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Defective Allelic Exclusion by IgD in the Absence of Autoantigen

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A considerable proportion of peripheral B cells is autoreactive, and it is unclear how the activation of such potentially harmful cells is regulated. In this study, we show that the different activation thresholds or IgM and IgD BCRs adjust B cell activation to the diverse requirements during development. We rely on the autoreactive 3-83 model BCR to generate and analyze mice expressing exclusively autoreactive IgD BCRs on two different backgrounds that determine two stages of autoreactivity, depending on the presence or absence of the cognate Ag. By comparing these models with IgM-expressing control mice, we found that, compared with IgM, IgD has a higher activation threshold in vivo, as it requires autoantigen to enable normal B cell development, including allelic exclusion. Our data indicate that IgM provides the high sensitivity required during early developmental stages to trigger editing of any autoreactive specificities, including those enabling weak interaction with autoantigen. In contrast, IgD has the unique ability to neglect weakly interacting autoantigens while retaining reactivity to higher-affinity Ag. This IgD function enables mature B cells to ignore autoantigens while remaining able to efficiently respond to foreign threats. ***The Journal of Immunology*****, 2022, 208: 293–302.**

To achieve the remarkable ability to recognize virtually any pathogen, an immune system evolved in which each of the billions of different B cell clones possesses a BCR with unique specificity; nevertheless, such a broad repertoire comes at a cost (1). Variable regions of Igs, which determine specificity, are generated by a random DNA rearrangement process, in which one V gene segment, one D segment (only in IgH), and one J segment are randomly chosen and brought into contiguity to form a single V(D)J exon that will encode for the Ag binding region of the BCR. The process, called V(D)J recombination, is random and therefore leads to generation of useful Abs that recognize invading pathogens as well as harmful, highly autoreactive Abs (2, 3). This issue is tackled by an ensemble of mechanisms, which fall under the term of central tolerance, that ensures unresponsiveness to self-antigens of B cells leaving the bone marrow by removing clones expressing membrane Igs with high affinity to self. The most relevant mechanism is receptor editing, in which B cells reactivate their Rag 1 and Rag 2 genes to trigger a new round of VJ recombination in the Ig κ or λ L chain (LC) locus, to modify the specificity of the Ig (4). If editing fails, autoreactive immature B cells undergo clonal deletion mediated by apoptosis (5, 6). According to the classical perspective, developing B cells that recognize self-antigens weakly may become functionally unresponsive and exit the bone marrow in an anergic state, implementing the so-called peripheral tolerance (7, 8). However, the mechanism of anergy is, to date, unclear (9), and the classical definition of peripheral tolerance seems too simplistic in that it fails to explain how a remarkable proportion of autoreactive clones persist in the periphery of healthy humans (3, 10, 11). Importantly, recent studies showed that self-reactivity is required for optimal and diversified B cell development (12–14). Also, a certain level of autoreactivity has been proposed to continue to play a positive role for the selection of B cells in the periphery (15). How this is conciliated with self-tolerance is currently a matter of discussion.

Ig gene recombination is governed by sophisticated epigenetic processes; indeed, differential structure of each locus ultimately controls the recombination machinery (16). In nonlymphoid cells, Ig loci are repressed by multiple targeted mechanisms that govern chromatin accessibility, such as histone acetylation and subnuclear localization (17, 18). In committed developing B cells, specifically at the pro–B cell stage, programmed stepwise release of Ig loci repression results in increased accessibility of recombination-specific loci, such as the recombination signal sequences (17, 19). This is achieved by hyperacetylation of specific histones (17). By means of these mechanisms, each B cell clone restricts rearrangement to only one immune-receptor allele during development. Whenever recombination of an IgH H chain (HC) gene locus on one chromosome is productive, this leads to generation of a μHC protein that forms a functional pre-BCR. Signals by the pre-BCR suppress the

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Abbreviations used in this article: ES, embryonic stem; Fo.B, follicular B; HC, H chain; HEL, hen egg lysozyme; KI, knock-in; KO, knockout; LC, L chain; MHC I, MHC class I; 4-OHT, 4-hydroxytamoxifen; Tg, transgenic; TKO, triple-deficient; WT, wild-type.

rearrangement of the Ig HC gene locus on the other chromosome, via a process known as allelic exclusion (20). Suppression of Rag1/2 gene expressions, key players in V(D)J recombination, implements allelic exclusion and prevents the expression of BCRs of two different specificities on the same clone. Moreover, allele-specific chromatin accessibility is believed to preserve allelic exclusion maintenance. Nevertheless, the second allele maintains the potential to become available to the recombination machinery, and defective signaling from the BCR can lead to secondary recombination (21).

During early stages of development, which occur in the bone marrow, B cells express the pre-BCR, which lacks a conventional LC and instead contains a preassembled and nonvariable surrogate LC (22). Subsequent to a productive Ig LC gene rearrangement, B cells express IgM-type BCR (23). The passage from the immature to the transitional stage is denoted by the movement from bone marrow to the periphery and by the acquisition of IgD-type BCRs. The two BCR isotypes are distinct by their HCs. Whereas IgM displays a rigid, canonical "Y" structure, IgD is characterized by the presence of a long hinge region that connects the crystallizable fragment to the Fab, providing remarkable flexibility and a peculiar "T" shape (24). Being coexpressed on one B cell clone, IgM and IgD share the same Ag specificity (25). Also, they seem to activate the same signaling cascade, and knockout (KO) mouse models for either IgM or IgD showed only mild phenotypes (26–29). The reason why IgD was conserved throughout evolution is still debated today (30, 31). However, a growing body of evidence indicates distinct functional roles for the two isotypes. A transgenic (Tg) mouse model expressing monoclonal hen egg lysozyme (HEL)–specific B cell population and soluble HEL constitutively showed that, under chronic exposure to Ag, follicular B (Fo.B) cells downregulate IgM expression but retain IgD on their surface (8). This regulation was thought to mediate peripheral tolerance by anergy (i.e., functional inactivation of the autoreactive B cells) (8). Our recent work provided a molecular explanation for the discrepancy in signaling between IgM and IgD isotypes by demonstrating that, in contrast to IgM, IgD responsiveness is restricted to multivalent/polyvalent Ag, whereas soluble, monovalent Ag interferes with this responsiveness (9, 32). Indeed, although not responding to Ag in soluble form, IgD-expressing cells retain full reactivity to cognate polyvalent Ag and anti-BCR stimulation, which suggests a unique regulation of IgD BCR and argues against functional inactivation of IgD-expressing cells (9, 32, 33). By employing the 3-83 BCR knock-in (KI) system, we show in the current study that the threshold for activation of autoreactive IgM or IgD BCR differs in vivo, thereby resulting in different repertoires of B cell specificities and failure of allelic exclusion establishment by IgD.

Materials and Methods

Mice

All mice used in this study were bred and housed either in the animal facility of Ulm University or in the animal facility of the Max Planck Institute of Immunobiology and Epigenetics (Freiburg, Germany), in both cases under specific pathogen–free conditions. All animal experiments were performed in compliance with the guidelines of the German law and were approved by the Animal Care and Use Committees of Ulm University and the local government. In this study, we used 3-83 IgM KI mice, which have been described previously (34), and the 3-83 IgD KI mice that we generated; both were bred onto the H2-K^b or H2-K^d background.

Cell culture conditions

Phoenix and triple-deficient (TKO) cells were cultured in Iscove's medium (Biochrom) containing 10% FCS (PAN-Biotech), 10 mM L-glutamine (Life Technologies), and 100 U/ml penicillin/streptomycin (Life Technologies). For culture of TKO cells, the medium was supplemented with 50 μM 2-ME (Life Technologies), and the supernatant of J558L mouse plasmacytoma cells was stably transfected with a murine IL-7 expression vector. TKO cells were cultured at a density of 2×10^5 to 4×10^5 cells/ml.

Plasmids and retroviral transduction

Ig HCs and LCs were expressed in TKO cells using the bimolecular fluorescence complementation vector system as previously described (14). Plasmid for expression of λ5 has been described previously (35). Retroviral transduction was performed as previously described (35). In brief, for generation of retroviral particles, the helper-free retrovirus producer cell line Phoenix was used (36). Twenty-four hours before transfection, Phoenix cells were plated on a new dish at a density of 2×10^5 cells/ml. Shortly before transfection, cells were supplemented with fresh medium. For transfection of a single well of a 6-well plate, 3 μl of GeneJuice Transfection Reagent (Merck Millipore) was added to 100 μl of plain Iscove's medium; the mix was then vortexed and incubated for 5 min at room temperature. One to two micrograms of the vector of interest were added to the resulting transfection mix, which was then vortexed again and incubated at room temperature for 15 min. Subsequently, transfection mixture with the DNA of interest was added dropwise to the well containing the Phoenix cells. Forty-eight hours after transfection, Phoenix cell supernatant was collected, filtered through a 0.22-μm filter, and directly used for transduction.

Real-time PCR

Quantitative real-time PCR was performed either with a 7500 Fast Real-Time PCR System (Applied Biosystems) using SYBR Green PCR Master Mix (Applied Biosystems) or with a StepOnePlus Real-Time PCR thermocycler (Applied Biosystems) using TaqMan probe mixes (Applied Biosystems) together with TaqMan Gene Expression Master Mix (Applied Biosystems). Cells were purified using a FACS Aria IIu Cell Sorter (BD Biosciences). RNA was extracted from FACS-sorted cells by using the ReliaPrep RNA Cell Miniprep System (Promega). Residual genomic DNA was digested using DNase I (Thermo Fisher Scientific). cDNA synthesis was performed with the RevertAid Reverse Transcriptase Kit (Thermo Fisher Scientific) as indicated by the manufacturer. Replicates for each sample were discarded from the analysis in case of nonexpected melting curves and inconsistency between the replicates. Amplification curves and ΔC_T values for each target gene relative to the reference gene (*mb-1*) for the same sample were exported into Microsoft Excel. With this software, ΔC_T data were transformed into 2 to the power of $-\Delta C_T$, and the resulting values were plotted in Prism. Sequences of oligonucleotides used for the real-time PCR are as follows: Rag1_qPCR_s1 (5′-GGC TAG GGT CAG CAA GGA-3′), Rag1_qPCR_as1 (5′-CAC GGG ATC AGC CAG AAT GTG TTC-3′), Rag2_qPCR_s1 (5′-TTT GAG TGA GGA TTG CAC TGG AGA C-3′), Rag2_qPCR_as1 (5′-CAG AAC TTC AGG ATG GGC TGT CTT T-3′), HPRT_qPCR_s (5′-TCC TCC TCA GAC CGC TTT T-3′), HPRT_qPCR_as (5′-CCT GGT TCA TCA TCG CTA ATC-3′), 3-83 CDR3_For (5′-TTC GGT AGT AGC TAC TAC TGG-3′), 3′Δ inner (5′-GAG GAC CAT TGT ATA ATT GCC ATT TCT CAT-3′), β-actin_m_RT_F (5′-TCC TGT GGC ATC CAT GAA ACT-3′), and β-actin_m_RT_R (5′-GAA GCA CTT GCG GTG CAC-3′).

Calcium measurement

Intracellular Ca^{2+} mobilization was measured as previously described (32, 37). TKO cells expressing exogenous BCR were stimulated with 2 μM 4-hydroxytamoxifen (4-OHT; Sigma-Aldrich) for measurement of autonomous signaling. For ex vivo measurements of murine primary lymphocytes, splenocytes were either sorted with MACS, as described below, to obtain a population of pure untouched mature B cells, or total splenocytes were used, and B cell discrimination was then performed by CD23 staining. Cells were then used to conduct the Ca^{+2} mobilization experiments following the same protocol used for TKO cells. Experiments shown in all figures are representative of triplicates. Ca^{2+} flux measurements were acquired at a FACS LSRFortessa flow cytometer (BD Biosciences).

Flow cytometry

Cell suspensions were blocked with anti-CD16/CD32 Fc Block (2,4G2; BD Biosciences) and stained by standard procedures. For flow cytometric analysis, cells were stained with the following: CD21/CD35–allophycocyanin (7E9; BioLegend), IgD–allophycocyanin (11-26C; eBioscience), IgM–allophycocyanin (RMM-1; BioLegend), IgD-allophycocyanin-Cy7 (11-26C.2a; BioLegend), IgM-allophycocyanin-Cy7 (RMM-1; BioLegend), SA-allophycocyanin-Cy7 (allophycocyanin-Cy7; BioLegend), CD23–biotin (B3B4; BD Biosciences), CD5–biotin (53-7.3; BD Biosciences), H2-K^b–biotin (AF6-88.5; BD Biosciences), Ig (3-83)-biotin (54.1; a kind gift from R. Pelanda), IgD–biotin (11-26; SouthernBiotech), IgM–biotin (R6-60.2; BD Biosciences), IgM^a–biotin (DS-1; BD Biosciences), Igκ-biotin (polyclonal; SouthernBiotech), CD45R–BV510 (RA3-6B2; BioLegend), IgG (H+L)–Cy3 (polyclonal; Invitrogen), B220–Cy5 (RA3-6P2; SouthernBiotech), CD19–eFluor V450 (1D3;

eBioscience), CD93–allophycocyanin (AA4.1; BioLegend), IgM–eFluor V450 (eB121-15F9; eBioscience), B220–FITC (RA3-6P2; SouthernBiotech), CD43–FITC (S7; BD Biosciences), H2-K^b–FITC (AF6-88.5; BD Biosciences), IgD–FITC (11-26C.2a; BD Biosciences), IgD–FITC (11-26C.2a; BD Biosciences), IgM-FITC (polyclonal; SouthernBiotech), IgM^b–FITC (AF7-78; BD Biosciences), Igκ–FITC (polyclonal; SouthernBiotech), IgD^a–Pacific Blue (AMS-9.1; BioLegend), SA–Pacific Blue (Invitrogen), H-$2D^d$–PE (34-2-12 BD Biosciences), CD23-PE-Cy7 (B3B4; eBioscience), CD5-PE-Cy7 (53-7.3; eBioscience), SA–V450 (BD Biosciences), Igλ5–biotin (LM34; produced in our laboratory), and Igλ5-DyLight 649 (LM34; AB-Direct). Analysis was performed using a FACSCalibur or an LSR II (Becton Dickinson). If not stated otherwise, numbers in the dot plots indicate percentages in the respective gates.

MACS cell isolation

Resting mouse B cells from the spleen were isolated from single-cell suspension using anti-mCD43 magnetically labeled beads according to the manufacturer's instruction. Separation of the unlabeled B lymphocytes was performed on an autoMACS column using the DepleteS program. Cells were counted with the automated CASY counter system.

Generation of V_H3-83δ KI mouse model

Cloning of the V_H3-83δ KI construct was performed with standard cloning techniques. To introduce the V_H3-83 segment into the J_H region of the murine Ig HC locus, we used a modified version of the IgV_H3-83 targeting construct (kindly provided by R. Pelanda) (34). Assembly of homology arms and V_H3-83 and Neo^R sequences, was designed the same way as in the IgV_HB1-8 KI targeting vector (38). To increase homologous recombination efficiency of the V_H3-83 targeting vector, the V_H3-83 gene segment was introduced between the Neo^R selection cassette and 5-kbps left homology arm. The long homology arm and the V_H3-83 VDJ sequence were cloned from the V_H3-83 targeting construct published previously (34). Frt-flanked Neo^R was cloned from the pFlrt vector (provided by Dr. Dimitry Penkov, Istituto FIRC di Oncologia Molecolare, Milan, Italy). The short homology arm, which was 800-bps long in the original plasmid, was extended to 2 kbps and it was cloned from two overlapping PCR fragments amplified from BALB/c tail gDNA template (Supplemental Fig. 1A). An auxiliary construct for optimizing the PCR-screening protocol contained the NeoR cassette and a 72-bp extended short homology arm. The vector was then employed to mediate replacement of the J_H segment, where naturally rearranged VDJ sequences are located. The optimized PCR was performed with the following primer pair: Neo_Frt3_fwd4 (5′-CAC TTC ATT CTC AGT ATT GTT TTG C-3′) and 383KI_gDNAup3 (5′-GTA GAG ACA GCA TCA GTA CCT CAA CTA-3′). Homologous recombination was detected by amplification of the 2140-bp PCR product. Homozygous IgM KO mice (26) on BALB/C background were used to generate an IgM KO embryonic stem (ES) cell line. Upon electroporation of the new V_H3-83 targeting construct into IgM KO ES cells, two clones were identified as positive in the PCR screen for recombination of the right homology arm. ES cells were injected into wild-type (WT) blastocyst, and few chimeric animals were raised. The male founder with a germline-transmitted V_H3-83 δ HC KI allele was mated with the Flp-Tg female (39), and double-Tg pups that excised Frt-flanked Neo^R sequence were further used to establish a mouse colony. Mice were genotyped by PCR using the following primers: 3-83:exon2 s (5′-GGT ATA CCT TCA CAA CTG CTG GAA TGC-3′), 3-83:WT3JH4 (5′-AGC CTC CAA AGT CCC TAT CCC ATC-3′), and 3-83:JH3 dir (5′-CAA GGG ACT CTG GTC ACT GTC-3′).

Results

Generation of V_H3-83 δ KI mice

As mentioned above, we have previously assessed with multiple in vitro experimental settings that IgD has a higher activation threshold than IgM, being selectively responsive to Ag in its polyvalent form (9, 32). In the current study, we aimed at translating our findings in vivo to understand their physiological implications. To compare the signaling of IgM and IgD BCR isotypes, we chose the 3-83 idiotype (40). The 3-83 idiotype specifically recognizes MHC class I (MHC I) with high and medium affinity to H-$2K^k$ and H-$2K^b$, respectively, whereas binding to H-$2K^d$ allotype ranked as the lowest tested (41). Although affinity of the 3-83 BCR to the H-$2K^d$ is identified as ultralow (41), conditional expression of the 3-83 IgM at the immature B cell stage on an H-$2K^d$ background can lead to receptor editing in some murine models (34). It was suggested that BCR expression requires a minimal threshold of basal signaling, also called "tonic" signaling, which is not reached on the H-$2K^d$ background. This would be due to Ag-independent background-related factors, which impact the expression level of the exogenous BCR (42). However, in our view, this is most likely caused by weak interaction of the 3-83 IgM BCR with unknown autoantigens.

Therefore, the 3-83 idiotype might be a powerful tool for comparing the signal strength of IgD and IgM BCR isotypes upon engagement with cognate autoantigen or upon weak nonspecific interaction with various autoantigens. To design an in vivo model with a 3-83 BCR expressed exclusively as an IgD isotype, we performed a V_H3-83 KI targeting in IgM KO ES cells (26). To this aim, we used an optimized version of the original 3-83 targeting vector (34) (Supplemental Fig. 1A). The resulting V_H3-83 KI IgH allele in the IgM KO locus is indicated as δH^i, and the original 3-83 KI (34) is referred to as μH^i. To achieve the expression of the 3-83 BCR idiotype as IgM or IgD at the immature B cell stage, mice carrying either μH^i or δH^i alleles were crossed with animals having the Vκ3-83 KI in the κ LC gene locus (34), indicated as κ^i. The resulting progenies, carrying homo- or hemizygous KI alleles in IgH and κ LC gene loci expressed 3-83 IgM or 3-83 IgD BCR at the immature cells stage (Supplemental Fig. 1B). To compare the capability of IgM and IgD to induce editing of autoreactive 3-83 idiotype, we bred these mice onto an H-$2K^b$ background to obtain exposure to specific autoantigens or onto an H-$2K^d$ background lacking a specific autoantigen.

V_H3-83 δ KI mice stably express IgD BCR only in the absence of Ag

To evaluate the expression of the 3-83 IgD KI and its stability in the presence or absence of Ag in B cells of our novel mouse model, bone marrow and spleen B lymphocytes from 3-83 IgD KI mice ($\delta H^{i/i}\kappa^{i/+}$) were stained with idiotype-specific Abs 54.1 (Fig. 1A). On the H2-K^d background lacking the cognate Ag for the 3-83 receptor a major fraction of 3-83 IgD KI B cells preserve the 3-83 idiotype, not only as immature cells in the bone marrow but also in the spleen as mature cells (Fig. 1A, 1B). Although 3-83 IgD-expressing cells retain the original idiotype, the responsiveness of the 3-83 IgD BCR is not abolished. Indeed, in the presence of Ag, namely on the H-$2K^b$ background, 3-83 IgD KI immature B cells undergo receptor editing, leading to loss of the 3-83 idiotype (Fig. 1A, 1B). To test whether 3-83 IgD KI mice with preserved idiotype acquire an anergic state, B cells were stimulated ex vivo with anti-IgD Ab, and the Ca^{2+}-mediated response was measured. The 3-83 idiotype-positive cells are activated upon BCR engagement, as shown by intracellular Ca^{2+} flux analysis (Supplemental Fig. 1C).

Data presented in Figure 1A and 1B refer to mice carrying two V_H3-83 δ HC KI alleles and V_K3-83 KI in one allele of the IgL gene locus ($\delta H^{i/i}\kappa^{i/+}$). To study the effects of hetero–homozygosity of the KI alleles, all genotype configurations were analyzed on the non-autoantigenic background (Fig. 1C). We found that the 3-83 IgD BCR is stable when the V_H3-83 δ HC KI is inserted in both copies of the IgH gene locus, with percentages of B cells preserving the idiotype above 80%, regardless of the KI configuration of the Igκ gene locus. In contrast, when the second allele of the IgH gene locus is unmodified, most B cells stain negative for the 54.1 Ab, which specifically recognizes the 3-83 idiotype. Loss of the 3-83 idiotype is caused by the inability of 3-83 IgD to sustain allelic exclusion, as explained later in this study. As only in the genotypic configuration $\delta H^{i/i}\kappa^{i/+}$ is the KI allele expressed on a considerable number of cells, allowing the analysis of the phenotype, this is used throughout this study as a reference genotype. Concerning the 3-83 IgM receptor, whereas 3-83 idiotype partially persists in mice with two V_H3-83 μ and two Vκ3-83 KI alleles, the vast majority of 3-83 BCR-expressing cells are modified by receptor editing in mice in which HC and/or LC KI is on one

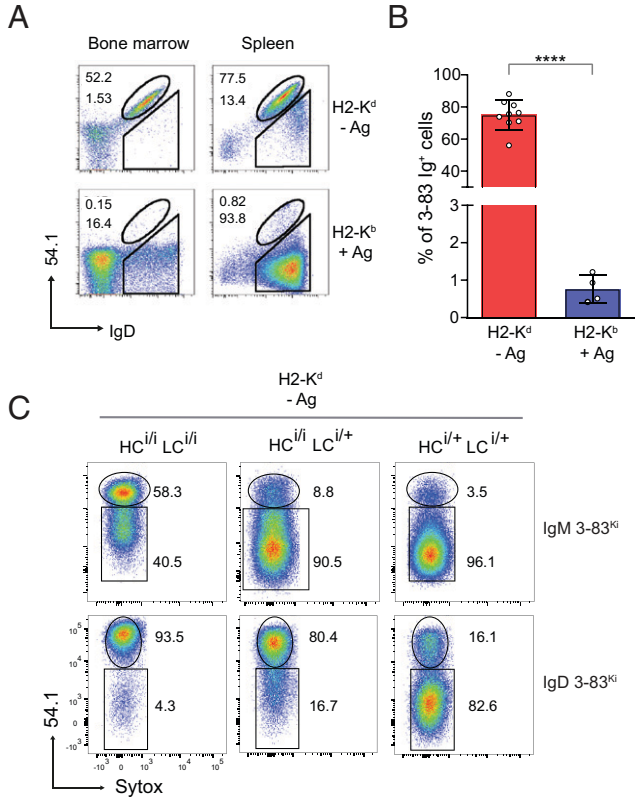

FIGURE 1. Autoreactive 3-83 IgD BCR does not support receptor editing on H-2K^d background. (**A**) Representative flow cytometric analysis of 3-83 idiotype expression using the idiotype-specific 54.1 Ab on bone marrow and splenic B cells (gated on B220$^+$) from mice of δH$^{i/i}$κ$^{i/+}$ genotype and the indicated background. Numbers in the plots indicate percentages of events in the respective gate. (**B**) Quantitative analysis of fraction (in percentage) of 3-83 idiotype-positive cells of all B220$^+$ lymphocytes in the spleen, as in (A). Analysis comprised results of four independent experiments. ****$p < 0.0001$ by Welch's t test. (**C**) Representative flow cytometric analysis of 3-83 idiotype expression using the idiotype-specific 54.1 Ab on splenic B cells (gated on B220$^+$) from mice of indicated genotype and background. Numbers on the plots correspond to the percentages of cells within respective gates.

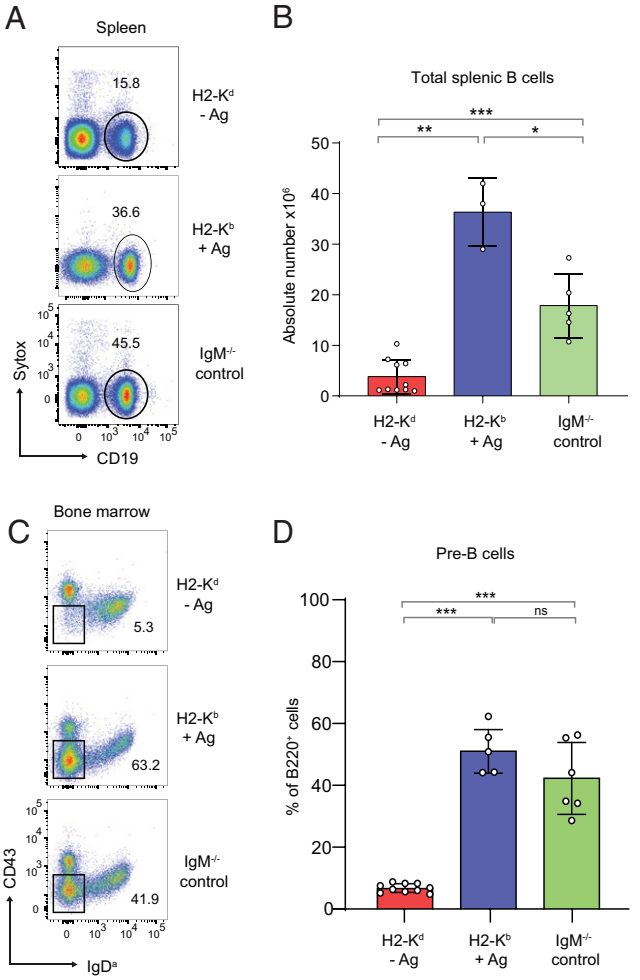

FIGURE 2. Absence of Ag causes lack of pre- and mature B cells in 3-83 IgD KI mice. (**A**) Quantification of B lymphocytes (CD19$^+$ lymphocytes) by flow cytometric analysis in the spleen of δH$^{i/i}$κ$^{i/+}$ H2-K^d mice. Data are representative of at least three experiments. Numbers on the plots correspond to the percentages of cells within the gate. (**B**) Quantitative analysis of absolute number of B lymphocytes (B220$^+$) in the spleen of 6- to 8-wk-old mice of indicated genotypes based on flow cytometric analysis. Results represent analysis of four independent experiments. *$p = 0.03$, **$p = 0.007$, ***$p = 0.0007$ by Mann–Whitney U test. (**C**) Flow cytometric analysis of pre-B (CD43$^-$IgD^{a-}) lymphocytes in the bone marrow. All displayed events were positively selected for pan–B cell marker B220 from the lymphocyte gate. Numbers on the plots correspond to the percentages of cells within respective gates. (**D**) Quantitative analysis of fraction (in percentage) CD43$^-$IgD^{a-} pre–B cells of all B220$^+$ B lymphocytes in the bone marrow, as in (C). Analysis is performed on results from four independent experiments. ***$p < 0.0005$ by Welch's t test.

allele only. This is in line with what has been previously observed (34), and it is probably due to effects related to gene dose and related impact on protein expression. In conclusion, differently from the IgM counterpart, the 3-83 IgD is less efficient in mediating receptor editing in absence of cognate Ag, indicating the requirement for higher-affinity autoantigen to mediate efficient editing of IgD 3-83 idiotype. All of this evidence is in agreement with our data showing a higher activation threshold for IgD and a recent study that proposed that IgD is less sensitive than IgM to endogenous autoantigen (9, 32, 33).

Pre- and mature B cell compartments are altered in the V_H3-83 IgD KI mice lacking Ag

Next, we sought to understand the impact of early IgD 3-83 idiotype expression on B cell development. To this end, we analyzed central and peripheral B cell compartments in V_H3-83 IgD KI mice of our reference genotype (δH$^{i/i}$κ$^{i/+}$). We found that the absence of Ag leads to strong reduction in the number of B lymphocytes in the periphery (Fig. 2A, 2B). Animals that preserve 3-83 IgD receptor on mature B lymphocytes show reduced numbers of CD23^{high} CD21^{low} Fo.B cells, whereas the marginal zone compartment (CD23^{low}CD21^{high}) seems unaffected (Supplemental Fig. 2A, 2B). The presence of Ag is sufficient to restore the peripheral B cell compartment, as exemplified by numbers of splenic B cells in mice of H-2K^b background. As decline of Fo.B cells may occur upon compromised B cell development (43), we analyzed bone marrow cells of the V_H3-83 IgD KI mice on H-2K^d (no autoantigen) and H-2K^b (with autoantigen) backgrounds. We observed that expression of IgD with the 3-83 idiotype in the absence of Ag results in contraction of the B cell niche in the bone marrow to an almost exclusively CD43$^+$ B cell population (Fig. 2C, 2D). When expression of 3-83 IgD takes place in the presence of Ag (H-2K^b), B cell development in the bone marrow proceeds normally from CD43$^+$ to CD43$^-$ pre–B cell stage (Fig. 2C, 2D).

According to genetic (44, 45), gene expression, and functional (46) studies, CD43$^-$ B cells in the bone marrow are represented by

pre– and immature B cells that undergo pre-BCR–mediated clonal expansion and LC recombination. As it was shown that δ HC cannot replace μ HC in a pre-BCR (47), we hypothesized that 3-83 IgD KI cells on H-2K^d background are blocked in their development at CD43$^+$ stage because of a defect in pre-BCR signaling.

Pre-BCR containing 3-83 δ HC is not functional

To test the hypothesis of a defective 3-83 δ pre-BCR, we studied the capacity of V_H3-83 δ HC to form a functional pre-BCR complex with surrogate LC components. To this aim, we made use of our TKO cell line (35). This murine pre–B cell line derives from a RAG2/λ5/SLP65 TKO mouse model on a mixed H-2K^b and H-2K^d background. These cells lack expression of any endogenous BCR, and endogenous SLP65 is substituted with an engineered tamoxifen-inducible ER^{T2}-SLP65 fusion protein (48). This makes the TKO cell line a powerful tool to test autonomous activation of exogenous BCRs, which can be introduced by retroviral transduction and are allowed to signal only upon addition of 4-OHT in solution. We transduced TKO cells with λ5 and either V_H3-83 δ- or μ HC to achieve expression of either δpre-BCR or μpre-BCR. As indicated by λ5 expression analysis, δpre-BCR expression on the cell surface was significantly lower than the μpre-BCR (Fig. 3A). Measurement of Ca^{2+} mobilization downstream of pre-BCR as a direct readout of pre-BCR function revealed strongly reduced autonomous activity of δpre-BCR with respect to the μpre-BCR counterpart (Fig. 3B). In contrast, when TKO cells were transduced with the 3-83κ LC in place of λ5, enabling assembly of either 3-83 IgM or 3-83 IgD, expression of 3-83 BCR was equally efficient regardless the HC isotype (Supplemental Fig. 2C). This indicates that TKO cells can express V_H3-83 δ- or μ HC with the same efficiency, implying that lower expression of δpre-BCR is specifically due to defective assembly of V_H3-83 δ HC with λ5. Therefore, improper pre-BCR function contributes to the loss of clonal expansion after the pro–B cell stage in the 3-83 IgD KI mice lacking Ag. In contrast, despite inefficient pre-BCR function, developing cells can proceed to the immature B cell stage by expression of the conventional LC, which is already provided as a KI of the functional 3-83κ LC KI. When conventional BCR is activated by an autoantigen at the immature B cell stage, downstream signaling functionally operates as upon autonomous activation of the pre-BCR (13) and therefore leads to clonal proliferation and expansion of the CD43$^-$ B cell pool. As shown in Fig. 2C and 2D, in the presence of H-2K^b autoantigen, a CD43$^-$ B cell population appears in the bone marrow, suggesting that a potent autoantigen can replace pre-BCR function in 3-83 IgD KI mice (13). A fundamental task performed by the pre-BCR is the suppression of Rag1/2 genes expression; this prevents further Ig gene rearrangement, thus enforcing allelic exclusion. Underlying the nonfunctionality of the 3-83 δ-pre-BCR, we found markedly increased expression of Rag1 and Rag2 genes in splenic B lymphocytes of V_H3-83 IgD KI mice, with respect to control, as shown by real-time quantitative PCR analysis of the correspondent transcripts (Fig. 3C).

Loss of allelic exclusion in V_H3-83 IgD-expressing cells

As shown in Fig. 1C, mice with two copies of the V_H3-83 δ HC KI allele retain a vast majority of 3-83 idiotype-positive cells in the absence of Ag. In sharp contrast, in animals without Ag and with one single copy of V_H3-83 δ HC KI allele, the majority of mature B cells lose the 3-83 idiotype and stain negative for the idiotype-specific 54.1 Ab (Fig. 1C). We sought to understand how these cells lose the 3-83 idiotype on the non-autoantigenic H2-K^d background. In these heterozygous mice, activity of the second WT allele can be monitored with allotype-specific staining, in that the unmodified allele belongs to IgH^b allotype (C57BL/6 background), whereas expression of V_H3-83δ HC KI allele is identified by staining with anti-IgD^a (IgH^a, BALB/C background) Ab. We found that in bone marrow and blood of V_H 3-83 IgD KI mice lacking Ag (δH$^{i/+}$κ$^{i/+}$ on H2-K^d background), expression of the second HC allele (detected by anti-IgM^b staining) takes place in a cell fraction that is negative or low for IgD^a staining (Fig. 4, Supplemental Fig. 3A). Strikingly, in the spleen of δH$^{i/+}$κ$^{i/+}$ mice on H2-K^d background, all B cells expressing the WT alleles stained weakly positive for IgD^a and were thus IgM^{b+} IgD^{a+} double-positive. To set a negative/positive threshold for expression of IgD^a and IgM^b, we have employed splenic B cells from a C57BL/6 mouse and a homozygous V_H 3-83 IgD KI mouse, δH$^{i/i}$κ$^{i/+}$, equivalent to IgM$^{-/-}$ (Fig. 4, lower panels). We identified cells above these thresholds as an allelically included population, as they express both the V_H 3-83 IgD KI (as IgD^a) and the unmodified WT allele (as IgM^b) as BCRs on their surfaces. The fact that B cells acquire this peculiar phenotype only in the spleen may indicate a correlation with ageing or maturation of the cells.

In contrast to the increased proportion of HC inclusion on the H-2K^d background lacking autoantigen, the autoreactive H-2K^b background showed only a minor cell fraction in bone marrow and blood expressing IgM^b. Consequently, the allelically included population in the spleen is extremely small (Fig. 4, Supplemental Fig. 3A). This is in agreement with the restored B cell development in 3-83 IgD KI mice when the autoantigen is present. Hence, 3-83 IgD KI B cells show lack of efficient and stable monoallelic expression of the IgH gene in the absence of Ag.

Together, 3-83 IgD KI B cells are unable to establish or maintain allelic exclusion in the absence of cognate Ag. Results obtained

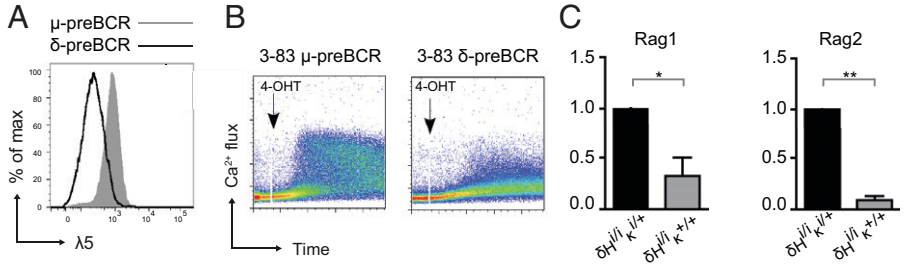

FIGURE 3. 3-83 δ HC cannot form a functional pre-BCR, and IgD 3-83 BCR fails to mediate downregulation of Rag genes in absence of Ag. (**A**) TKO-Est cell line was reconstituted with constructs for expression of pre-BCR with 3-83 μ and 3-83 δ HC. The histogram depicts λ5 expression in the pre-BCR complex with 3-83 μ HC and 3-83 δ HC. (**B**) Flow cytometric analysis of intracellular Ca^{2+} concentration upon application of 4-OHT (arrow). TKO-Est cell line was reconstituted with constructs for expression of pre-BCR with either 3-83 μ (left panel) or 3-83 δ HC (right panel). (**C**) Real-time PCR analysis of Rag1 and Rag2 expression in B cells from spleen of mice with H2-K^d background and indicated genotype. *$p < 0.05$, **$p < 0.01$ by Mann–Whitney U test.

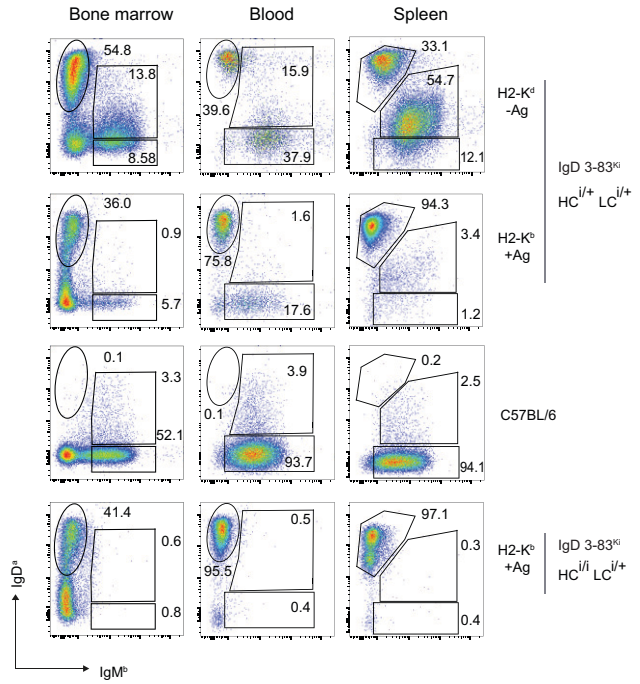

FIGURE 4. In absence of Ag 3-83 IgD KI fails to sustain monoallelic expression. Representative flow cytometric analysis of cells from indicated compartments of mice of the indicated genotypes (pregated on B cells: $B220^+/CD19^+$) for surface expression of IgD^a and IgM^b. Shown data are representative of three independent experiments. Numbers on the plots indicate percentage of events in the respective gate.

from the analysis of mice with different genotypic configuration and presence or absence of Ag, which are presented in Figs. 1 and 4, are summarized in Table I.

V_H3-83 IgD KI transcription is diminished in allelically included cells

To genetically characterize the allelically included cells, we FACS-sorted this population, which we refer to as biallelic population (IgD^{a+} IgM^{b+}), as well as the monoallelic population (IgD^{a+} IgM^{b-}), whose cells only use IgH^a KI-derived HCs in their BCRs, as depicted in Fig. 5A. Analysis of sorted cells showed purity of the two populations (Supplemental Fig. 3B). PCR analysis on genomic DNA extracted from the two target populations showed the presence of the WT allele (in germline configuration) and the KI allele (HC) in both cellular populations, implying that biallelic cells retain the KI at the genomic level (Fig. 5B). We next investigated gene expression of the KI allele. Quantitative real-time and standard PCR analyses performed on cDNA showed that the transcript is present in both populations, although in smaller quantities in the allelically included population. This indicates that the KI allele is transcriptionally repressed in these cells, thereby resulting in reduced IgD^a expression (Fig. 5C, 5D). Therefore, cells belonging to the biallelic population, retain both the KI and the WT allele at the genomic as well as at the transcriptional level. After assessing that both alleles are transcribed and that the respective proteins are expressed on the cell surface, we investigated functionality of these receptors. To this aim, we employed intracellular Ca^{2+} release as direct readout of BCR-mediated cellular activation. To discriminate between the IgD^{a+} IgM^{b+} biallelic cells and the IgD^{a+} IgM^{b-} monoallelic population without stimulating the BCRs, we employed IgM^b-Fab staining (Fig. 5E, left panel). Calcium flux in biallelic cells upon treatment with anti-κ Ab is comparable with that of the monoallelic population (Fig. 5E, right panels). This analysis bears a limitation in that it did not permit us to distinguish signaling triggered by IgD^a or IgM^b BCRs on biallelic cells. We detected similar intracellular Ca^{2+} release in mono- and biallelic cells upon stimulation with anti-IgD^a Abs (Supplemental Fig. 3C); however, signaling competence of these receptors needs to be further characterized with more experiments.

In summary, these data suggest that, in absence of cognate Ag 3-83 IgD, KI B cells cannot suppress gene recombination of the WT allele. After successful recombination, the IgM BCR expressed by the WT allele leads to suppression of the 3-83 IgD KI in the biallelic B cell population.

Allelic inclusion is found mainly in mature aged 3-83 IgD KI cells

As in heterozygous $\delta H^{i/+}\kappa^{i/+}$ mice on an H2-K^d Ag-lacking background we assessed a prevalence of allelically included cells in the spleen, where most mature and aged B cells reside, we next tested an eventual correlation of the phenotype with ageing. To this aim,

Table I. Summary of genotype configurations with related phenotypes and molecular mechanism

3-83 μ Ki	3-83 κ Ki	Ag	Idiotype Retention	Mechanism Involved	Conclusion
i/i	i/+	+	−	Cognate Ag induces signaling above threshold of activation.	IgM has a lower activation threshold than IgD.
i/i	i/+	−	−	Unspecific Ag induces signaling above threshold of activation.	
i/i	i/i	−	+/−	Gene dose	Excessive gene dose partially inhibits editing.
i/+	i/+	−	−	Unspecific Ag induces signaling above threshold of activation.	IgM has a low activation threshold.
3-83 δ Ki					
i/i	i/+	+	−	Cognate Ag induces signaling above threshold of activation.	IgD has a higher activation threshold than IgM.
i/i	i/+	−	+	Unspecific Ag induces signaling below threshold of activation.	
i/i	i/i	−	+	Gene dose and absence of specific Ag	Excessive gene dose and signaling below activation threshold.
i/+	i/+	−	−	Reduced tonic signaling by IgD	Loss of allelic exclusion causes replacement of KI allele with WT allele.
i/+	i/+	+	+	BCR engagement by Ag increases tonic signaling by IgD.	BCR engagement by Ag restores allelic exclusion.

Conclusions drawn by analysis of mice with different genotypes are summarized in the table. Each genotype configuration is described, and the molecular mechanism causing the observed phenotype (idiotype retention or not) is explained. Ag presence (indicated by "+") refers to H2-K^b background; Ag absence (indicated by "−") indicates H2-K^d background.

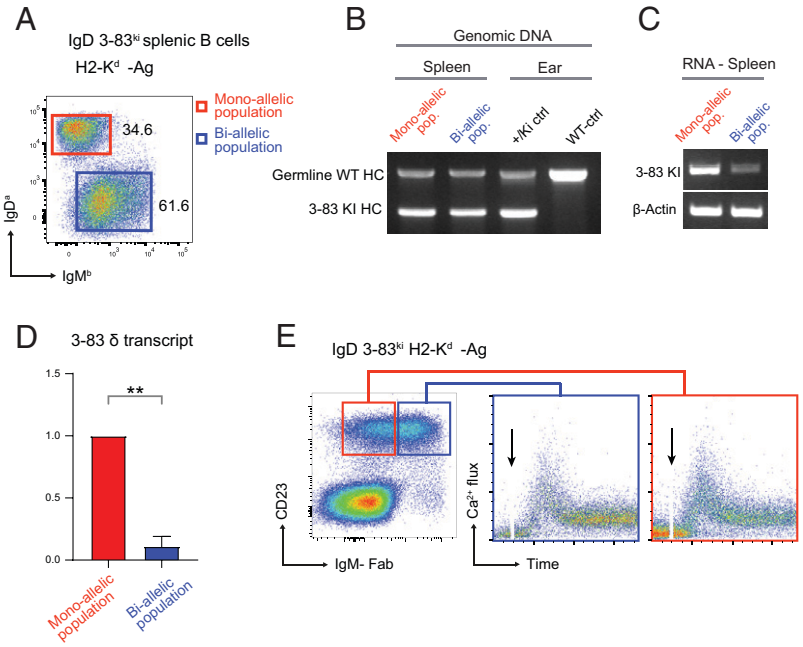

FIGURE 5. KI allele is transcriptionally repressed in allelically included cells. (**A**) Flow cytometer analysis scheme for sorting of monoallelic (IgD^{a+}, red gate) and biallelic (IgD^{a+} IgM^{b+}, blue gate) B cells from spleen of $\delta H^{i/+}\kappa^{i/+}$H2-$K^d$ mice. (**B**) PCR fragments amplified with specific primers for JH3 and JH4 (HC of WT allele in germline configuration) and 3-83 V_H VDJ and JH4 (HC of KI allele) from genomic DNA of purified splenic B cells of the indicated population [as per scheme in (A)] from $\delta H^{i/+}\kappa^{i/+}$H2-$K^d$ mice. Genomic ear DNA from a $\delta H^{i/+}\kappa^{i/+}$ mouse (indicated as +/KI ctrl) and from a C57BL/6 (WT-ctrl) were used as controls. The results are representative of three independent experiments. (**C**) PCR analysis on cDNA generated from RNA from samples of the indicated B cell population [as per scheme in (A)] from a $\delta H^{i/+}\kappa^{+/i}$ H2-K^d mouse. For amplification of 3-83 KI allele, the same primers were used as in (B). β-actin was used as loading control. (**D**) Real-time PCR analysis of 3-83 KI allele expression in the two target populations from spleen of $\delta H^{i/+}\kappa^{+/i}$ H2-K^d mice. Data were collected from three independent experiments. $^{**}p < 0.01$ by Mann–Whitney *U* test. (**E**) Intracellular calcium flux analysis in target populations of $\delta H^{i/+}\kappa^{i/+}$ H2-K^d upon treatment with anti-κ Ab (indicated by the arrow). The left panel, showing all cells contained in lymphocytes gate, shows pregating used for calcium flux-monitoring experiment to distinguish between the monoallelic ($CD23^+IgM^{b-}$) cells and the biallelic ($CD23^+IgM^{b+}$) splenic populations.

we analyzed mice belonging to different age groups. We detected a clear trend, with the proportion of monoallelic splenic B cells (expressing the 3-83 KI allele only) gradually decreasing with ageing and the biallelic population following an opposite tendency, increasing in spleens of aged mice (Fig. 6A). In sharp contrast, when V_H 3-83 IgD KI was expressed in the presence of Ag (on a H2-K^b background), the proportion of allelically included cells was extremely small and constant throughout the age groups (Fig. 6B). Moreover, in mice lacking the Ag, the great majority of allelically included cells do not express CD93, a marker specific for early B cell immigrants in spleen (Fig. 6C) (49). This is further proof for an association of the allelic-inclusion phenotype with maturation of cells. Together, the data show that allelically included cells accumulate with age in V_H 3-83 IgD KI mice lacking cognate autoantigen, suggesting that the expression of WT allele enables the development and maintenance of these cells as compared with the monoallelic cells.

Discussion

In this study, we tackle the enigma of the dual expression of IgM and IgD BCRs on mature naive B cell surfaces. We address the hypothesis of a different sensitivity to endogenous Ag for IgM and IgD (33) by employing the well-studied 3-83 BCR, which recognizes MHC I allele H2-K^b with low affinity. We generated and analyzed a mouse model that expresses 3-83 idiotype as IgD BCR isotype only. These mice were bred onto an autoreactive H2-K^b background as well as a non-autoreactive H2-K^d background. A counterpart model that carries Tg rearranged V region genes in the IgH and Igκ loci to encode 3-83 idiotype as both IgM and IgD, which we refer to as 3-83 KI mouse, was already available (34). Therefore, we could compare how different HC isotypes impact recognition of low-affinity autoantigen(s) and its effect on B cell development, central tolerance, and allelic exclusion establishment and sustenance. In agreement with previous work (9, 32, 33), we found that IgD, with respect to IgM, requires higher-affinity Ag to activate downstream signaling, rendering it tolerant to weak (auto)antigen. Indeed, whereas IgM edits the 3-83 specificity already on a H2-K^d background because of unspecific interactions with unknown autoantigens, IgD requires the presence of the cognate Ag, which is achieved on the H2-K^b background, to trigger receptor editing. Distinctive reactivity of the same 3-83 idiotype BCR, expressed as IgM or IgD at the immature B cell stage, profoundly determines B cell development in the 3-83 IgD KI mouse model. In contrast to the 3-83 KI mouse model and IgM KO animals (34), mice with 3-83 IgD KI display a poor B cell development on H-2K^d background because of the lack of the pre–B cell stage, which leads to a strong reduction in mature B cell pool. This is the result of the inability of the δ HC to make a functional pre-BCR. In turn, this leads to dysregulation of Rag 1/2 gene transcription in 3-83 IgD KI mice (45, 50). Because 3-83 IgD KI B cells lack both the primary Ig rearrangement and the receptor editing stage, it is possible that these B lymphocytes leave the bone marrow prematurely. Continuous expression of Rag genes may lead to recombination and activation of the second HC allele, leading to the appearance of allelically included cells in the spleen. Additionally, it was shown that tonic signaling from the receptor is essential to silence Rag genes in peripheral B cells and that loss of surface BCR leads to Rag1/Rag2 expression (51, 52). Because of its intrinsic high-activation threshold, it is conceivable that 3-83 IgD does not obtain those loose

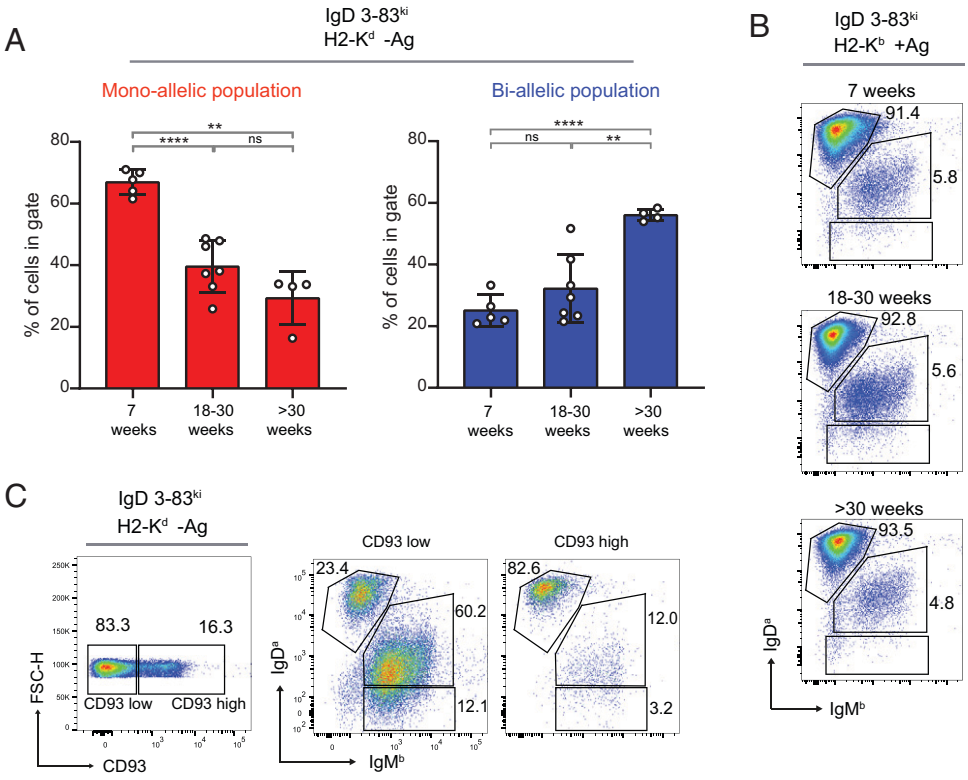

FIGURE 6. WT allele prevails on 3-83 IgD KI in aged mice. (**A**) Quantitative analysis of fraction (as percentage) of B lymphocytes ($B220^+$) belonging to the monoallelic or the biallelic population in the spleen of $\delta H^{i/+}\kappa^{i/+}$H2-$K^d$ mice as defined in Fig. 5A. Results comprise analysis of 16 mice. $^{**}p = 0.007$, $^{****}p = 0.0007$ by Mann–Whitney *U* test. (**B**) Representative flow cytometric analysis of cells from spleen of $\delta H^{i/+}\kappa^{i/+}$H2-$K^b$ mice. Anti-IgD^a and anti-IgM^b Abs are used to distinguish between the mono- and the biallelic population. Mice belonged to different age groups as indicated. Shown data are representative of at least three independent experiments. Numbers on the plots indicate percentage of events in the respective gate. (**C**) Representative flow cytometric analysis of splenic B cells from $\delta H^{i/+}\kappa^{i/+}$ H2-K^d mice in which CD93 is used to distinguish between mature lymphocytes and early immigrants in the spleen (left panel). The two subpopulations ($CD93^{low}$, mature B cells; $CD93^{high}$, early immigrants) are then analyzed with allotype-specific Abs.

antigenic interactions that are presumably important for the generation of survival/tonic signaling provided by the BCR to sustain B cell survival (33). This leads to failure in the prevention of expression of the second HC allele, causing the emergence of the WT allele. IgM BCR generated by physiologically random recombination of the WT allele provides better survival signals that, in the long-term, can induce this allele to prevail on the IgD KI allele. In heterozygous mice, in which μ HC is available and a μ-pre-BCR can provide the required autoreactivity (13), generation of B cells is more efficient. This leads to accumulation of cells derived from the WT allele, over the KI allele, which can only provide δ HC, not suitable for formation of a functional pre-BCR. Therefore, the IgD BCR fails to promote its own expression, and the corresponding allele is outcompeted in the race for allelic exclusion. In this scenario, the expression of the artificially provided KI allele, which drove development of the clone, slowly attenuates in time in favor of the second allele, leading to the emergence of a "hidden specificity." This would be the result of epigenetic opening of the second allele mediated by de-heterochromatization, histone reacetylation, and DNA demethylation (21). Allelic inclusion, causing the presence of multispecific lymphocytes, has been previously described in the T cell repertoire of normal mice (53).

As discussed, we show that the V_H3-83 δ HC is not able to form a functional pre-BCR complex with the surrogate LC, resulting in compromised pre–B cell development in the 3-83 IgD KI mice on a non-autoantigenic H-$2K^d$ background and consequent scarcity of lymphocytes in the bone marrow and peripheral niches. It was not tested whether clonal deletion contributes to this phenotype. Two elegant studies addressing the contribution of receptor editing and clonal deletion of IgHEL (54) and Ig3-83 (55) BCRs convincingly showed that, in contrast to the respective conventional Tg models, mice with KI HC and LC use receptor editing as the main mechanism to eliminate autoreactive BCRs.

Intriguingly, despite the evidence that IgD receptors have higher threshold for editing the autoreactive BCRs, neither 3-83 IgD KI animals nor IgM-deficient mice with polyclonal repertoire show signs of autoimmunity (data not shown). Retention of the 3-83 idiotype on mature B lymphocytes does not lead to accumulation of self-specific Abs; neither does it lead to the MHC I autoantigen nor to other self-antigens.

It was previously shown that, whereas IgD is more efficient than IgM in the recruitment of B cells into germinal centers and consequent maturation of Ab responses (9), IgM is specialized for induction of short-lived $IgG1^+$ plasma cells (33). This indicates that IgD activation directs lymphocytes toward differentiation into IgG-secreting plasma and memory B cells, whereas IgM-induced signaling mediates rapid terminal differentiation with consequent failure to enter the memory repertoire. In this view, IgD, with its high-activation threshold, acts as a gatekeeper for memory response. In contrast, IgM is more easily activated and, accordingly, more prone to generation of autoreactive clones than IgD. However, IgM is specialized for quick, short-lasting response, not for induction of memory response, and this, along with spatially and temporally restricted expression, prevents uncontrolled activation of IgM^+ cells. Consequently, IgD-deficient mice do not develop autoimmunity in that IgM-only cells fail to trigger the generation of

anti–self-IgG–producing plasma cells. It was suggested that IgM is the preferred BCR isotype in some B cell malignancies, such as activated B cell–like diffuse large B cell lymphoma (56). As it was shown that activated B cell–like diffuse large B cell lymphoma is BCR signaling driven (57), it is conceivable that, because of its ease to activate the signaling pathway (9, 32) and its better ability to induce prosurvival and mitogenic signals (58), IgM outcompetes other isotypes in induction of survival and proliferation in transformed B cells.

The incompetence of IgD in establishing allelic exclusion, unless an Ag is available, which we report in the current study, impacts interpretation of data in the literature. If the LC gene is free to recombine, as in the case of Tg animals with random integration of the Tg cassette, it is likely that all sorts of specificities, including the mildly autoreactive ones that are necessary for driving early B cell development, are generated. This might explain how B cells in Tg mice expressing IgD transgenes establish allelic exclusion in the absence of Ag. Therefore, all experiments that rely on the monospecificity of the repertoire of these mice have to be re-evaluated. To prevent endogenous genes from recombining, introducing new specificities in the repertoire, these mouse lines should be crossed to a Rag 1/2–deficient mouse line. The resulting line could be used as a reliably monoclonal repertoire mouse model.

In recent years, several studies that helped shape the opinion of the community about the roles of the two BCR isotypes in tolerance and Ag recognition have contributed major advances to this research field. Wilson and colleagues (10) identified a novel subpopulation of naturally occurring mature naive autoreactive B cells in humans that downregulated IgM but maintained high levels of surface IgD. Mature naive autoreactive B cells were found in healthy donors that showed no signs of autoimmunity, indicating that downregulating IgM is sufficient to prevent these cells from attacking self-structures. Recently, Zikherman and colleagues (59) exploited an in vivo reporter of Ag recognition, Nur77-eGFP BAC Tg, and found that, unexpectedly, a large proportion of B cells do encounter Ag during late development in the spleen. Also, they found that the extent of Ag recognition during development correlates with IgM downregulation, whose expression varies greatly among Fo.B cells. Later, the same group generated Nur77-eGFP reporter mice deficient for either IgM or IgD and found that, on the same genetic background, IgD-only cells show weaker BCR activation, indicating that IgD is less sensitive than IgM to bona fide endogenous Ags in vivo (33). In light of these data, it is clear that an alarmingly high amount of B cells reactive to endogenous Ags reach the periphery, are unresponsive to self, but retain responsiveness to foreign Ags. This seems to be achieved by responsiveness tuning via shift in the IgM/IgD ratio. In our view, this shift serves as an adaptive system for adjusting sensitivity to the available Ags in the course of B cell development. IgM guarantees the high sensitivity required at the immature stage for receptor editing. IgD as a dominant type of BCR guarantees the quiescence of B cells bearing mild autoreactive BCRs in the encounter of low-affinity self-antigens in the periphery. This would ensure maintaining the broadest possible spectrum of specificities in the repertoire while avoiding autoimmunity. As polyreactivity of BCR has been previously described (60), it is legitimate to assume that mildly autoreactive specificities would be of value. Indeed, mildly autoreactive clones might recognize nonself molecules as well, thereby expanding the repertoire of useful specificities, explaining the evolutionary advantage provided by retention of anti–self-clones.

Polyreactive B cell clones that have mild affinity to a range of endogenous and exogenous Ags might bring great benefit, considering how important timing of immune reaction is and that low affinity could be resolved by germinal center reaction and related somatic hypermutation. The shift to δ HC provides polyreactive BCRs, which partially fit autoantigens in their Ag binding pocket, with the capability to remain passive upon encounter of such Ags while preserving ability to signal when exposed to Ag that fully engages the receptor. In this picture, activation of innate immunity that occurs upon infection, which is often essential for triggering a proper reaction, would assist auto-polyreactive B cell clones in the distinction between self and nonself, functioning as a further regulatory mechanism. Our findings contribute fundamental evidence for an essential role of BCR isotype shift along the life of a B cell, and in conjunction with other recent studies (10, 33, 59), ultimately establish the role of IgD in tolerance and Ag recognition. Also, our novel, to our knowledge, mouse model will allow in depth study of allelic exclusion in that it provides a system were a KI allele systematically drives the B cell clone through development to then diminish its expression in favor of a more autoreactive second allele along maturation of the cell.

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Disclosures

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