96}, germline) →TTCAGAAAC (F^{94} R^{95} N^{96}, modified) exchange should result in a conversion to the MOPC315 λ2 chain (λ2^{315}) sequence except for position 38, which is the mutated a.a. Ile in λ2^{315}, but remained the germline-encoded a.a. Val in Vλ2^{315m} (8). The targeting vector included a Neomycin resistance cassette positioned 5' of the Vλ2^{315} modified exon 2 sequence (See Fig. S3A above). Genomic targeting of BALB/c BL1 ES cells was performed at Ozgene as described (8). Transformed cells were selected and genomic integration was verified by Southern blots. ES clones were then injected into blastocysts of pseudopregnant mice. The resulting chimeric mice were crossed and the offspring typed by Southern blotting to verify germline transmission. The Neo^r cassette was removed through breeding to an Flp-deleter line (Oz_Flp). The Flp gene was then removed through crossing with BALB/c mice. Thereafter, homozygous offspring (Vλ2$^{315m+/+}$) mice were generated, and colonies were maintained in the homozygous state. The founder colony was verified by Southern blot analysis. Probes for Southern blotting bound either 5' (Chr. 16, - strand; positions 19264489 to 19264899) or 3' (mouse Chr. 16, - strand; positions 19256392 to 19256791) of the targeted locus, or to the Neo^r gene.

B cell phenotyping

Characterization of splenic, lymph node and femoral bone marrow B cells was done by flow cytometry and analyzed essentially as in (7). The 2B6 mAb (9) was used to select B cells that expressed a λ2/3 L chain BCR.

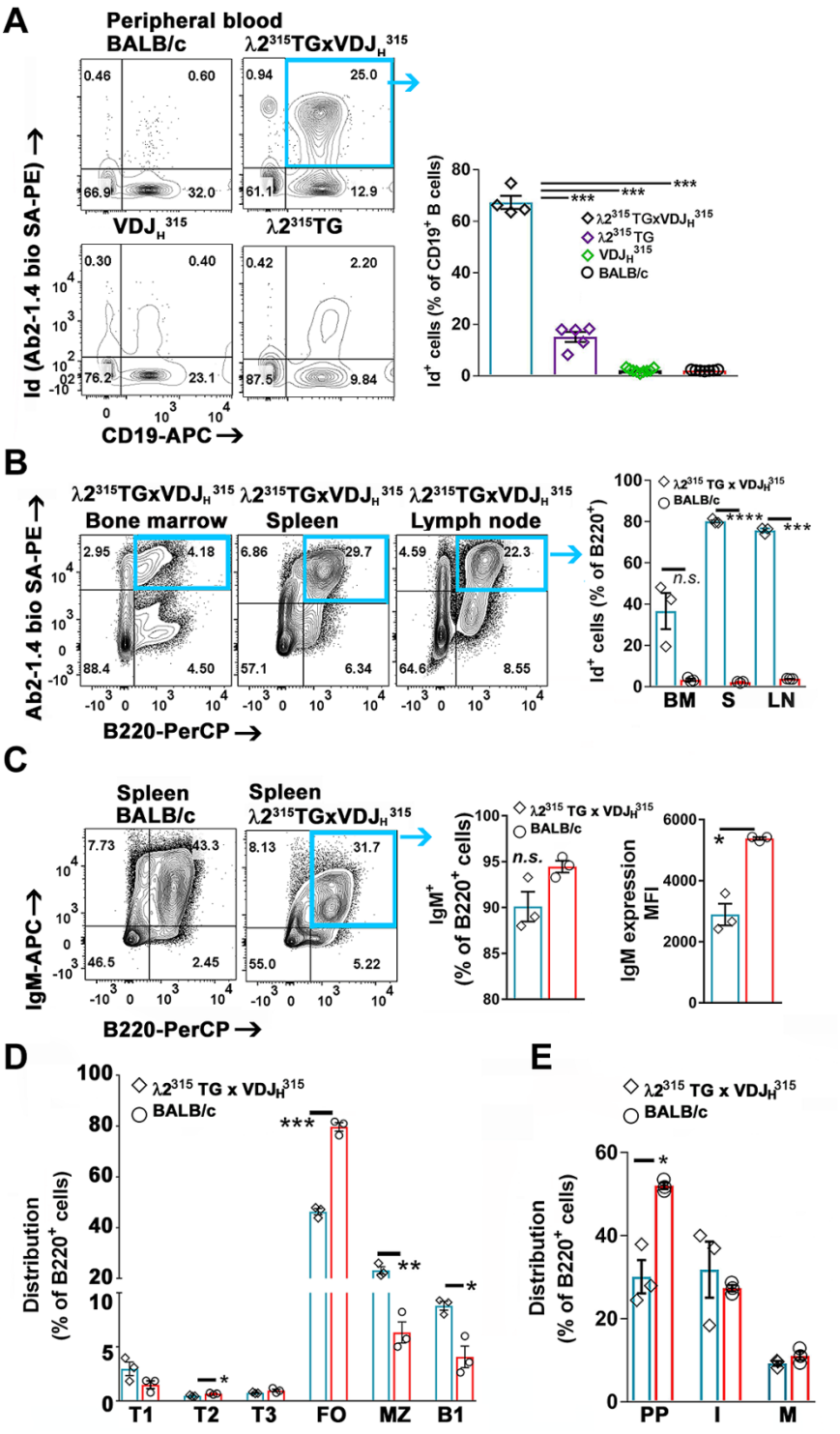

Fig.S4. High frequencies of Id$^+$ B cells in the lymphoid organs λ2^{315} TG x VDJ$_H^{315}$ F1 mice. (A) Left: representative FACS plots show reactivity of circulating B cells with Ab2-1.4 (anti-Id IgG) in the indicated strains of mice. Right: scatter plot summary of the data (λ2^{315} TG x VDJ$_H^{315}$ n=4, λ2^{315} TG n=5, VDJ$_H^{315}$ n= 9, BALB/c n=7). (B) Representative FACS plots showing Id$^+$ B

cells among femoral bone marrow cells, splenocytes and pooled lymph node cells of a ($\lambda2^{315}$ TG x VDJ_H^{315}) F1 mouse. Right: Summary of data for ($\lambda2^{315}$ TG xVDJ_H^{315}) F1 mice and BALB/c (n=3/strain). (C) FACS plots show IgM expression on splenic B cells from ($\lambda2^{315}$ TG xVDJ_H^{315}) F1 versus BALB/c mice (n=3/strain). Center: percentages of IgM^+ cells among $B220^+$ cells, gated as indicated. Right: scatter plot shows the mean fluorescence intensity of IgM signal on the $B220^+$ population. (D) B cell subsets in the spleens of ($\lambda2^{315}$ TG x VDJ_H^{315}) F1 and BALB/c mice (n=3/strain). T1-T3: Transitional cells, FO: Follicular B cells, MZ: Marginal zone B cells, B1: B1 B cells. (E) Distribution of $B220^+$ bone marrow cells in ($\lambda2^{315}$ TG x VDJ_H^{315}) F1 and in BALB/c mice (n=3/strain). PP: Pre-B cells and Pro-B cells, I: Immature B cells, M: Mature B cells. Statistical comparisons: Tukey′s multiple comparisons test (A), Unpaired T tests (B-E).

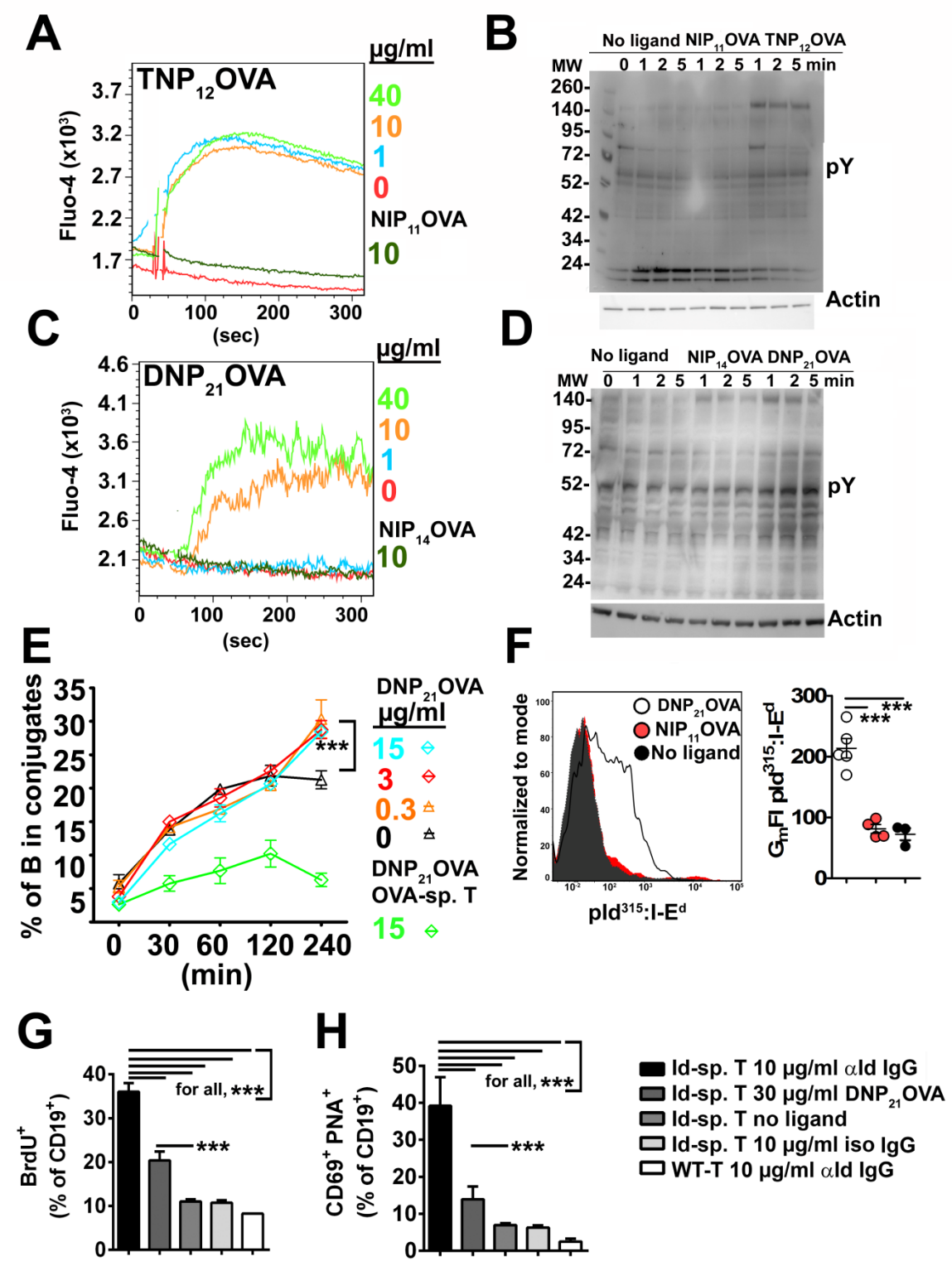

Fig.S5. In vitro responses of Id$^+$ B cells to TNP-OVA and DNP-OVA. Id$^+$ B cells (enriched from $\lambda2^{315}$TG xVDJ_H^{315} mice by immunomagnetic depletion) were stimulated in culture with specific BCR ligands $TNP_{12}OVA$ (A, B) or $DNP_{21}OVA$ (C-H), or specificity control $NIP_{14}OVA$

(C, D) or $NIP_{11}OVA$ (A, B, F). (A) Ca^{2+} flux assay using $TNP_{12}OVA$. Concentrations are indicated (range: 1-40 µg/ml). (B) Phosphotyrosine blots of cell lysates. Cells were stimulated using 15 µg/ml $TNP_{12}OVA$ or $NIP_{11}OVA$ or no ligand. (C) Ca^{2+} flux assay using $DNP_{21}OVA$. Concentrations of ligand are indicated (range: 1-40 µg/ml). (D) Phosphotyrosine blots of cell lysates. Cells were stimulated using 15 µg/ml $DNP_{21}OVA$ or $NIP_{14}OVA$ or no ligand. (E) Conjugate formation between fluorochrome-labeled Id^+ B cells and Id-sp. T cells in the presence of titrated doses of $DNP_{21}OVA$. Conjugate formation with OVA-sp. $CD4^+$ T cells enriched from $DO11.10^{+/+}$ TCR-transgenic mice in the presence of $DNP_{21}OVA$ is indicated (green diamonds). (F) pId:MHCII display on Id^+ B cells after 18 hours of stimulation using 10 µg/ml $DNP_{21}OVA$, or $NIP_{11}OVA$ or no antigen. Right: quantification. (G) BrdU incorporation into Id^+ B cells in T-B cell co-cultures stimulated with 10 µg/ml of anti-Id IgG or with 30 µg/ml of $DNP_{21}OVA$ (comparable molar concentrations). BrdU incorporation was measured during the last 14 hours of a four-day co-culture. (H) Activation measured by peanut agglutinin (PNA) binding and CD69 co-expression on Id^+ B cells. Statistical comparisons: Unpaired two-tailed T test (E), Dunn's multiple comparisons test (F), Tukey's multiple comparisons tests (G-H).

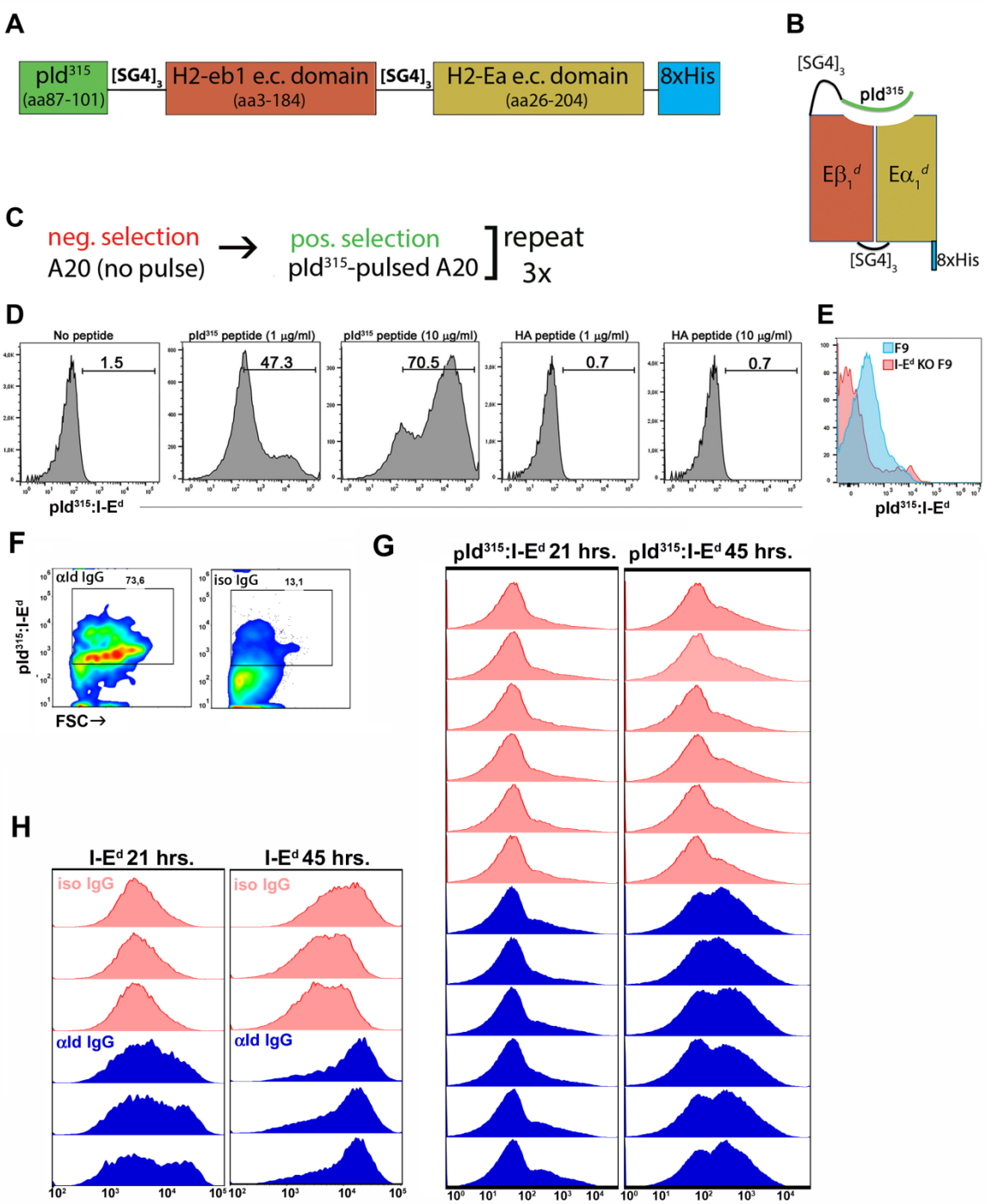

Fig.S6.Generation and validation of a TCR mimetic (TCRm) with specificity for pId^{315}:I-E^{d}. (A) Schematic of the pId^{315}:I-E^{d} construct used to generate recombinant protein for immunization. (B) Schematic of the recombinant protein produced by non Ig-producing MOPC315.36 myeloma cells transfected with the construct depicted in (A). (C) A scFv phage display library was generated from the spleen of a mouse immunized with the purified pId^{315}:I-E^{d} protein depicted in B (for details, see Methods below). Phages were selected by negative and positive selection on A20 lymphoma cells that were either unpulsed or pulsed with synthetic pId^{315} (λ2^{315} a.a. 89-105). The sequence from a positive binder was reformatted to a His-tagged scFv format, expressed in

E. coli, purified, biotinylated and used for staining. This reagent is referred to as TCRm (TCR mimetic). (D) Flow cytometry histograms show staining of A20 lymphoma cells that were pulsed with pId^{315} peptide (a.a. 89-105) or an irrelevant control peptide similarly restricted by $I\text{-}E^d$ (hemagglutinin a.a. 110-120; strain A/Puerto Rico/8/1934 H1N1; HA) at the indicated concentrations. Non-pulsed A20 cells are shown as a reference. (E) Staining of F9 cells (A20 cells transfected with the $\lambda 2^{315}$ gene) with the TCRm (blue histogram) compared to F9 cells where $I\text{-}E^d$ was deleted using CRISPR-Cas9 technology. (F) Representative plots of forward scatter vs. pId^{315}:$I\text{-}E^d$ expression on splenic Id^+ B cells in Id^{315} mice 24 hours after i.v. injection with anti-Id IgG or isotype control IgG. (G, H). Related to Figures 2C, 2E and 2F. Histograms show expression of pId^{315}:$I\text{-}E^d$ (G, 6 wells/group) and $I\text{-}E^d$ (H, 3 wells/group) on Id^+ B cells in *in vitro* cultures. Time lengths of stimulation are indicated.

Materials and Methods

Generation of a TCR mimetic specific for pId:MHCII

To generate a single chain pId^{315}:$I\text{-}E^d$ peptide fusion protein for immunization, a synthetic gene sequence (Genscript) was generated that encoded (i) the *H2-eb* and *H2-ea1* extracellular domains ($E\beta 1^d$ and $E\alpha 1^d$) fused via an $[SG4]_3$ linker, (ii) a pId^{315} fragment encoding a.a. 87-101 of the $\lambda 2^{315}$ light chain variable region (MYFCALWFRNHFVFG) fused to the 5' end of H2-eb through an $[SG4]_3$ linker and (iii) a polyhistidine tag inserted 3' of *H2-ea1*. The sequence was inserted into a pMSCV-IRES-GFP vector (Addgene #20672), and stably expressed in the Ig non-producing MOPC315.36 myeloma cell line (10). The resulting fusion protein was purified from cell culture supernatant by immobilized metal affinity chromatography using a HisTrap HP column (GE Healthcare). The purified protein was mixed with RIBI adjuvant (Sigma Aldrich) according to the supplied protocol, and the emulsion was used for vaccination of BALB/c mice by s.c. injection of 2x100 µg antigen at separate sites. Booster doses were administered on day +21 and +35. cDNA was prepared from splenocytes of the mouse with the highest titers of serum antibodies that bound pId^{315}-pulsed A20 ($I\text{-}E^{d+}$) B lymphoma cells, and a scFv phage library was constructed essentially as described using a high valence pIX display system (11). scFv constructs reactive against pId^{315}:$I\text{-}E^d$ complexes were selected by cell-based biopanning of phages using A20 cells loaded with synthetic pId^{315} (a.a. 89-105) peptide. Top candidates were screened by flow cytometry. Briefly, pId^{315}-pulsed and unpulsed A20 cells were incubated on ice for 45 min with phage-containing 2xYT bacterial supernatants diluted in PBS, followed by 30 min incubation with biotinylated rabbit anti-fd Bacteriophage antibodies (B2661, Sigma-Aldrich), and finally 25 min with Streptavidin-Phycoerythrin (BD). Binders showing preferential staining with peptide-pulsed A20 cells were reformatted into a His-tagged scFv format and biotinylated using EZ-Link NHS-Biotin (Thermo Scientific) followed by assessment for binding to cells by flow cytometry. The selected clone was sequenced and transferred to a pHOG21 periplasmic expression vector (12). The scFv was expressed in *E. coli*, purified using a HiTrap protein L affinity chromatography column (GE Healthcare), and biotinylated using the EZ-Link Sulfo-NHS-Biotin reagent (Thermo Fischer Scientific).

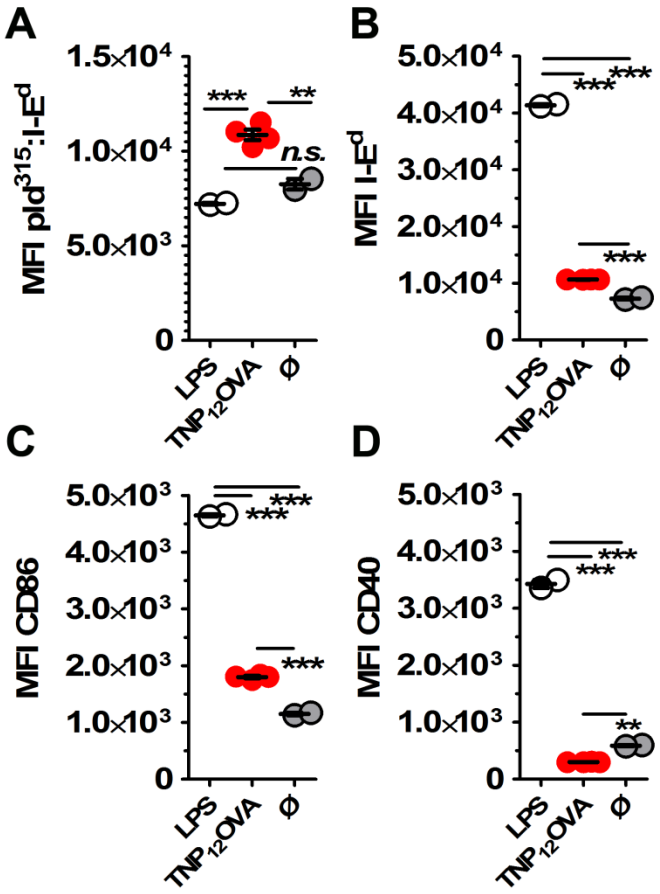

Fig.S7. Ligation of the Id$^+$ BCR induces a selective increase in pId^{315}:I-E^d cell surface expression. Id$^+$ B cells were stimulated *in vitro* for three days in the presence of either LPS (10 µg/ml) or TNP-OVA (10 µg/ml) or no additions (unstimulated, indicated by Ø). On day 3, cells were analyzed by FACS. MFI values measured for (A) pId^{315}:I-E^d (B) I-E^d (C) CD86 (D) CD40 are given.

Materials and Methods

B cells were enriched from the spleens of λ2^{315}TG x VDJ$_H^{315}$ mice by immunomagnetic depletion. Cells were seeded at a density of 5x10^5 cells/well. The cells were stimulated for three days *in vitro* in complete culture medium (RPMI1640, 10% FCS and supplements) with addition of either 10 µg/ml of LPS (*E. coli* O111:B4, L2630, Sigma) or 10 µg/ml of TNP$_{12}$OVA, or no addition. The cells were analyzed on day 3 by FACS.

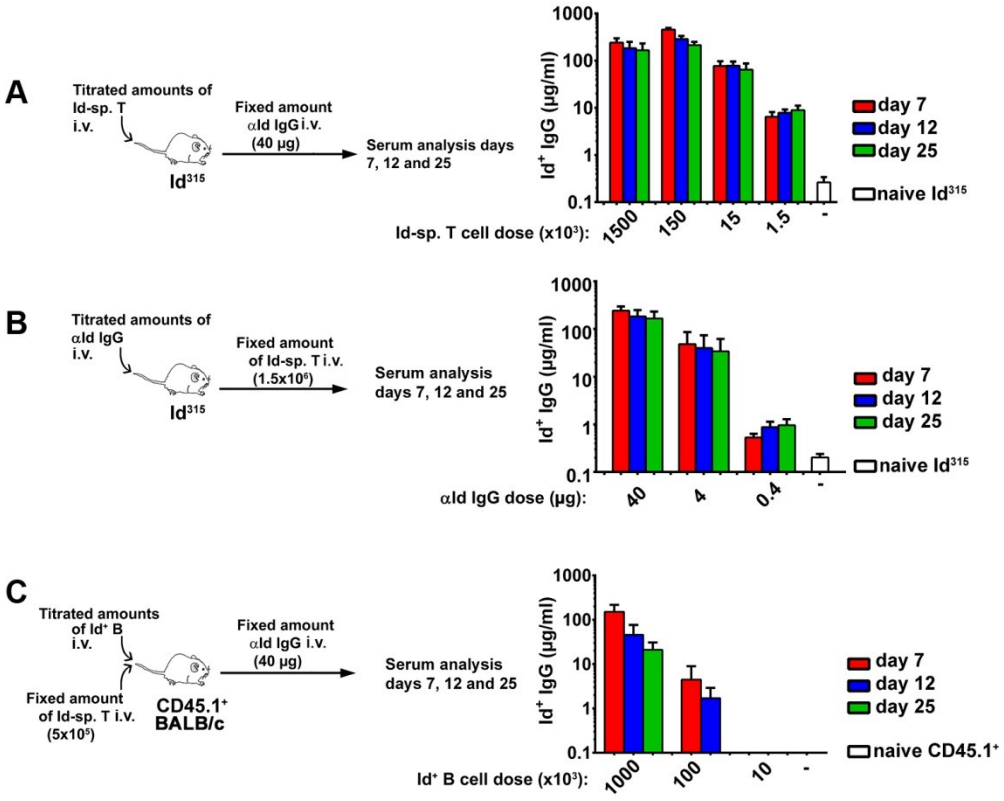

Fig.S8. Id^+ IgG responses depend on transferred cell numbers and on the delivered BCR ligand dose. (A-C) Left: experimental setup. Right: serum levels of Id^+ IgG in recipient mice on days 7, 12 and 25 as a function of titration. (A) Titration of naïve Id-specific $CD4^+$ T cells in Id^{315} mice accompanied by a saturating dose of anti-Id IgG BCR ligand (n=3 per cell dose). (B) Titration of BCR ligand (anti-Id IgG) in Id^{315} mice that had received a saturating dose of naïve Id-specific T cells (n=3 per dose, except for 4 µg/mouse n=4). (C) Titration of naïve Id^+ B cells (from $\lambda2^{315}$TG xVDJ_H^{315} mice) into $CD45.1^+$ congenic mice that received a saturating dose of Id-specific T cells and anti-Id IgG BCR ligand (n=3 per cell dose). Levels of Id^+ IgG in the sera of naïve Id^{315} mice (A, B) are shown for comparison. $CD45.1^+$ congenic WT mice had undetectable levels of Id^+ IgG (C).

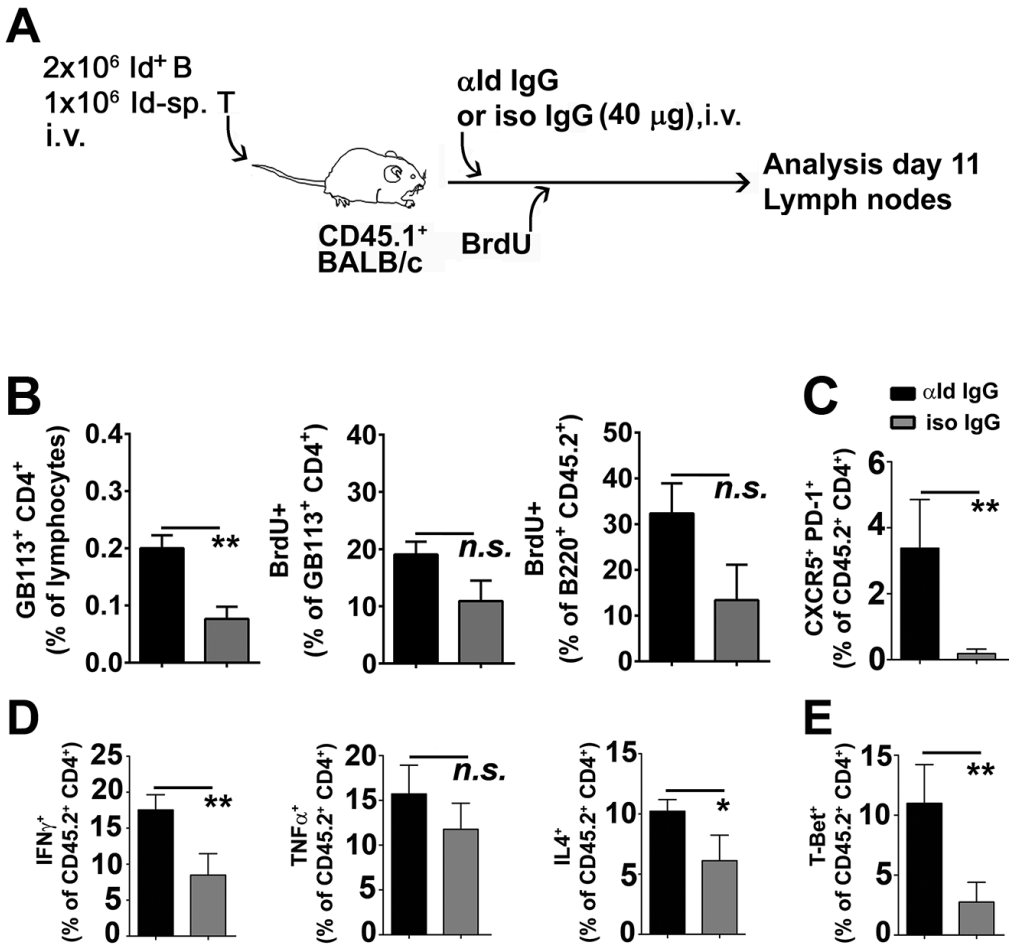

Fig.S9. Id-specific T cells in the lymph nodes of adoptively transferred mice show preferential expansion and differentiation when the Id$^+$ BCR is ligated. (A) Experimental setup, related to Figure 3. The samples were stained in the same experiment as in Figure 3 and analyzed together. (B) Frequencies and BrdU incorporation of Id-specific T cells and Id$^+$ B cells. (C) Frequencies of Id-specific follicular T helper cells among transferred T cells. (D, E) Frequencies of transferred T cells that stained for the indicated cytokines and the T-Bet (T_H1) transcription factor as analyzed by flow cytometry. Expression of GATA-3 (T_H2-cells) and Foxp3 (T_{reg} cells) was analyzed, but not detected. Statistical comparisons: Mann-Whitney *U* tests.

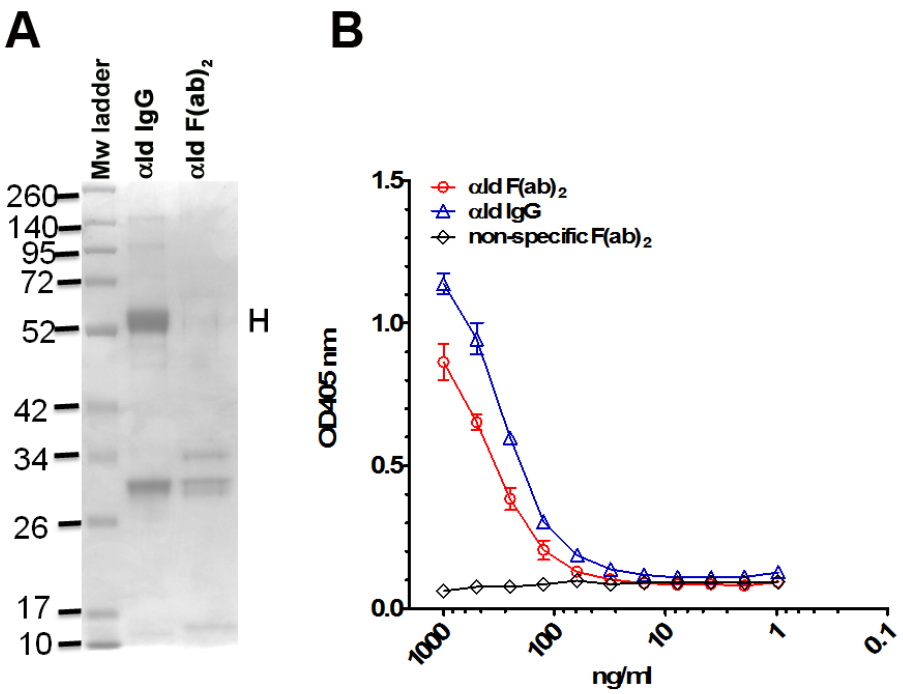

Fig.S10. Characterization of the anti-Id F(ab)$_2$ fragment (A) Purity analysis of the complete Ab2-1.4 (anti-Id IgG) vs. the Ab2-1.4-derived anti-Id F(ab)$_2$ fragment on SDS-PAGE (reducing condition) stained with Coomassie Blue. Relative absence of heavy chain (H) at around 50 kDa is seen in the F(ab)$_2$ preparation. (B) ELISA comparing the binding abilities of anti-Id F(ab)$_2$ and complete anti-Id IgG to M315-coated plates. A commercial mouse IgG F(ab)$_2$ was used as a specificity control (open black diamonds).

Materials and Methods

Generation of F(ab)$_2$ fragments

The anti-Id IgG Ab2-1.4 was digested with immobilized Ficin and purified using a Protein A Spin column (Mouse IgG1 F(ab)$_2$ preparation kit, #44980, Pierce, Rockford, IL). Further removal of undigested IgG was done on a protein G column. SDS-PAGE was run under reducing conditions to verify relative absence of residual Fc fragments. Antigen binding was verified on ELISAs. Plates were coated with 2 µg/ml of M315 (IgA, λ2). Anti-Id IgG and anti-Id F(ab)$_2$ fragments were added followed by detection with anti-mouse-κ-biotin (187.1). A commercial mouse IgG F(ab)$_2$ (Novusbio, NBP1-97018) was used as a control.

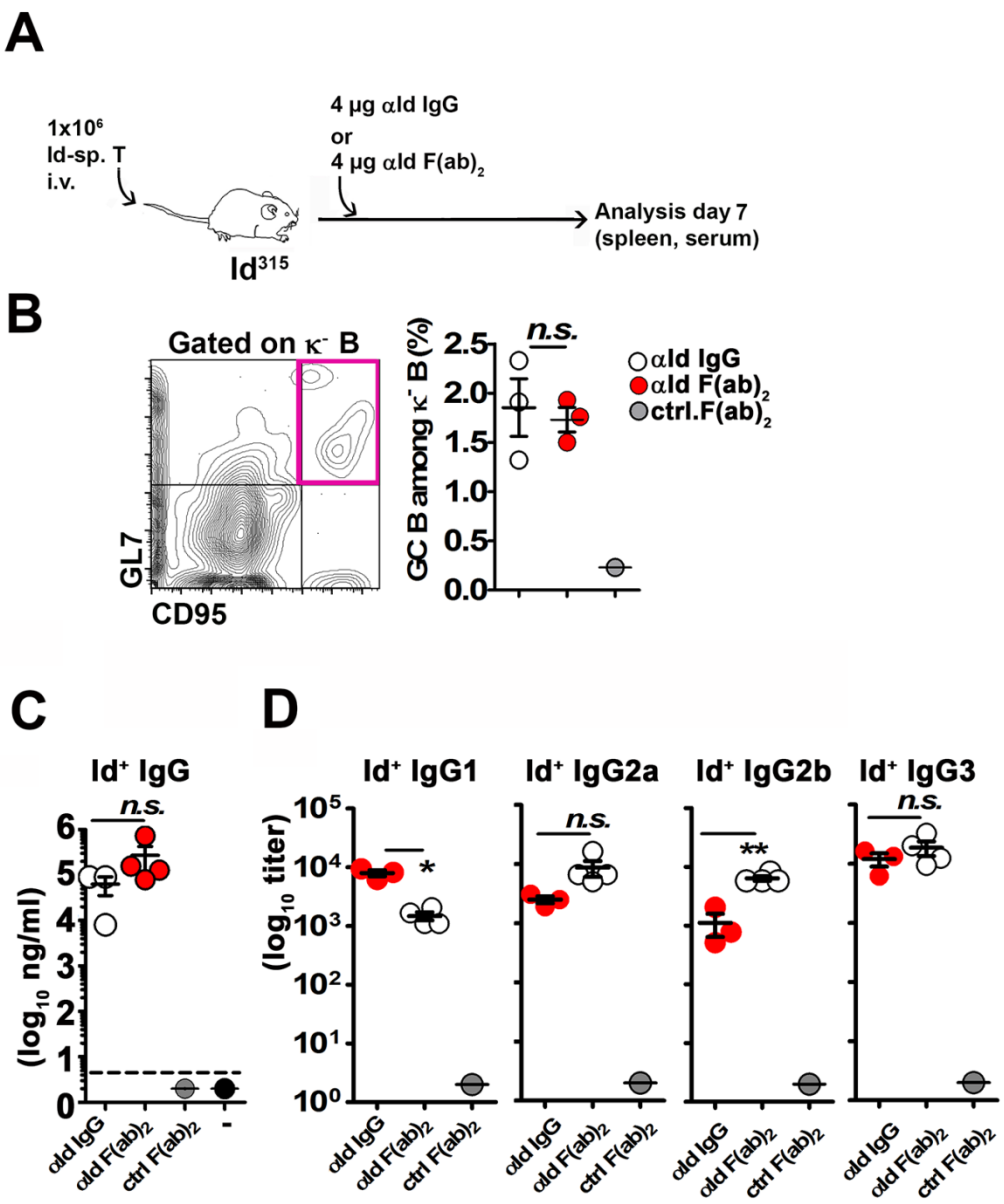

Fig.S11. In vivo responses in Id^{315} mice to ligation of the Id^{+} BCR by anti-Id F(ab)$_2$. (A) Experimental setup. (B) Germinal center B cells (CD95^{+} GL7^{+}) among κ L chain negative B cells. (C, D) Serum ELISAs on day 7. Experimental groups: Id-specific T cells / anti-Id IgG n=6, Id-specific T cells / anti-Id F(ab)$_2$ n=4, Id-specific T cells / control F(ab)$_2$ n=1. Statistical comparisons: Unpaired T-tests (B-D).

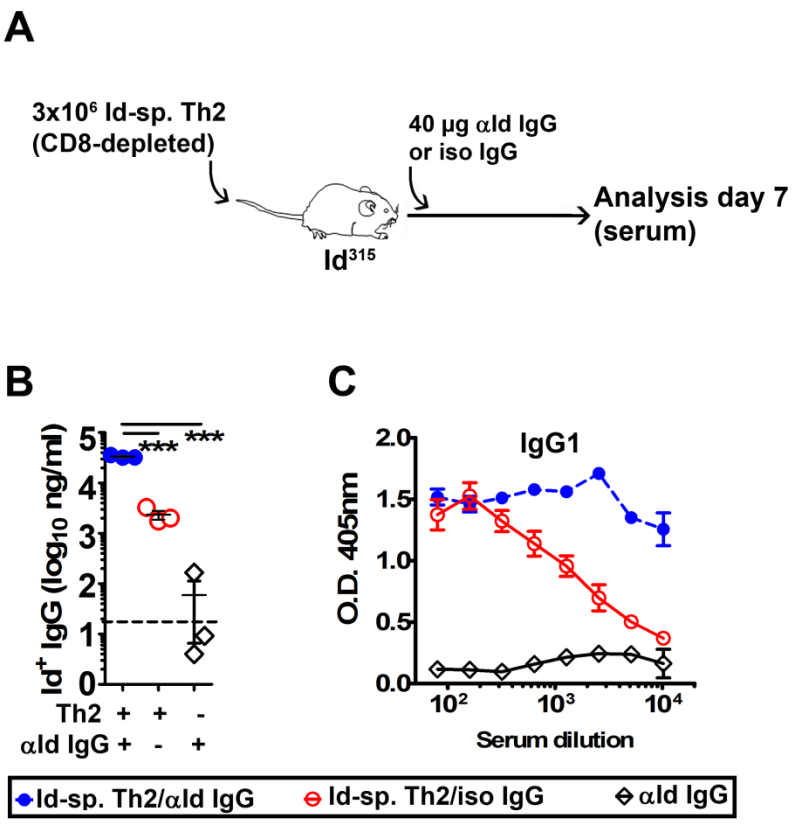

Fig.S12. BCR ligation enhances Id$^+$ B cell responses to Id-specific Th2 cells in Id^{315} mice. (A) Experimental setup (n=3/group). (B) Total Id$^+$ IgG serum concentrations. (C) Serum titers of Id$^+$ IgG1. Statistical test: Tukey's multiple comparisons test (B).

Materials and Methods

In vitro generation of memory T cells

Id-specific Th2 cells were generated as in Munthe et al. (13). Briefly, splenocytes and pooled LN cells from Id-specific TCR-TG mice were isolated by CD4$^+$ T cell enrichment (negative selection, Miltenyi kits and columns) and stimulated in the presence of irradiated BALB/c splenocytes (APCs) added 1 µg/ml Id^{315} peptide (FAALWFRNHFVFGGGTK) in the presence of 40 U/ml rec. murine IL-4 (#214-14, Peprotech) and 20 U/ml rec. murine IL-2 (#212-12, Peprotech). For *in vivo* studies, Th2 cultures (on day 8-10) were CD8-depleted using Lyt2 Dynabeads (11447D, Invitrogen), counted and assessed for viability and purity. Recovered cells were washed extensively in cell culture-grade 1X PBS before injection.

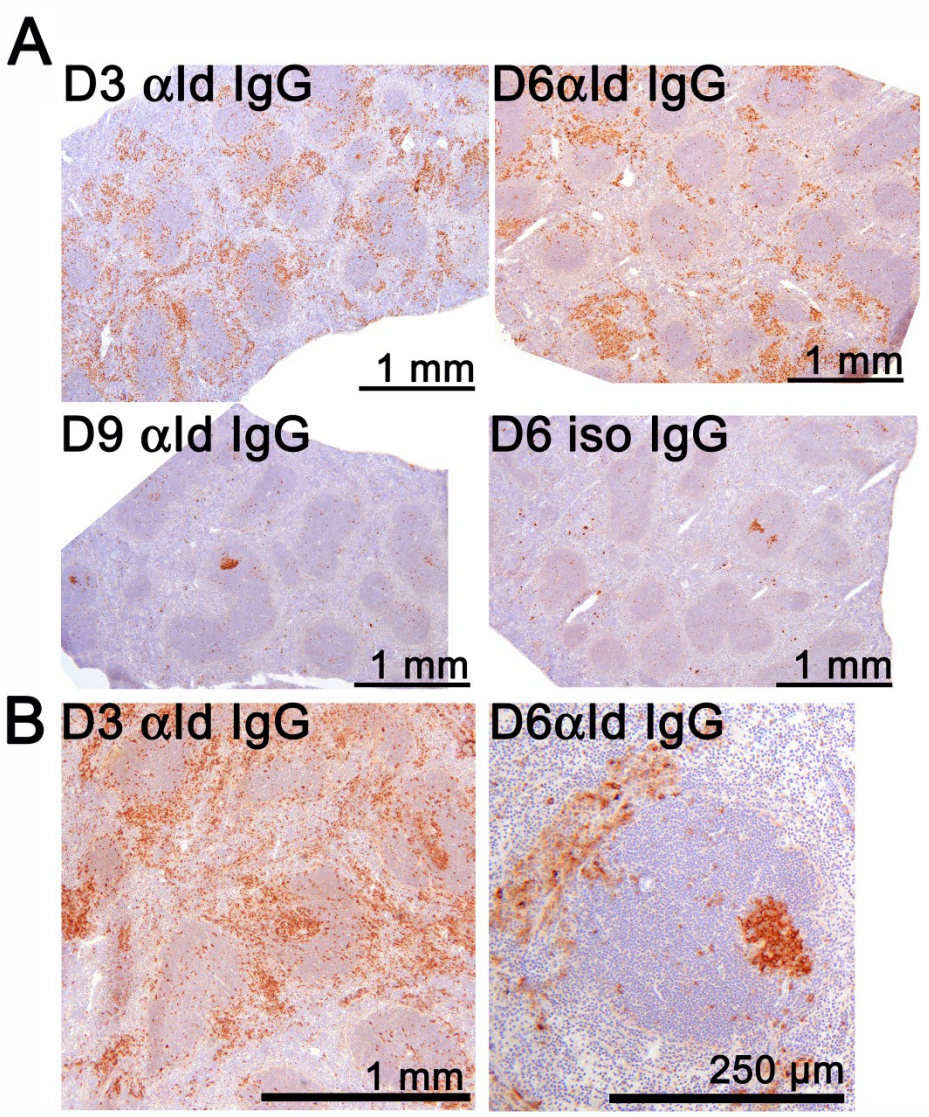

Fig.S13. Localization of GL7$^+$ cells in response to BCR ligation and T cell help in the spleens of Id^{315} mice. Microscopic images show spleen cryosections stained for the T- and B cell activation antigen and GC B cell marker GL7. Counter stain was hematoxylin. Id^{315} mice were injected i.v. with naive Id-specific T cells and anti-Id IgG BCR ligand (experiment of Fig. 4A). Shown are representative comparisons of spleens on day 3, day 6 and day 9. D6 iso IgG represents staining of a spleen of a control Id^{315} mouse given T cell help but no BCR ligation (but isotype-matched IgG) on day 6. (A) Representative low-power images. (B) Left: Higher magnification image shows the diffusely scattered distribution of GL7$^+$ cells, predominantly localizing to extrafollicular areas (day 3 after BCR ligation). Right: On day 6 after BCR ligation, GL7$^+$ cells are localized in extrafollicular areas (upper left quadrant) as well as in the follicle as a germinal center (lower right quadrant).

Materials and Methods

Immunostaining for the GL7 activation marker

Frozen spleen sections were allowed to air dry, fixed in 4% neutral-buffered formalin, serum blocked for 45 minutes at room temperature, and incubated with rat-anti-mouse GL7-FITC (BioLegend, San Diego, CA, USA, #144603, clone GL7, 1:100) for 1hr at room temperature. Thereafter, the sections were rinsed and probed with anti-FITC-biotin (Sigma-Aldrich Corp., St. Louis, MO, USA, clone FL-D6, 1:400) for 1 hr. at room temperature, before rinsing again and peroxidase-quenching with 1% H_2O_2 in PBS. The sections were finally incubated with peroxidase-conjugated ABC reagent (Vectorlabs, Burlingame, CA, USA) for 30 min. at room temperature. The antibody label was visualized by using 3'3'-diaminobenzidine (DAB, Sigma Aldrich) and nuclei were counterstained with hematoxylin.

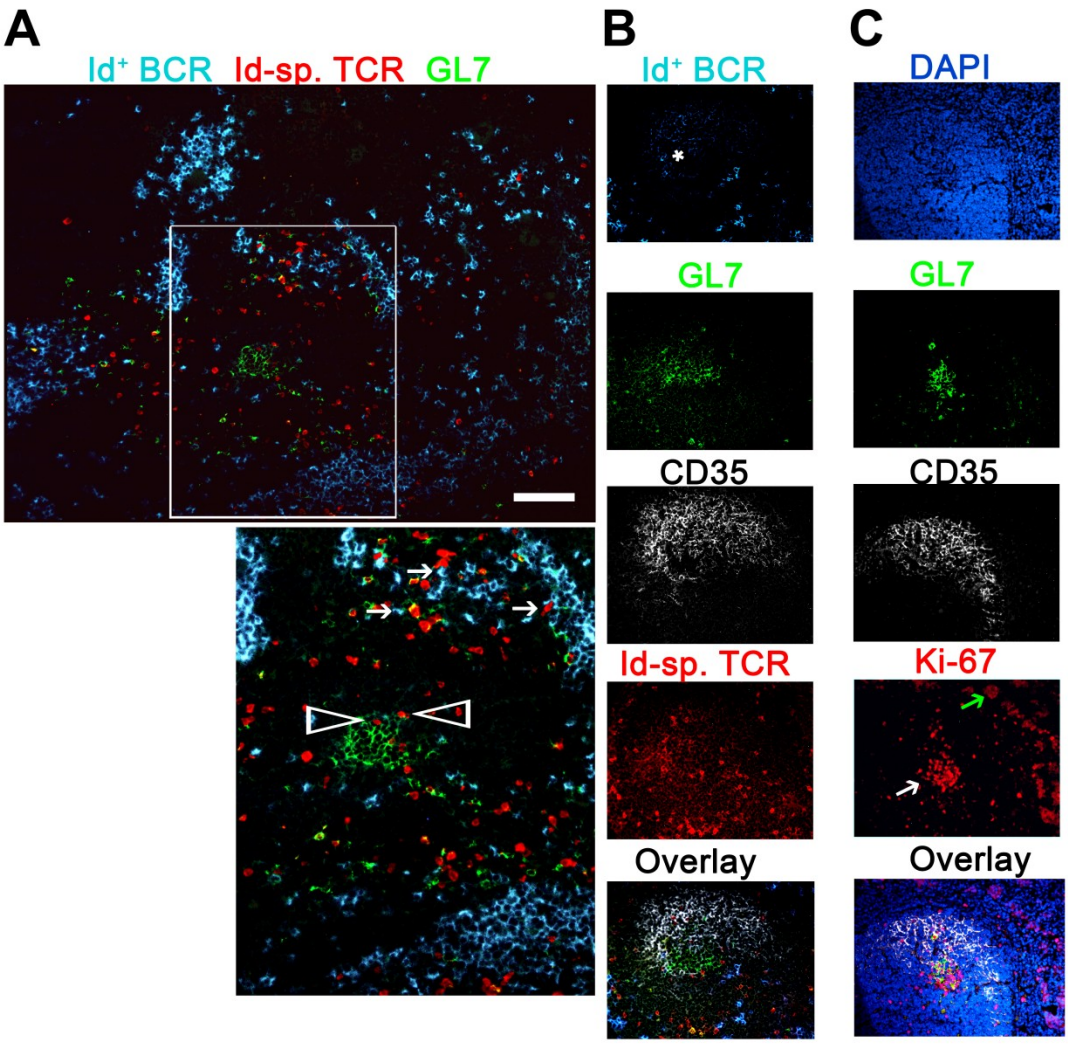

Fig.S14. Immunofluorescent staining of spleens in Id^{315} mice that had received Id-specific T cells and BCR ligand (anti-Id IgG) three days earlier (see Figure 4A). (A) Evidence of extra-follicular expansion of Id$^+$ B cells (top: scale bar 100 μm; below: magnified image of the region of interest). A small germinal center (GL7$^+$ cluster, green) is surrounded at considerable distances by sheets of strongly staining Id$^+$ B cells (cyan). Some of the B cells appear to have intracellular Id$^+$ Ig. Id-specific T cells (red) are scattered throughout the section. Some Id-specific T cells make contacts with germinal center Id$^+$ B cells (open arrowheads), others with extrafollicular B cells (arrows). (B) Single fluorescence- and overlay pictures of the image in Fig. 4B. Top: GC B cells have a weak expression of the Id$^+$ BCR (the GC area is marked by asterisk), whereas peripherally located Id$^+$ B cells have a strong Id signal (bottom section of the figure). CD35 marks the GC light zone (follicular dendritic cells, white). Id-specific T cells are scattered throughout. Overlay (bottom) shows the localization of Id$^+$ B and Id-specific T cells relative to the GC. (C) Evidence of proliferation (Ki-67, red) both in GC B cells (GL7, green) and outside the follicle (top right part of the image). The follicle is delineated by a denser DAPI nuclear stain

(blue) and includes the $CD35^+$ light zone. On the Ki-67 single single fluorescence image, proliferation of cells in the GC is indicated by a white arrow and proliferation outside the follicle by a green arrow. (B, C) Images were taken at x20 original magnification. Of note, we could not detect any of the injected anti-Id IgG in the sera on day 3 by ELISA; thus staining for the Id^+ BCR on the sections is expected to be complete with no blocking by residual anti-Id IgG.

Materials and Methods

Immunofluorescence and quantification on tissue sections

Frozen spleen sections were equilibrated to room temperature, fixed in acetone and blocked using 30% normal mouse serum and 10 µg/ml purified anti-FcRγ mAb (clone 2.4G2, HB-197, ATCC, in-house purified). All dilutions were made with 1% BSA in PBS. Incubation with the primary antibodies Ab2-1.4-biotin (1:150, 10 µg/ml), GB113-PE (1:100, Diatec, Oslo, Norway, 0.2 µg/ml), GL7-FITC (BioLegend, San Diego, CA, USA, #144603, 1:100), rat anti-mouse CD35-biotin (BD Pharmingen, San Diego, CA, USA, #553816, 3 µg/ml), rabbit-anti-Ki-67 (Lab Vision, Fremont, CA, USA, 1:200) was performed overnight at 4 °C. Incubation with the secondary reagents goat anti-PE-Texas Red (red, 1:1000, Abcam, Cambridgeshire, UK, ab34734), SA-Cy5 (far red, GE LifeSciences, Uppsala, Sweden, PA45001, 1µg/ml), a 1:1 mixture of SA-AMCA and SA-AF350 (blue; 1µg/ml each, Molecular Probes, Eugene, OR, USA, S-6364 and S-11249) and mouse anti-FITC-Cy2 (green, 1:200, Jackson Immunolabs, Cambridgeshire, UK, 200-222-037) were done for 2 hrs at RT. A blocking step with avidin and biotin (Ventana Medical Systems, Tucson, AR, USA, #760-050, 30 min each) was performed before the incubation with the second round with biotinylated antibody. Three-color fluoresce was examined using a Nikon Eclipse T*i*-S microscope fitted with Nikon Plan Fluor objectives. DAPI, FITC and ET-DSRed filter blocks were used. Images were acquired using a DS-Ri1 camera and captured using NIS-Elements BR 3.2 software. Four-color fluorescence was captured using a Nikon Eclipse 90i microscope fitted with Nikon Plan Fluor objectives. DAPI, FITC, Texas Red and Cy5 filter blocks were used. Images were acquired using a DS-Ri1 camera and captured using NIS-Elements F4.30.01 software.

A

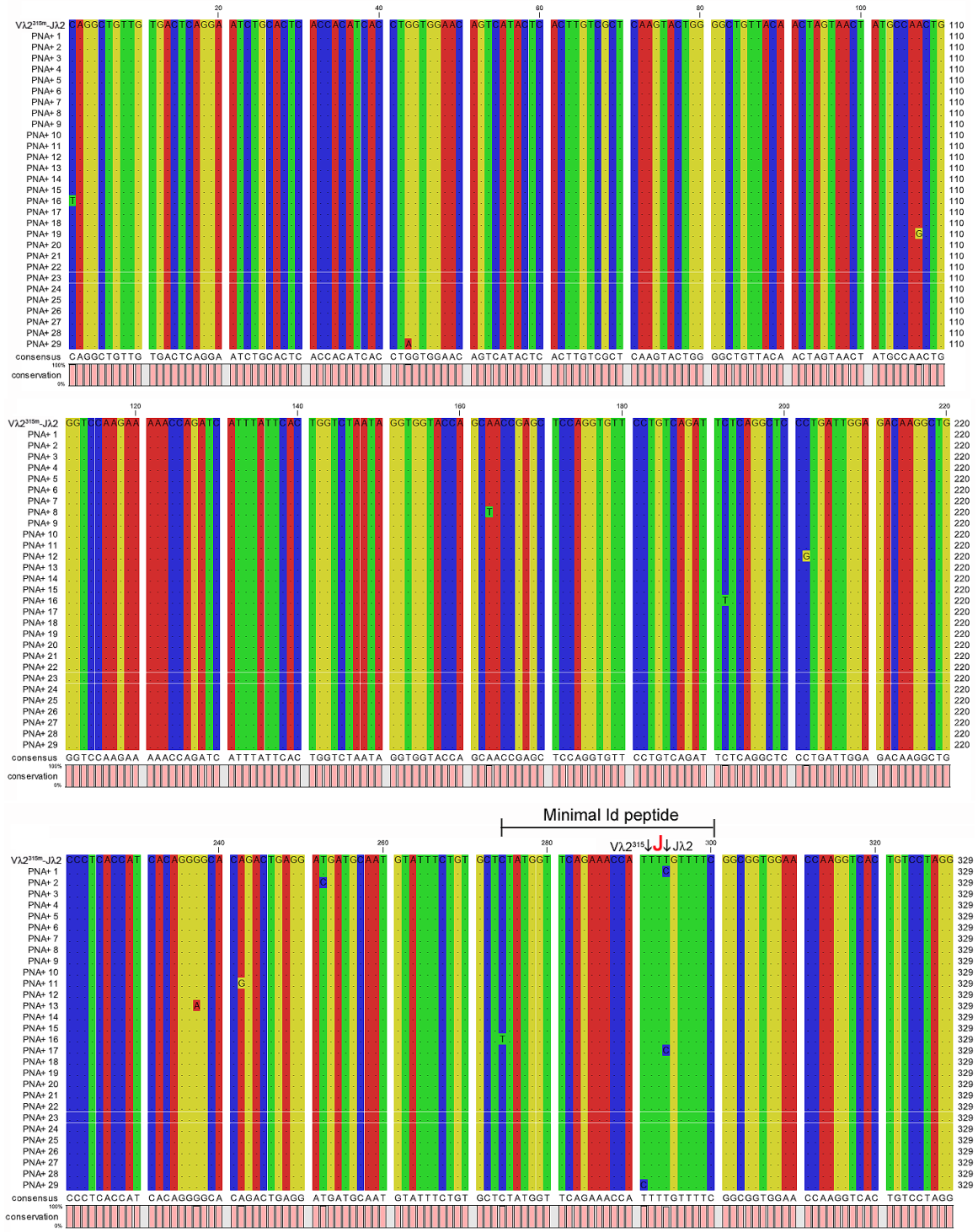

Fig.S15A. Nucleotide sequence alignment from PNA^{+} sorted B cells. The various clones were aligned to the λ2^{315m}-Jλ2 sequence shown on top. The Vλ2^{315m} and Jλ2 as well as the stretch of nucleotides encoding the minimal Id peptide (amino acid 92-100) are indicated. The V-J junction at codon 98 is indicated (between the black arrows). Red "J" demarks the junctional amino acid.

B

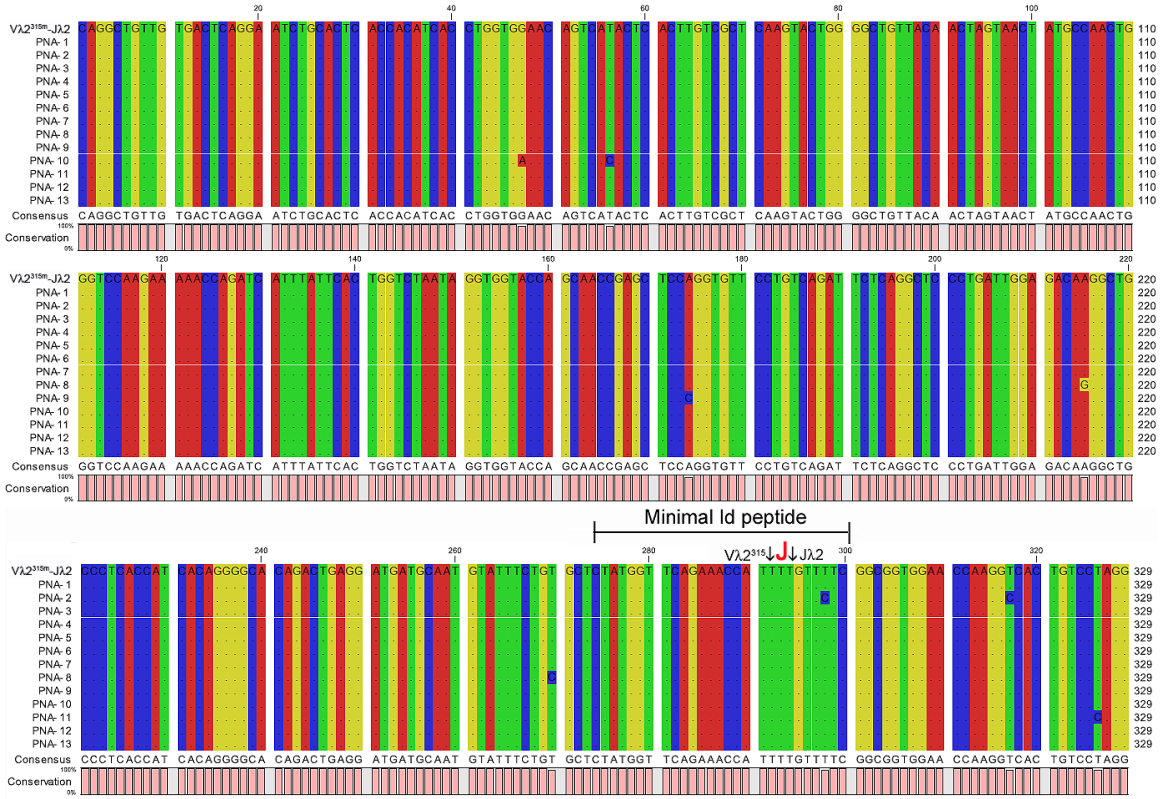

Fig.S15B. Nucleotide sequence alignment from PNA⁻ (column flow-through) B cells. The various clones were aligned to the $\lambda 2^{315m}$-Jλ2 sequence shown on top. $V\lambda 2^{315m}$ and Jλ2 as well as the stretch of nucleotides encoding the minimal Id peptide (spanning amino acids 92-100) are indicated. The V-J junction at codon 98 is indicated (between the black arrows).

C

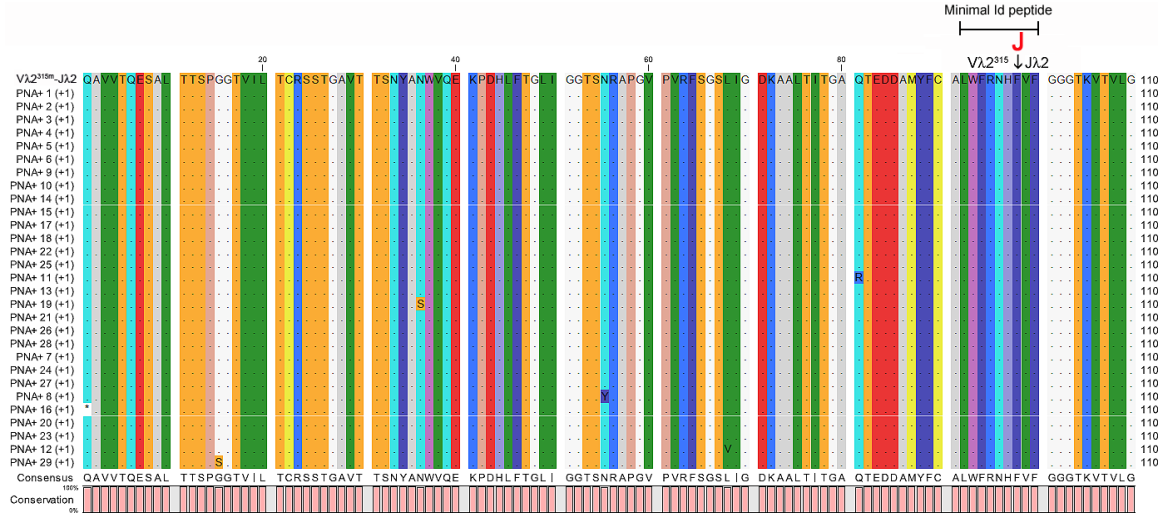

Fig.S15C. The Vλ2^{315m}-Jλ2 amino acid sequences cloned from PNA^{+} purified B cells. The amino acid sequences are based on the nucleotide sequences presented in Fig. S15A. Sequences from the various clones were aligned to the Vλ2^{315m}-Jλ2 sequence shown on top. The start of Vλ2^{315m} and Jλ2 are indicated as well as the junctional residue 98 and the minimal Id peptide (amino acid 92-100).

D

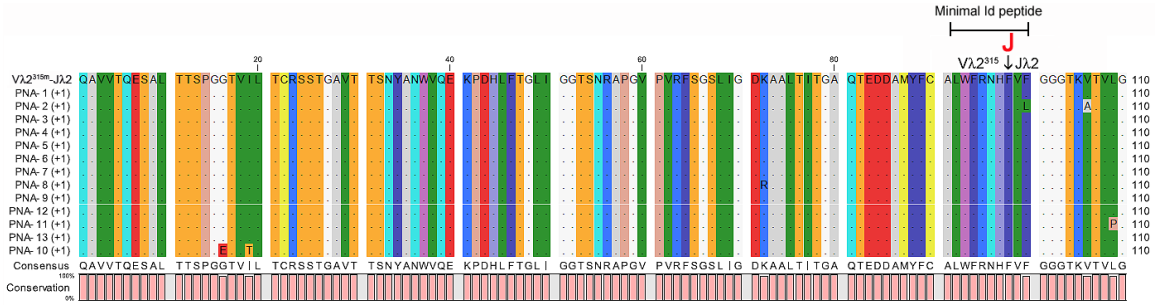

Fig.S15D. The Vλ2^{315m}-Jλ2 amino acid sequences cloned from PNA^{-} (column flow-through) B cells. The amino acid sequences are based on the nucleotide sequences presented in Fig. S15B. Sequences from the various clones were aligned to the Vλ2^{315m}-Jλ2 sequence shown on top. The start of Vλ2^{315m} and Jλ2 are indicated as well as the junctional residue 98 and the minimal Id peptide (amino acid 92-100).

Materials and Methods

V_L and V_H sequencing in GC B cells

Pooled splenocytes from three Id^{315} mice primed with naïve Id-sp. T cells and anti-Id F(ab)$_2$ as a BCR ligand were prepared on day 7 post-ligation. Splenocytes were incubated on ice with biotinylated peanut agglutinin (B-1075, VectorLabs) followed by anti-biotin magnetic microbeads (#130-105-637, Miltenyi) and positively sorted on MS columns (#130-042-201, Miltenyi). Flow-through (non-PNA-reactive) cells were also collected. Cells were rinsed in PBS and cell pellets were frozen at -80°C. Total RNA was isolated using the RNA Easy kit (Qiagen) and cDNA prepared using a kit (K1612, Thermo Scientific). The λ transcripts were PCR-amplified using the forward primers mVλ1/2 (5'-CAGGCTGTTGTGACTCAGGAATC-3') together with the downstream primers mCλ-outer (5'-GTACCATYTGCCTTCCAGKCCACT-3') that anneal to Cλ1, Cλ2 and Cλ3, as published (14). PCR products were TOPO-cloned using Zero blunt TOPO PCR cloning kit (450245, Thermo Fisher Scientific). Colonies were selected on LB plates containing Kanamycin. Small scale DNA preps were made from overnight cultures of single colonies using SV miniprep kit (Promega). Heavy chain transcripts were amplified using an upstream primer that aligns to the FR1 region of V_H^{315} and of VH3-6*02 (5′-GATGTACAGCTTCAGGAGTCAGGACCT-3') in combination with downstream primers against IgG C-regions: 5′-ATAGACAGATGGGGGTGTCGTTTTGGC-3' (IgG1) and 5′-AGGGACCAAGGGATAGACAGATGG-3' (IgG3). Small-scale DNA preps were sent for sequencing and analyzed. Sequences were analyzed using CLC Sequence Viewer v8.0 (Qiagen).

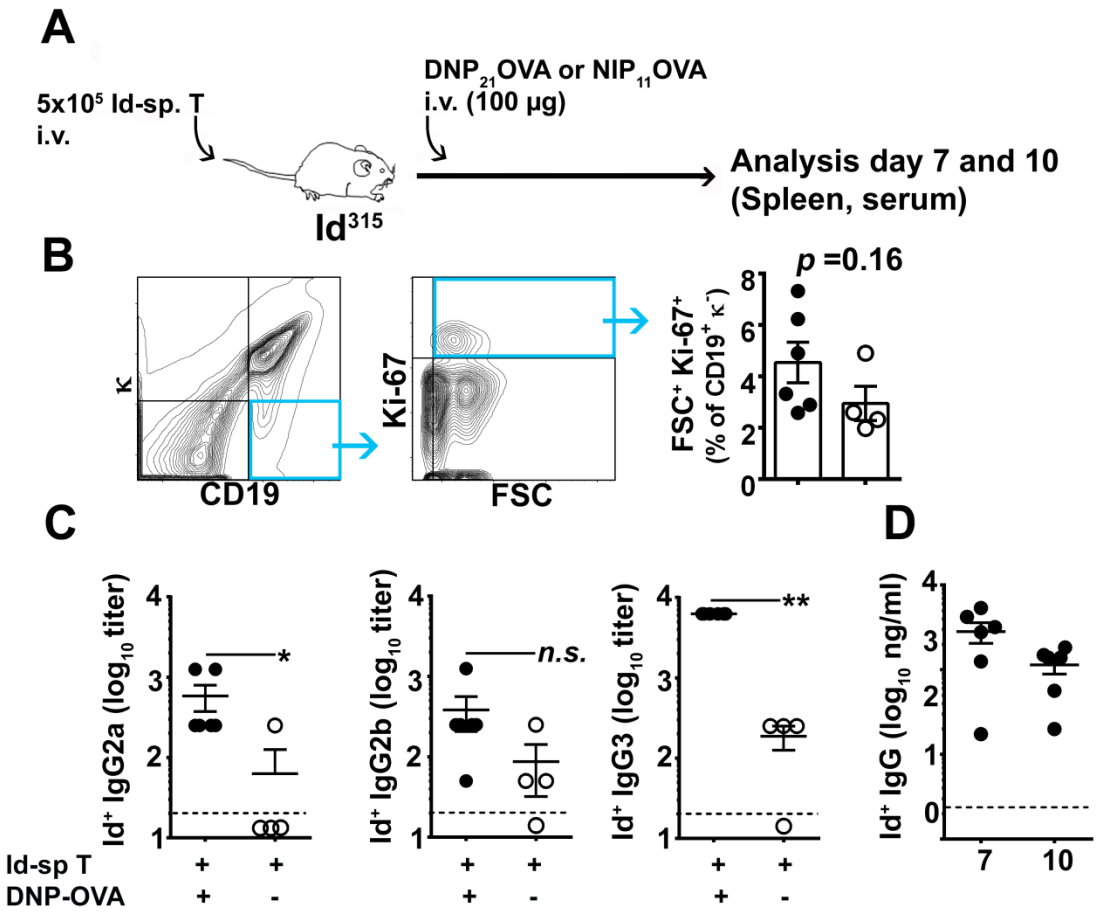

Fig.S16. In vivo responses to DNP-OVA. (A) Experimental set-up. (B) Gating strategy to resolve κ L chain-negative B cells (left) and therein the Ki-67$^+$ population (center). Ki-67 stains cells in all active phases of the cell cycle: G1, S, G2, and mitosis. Statistical comparison of the frequencies of Ki-67$^+$ cells among mice primed with Id-specific T cells and DNP-OVA vs. Id-specific T cells and NIP-OVA. (C) Serum Id$^+$ IgG subclass titers on day 10. (D) Total IgG concentrations on day 7 and day 10 when transferring Id-specific T cells and DNP-OVA. Experimental groups: Id-specific T cells / DNP-OVA n=6 (black filled circles), Id-specific T cells / NIP-OVA n=4 (open circles). Statistical analyses: Unpaired T tests (B, C).

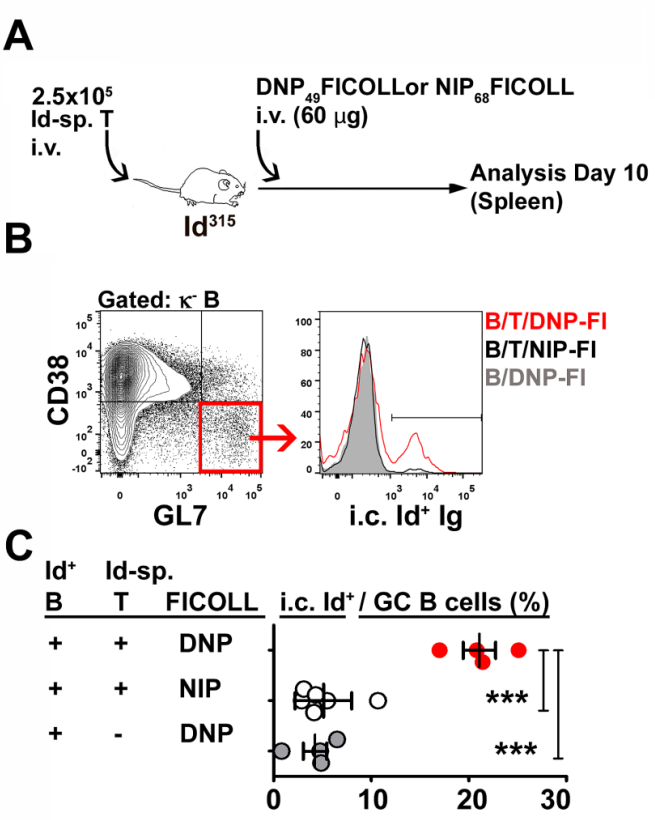

Fig.S17. Germinal centers in Id^{315} mice resolved by intracellular staining for the Id$^+$ Ig. (A) Experimental setup. (B) Left: contour plot shows gating strategy for GL7$^+$ CD38$^-$ germinal center B cells among surface κ L chain negative CD19$^+$ B cells in the spleen. Right: overlay histogram of the representative distributions of intracellular Id$^+$ Ig fluorescence from each group of mice, gated on the GC population. Id$^+$ Ig was detected with the Ab2-1.4 mAb. Experimental groups are indicated. (C) Scatter plot the shows percentages of cells that express Id$^+$ Ig intracellularly, gated as above. Experimental groups: Id-specific T cells / DNP-FICOLL n=4, Id-specific T cells / NIP-FICOLL n=6, DNP-FICOLL n=4. Statistical analysis: Tukey's multiple comparison test (C).

Materials and Methods

Intracellular staining for Id$^+$ Ig

Splenocytes isolated from individual mice were cell surface-stained and then rinsed, fixed, permeabilized and stained using BD Cytofix/Cytoperm^{TM} Kit (Cat. #554714) according to the manufacturer's instructions. Intracellular Id$^+$ Ig was detected using the Ab2-1.4 mAb directly conjugated to Alexa Fluor-488.

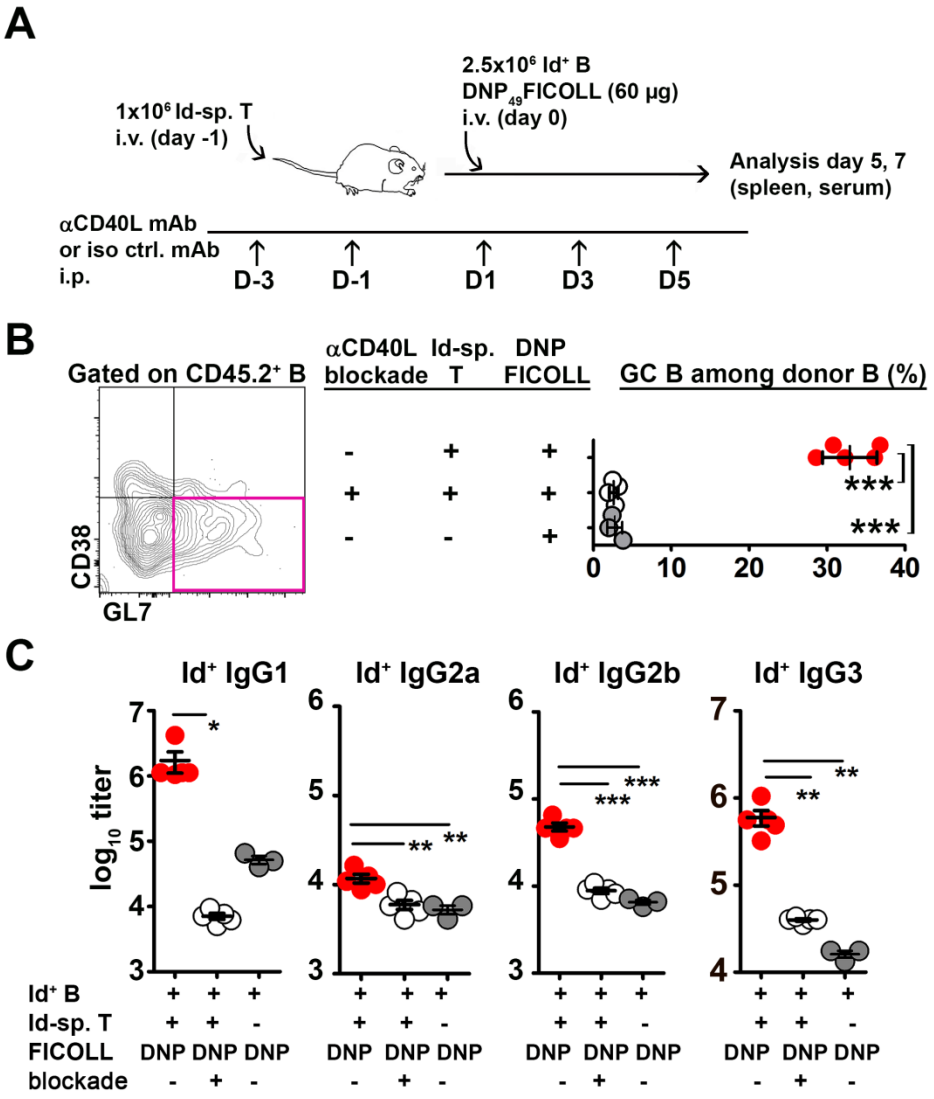

Fig.S18. Blockade of CD40:CD40L interactions between Id$^+$ B cells and Id-sp. T cells interferes with germinal center responses and Id$^+$ IgG production. (A) Experimental setup. Groups of CD45.1 congenic BALB/c mice were treated i.p. with 250 µg/recipient of anti-CD40L mAb (clone MR1) or isotype-matched control hamster IgG on the indicated days, given relative to BCR ligation (D0). Naïve Id-specific T cells (CD45.2$^+$), Id$^+$ B cells (CD45.2$^+$) and BCR ligand, DNP_{49}FICOLL were all transferred i.v. Mice were analyzed on day 5 and 7 by ELISAs and on day 7 by FACS (spleens). (B) Germinal center B cells (CD38$^-$ GL7$^+$) among donor (CD45.2$^+$) B cells. (C) Id$^+$ IgG subclass serum titers on day 7. Experimental groups: CD45.1$^+$ mice given anti-CD40L mAb / Id$^+$ B cells / Id-specific T cells / DNP_{49}FICOLL n=5, mice given control hamster IgG /Id$^+$ B cells /Id-specific T cells / DNP_{49}FICOLL n=5, mice given Id$^+$ B cells / DNP_{49}FICOLL n=3. Statistical analyses: Tukey's multiple comparison tests (B, C).

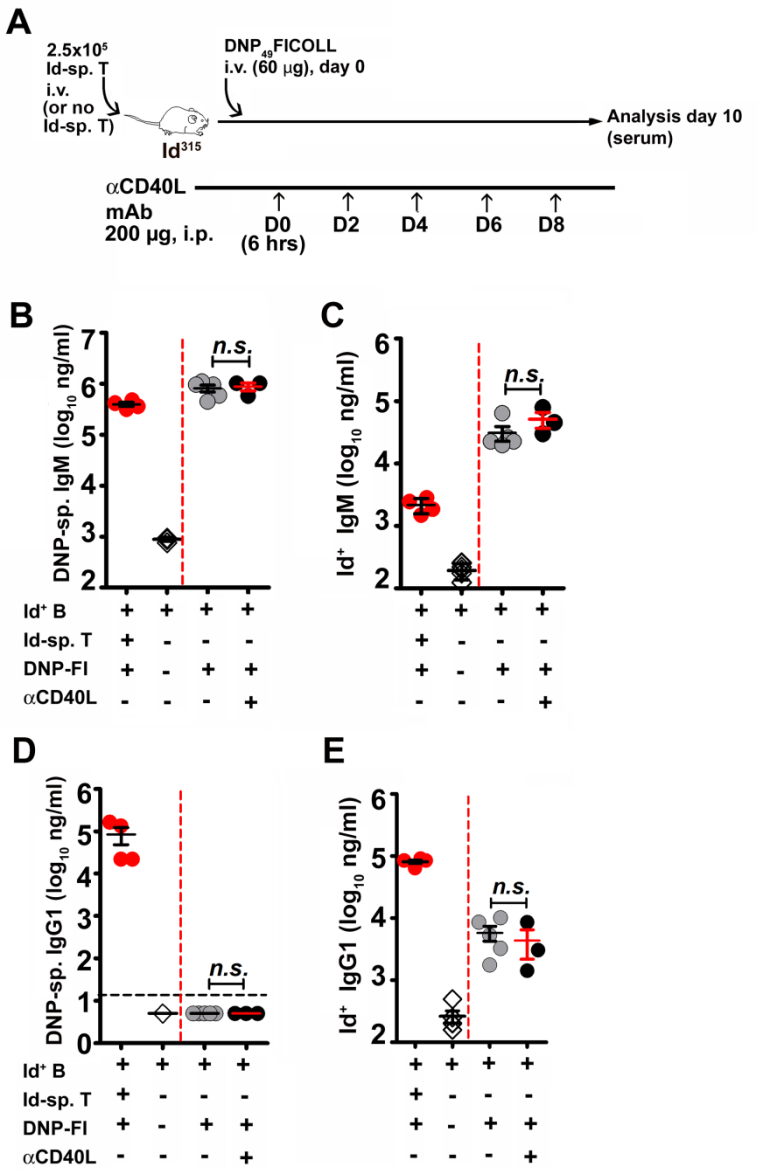

Fig.S19. In the absence of Id-specific T cells, blockade of the CD40:CD40L interaction does not diminish Id$^+$ IgM and IgG1 serum levels induced by the TI-2 antigen DNP-FICOLL. (A) Experimental setup. Groups of Id^{315} mice received Id-specific T cells or not on day -1. The next day (D0), all mice were given DNP-FICOLL. Six hours later, one group of mice received anti-CD40L mAb (clone MR1). The treatment was repeated every other day. Day 10 sera were analyzed for antibodies by ELISA. (B-E) Serum levels of (B) DNP-specific IgM, (C) Id$^+$ IgM, (D) DNP-specific IgG1 (horizontal stippled black line indicates level of detection), (E) Id$^+$ IgG1. Control groups are shown to the left of the vertical stippled red line, the experimental groups to be compared to the right. Experimental groups: Id-specific T cells / DNP-FICOLL, n=5; DNP-FICOLL, n=5; DNP-FICOLL / anti-CD40L mAb, n=3. Diamonds show values for untreated naive Id^{315} mice (n=5-7). Statistical analyses: Mann-Whitney U tests compared the effect of CD40L blockade in Id^{315} mice given DNP-FICOLL.

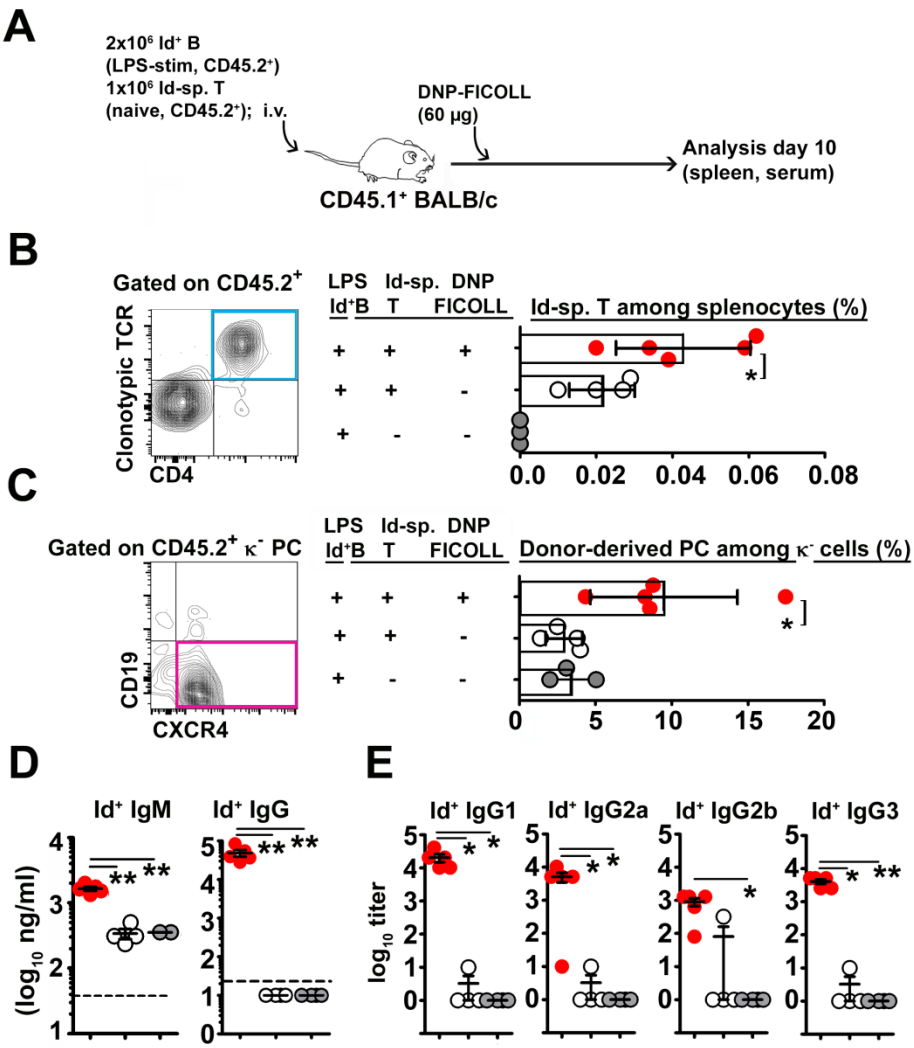

Fig.S20. BCR ligation of LPS-stimulated Id$^+$ B cell blasts leads to the expansion of Id-specific T cells, generation of plasma cells and induction of Id$^+$ Ig. (A) Experimental setup. (B) Id-specific T cells (gated as CD45.2$^+$ CD4$^+$ GB113$^+$ cells) among splenocytes. (C) Plasma cells among κ L chain negative splenocytes. Gating strategy: κ L chain negative splenocytes were gated for CD45.2 and CD138 expression; this population was then further resolved for CD19 vs. CXCR4 (see gating example). κ L chain negative CD45.2$^+$ CD19$^-$ CD138$^+$ CXCR4$^+$ plasma cells were enumerated. Frequencies of plasma cells among κ L chain negative splenocytes are given. (D) Serum concentrations of Id$^+$ IgM and total Id$^+$ IgG, and (E) serum titers of Id$^+$ IgG subclasses. Broken lines in (D) indicate the sensitivity of the assay. Experimental groups: Id$^+$ B cells / Id-specific T cells / DNP-FICOLL n=5, Id$^+$ B cells / Id-specific T cells n=4, Id$^+$ B cells / DNP-FICOLL n=3. Statistical comparisons: Unpaired T tests (B, C) Tukey's multiple comparisons test (D), Dunn's multiple comparisons tests (E).

Materials and Methods

LPS stimulation and transfer of blasts

B cells were enriched from the spleens of $\lambda 2^{315}$TG x VDJ_H^{315} mice ($CD45.2^+$) by immuno-magnetic depletion and were stimulated for 68 hours *in vitro* using similar conditions as published by Hikida et al. (15). Briefly, we incubated the cells in complete RPMI culture medium supplemented with 10 μM β-mercaptoethanol and 20 μg/ml LPS (*E. coli* O111:B4, L2630, Sigma). The recovered cells were blasts, had expanded nearly two-fold, and had up-regulated the cell surface expression of MHCII ($I\text{-}E^d$) and CD86 (see also Fig.S7). The cells were extensively rinsed in sterile cell culture-grade PBS; viability assessed, counted and injected i.v. into $CD45.1^+$ BALB/c mice. Six hours later, the same mice received a new i.v. injection with naive Id-sp. T cells ($CD45.2^+$) and DNP_{49}FICOLL. Control mice, where Id-specific T cells or Id-specific T cells and DNP-FICOLL were omitted, were included.

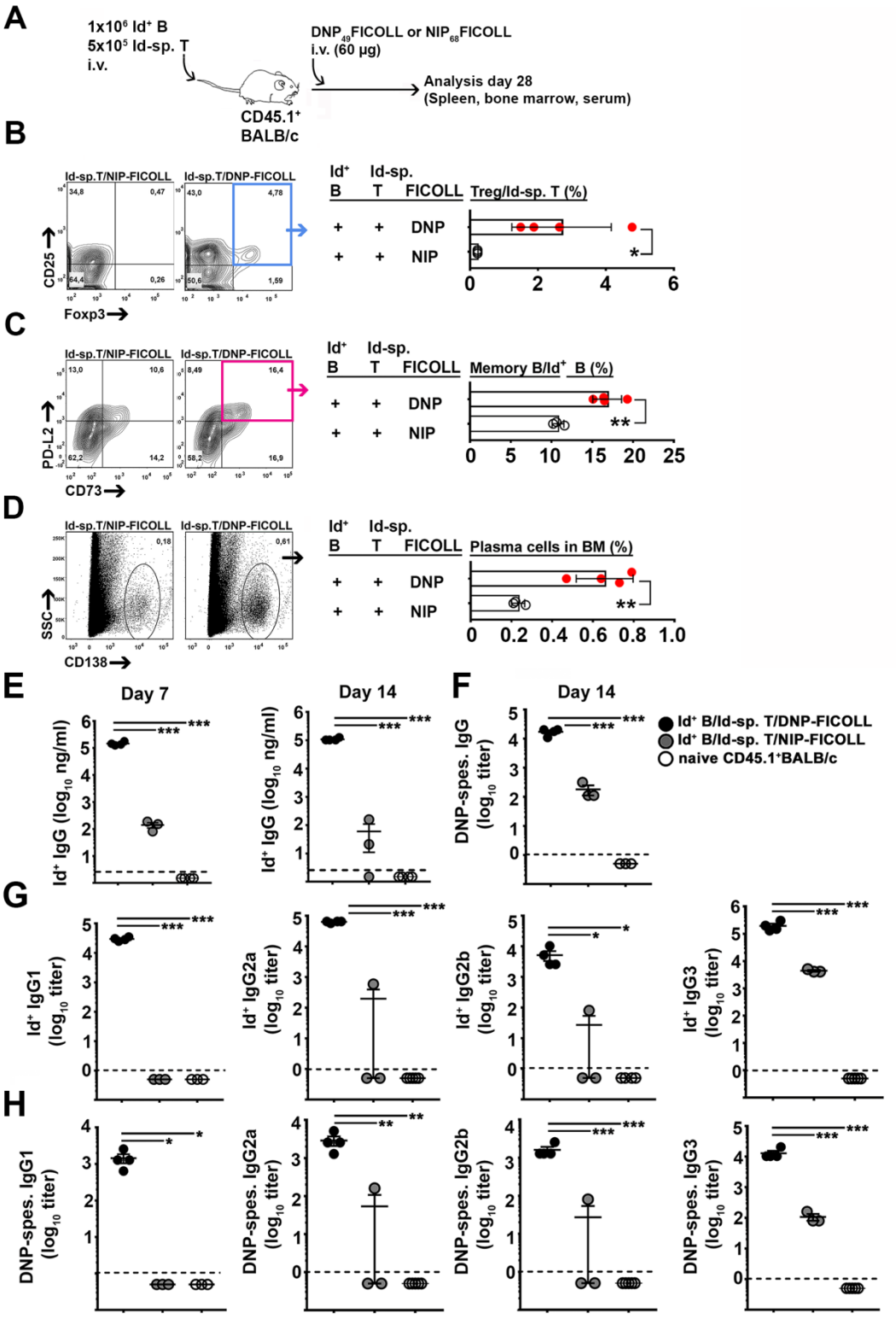

Fig.S21. BCR ligation induces the formation of Id-specific regulatory T cells, Id$^+$ memory B cells, and bone marrow plasma cells. (A) Experimental setup. (B) Regulatory T cell differentiation (CD25$^+$ Foxp3$^+$) of Id-specific T cells (CD45.2$^+$ CD4$^+$ GB113$^+$ gated). (C)

Memory B cell differentiation ($CD73^+$ $CD273^+$) of Id^+ B cells ($CD45.2^+$ $CD19^+$ κ L chain negative cells were gated). (D) Plasma cell ($CD138^+$) development in the bone marrow. (E) Serum concentrations of anti-Id mAb reactive IgG antibodies on day 7 and 14. (F) DNP-reactive IgG titers on day 14. (G) Titers of anti-Id Id mAb reactive IgG subclasses on day 14. (H) Titers of DNP-reactive IgG subclasses on day 14. In E-H, broken lines represent the detection limit of the assay. The sera of naïve $CD45.1^+$ BALB/c mice (open circles) produced signal below the detection limit (broken lines). Experimental groups: Id^+ B cells / Id-sp. T cells / DNP-FICOLL n=4, Id^+ B cells / Id-sp. T cells / NIP-FICOLL n=3. Statistical comparisons: Unpaired T tests (B-D), Tukey's multiple comparisons tests (E-H).

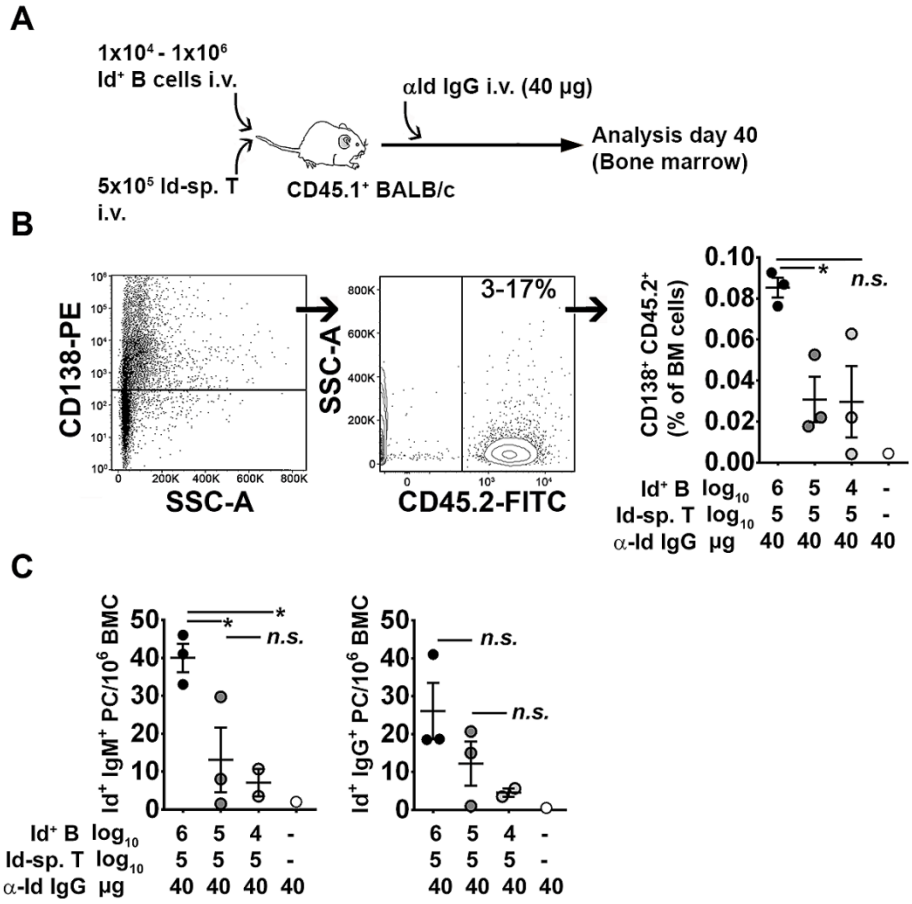

Fig.S22. Adoptively transferred Id$^+$ B cells differentiate into bone marrow plasma cells in CD45.1$^+$ congenic BALB/c mice after BCR ligation and provision of Id-specific T cell help. (A) Experimental setup (n=3/group; Id$^+$ B cell doses: 1x10^4, 1x10^5 or 1x10^6). (B) Donor-derived plasma cells (frequencies among bone marrow cells) in recipient mice 40 days after cell transfer and BCR ligation. (C, left) Frequencies of Id$^+$ IgM-producing plasma cells per one million viable bone marrow cells among the groups. (C, right) Frequencies of Id$^+$ IgG-producing plasma cells per one million viable bone marrow cells. Statistical comparisons: Tukey's multiple comparisons tests.

Materials and Methods

ELISPOT assays

Bone marrow cells from both tibia were harvested from mice (n=3 per group) and single cell suspensions were prepared. MultiScreen HTS filter plates (MSIPS45, Merck Millipore Ltd., Tullagreen, Ireland) were coated with 12µg/well of AM5 (anti-Id IgM) or Ab2-1.4 (anti-Id IgG) over night at 4° C. Bone marrow cell suspensions were seeded and incubated for 20h. Spots were detected with anti-mouse IgG-AP (A1418, Sigma-Aldrich) or anti-mouse IgM-AP (A9688) at 1:5000 and developed with BCIP®/NBT (B3679, Sigma Aldrich). ELISPOT plates were analyzed in CTL-ImmunoSpot® analyzer (CTL, Shaker Heights, OH, USA).

Table S1. Specificities of Ab2-1.4 and Ab2-TH anti-Id mAbs and TNP/DNP haptens: influence of H chains and positions 94, 95, 96 and 98 in Vλ2^{315m} and Vλ2

						V_H chains								
						V_H^{315}	VH3-06[d]	V_H^{A20}	V_H^{315}	VH3-06	V_H^{A20}	V_H^{315}	VH3-06	V_H^{A20}
	Position[a]	94	95	96	98									
V_L	Vλ2^{315m}-Jλ2	F	R	N	Y	+[b]	+	–	+	–	–	+/+	+/+	–/–
		F	R	N	F	+	+	–	+	–	–	+/+	+/+	–/–
		F	R	N	L	+	–	–	–	–	–	+/+	+/+	–/–
	Vλ2-Jλ2	Y	S	T	Y	–[c]	–	–	+	–	–	+/+	+/+	–/–
		Y	S	T	F	–	–	–	+	–	–	+/+	+/+	–/–
		Y	S	T	L	–	–	–	–	–	–	+/+	+/+	–/–
						Binding Ab2-1.4			Binding Ab2-TH			Binding TNP/DNP		

[a] amino acid residues of the various λ light chains in position 94-96 and 98 (Kabat numbering) are shown.
[b] + = positive signal in the ELISA.
[c] - = negative signal in the ELISA.
[d] = VH3-06 denotes the germline IGVH3-06*2 (1).

Materials and methods

Expression of V_H and V_L regions for specificity studies

V_H (VDJ) and V_L (VJ) were cloned into heavy and light chain vectors (16) and expressed as hIgG3 by cotransfection of HEK293E cells. The supernatant was purified on a protein G HP SpinTrap column (GE Healthcare). The indicated combinations of V_H and V_L were analyzed in ELISAs for the recognition of Ab2-1.4 and Ab2-TH mAbs, and TNP/DNP, as indicated in the Table. The ELISAs used either anti-hIgG3 as coat (MCA878G, AbD Serotec, 1:1000) when detected with Ab2-1.4-biotin and Ab2-TH-biotin (both at 1 µg/ml) or anti-hIgG3-biotin as a detection reagent (HP6017, Sigma, 1:1000) with $TNP_{12}OVA$ or DNP_5BSA as coat. The biotinylated antibodies were probed using streptavidin-HRP and developed with TMB substrate solution (Calbiochem) and stopped with 1M H_2SO_4. Absorbance was measured at 450nm.

Extended Materials and Methods

Ethics statement, animal experiments
The *in vivo* procedures followed institutional guidelines of the Department of Comparative Medicine, Oslo University Hospital. The project was approved by the Norwegian Food Safety Authority (ID#7414).

Generation of an anti-idiotypic IgM specific for M315
To generate a detection reagent specific for Id^+ IgG, Id^{315}-specific B cell hybridomas were established from aId^{DKI} mice (7) using standard procedures. Individual clones were isolated and assayed for heavy chain isotype and binding to M315. Clones producing anti-Id^{315} antibodies with IgM heavy chains were isolated and expanded for large scale production. PCR products were generated from one clone (AM5-1-3) of a hybridoma using specific primers for the heavy- and light chain sequences in aId^{DKI} mice (7), which were then sequenced. IgM was purified on 187.1 (rat anti-mouse κ light chain) coupled Sepharose 4B columns. SDS-PAGE was done to ascertain purity, and specificity was confirmed by ELISA after biotinylation.

Generation of targeted VDJ_H^{315} knock-in mice
Mice that express the V-region of the M315 myeloma protein heavy chain, produced by the MOPC315 multiple myeloma cell line (6) were generated by inserting the rearranged VDJ_H^{315} sequence into the D-J1-J4 region of the IgH locus through homologous recombination. For a thorough description, see Supporting Online Material section under Supplemental Figure 2.

$V\lambda2^{315m}$ mice
$V\lambda2^{315}$-modified mice were generated by targeted replacement of the exon 2 of Vλ2 with a synthetic $V\lambda2^{315}$, thereby modifying the germline amino acid coding in positions 94, 95 and 96 from YST to FRN. A thorough description is given in Supporting Online Material section under Supplemental Figure 3.

Id^{315} mice and $\lambda2^{315}$TG x VDJ_H^{315} mice
To obtain mice that express the M315 V regions, homozygous VDJ_H^{315} mice were crossed with homozygous $V\lambda2^{315m}$ mice to obtain heterozygous offspring, designated Id^{315} mice. Alternatively, $VDJ_H^{315+/+}$ mice were crossed with $\lambda2^{315}$ $TG^{+/+}$ mice to obtain offspring designated $\lambda2^{315}$TG x VDJ_H^{315} mice. $\lambda2^{315}$TG mice are transgenic for the Ig L chain produced by the MOPC315 plasmacytoma (17). Both Id^{315} mice and $\lambda2^{315}$TG x VDJ_H^{315} mice were typed by staining PBMC with a combination of anti-Cλ2/λ3 mAb 2B6 (9) and the anti-Id^{315} mAb Ab2-1.4 (5). Id^{315} mice expressed physiological levels of the mutated $\lambda2^{315}$ L chain and served as cell transfer recipients. $\lambda2^{315}$TG x VDJ_H^{315} mice that expressed a high proportion of Id^+ B cells (≥70%), were used as a source of Id^+ B cells for adoptive transfer experiments and in *in vitro* cultures.

Cell enrichment
Spleens from $\lambda2^{315}$TG x VDJ_H^{315} or BALB/c mice were a source of B cells; whereas spleens and lymph nodes from Id-specific TCR transgenic mice ($TG46^{+/+}$ or $TG46^{+/-}$) were a source of $CD4^+$ T cells. B cells or $CD4^+$ T cells were enriched by negative selection using immunomagnetic depletion (Miltenyi, Bergisch-Gladbach, Germany, #130-090-862 and #130-095-248, respectively) and thus presumed to be naive. Viable cells were counted and FACS stained to assess purity. CD19 and Ab2-1.4 mAb was used for B cells; combinations of CD4, anti-TCRVβ8 (F23.1) and clonotype-specific GB113 mAbs for Id-specific $CD4^+$ T cells. After enrichment, the proportion of Id^{315} L chain expressing cells among splenic B cells was ≈75%. The proportion of Id-sp. T cells among enriched $CD4^+$ T cells was ≈70%.

BCR ligands and controls

The following hapten-conjugated antigens were obtained from LCG Biosearch Technologies Inc. (Petaluma, CA); DNP_5BSA, $DNP_{21}OVA$, $TNP_{13}OVA$, DNP_{49}AECM-FICOLL, NIP_7BSA, $NIP_{11}OVA$, $NIP_{14}OVA$, NIP_{68}AECM-FICOLL, TNP_{67}AECM-FICOLL-Fluorescein, NP_{122} AECM-FICOLL-Fluorescein; (AECM: AminoEthylCarboxyMethyl). DNP-albumin (Sigma**,** 324101) was biotinylated and used as a detection reagent. Ligands were resuspended in cell culture grade PBS (to 1 or 5 mg/ml) and stored at -20°C (proteins) or at 4°C (FICOLL). The anti-Id mAbs Ab2-1.4 (IgG1κ) and Ab2-TH (IgG1κ) specific for the binding site of M315 myeloma protein (5) were purified from hybridoma culture supernatants on anti-κ Sepharose columns. Ab2-TH was only used to characterize VDJ_H^{315} mice (Supplemental Figure 2F-H) and to investigate binding specificities (Supplemental Table 1); otherwise, Ab2-1.4 was used. As a non-specific isotype-matched control, we used purified MOPC21 IgG1κ (M9269, Sigma, St. Louis, Missouri). $F(ab)_2$ fragments prepared from complete Ab2-1.4 were purified and characterized in the laboratory (see Supporting Online Material, Supplemental Figure 10). Mouse IgG $F(ab)_2$ (Novusbio, NBP1-97018) served as a specificity control.

In vitro stimulation cultures

In vitro stimulation cultures were set up using $2x10^5$ Id-sp. T cells and $5x10^5$ Id^+ B cells per well in flat-bottom 24-well plates (800 µL volume). BCR ligands or their controls were added to the cultures in titrated doses, typically ranging from 0.01-10 µg/mL. The cultures were incubated for four days before analysis. Where used, BrdU to 1mM was added for the last 14-16 hours before staining for cell surface markers and BrdU incorporation (BD Biosciences, #552598). For conjugate assays, enriched B and T cells were labeled with CTV and CFSE, respectively. B cells were incubated with anti-Id IgG for 60 minutes at 37 °C before mixing with Id-sp. T cells, followed by centrifugation at 70G and incubation in a humidified cell culture incubator to allow conjugate formation. Non-specific conjugates were broken up by mild shaking for 10 seconds (300 rpm, tabletop shaker) before fixing in 1% PFA and FACS analysis.

In vivo transfer experiments

Cells were enriched as described above, and the numbers of viable, specific cells (Id^+ B or Id-specific T) were calculated using trypan dye exclusion and by FACS staining. Enriched cells were extensively rinsed in cell culture grade PBS and re-suspended to yield the desired dose of viable, specific cells in 200 µL volume for injection into the tail vein. When both purified B and T cells were injected, these were mixed immediately before injection and kept on ice. BCR ligands and appropriate controls were dialyzed against two changes of sterile cell culture grade PBS for 4 hrs or over night at 4°C. BCR ligands (in 200 µL volume) were injected i.v. the day after cell transfer. For BrdU labeling, the mice received one i.p. injection of 1 mg BrdU the day after ligand injection, followed by provision of 600 µg/mL of BrdU (Sigma Aldrich) in the drinking water until termination of the experiment. Short term experiments proceeded for 7-13 days post-ligation. Recipients for transfer were Id^{315} mice (described above) and $CD45.1^+$ congenic mice (CByJ.SJL (B6)-Ptprca/J, #006584, JaxLabs, USA), all on the BALB/c background.

ELISAs for Id^+ IgG and Id^+ IgM

Blood was harvested from the mice by saphenous vein puncture and sera prepared by centrifugation at 17,000 rpm in a tabletop centrifuge for 10 minutes. For detection of Id^+ IgG that bound the Ab2-1.4 IgM analogue AM5 (see above), 96-well ELISA plates (Costar 3590, Corning, NY, USA) were coated at 4° C over night with an equivalent mixture of rat-anti-mouse IgG1, IgG2a, IgG2b and IgG3 antibodies (1 µg/ml each; Southern Biotech, #1144-01, #1155-01, #1186-01, #1191-01). Captured IgG was detected using biotinylated AM5 (see above). Standard curves

for total IgG and for IgG1 were based on an affinity-purified Id+ IgG1 from a $\lambda 2^{315}$TG x VDJ_H^{315} mouse-derived hybridoma line (clone PK2). For Id^+ IgG isotype-specific detection, 2 µg/mL of the appropriate isotype specific rat anti-mouse IgG was used as coat and detection was performed as for total IgG. For Id^+ IgG2a, IgG2b and IgG3, serum cutoff values were established on each ELISA plate from several (≥3) individual BALB/c mice. For detection of Id^+ IgM, plates were coated with anti-Id IgG (Ab2-1.4, 2 µg/ml) and probed using anti-IgM-AP (2 µg/ml, A9688, Sigma Aldrich) or by coating plates with rat anti-mouse anti-IgM (2 µg/ml; Southern Biotech, 1139-01) and detected using Ab2-1.4-biotin. An IgM standard curve was generated using a purified IgM (clone PK8) from a $\lambda 2^{315}$TG x VDJ_H^{315} mouse hybridoma. For measurements of DNP-specific Ig, the plates were coated using DNP_5BSA (5 µg/ml) and captured antibodies were detected using reagents directed against mouse H chain isotypes and subclasses. Biotinylated secondary antibodies were probed using streptavidin-alkaline phosphatase (RPN1234, GE Healthcare, Buckinghamshire, UK). Plates were developed using AP substrate (P4744, Sigma Aldrich).

Antibodies and flow cytometry

The following mAbs were produced and affinity purified in the laboratory, followed by conjugation to biotin, Alexa Fluor 488, Alexa Fluor 546 or PE using standard procedures: anti-Id mAb Ab2-1.4 (IgG1κ), clonotype-specific GB113 mAb that binds the TCR of Id-specific T cells from TCR-TG mice (IgG2aκ, (18)), 2B6 (anti-Cλ2/3 (9)), 187.1 (anti-κ), 9A8 (anti-Vλ1/2 (9)). mAbs with the following specificities were purchased from eBioscience: CD25 (PC61.5), CD273 (TY25/PD-L2), CD93 (AA4.1), CD23 (B3B4), Foxp3 (FJK-16s), GATA-3 (TWAJ), T-bet (4B10), CXCR4 (2B11), $I\text{-}E^k$/$I\text{-}E^d$ (14-4-4S); from BD Biosciences: IgM (II/41), B220 (RA3-6B2), CD21/35 (7G6), IgM^a (DS-1), IgM^b (AF6-78), BrdU (BU-1), CD4 (RM4-5), CD40 (3/23), CD45.1 (A20), CD45.2 (104), CD95 (Jo2), CD138 (281-2), CD279 (J43), CXCR5 (2G8), I-A/I-E (2G9), Vβ8 TCR (F23.1), κ (187.1); from Southern Biotech: CD80 (1G10), CD86 (GL1); from Invitrogen: CD4 (RM4-5); from BioLegend: CD19 (6D5), CD69 (H1.2F3), CD93 (AA4.1), λ (RML-42), κ (187.1), CD73 (TY/11.8), CD38 (90) T and B cell activation Ag (GL7). The following fluorochromes were used for seven-color staining in different combinations: V405, V450, PE, FITC, APC, APC–Cy7, PE-Cy7, and PerCP or PerCP-Cy5.5. Cells were analyzed on an LSR II (BD Biosciences) or an Attune NxT flow cytometer (Thermo Fisher Scientific). For quadruple staining, we used PE, FITC, PerCP, and APC, and analyzed stained cells on a FACS Calibur (BD Biosciences). Flow cytometry data were analyzed with FlowJo version 10 (TreeStar, Ashland, OR) software.

Peptide stimulation experiments

Peptides for stimulation of Id-specific T cells had the following sequences: FAALWFRNH$\underline{X}$VFGGGTK where X_{98} is F, Y, L or W. Peptides were synthesized >98% pure and verified by mass spectrometry (Think Peptides, Oxford, UK). Peptides were reconstituted at 10 mg/ml in cell culture grade DMSO and stored at -20 °C. For stimulation experiments, the peptides were diluted in tissue culture medium.

Lambda amplicon sequencing of B cells from the $V\lambda 2^{315m}$ mouse

B cells were positively sorted from the spleens of a young naïve heterozygous $V\lambda 2^{315m}$ mouse using a CD19-selection kit (Miltenyi) and total RNA was isolated using an RNA Easy Kit (Qiagen). 5 µl RNA was used for cDNA generation using kit (K1612, Thermo Scientific). cDNA quality was evaluated using primers for GAPDH included in the kit. The λ gene was extended for 20 cycles using primers mVλ1/2 (5'-CAGGCTGTTGTGACTCAGGAATC-3') and mCλ-outer (5'-GTACCATYTGCCTTCCAGKCCACT-3') as published (15). The purified PCR products were subjected to purity check using gel electrophoresis and $A_{260/280}$ and $A_{260/230}$ measurements.

The amplicons (about 1 µg) were subject to Illumina miSeq sequencing. The amplicon sequences ($2.7x10^6$ reads) in a bam file were used to align the sequences and assign them to V, J and C segments. Counting and plotting was done in Rstudio and the software tool Knitr. Low level filtering was done in Rstudio to exclude sequences with somatic mutations in V-segments, which were assumed to be from non-naïve B cells. 2009488 reads were mapped in the bowtie2 alignment program using relaxed parameters.

Immunofluorescence and quantifications on sections

Spleen cryosections were air-dried, fixed in acetone and blocked using 30% normal rat serum added 5% BSA and 5% FCS in PBS. Primary reagents: goat anti-mouse λ-FITC (1:200, GLBF-90F, Immune Systems Ltd.) and peanut agglutinin lectin (1:600, PNA-Alexa Fluor 594, Molecular Probes) were added for 1 hour at room temperature, whereas rat-anti mouse ER-TR7 (1:100, BioRad, MCA2402) was incubated at 4°C overnight. Secondary reagent was rabbit anti-rat $F(ab)_2$-DyLight 405 (Star16D405GA, BioRad). For quantification of germinal centers in spleens, each data point represented the average number of GCs counted per x10 microscopic field, pooled group-wise (d3, d6 or d9 and isotype-matched IgG control). 10-12 transversal sections from each mouse spleen spanning over a 1-2 mm were used to give a representative overview. For quantifications of T-B synapses, immunofluorescence was performed as given in Supplemental Figure 14. Each data point on the graph represents the number of cell-to-cell contacts between Id^+ B and Id-specific T cells counted in one x20field. Data were pooled and compared group-wise (d3, d6 or d9 and isotype-matched IgG control on day 6).

Ca-flux measurements

B cells from $\lambda2^{315}$ TG x VDJ_H^{315} or BALB/c mouse spleens were enriched through depletion and incubated in complete media for 60 min at 37°C ($2.25x10^6$ c/ml) with Fluo-4 Direct™ calcium reagent (Fluo-4 Direct™ Calcium Assay Kit, F10471, Thermo Fisher Scientific) according to manufacturer's instructions. Baseline was recorded for 30 seconds, then ligand was added and the sample was recorded for 300 seconds. Samples were acquired on BD LSR II flow cytometer (BD Biosciences).

Phosphotyrosine blotting

B cells were enriched from $\lambda2^{315}$ TG x VDJ_H^{315} mouse spleens, washed and the pellet was stored on ice until stimulation. For stimulation, $\approx2x10^6$ B cells were re-suspended in warm (37°C) 1X PBS, and either anti-Id IgG or isotype control IgG was added (both 10 µg/ml) for the indicated time points. Stimulation was stopped by adding 1ml of ice-cold PBS and the cells were centrifuged at 4°C immediately. Cells were lysed for 45 min at 4°C in NP-40 lysis buffer mixed with freshly prepared 0.5 mM Phenylmethylsulfonyl fluoride (P-470, GoldBio, St. Loius, MO, USA), 1mM sodium orthovanadate (450243, Sigma Aldrich), and protease inhibitor cocktail (P8340, Sigma Aldrich). Denatured samples were run on a 4-12% gel (NW04125BOX, Novex, Carlsbad, CA, USA) and transferred to a PVDF membrane (IB24001, Invitrogen). The membrane was blocked with 3% BSA (805095, Bio-Rad, Hercules, CA, USA) or milk powder (A0830, AppliChem, Darmstadt, Germany) in PBS-T, and incubated with either antiphosphotyrosine (clone 4G10, 1.0 µg/ml) in 3% BSA or anti-actin (612656, BD Biosciences, 0.5 µg/ml) in 3% milk powder. Primary antibodies were detected with goat anti-mouse IgG HRP (1030-05, Southern Biotech) and developed with ChemiGlow West Chemiluminescence Substrate Kit (60-12596-00, Proteinsimple, San Jose, CA, USA).

Statistics

Graph Pad Prism v5.01 and v7.02 was used for data plotting and analysis. The variables were inspected for normality. Flow cytometry data that passed were analyzed using two-tailed unpaired

T tests with Welch′s correction for two groups, or using one-way ANOVA (Dunnett's test, two or more groups against a single control group) or Tukey's test (multiple comparisons). For values that did not pass, two-tailed Mann-Whitney U test, or Dunn's test (Kruskal-Wallis test, multiple comparisons) were used. Serum antibody concentrations were compared using non-parametric tests, unless otherwise indicated. Means (±SEM or ±SD) are indicated on the scatter plots or bar graphs. All data points represent distinct biological samples. Significance levels: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.005$, **** $p \leq 0.001$. *n.s.* not significant.

References

1. Collins AM, Wang Y, Roskin KM, Marquis CP, & Jackson KJ (2015) The mouse antibody heavy chain repertoire is germline-focused and highly variable between inbred strains. *Philos Trans R Soc Lond B Biol Sci* 370(1676).
2. Lauritzsen GF & Bogen B (1993) The role of idiotype-specific, CD4+ T cells in tumor resistance against major histocompatibility complex class II molecule negative plasmacytoma cells. *Cell Immunol* 148(1):177-188.
3. Hofgaard PO*, et al.* (2012) A novel mouse model for multiple myeloma (MOPC315.BM) that allows noninvasive spatiotemporal detection of osteolytic disease. *PLoS One* 7(12):e51892.
4. Rinfret A, Horne C, Dorrington KJ, & Klein M (1989) Cloning, sequencing and expression of the rearranged MOPC 315 VH gene segment. *Mol Immunol* 26(4):431-434.
5. Kristoffersen G, Hannestad K, & Hansen T (1987) Two M315 idiotopes defined by isologous monoclonal antibodies: one depends on germline and the other on mutated murine lambda 2 light chain sequences. *Scand J Immunol* 26(5):535-546.
6. Eisen HN, Simms ES, & Potter M (1968) Mouse myeloma proteins with antihapten antibody acitivity. The protein produced by plasma cell tumor MOPC-315. *Biochemistry* 7(11):4126-4134.
7. Jacobsen J*, et al.* (2014) Naive idiotope-specific B and T cells collaborate efficiently in the absence of dendritic cells. *J Immunol* 192(9):4174-4183.
8. Bothwell AL*, et al.* (1982) Somatic variants of murine immunoglobulin lambda light chains. *Nature* 298(5872):380-382.
9. Bogen B (1989) Monoclonal antibodies specific for variable and constant domains of murine lambda chains. *Scand J Immunol* 29(3):273-279.
10. Winberry L, Marks A, & Baumal R (1980) Immunoglobulin production and secretion by variant clones of the MOPC 315 mouse myeloma cell line. *J Immunol* 124(3):1174-1182.
11. Hoydahl LS*, et al.* (2016) Multivalent pIX phage display selects for distinct and improved antibody properties. *Sci Rep* 6:39066.
12. Kipriyanov SM, Moldenhauer G, & Little M (1997) High level production of soluble single chain antibodies in small-scale Escherichia coli cultures. *J Immunol Methods* 200(1-2):69-77.
13. Munthe LA, Kyte JA, & Bogen B (1999) Resting small B cells present endogenous immunoglobulin variable-region determinants to idiotope-specific CD4(+) T cells in vivo. *Eur J Immunol* 29(12):4043-4052.
14. Busse CE, Czogiel I, Braun P, Arndt PF, & Wardemann H (2014) Single-cell based high-throughput sequencing of full-length immunoglobulin heavy and light chain genes. *Eur J Immunol* 44(2):597-603.
15. Hikida M, Mori M, Kawabata T, Takai T, & Ohmori H (1997) Characterization of B cells expressing recombination activating genes in germinal centers of immunized mouse lymph nodes. *J Immunol* 158(6):2509-2512.
16. Norderhaug L, Olafsen T, Michaelsen TE, & Sandlie I (1997) Versatile vectors for transient and stable expression of recombinant antibody molecules in mammalian cells. *J Immunol Methods* 204(1):77-87.
17. Bogen B & Weiss S (1991) A rearranged lambda 2 light gene chain retards but does not exclude kappa and lambda 1 expression. *Eur J Immunol* 21(10):2391-2395.
18. Bogen B, Gleditsch L, Weiss S, & Dembic Z (1992) Weak positive selection of transgenic T cell receptor-bearing thymocytes: importance of major histocompatibility complex class II, T cell receptor and CD4 surface molecule densities. *Eur J Immunol* 22(3):703-709.