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

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Edited by Klaus Rajewsky, Max Delbrück Center for Molecular Medicine, Berlin, Germany, and approved November 5, 2019 (received for review February 19, 2019)

The B cell receptors (BCRs) for antigen express variable (V) regions that are enormously diverse, thus serving as markers on individual B cells. V region-derived idiotypic (Id) peptides can be displayed as pId:MHCII complexes on B cells for recognition by CD4+ T cells. It is not known if naive B cells spontaneously display pId:MHCII in vivo or if BCR ligation is required for expression, thereby enabling collaboration between Id+ B cells and Id-specific T cells. Here, using a mouse model, we show that naive B cells do not express readily detectable levels of pId:MHCII. However, BCR ligation by Ag dramatically increases physical display of pId:MHCII, leading to activation of Id-specific CD4+ T cells, extrafollicular T–B cell collaboration and some germinal center formation, and production of Id+ IgG. Besides having implications for immune regulation, the results may explain how persistent activation of self-reactive B cells induces the development of autoimmune diseases and B cell lymphomas.

Each B cell expresses unique BCR variable (V) regions due to V(D)J recombination and somatic hypermutation (1). The highly diversified V regions express idiotypic (Id) determinants that can be recognized by antibodies (2) and by CD4+ T cells (3). B lymphoma cells constitutively antigen-process their BCR and present Id peptides on their MHC class II molecules (pId:MHCII) to Id-specific CD4+ T cells (4, 5). Consistent with this, Id peptides were eluted from MHC class II molecules of tumor B cells (6). On the basis of these results, it was proposed in 1993 that Id-specific T cells help B cells that display pId:MHCII complexes on their surface (7). Such Id-driven T–B collaboration appears to be limited to rare Id peptides that express somatic mutations or unique N-region sequences (3, 8–11), since T cells are tolerant to germline-encoded V region sequences (8, 12). The existence of Id-driven T–B collaboration has been supported by studies using paired Ig/TCR-transgenic mice in 2 independent models (7, 13, 14). Chronic Id-driven T–B collaboration in these models has been associated with development of SLE-like autoimmune disease (13–16) and even B cell lymphomas (17).

The relevance of Id-driven T–B collaboration to disease development has been supported by recent observations in humans. First, bioinformatic analysis has indicated that human Ig V-regions are enriched for sequences that bind MHC molecules (18). Second, signs of Id-driven T–B collaboration have been observed in multiple sclerosis patients (19–21). Third, evidence of Id-driven T–B collaboration was obtained in chronic lymphatic leukemia (CLL) patients (22). Fourth, Id peptides were readily isolated from MHC class II molecules of mantle cell B lymphomas (23), as well as follicular B cell lymphomas, diffuse large B cell lymphoma, and CLL (24).

As an explanation for the pathogenicity of Id-driven T–B collaboration, it has been hypothesized that autoreactive B cells, in lieu of help from self-antigen-specific T cells (that are tolerated), could instead receive help from Id-specific CD4+ T cells (13, 25). It was further hypothesized that BCR ligation by self antigen could contribute to such pathogenic Id-driven T–B collaboration (13, 25). In support of the hypothesis, BCR ligation caused a GC reaction and isotype switch; however, the experiments employed memory B cells and Th2 cells, not naive cells (13).

To investigate the unresolved issue of whether BCR ligation is required for Id-dependent collaboration between naive B and T cells in vivo, we have here generated a strain of mice that have a low frequency of B cells with a BCR that (i) can be deliberately ligated by antigen and (ii) contains a particular Id sequence in its V region. The model employs a type of V gene segment-modified mice that yields physiological expression of the Id sequence only subsequent to a VJ2→J3 rearrangement in developing B cells. The surface display of pId:MHCII was physically detected by a staining reagent. The results show that naive B cells do not express

Significance

B and T lymphocytes collaborate during immune responses to antigens. B cells use membrane-bound antibody as part of their antigen receptor while T cells use a different receptor that recognizes antigen fragments bound to MHC molecules. We show here that T cells can recognize the variable parts of the B cell receptor when these are presented on MHC molecules. A prerequisite for such receptor cross-talk is that the B cell receptor binds antigen. The cross-talk results in collaboration between B and T cells and production of antibodies directed against the antigen. The findings have implications for basic immune regulation. The results may also help us understand the mechanism behind the development of SLE-like autoimmune diseases and B cell lymphomas.

Author contributions: P.C.H., J.T.J., and B.B. designed research; P.C.H., R.P.G., J.T.J., O.A.W.H., R.B., K.S., L.A.M., and B.B. analyzed reagents/analytic tools; P.C.H., R.P.G., J.T.J., O.A.W.H., R.B., K.S., L.A.M., and B.B. analyzed data; and P.C.H. and B.B. wrote the paper.

The authors declare no competing interest.

This article is a PNAS Direct Submission.

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Data deposition: Sequence Read Archive accession ID PRJNA495162.

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This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.1902836116/-/DCSupplemental.

First published December 3, 2019.
detectable levels of pld:MHCII. However, BCR ligation induces pld:MHCII display, thereby enabling Id-driven T–B collaboration. The findings support a mechanism where BCR ligation by self antigen is required for display of pld:MHCII on autoreactive B cells and solicitation of help from Id-specific T cells.

Results

A Model System for Studying the Influence of BCR Ligation on Id-Driven T–B Collaboration. The M315 myeloma protein produced by the MOPC315 myeloma cell line binds defined ligands such as anti-Id mAbs (26) and DNP/TNP haptons (27) (Fig. 1A). Its L chain (\(\lambda\)) contains a mutated Id sequence that spans the V–J junction in the CDR3 loop. Upon antigen processing by APCs, the Id peptide is released and binds to the MHC class II molecule I-E\(^+\) for presentation to Id-specific CD4\(^+\) T cells (3, 4) (Fig. 1A).

To study the role of BCR ligation in Id-driven T–B collaboration, we developed two strains of mice that express the VH and the VL of M315, respectively. Upon cross-breeding, the offspring should express an M315-like BCR on a low proportion of their B cells. For the VH, we used a conventional BCR knock-in mouse that should express an M315-like BCR on a small subset of their B cells. Such rare Id\(^+\)B cells were tested for collaboration with Id-specific CD4\(^+\) T cells from previously described TCR-transgenic mice (28), either in the presence or the absence of BCR ligation by defined ligands (Fig. 1C).

Characterization of Gene-Modified Strains of Mice. In the VDJ\(_{\lambda}^{315}\) mouse, allelic exclusion was pronounced, and VDJ\(_{\lambda}^{315}\) was expressed by most B cells [SI Appendix, Fig. S2 C–H]. B cell development in the bone marrow was slightly accelerated [SI Appendix, Fig. S3G], as is commonly found in VDJ knock-in mice. In the V\(_{\lambda}^{2315}\) mouse, codons 94, 95, and 96 of the germline V\(_{\lambda}\)2 were exchanged with those of V\(_{\lambda}^{2315}\) expressed by MOPC315. Since the FRT recombination left a short residual sequence within the intron immediately downstream of V\(_{\lambda}^{2315}\) (SI Appendix, Fig. S3A), the

![Fig. 1](https://example.com/figure1.jpg)

**Fig. 1.** A model system for studying the influence of BCR ligation on idiotype-driven T–B collaboration. (A) The M315 monoclonal Ig binds the indicated ligands. The \(\lambda\) chain expresses a mutated CDR3 Id peptide (residues 92 to 100; red triangle) that, after antigen processing, is presented on the MHC class II molecule I-E\(^d\). (B) In V\(_{\lambda}^{2315}\) mice, the germline V\(_{\lambda}\)2 gene segment was modified by the exchange of 9 nucleotides so that the modified V\(_{\lambda}\)2 allele encodes the mutated residues F\(^94\)R\(^95\)N\(^96\) within the central part of the Id sequence. (C) Offspring from a V\(_{\lambda}^{2315}\) x VDJ\(_{\lambda}^{2315}\) cross, called Id\(^{315}\) mice (left), should have a low frequency of B cells with an Id\(^+\)M315-like BCR that can be deliberately ligated. Id-driven T–B collaboration (Center) can be tested using Id-specific CD4\(^+\) T cells from previously established TCR-transgenic mice (Right). (D) The prevalence of germ line (YST) and mutated (FRN) sequences in the bone marrow was slightly accelerated (SI Appendix, Fig. S3G), as is commonly found in VDJ knock-in mice. In the V\(_{\lambda}^{2315}\) mouse, codons 94, 95, and 96 of the germline V\(_{\lambda}\)2 were exchanged with those of V\(_{\lambda}^{2315}\) expressed by MOPC315. Since the FRT recombination left a short residual sequence within the intron immediately downstream of V\(_{\lambda}^{2315}\) (SI Appendix, Fig. S3A), the
that express VH
To investigate this, we established a panel of recombinant IgGs of circulating B cells expressed an M315-like BCR detected by differences between these two BCR ligands (Fig. 1). We found that the rearranged WT and VH2/315m alleles had an identical frequency of different amino acids in position 98 with the ranking order Tyr->Phe->Leu (Fig. 1E). While Leu98 was not (Fig. 1F). This result indicates that most B cells in which a VH2/315m -> JH2 rearrangement occurs should potentially be able to stimulate Id-specific T cell responses. The frequencies and specific lineages of Jα2/3* B cells (detected by a C1D/C3 cross-reactive mAb, 2B6) and the levels of total Jα2/3* serum IgG did not differ between VH2/315m and BALB/c mice (SI Appendix, Fig. S3 D-G). These results indicate that the gene segment modification in VH2/315m did not influence B cell development, so the mouse strain displayed a physiological B cell compartment.

Offspring of homozygous VDJH2/315m and VDJH315m mice, called ID315m, had a slightly accelerated development of B cells in the bone marrow and a small increase in Jα2/3* T2 cells in the spleen, but were otherwise normal (SI Appendix, Fig. S3 F and G). These changes are most likely due to the prerearranged VDJH2/315 component of ID315 mice (SI Appendix, Fig. S2A). In ID315m mice, ~3 to 4% of circulating B cells expressed an M315-like BCR detected by binding of DNP (Fig. 1G), while ~1.5% were detected by the anti-Id IgG1 Ab2-1.4 (26) (Fig. 1H), consistent with the fine specificity differences between these two BCR ligands (SI Appendix, Table S1) (26) (also detailed later). The result with the highly specific Ab2-1.4 mAb (Fig. 1H) is as expected, since VH2/315m -> JH2 rearrangements should only occur in ~1 to 2% of peripheral B cells (30). ID315m mice had about ~2 pg/mg of serum IgG that bound DNP (Fig. 1J) and ~0.3 to 0.8 µg/mL that bound anti-Id Ab2-1.4 mAb (Fig. 1J), again consistent with the fine specificity of these reagents (SI Appendix, Table S1). Collectively, these results indicate that VDJH2/315m, VDJH315m, and ID315m mice displayed the expected features.

ID315 mice were used in most of the in vivo experiments described herein. However, in some experiments (e.g., adoptive transfers to CD45.1* congenic BALB/c mice), large numbers of ID315 B cells (hereafter called ID* B cells) were needed. For this purpose, VDJH315m mice were crossed with previously described Jα2/315 transgenic mice (31). In the progeny, as much as 70 to 80% of peripheral B cells expressed an M315 BCR (SI Appendix, Fig. S4 A and B). In Jα2/315TG × VDJH315m mice, splenic B cells had slightly reduced IgM expression levels (SI Appendix, Fig. S4C), and marginal zone (MZ) B cells were increased relative to follicular (FO) B cells (SI Appendix, Fig. S4D). Nevertheless, ID* B cells from the progeny were fully responsive to stimulation. Pre-B and pro-B cells in the bone marrow were reduced, but mature B cells were found to be at normal levels (SI Appendix, Fig. S4E). Since activated B and T cells may modulate their surface antigen receptors, the use of CD45.1* congenic mice facilitated the detection of the transferred lymphocyte populations upon recovery.

**Characterization of Specificity of BCR Ligands.** The amino acid exchange introduced in positions 94, 95, and 96 of VH2/315m as well as the junctional variation present in position 98 in VH2/315m-JH2 joints (Fig. 1E), could influence the binding of anti-Id mAb Ab2-1.4 and DNP/TNP used as ligands for the ID* BCR (Fig. 1A). To investigate this, we established a panel of recombinant IgGs that express VH14 together with various Vα2-regions and tested these for binding to the anti-idiotypic mAb Ab2-1.4 as well as DNP and TNP haptens (SI Appendix, Table S1). In brief, the Ab2-1.4 bound VH14 associated with VDJH2/315m but not with germline VH2. Moreover, Ab2-1.4 binding was compatible with the three most frequent amino acids found in position 98: Tyr, Phe, and Leu (Fig. 1E). The germline equivalent of VH14, VH3-6*02, was also compatible with binding, while a quite different VαH, VαH2/315m, was not. However, VH3-6*02 is probably poorly expressed in ID* mice due to the prerearranged VDJH315m. In conclusion, Ab2-1.4 is a highly specific ligand for the BCR of ID* B cells and should bind all BCRs independent of junctional variation. Its affinity for ID* M315 is Kd = 7.7 × 10^6 M^-1 (32).

TNP and DNP BCR ligands have a broader specificity since either of the haptens bound to VDJH2/315m and VH3-6*02 (but not VH3-6*02) associated with either VDJH2/315m or VDJ2 (SI Appendix, Table S1). Junctional variation in position 98 did not influence binding. These results extend a previous study demonstrating that TNP and DNP bind VH14 associated with either λ1, λ2, and λ3 light chains, but not k light chains (26). Thus, TNP/DNP should bind BCRs that express endogenous λ-chains together with VDJH315m in ID315 mice. The affinity of DNP for M315 has been measured to be in the range of Kd = 1.6 × 10^9 to 3.9 × 10^9 M^-1 (33), and the affinities of DNP and TNP are in a similar range (34).

In the course of the experiments described later, TNP/DNP and Ab2-1.4 were used not only as BCR ligands but also to measure B cell and antibody responses. Somatic hypermutation could have influenced binding and detection, which is difficult to control for. However, this does not seem to have been a major issue, since potent responses were detected by the BCR ligands over prolonged periods of time.

**BCR Ligation Is Required for Id-Driven T--B Collaboration in Vitro.** We performed a series of in vitro experiments on naïve splenic ID* B cells enriched through immunomagnetic depletion. Exposure of ID* B cells to Ab2-1.4 (hereafter referred to as anti-Id IgG) as BCR ligand resulted in intracellular Ca2+ mobilization (Fig. 2A), protein phosphorylation (Fig. 2B), and up-regulation of MHC class II (Fig. 2C) and CD86 (Fig. 2D), but not CD80, cell surface expression. Similar, although slightly lower, responses were obtained with TNP-OVA and TNP-OVA as BCR ligands (SI Appendix, Fig. S5). Importantly, BCR ligation resulted in an increased display of Id peptide--MHC class II complexes on the B cell surface, detected by an scFv reagent specific for the pId315:I-Ed complex (hereafter called pId:I-Ed or pId:MHCII; the reagent detecting these for binding to the anti-idiotypic mAb Ab2-1.4). BCR ligation resulted in a specific up-regulation of pId presentation on BCR by anti-Id IgG resulted in the proliferation of B cells and Id-specific T cell conjugate formation within 30 min (Fig. 2G). In cocultures of Id* B cells and Id-specific T cells, BCR ligation induced T cell–B cell conjugate formation within 30 min (Fig. 2G).

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BCR Ligation Is Required for Id-Driven T–B Collaboration In Vivo. We first tested the effect of BCR ligation on Id+ B cells in vivo in the absence of Id-specific T cells. A cohort of Id15 mice were injected i.v. with anti-Id IgG or isotype-matched specificity control mAb, and splenic Id+ B cells were analyzed by FACs at the indicated time points. Within 24 to 48 h, BCR ligation enhanced the surface expression of MHC class II molecules, CD86, and pld:MHCII complexes on Id+ B cells (Fig. 2 J–L and SI Appendix, Fig. S6F). The increased expression was still detectable after 72 h. These results suggest that in vivo provision of BCR ligand prepares Id+ B cells for efficient interaction with Id-specific T cells through surface display of both pld:MHCII and costimulatory molecules. Up-regulation of pld:MHCII was observed already at 24 h (Fig. 2L), while a general up-regulation of MHCII was seen first after 48 h (Fig. 2F), again arguing that BCR ligation has a specific contribution to increased display of pld:MHCII.

To probe the sensitivity of the model for Id-driven T–B collaboration, we titrated the amounts of Id-specific T cells, anti-Id IgG, and Id+ B cells needed to obtain Id+ IgG responses in Id15 and in CD45.1 congenic BALB/c recipients. Transfer of 15,000 naïve T cells and 4 µg anti-Id IgG sufficed to elicit full Id+ IgG responses in Id15 recipients (SI Appendix, Fig. S8A and B). Even as little as 1,500 Id-specific T cells induced Id+ IgG levels above background levels. Assuming a 10 to 15% parking efficiency, this cell dose is estimated to result in a physiological frequency of the Id-specific T cells in the recipient (35). Naïve Id+ B cells were titrated by transfer into CD45.1+ congenic BALB/c mice together with saturating dose of naïve Id-specific T cells and anti-Id IgG. About 103 Id+ B cells were required to elicit Id+ IgG responses (SI Appendix, Fig. S8C).

Based on these results, we transferred naïve Id-specific T cells and Id+ B cells, enriched through immunodepletion of nondesired populations, into CD45.1+ congenic BALB/c mice, followed by anti-Id IgG or isotype-matched control IgG and a continuous BrdU administration (Fig. 3A). A number of conclusions could be made based upon analysis of recipient spleens (Fig. 3) and lymph nodes (SI Appendix, Fig. S9). In the spleen, BCR ligation increased the numbers of (i) donor-derived CD45.2+ cells (Fig. 3B), (ii) Id-specific CD4+ T cells that had incorporated BrdU (Fig. 3 C and D), (iii) follicular T helper cells (Tfh; Fig. 3E), (iv) Id+ B cells that had incorporated BrdU (Fig. 3F), and (v) germinal center (GC) B cells (Fig. 3G). Intracellular staining of recovered Id-specific T cells revealed that BCR ligation increased expression of IFN-γ, TNF-α, and IL-4 cytokines and the transcription factor T-Bet (Fig. 3H), whereas GATA-3 and Foxp3 were not detected. Responses were stronger in the spleen than in the lymph nodes (SI Appendix, Fig. S9). BCR ligation induced a 3- to 4-log10 increase in serum levels of Id+ IgG of all subclasses (Fig. 3I).

Some of the anti-Id mAb Ab2-1.4 (IgG1) used in the aforementioned experiments could have been internalized via FcγRIIB on B cells rather than through receptor-mediated uptake, which could have contributed to pld:MHCII presentation. To exclude this possibility, we generated F(ab)2 fragments of the anti-Id IgG (SI Appendix, Fig. S10). F(ab)2 fragments were at least as stimulatory as intact anti-Id mAb to initiate Id-driven T–B cell collaboration, demonstrating that the Fc region and uptake via FcγRIIB is dispensable for responsiveness to BCR ligation (SI Appendix, Fig. S11).

In these experiments, the necessity of BCR ligation for initiation of Id-driven T–B collaboration was demonstrated with naïve B and T cells. However, it has been previously demonstrated that naïve B cells, although unable to stimulate naïve T cells, can stimulate memory T cells (36). Consistent with this, it was shown that naïve B cells from I2–15 transgenic mice could stimulate Id-specific Th2 cells in the absence of BCR ligation (37). We therefore tested if naïve Id+ B cells in Id15 mice could collaborate with in vitro-polarized Id-specific Th2 cells after adoptive transfer. The results show that naïve Id+ B cells can collaborate with Th2 cells in vivo; however, addition of BCR ligation clearly enhanced responses (SI Appendix, Fig. S12).

Localization of Id-Driven T–B Collaboration. To study the histological correlates of BCR ligation, we transferred naïve Id-specific T cells into Id15 mice, injected anti-Id IgG or isotype-matched
control IgG the next day, and analyzed spleens at different time points after ligation (Fig. 4 and SI Appendix, Figs. S13 and S14). Staining of sections for the GL7 lymphocyte activation marker showed that BCR ligation induced a robust extrafollicular activation of B and T cells on day 3 and day 6; however, activation had considerably subsided by day 9. Germinal centers (GCs) also peaked early (day 3), but these still remained on day 9, although they were fewer (SI Appendix, Fig. S13A and Fig. 4D). Germinal center differentiation of Id⁺ B cells. (H) Intracellular cytokine and intranuclear transcription factor expression by Id-specific CD4⁺ T cells. (I) Serum titers of Id⁺ (anti-id Ig-reactive) IgG antibody subclasses on day 11. Statistical comparisons: unpaired t tests (B–G), Mann–Whitney U tests (H and I). *P ≤ 0.05, **P ≤ 0.01, and ***P ≤ 0.005.

Quantification revealed that BCR ligation increased specific T–B synapses as well as GCs in spleen sections (Fig. 4 C and D). Synapses as well as GCs were most frequent already on day 3, and declined thereafter (Fig. 4 C and D). Synapses and GC formation preceded the peak expansion of Id⁺ B cells (day 6; Fig. 4E) and the peak of Id⁺ serum IgG (Fig. 4F). In summary, Id-driven T–B collaboration appeared to occur predominantly in extrafollicular sites and to a smaller extent in GCs. The latter appeared small and of short duration, perhaps indicative of abortive GC reactions. Analysis of stained sections (Fig. 4B and SI Appendix, Figs. S13 and S14) revealed less robust GC responses than did the FACS analysis (Fig. 3), where a preponderance of GC B cells and TFH was found. Of note, for the FACS analyses, we used the CD45.2 congenic marker, which is expressed irrespective of down-modulation of the BCR or the TCR, thereby facilitating recovery and enumeration of a larger proportion of the transferred cells. Furthermore, the anatomical location of Id⁺ B cells could differ in Id315 mice (natural positioning) compared to their location after injection i.v. into CD45.1 congenic recipients, thereby influencing the results.
Fig. 4. Germlinal centers form rapidly in Id<sup>315</sup> mice upon ligation of the Id<sup>+</sup> BCR. (A) Experimental setup (Id-sp. T/anti-Id IgG, n = 14; Id-sp. T/isotype control IgG, n = 4). (B) Representative germlinal center in the spleen of a recipient mouse on day 3. Id-specific TCR (clonotype-specific mAb GB113, red) and Id<sup>+</sup> BCR (anti-id BCR Ab2-1.4 mAb, blue) staining shows an area of T cell–B cell interaction. Germlinal center B cells were identified as GL7<sup>+</sup> clusters (green) with the light zone indicated by the presence of follicular dendritic cells (CD35, white). Examples of T-B synapses are magnified in 1 and 2. SI Appendix, Fig. S14 shows single stains contributing to the overlay. (C) Quantification of specific T-B synapses based on transgenic receptor expression in sections. Each data point represents the number of Id<sup>+</sup> B/Id-sp. T synapses counted in one x20 field. (D) Quantification of germinal centers from spleen cryosections (means per x10 field). (E) Id<sup>+</sup> B cells (Ab2-1.4-reactive) among L light chain negative splenic B cells by FACS analysis. (F) Id<sup>+</sup> IgG serum levels. (G) Frequencies of the different λ transcripts recovered among PNA<sup>+</sup> and PNA<sup>−</sup> fractions of sorted B cells. The B cells were sorted on day 7 from spleens of Id<sup>315</sup> mice primed with Id-specific T cells and anti-Id F(ab), fragments (SI Appendix, Fig. S11). (H) Comparisons of mutational load among V<sub>λ</sub>2315m-J2, L chain transcripts recovered from the PNA-positive and PNA-negative fractions. Statistical comparisons: Dunnett’s multiple comparisons test (C and D), unpaired t tests (F, E, and H). *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.005, and ****P ≤ 0.001; n.s., not significant.

Analysis of Id-Driven T–B Collaboration by Ig Gene Sequencing. On day 7 of Id-driven T–B collaboration, using anti-Id F(ab); fragments as BCR ligand (SI Appendix, Fig. S11), we purified PNA<sup>+</sup> and PNA<sup>−</sup> B cells from Id<sup>315</sup> mouse spleens and PCR-amplified and sequenced light chain transcripts with primers specific for λ1, λ2, and λ3 chains. The PNA<sup>+</sup> fraction contained 20% GL7<sup>+</sup> CD95<sup>+</sup> cells by FACS, indicating ~15 fold enrichment of GC B cells. Sequence analysis showed that the PNA<sup>+</sup> population was almost completely dominated by V<sub>λ</sub>2315m sequences having the idiotypic FRN sequence in positions 94 to 96 (Fig. 4G). A similar but less pronounced result was obtained for the PNA<sup>−</sup> population. These observations demonstrate a strong expansion of V<sub>λ</sub>2315m B cells, not only in the GC (PNA<sup>+</sup>) population, but also in the non-GC (PNA<sup>−</sup>) population. The results support the functional data (Figs. 3 and 4). Interestingly, the junctional amino acid in V<sub>λ</sub>2315m-J2 sequences was exclusively Y<sup>98</sup> even though Y<sup>98</sup> was by far the most frequent junctional residue in V<sub>λ</sub>2315m-J2 sequences of unstimulated B cells (Fig. 1E). The level of nucleotide substitutions was surprisingly low, with most sequences being unmutated or having only one mutation (Fig. 4H and SI Appendix, Fig. S15). The level of nucleotide substitutions in V<sub>λ</sub>2315m-J2 transcripts was not significantly different between the PNA<sup>+</sup> population and the PNA<sup>−</sup> population (Fig. 4H). The paucity of mutations in the PNA<sup>−</sup> cell population is surprising and appears consistent with a limited GC response.

Ligation of the Id<sup>+</sup> BCR Using a Hapten-Protein Conjugate Promotes Id-Driven T–B Collaboration. M315 has a specificity for the structurally similar hapten DNP and TNP (27), but does not bind the NIP hapten. We therefore tested if BCR ligation by DNP- and TNP-conjugated proteins could promote Id-driven T–B collaboration. CD45.1<sup>+</sup> BALB/c mice were transferred with Id<sup>+</sup> B cells and Id-specific T cells, followed by TNP-OVA or NIP-OVA (specificity control). Thereafter, mice continuously received Brdu (Fig. 5A). High doses of hapten–OVA conjugates were used (200 μg), since preliminary experiments suggested that DNP-OVA is relatively inefficient at promoting Id-driven T–B collaboration in vivo compared to anti-Id IgG (SI Appendix, Fig. S16). Several factors could contribute to this difference. First, OVA is filtrated in the kidneys (38), while the higher-MW anti-Id IgG is not. Second, B cells expressing VDJ<sub>λ</sub>2315m-J2 together with other λ chains than λ2315m could bind DNP/TNP conjugates (SI Appendix, Table S1) and thus serve as a sink for DNP/TNP-OVA. Finally, the Ig structure of the anti-Id IgG ligand could be particularly efficient at cross-linking BCR due to matching distances between antigen binding sites of Id<sup>+</sup>-anti-Id Iggs.

Despite the decreased sensitivity, BCR ligation by TNP-OVA in vivo increased Brdu incorporation into both Id-specific T cells and Id<sup>+</sup> B cells compared to that seen with NIP-OVA (Fig. 5B and C). Further, BCR ligation increased the frequency of GC-like Id<sup>+</sup> B cells (Fig. 5D). Finally, BCR ligation enhanced the serum levels of Id<sup>+</sup> IgG of all subclasses (Fig. 5E). These results show that BCR ligation with TNP-OVA enhances Id-driven T–B collaboration.

BCR Ligation with a T Cell-Independent Type 2 Antigen Enhances Id-Driven T–B Collaboration. In the experiments described here earlier, endogenous T cell responses directed against the BCR ligand, either anti-Id IgG or OVA, could have influenced the results. To exclude this possibility, we tested DNP-FICOLL as a BCR ligand, the rationale being that FICOLL is a polysaccharide that should not be presented on MHC class II molecules to endogenous T cells. Moreover, since DNP-FICOLL is a T cell-independent type 2 (TI-2) antigen, the experiment addresses whether Id-specific DNP<sup>+</sup> T cells influence B cell responses to a TI-2 antigen.

Id-specific T cells were transferred to Id<sup>315</sup> mice followed by DNP-FICOLL or NIP-FICOLL the next day (Fig. 6A). Responses in the spleens were analyzed on day 10. Immunohistochemical staining revealed an enhanced generation of GCs on day 10 when using DNP-FICOLL compared to NIP-FICOLL (Fig. 6B), which was verified by quantitative assessment (Fig. 6C). In the absence of T cell help, BCR ligation by DNP-FICOLL elicited a slight

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expansion of Id\(^+\) (λ\(^+\)) B cells, including GC B cells and plasma cells, as determined by flow cytometry (Fig. 6 D and E and SI Appendix, Fig. S17). Addition of Id-specific T cells enhanced the expansion of Id\(^+\) B cells and promoted their differentiation into GC B cells and plasma cells (Fig. 6 D and E and SI Appendix, Fig. S17). Finally, the concentrations of serum IgM and IgG that bound anti-Id mAb or DNP were enhanced in the presence of DNP-FICOLL and Id-specific T cell help compared to control (Fig. 6F). Taken together, the results show that a TI-2 BCR ligand alone stimulates Id\(^+\) B cell responses weakly, but that provision of Id-specific T cell help significantly augments responses. Further, recognition of the BCR ligand by endogenous polyclonal T cells is not required for Id-driven T–B collaboration.

To test if the CD40L–CD40 axis was involved in Id-driven T–B collaboration, we next tried to block responses to DNP-FICOLL by repetitious injections of anti-CD40L mAb (Fig. 6G and SI Appendix, Fig. S18). The results show that CD40L blockade increased the levels of Id\(^+\) IgM while Id\(^+\) IgG of all subclasses were decreased (Fig. 6G and SI Appendix, Fig. S18). Thus, CD40L is involved in the IgM → IgG switch in the presence of Id\(^+\) B and Id-specific T cells. Anti-CD40L mAb did not inhibit the IgM response by Id\(^+\) B cells induced by DNP-FICOLL in the absence of Id-specific T cells, consistent with DNP-FICOLL being a TI-2 antigen (SI Appendix, Fig. S19).

The experiment was extended to adoptive transfer of Id\(^+\) LPS blasts and naive Id-specific T cells to CD45-congenic recipients, followed by injection of DNP-FICOLL (SI Appendix, Fig. S20A). BCR ligation enhanced responses of Id\(^+\) LPS blasts, both in terms of plasma cell differentiation and IgG production (SI Appendix, Fig. S20 C–E). A slight expansion of Id-specific T cells was observed in response to LPS blasts in the absence of ligation (SI Appendix, Fig. S20B), but this did not result in IgG serum levels above that observed in mice transferred LPS blasts only (SI Appendix, Fig. S20 D and E). These results indicate that nonspecific activation of Id\(^+\) B cells through TLR4 is not sufficient to induce collaboration with naive Id-specific T cells, but that BCR ligation is required. This may well relate to the importance of recognition of pld:MHCII by Id-specific T cells, since the level of pld:MHCII remained unchanged after LPS stimulation (SI Appendix, Fig. S7).

**Ligation of the Id\(^+\) BCRPromotes the Development of Memory B Cells, Bone Marrow Plasma Cells and Id-Specific Regulatory T Cells.** After having evaluated early responses to BCR ligation, we performed experiments where responses were analyzed at later time points, e.g., day 28 (SI Appendix, Fig. S21). Id\(^+\) B cells and Id-specific T cells were transferred to CD45.1+ BALB/c mice followed by specific BCR ligation (DNP-FICOLL) or nonspecific control antigen (NIP-FICOLL). Four weeks later, the spleens and bone marrow cells from femurs were analyzed (SI Appendix, Fig. S21A). Id-specific regulatory T cells were found on day 28, and had increased to ~2% of recovered Id-specific T cells in the spleen (SI Appendix, Fig. S21B). The development of Tregs required BCR ligation. Formation of Id\(^+\) memory B cells (CD73\(^+\) CD273\(^+\)) in the presence of BCR ligation was also increased (SI Appendix, Fig. S21C). Finally, bone marrow plasma cells were significantly expanded in recipients that had received the BCR ligand (SI Appendix, Fig. S21D), and serum antibodies specific for anti-Id IgG and DNP were significantly elevated (SI Appendix, Fig. S21 E–F). Id\(^+\) plasma cells secreting Id\(^+\) IgM and Id\(^+\) IgG were also found on day 40 in another experiment, where anti-Id IgG was used as BCR ligand (SI Appendix, Fig. S22). These results show that BCR ligation in Id-driven T–B collaboration has a long-lasting influence.

**Discussion**

The present results demonstrate that BCR V region-derived Id peptides are undetectable on MHC class II molecules of naive B cells, but that BCR ligation induces display. Further, in the presence of naive Id-specific CD4\(^+\) T cells, BCR ligation initiates the full panoply of events of T cell–B cell collaboration, including T–B synapse formation, mutual activation and proliferation, GC formation with generation of GC B cells and TFH, extrafollicular T–B cell responses, and an increase in plasma cells and antibody production. Despite the expansion of Id\(^+\) B cells, the level of mutations in their BCR L chain V region was surprisingly small. Whether BCR-ligated Id-driven T–B cell collaboration requires dendritic cells has not been addressed in the current work, although DCs were dispensable during conventional T–B collaboration using anti-Id BCR knock-in mice and Id\(^+\) M315 as antigen (39).

These results seemingly contradict previous functional studies in mice (4, 5, 37) and MHCII elution studies in mice (6) and humans (23, 24, 40), which have demonstrated spontaneous pld:MHCII presentation without deliberate BCR ligation. However, these previous studies employed malignant B cells of unknown specificity cultured in vitro, and a number of uncontrolled factors could have contributed to constitutive pld:MHCII display. In more
Id-specific CD4 \(_L\)-transgenic mice stimulated Id-specific Th2 cells, but not naive chain (from VDJH knock-in mice), and thus had a BCR of known frequency of B cells, which, subsequent to a V\(_\lambda\) gene segment-modified mouse was created that had a low frequency of BCR ligands to unknown antigens to pId:MHCII display. The present study thus makes it difficult to exclude a contribution of BCR ligation.

Physiologically relevant experiments, naïve B cells from \(\lambda^2\text{315}\) Ig L-transgenic mice stimulated Id-specific Th2 cells, but not naïve Id-specific CD4\(^+\) T cells (37). In a second Ig L-chain transgenic model, B cells had to be activated in order to stimulate Id-specific T cell hybridomas in vitro (34). In these studies, the Ig L chain transgenic B cells had a polyclonal H chain repertoire, specific T cell hybridomas in vitro (41). In these studies, the Ig L chain transgenic B cells had a polyclonal H chain repertoire, specific T cell hybridomas in vitro (37). In a second Ig L-chain transgenic model, B cells had to be activated in order to stimulate Id-specific T cell hybridomas in vitro (41). In these studies, the Ig L chain transgenic B cells had a polyclonal H chain repertoire, specific T cell hybridomas in vitro (41). In these studies, the Ig L chain transgenic B cells had a polyclonal H chain repertoire, specific T cell hybridomas in vitro (37).

The mechanism by which BCR ligation by antigen enhances BCR of a B cell, that B cell is anticipated to display both Ag-specific and Id-specific T cells. Such a mechanism would effectively merge conventional (42, 46) and Id-driven (7, 13) T cell collaboration. Thus, whenever Ag ligates the BCR L chain, that B cell is anticipated to display both Ag-specific and Id-specific T cells. Such a mechanism would effectively merge conventional (42, 46) and Id-driven (7, 13) T–B collaboration.

The observation that DNP- and TNP-conjugated OVA were somewhat less potent at inducing B cell responses than was anti-Id peptide.
IgG may be related to the slightly lower affinity of the haspens for the Id3 BCR. Further, filtration of TNP/DNP-OVA in the kidney, and a binding of TNP/DNP to BCRs having irrelevant (non-Id) 2 chains, could also contribute. DNP-FICOLL was highly efficient, but this may relate to the TI-2 nature of FICOLL. It should be noted that DNP-FICOLL elicited much more potent responses in the presence of Id-specific T cells, even when employing LPS-stimulated B cells.

It has been hypothesized that Id-driven T–B collaboration could cause development of autoimmune diseases, since autoreactive B cells that have their BCR ligated by autoantigen could receive help from Id-specific T helper cells (13, 25). Such Id-driven T–B collaboration could easily become chronic, since self-antigens are not readily eliminated, in contrast to exogenous antigens. The persistent nature of Id-driven T–B collaboration could cause development of autoimmune diseases and, after acquisition of oncogenic events, B cell lymphomas. Supporting the hypothesis, chronic Id-driven T–B collaboration has been shown to elicit SLE-like disease in mice (16, 25), with hallmarks of human SLE (15). Moreover, Id-driven T–B collaboration has been associated with multiple sclerosis in humans (19–21). Finally, chronic Id-driven T–B collaboration initiated development of B cell lymphomas in mice (17) and possibly CLL in humans (22). The influence of BCR ligation could not be addressed in these studies. Here, in support of the hypothesis, we demonstrate that BCR ligation is required for efficient Id-driven T–B cell collaboration. This suggests that (i) chronic ligation of the BCR by autoantigen and (ii) persistent help from Id-specific T cells may synergize in causing chronic proliferation of B cells and the development of disease. This scenario is consistent with the observations that not only autoimmune diseases (47) but also B cell cancers (48–51) are associated with self-reactive BCRs. It should be stressed that the hypothesis relates to the induction of B cell disorders. In later phases of the disease, the requirement for BCR ligation and for Id-specific T cell help could diminish as the pathologic B cells attain a more differentiated and (52), deletion of Id-specific thymocytes (12), and perhaps through the action of Id-specific T suppressor cells (53).

Autoimmune diseases and B cell malignancies are relatively rare disorders. Therefore, in most individuals, Id-driven T–B collaboration does not escalate beyond control but is down-regulated over time. Such down-modulation might occur through a number of suppressive mechanisms, such as peripheral exhaustion of Id-specific T cells, deletion of Id-specific thymocytes (12), and perhaps through the action of Id-specific T suppressor cells (53). We here demonstrate the induction of Foxp3+ Id-specific regulatory T cells in the wake of Id-driven T–B collaboration; such cells could contribute to down-regulation of the response. Presumably, only when one or more of these suppressive mechanisms fail may unchecked Id-driven T–B collaboration result in development of autoimmunity and B cell cancers.

Experimental Outline. Extended materials and methods have been added at the end of the SI Appendix after SI Appendix, Table S1 (p. 37–41). SI Appendix describes the generation of VDJ3315 mice (SI Appendix, Fig. S2, p. 5) and Vλ2315m mice (SI Appendix, Fig. S3, p.7). Pages 7 and 8 describe the Id315 and λ2315 TG × VDJ3315 mice. Page 7 describes FACS characterization of splenic and bone marrow B cell lineages. SI Appendix, Fig. S6 (p. 13) shows generation of the anti-IdF(ab)2 scFv. SI Appendix, Fig. S10 (p. 17) shows preparation and validation of the anti-Id F(ab)2. SI Appendix, Figs.S13 and S14 show GL7 immunostaining (p. 21) and immunofluorescence (p. 23) procedures. SI Appendix, Fig. S15 (p. 26) shows germinal center B cell sorting and sequencing procedures. Cell enrichment, in vitro stimulation cultures, and in vivo transfer experiments are described in SI Appendix, Extended Materials and Methods (p. 37–38). ELISAs for IdG and IgG are described in SI Appendix, Extended Materials and Methods (p. 38–39). All antibodies for flow cytometry are described in SI Appendix, Extended Materials and Methods (p. 39). λ Amplex sequencing in naïve Vλ2315 mice is described in SI Appendix, Extended Materials and Methods (p. 39). Analyses of in vitro B cell responses, including of Ca2+ flux measurements and phosphoarrays Western blotting are described in SI Appendix, Extended Materials and Methods (p. 40).

Data and Materials Availability. The V-gene modified mice and the TCRm reagent may be obtained on a collaborative basis. Sequencing raw data from λ amplex sequencing from the Vλ2315m SRA, mouse are available at the Sequence Read Archive. Identifiers are BioSample SAMN10228988; sample name, VL2-315 B9’; SRA, SRS3891429; BioProject, PRJNA495162.

ACKNOWLEDGMENTS. Hilde Omholt, Peter Hofgaard, Keith M. Thompson, Marte Fauskanger, Kristina Randjelovic, Elisabeth Vikse, Nikolay Rustad Nilsen, and Olaf F. Schreurs are thanked for technical help; Vegard Nygaard and Eivind Hovig at the Oslo University Hospital Bioinformatics Core Facility for help with analyzing sequence data; Omri Snir for help with mRNA QC; and the staff at the Department of Comparative Medicine, Rikshospitalet, for assistance with mouse experiments. We are indebted to Drs. Robert Bremel and Jane Homan for critically reviewing the manuscript. Funding: The Norwegian Research Council (NFR, project 221709, to B.B.) and South-East Health Authority (Helse Sør-Ost, project 2017082, to B.B.) are acknowledged for funding.

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