Antibody repertoire diversification through VH gene replacement in mice cloned from an IgA plasma cell

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In mammals, VDJ recombination is responsible for the establishment of a highly diversified preimmune antibody repertoire. Acquisition of a functional Ig heavy (H) chain variable (V) gene rearrangement is thought to prevent further recombination at the IgH locus. Here, we describe V_HQ52^{NT}; $V\kappa gr32^{NT}$ Ig monoclonal mice reprogrammed from the nucleus of an intestinal IgA^+ plasma cell. In V_HQ52^{NT} mice, IgA replaced IgM to drive early B-cell development and peripheral B-cell maturation. In V_HQ52^{NT} animals, over 20% of mature B cells disrupted the single productive, non-autoimmune IgH rearrangement through VH replacement and exchanged it with a highly diversified pool of IgH specificities. VH replacement occurred in early pro-B cells, was independent of pre–B-cell receptor signaling, and involved predominantly one adjacent V_H germ-line gene. VH replacement was also identified in 5% of peripheral B cells of mice inheriting a different productive V_H rearrangement expressed in the form of an IgM H chain. In summary, editing of a productive IgH rearrangement through VH replacement can account for up to 20% of the IgH repertoire expressed by mature B cells.

B cells | VH replacement | antibody repertoire | IgA | nuclear cloning

A functional immune system relies on the ability of B-lymphocytes to recognize foreign antigens in a highly specific fashion through the Ig receptor (also called B-cell receptor, or BCR). Each BCR consists of two identical Ig heavy (H) and light (L) chains, which are expressed on the surface of the B cell together with an Igα/Igβ heterodimer to form a signaling unit. In most vertebrates, the ability of the immune system to generate a highly diversified repertoire of antibody specificities relies on the stochastic assembly of variable (V), diversity (D), and joining (J) gene segments that encode for the antigen-binding domain of IgH and IgL chains of the BCR. This process, called V(D)J recombination, is mediated by the ordered recruitment at Ig loci of the RAG-1/2 endonucleases, followed by nonhomologous end-joining factors that catalyze the joining of the cleaved DNA segments (1). The latter processes are often accompanied by trimming and insertion of *n*-nucleotides at junctional ends. All together these mechanisms contribute to generate a highly diversified pool of Ig specificities.

Ig receptor editing provides B cells with the opportunity to exchange BCR specificity through secondary VDJ recombination. Whereas IgL chain editing was shown to play a central role in the neutralization of autoimmune BCR specificities expressed by newly generated immature B cells (2), the contribution of IgH receptor editing to antibody repertoire diversification has remained largely unappreciated. Two mechanisms promote secondary IgH rearrangements. In V_H-to-J_H direct joining, RAG proteins cleave at Recombination Signal Sequences (RSSs) of V_H and J_H elements lying, respectively, upstream and downstream of the original V_H rearrangement, followed by microhomology-driven joining of the Ig gene segments. This mechanism has been mainly observed in IgH knock-in mice carrying nonproductive IgH rearrangements (3, 4). VH replacement relies, instead, on evolutionary conserved cryptic RSSs embedded within the framework region 3 of most V_H germ-line genes (5). During VH replacement, cryptic recombination signal sequences (cRSS) within the V gene of a preexisting V_H rearrangement are engaged in a RAG-mediated recombination reaction together with RSSs of an upstream V_H germ-line gene, in accordance with the 12/23 rule (reviewed in ref. 6). VH replacement generates a novel V_H rearrangement that carries a different V gene and shares with the original one part of its Complementary Determining Region 3 (CDR3). Studies in B-lymphoma cell lines were the first to identify VH replacement as a mechanism to edit both in-frame (IF) and out-of-frame (OF) IgH rearrangements (7, 8). Later analyses with IgH knock-in mice confirmed in vitro studies, unraveling the potential of VH replacement to rescue progenitor B cells carrying nonproductive V_H rearrangements (4, 9). VH replacement has also been proposed to diversify the preimmune repertoire of productive IgH specificities in both human and mice (10–12). Bioinformatic analyses of IgH V gene repertoires obtained through next-generation sequencing have shown limitations to identify VH replacements (13). Studies on IgH transgenic mice have in part overcome such limitations (9, 11, 14–17). However, the targeting strategy to generate most IgH knock-in mice may severely limit the interpretation of VH replacement data obtained from such models. Indeed, in most IgH knock-in mice, prerearranged V_H genes replace the four J_H segments of the IgH locus. This atypical

Significance

Antibodies produced by B cells provide a protective barrier to our organism against the penetration and dissemination of microorganisms. Each antibody recognizes a specific antigen through variable (V) region domains of pairs of immunoglobulin (Ig) heavy (H) and light (L) chains. In mammals, VDJ recombination generates a highly diversified preimmune pool of V_H and V_L domains. Acquisition of a functional V_H rearrangement is thought to prevent further VDJ recombination at the IgH locus. Instead, mice cloned from a terminally differentiated B cell unravel the ability of VDJ recombination to revise a functionally rearranged V_H gene through VH replacement. We show that up to 20% of the antibody V gene repertoire of mature B-lymphocytes can be generated through VH replacement.

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chromosomal configuration may affect the rate and nature of secondary IgH rearrangements. Intergenic Control Region 1 (IGCR1), which is crucial for ordered and lineage-specific VDJ recombination (18), represents one example of a *cis* regulatory element that is excised from the IgH locus during physiologic V-to-DJ recombination, but is retained in most IgH knock-in animals.

Recent advancements in ES gene targeting strategies have allowed the establishment of next-generation IgH knock-in mice where the insertion of a particular V_H rearrangement into the J_H locus is coupled to Cre recombinase-assisted deletion of the intervening region between D_H-proximal V_H genes and the J_H locus (4). This elegant approach relies on multiple targeting steps that are time consuming and may preclude germ-line transmission of targeted ES cells. Instead, somatic cell nuclear transfer (SCNT) technology applied to B-lymphocytes allows the rapid generation of IgH monoclonal mice carrying V_H rearrangements placed in their physiologic location (19).

Here, we applied SCNT to establish a novel mouse strain (V_HQ52^{NT}; $V\kappa gr32^{NT}$) starting from the nucleus of a terminally differentiated IgA$^+$ intestinal plasma cell (PC). Cloned mice allowed the investigation of B-cell development and IgH repertoire diversification under conditions where a single, productive IgH rearrangement consisting of a D_H-proximal Q52 V_H gene was expressed from an IgA class-switched IgH locus. We could show that a BCR specificity selected in the lamina propria (LP) of the small intestine and expressed in the form of IgA could effectively drive early B-cell development and instruct peripheral B-cell subset differentiation. V_HQ52^{NT} mice allowed the study of the contribution of VH replacement to the diversification of the IgH antibody repertoire in mice starting with a single productive nonautoimmune IgH specificity. Surprisingly, our results indicate that up to 20% of IgH specificities expressed in the pool of mature B cells can be generated through VH replacement.

Results

Nuclear Reprogramming of Intestinal PCs. We applied SCNT to reprogram terminally differentiated IgA$^+$ PCs isolated from the LP of the small intestine of mice housed under specific pathogen-free conditions. Nuclear transferred ES (ntES) cell lines were established from independent IgA cloned embryos. Derivation of ntES lines from IgA PCs was confirmed by genomic PCR amplification of Ig H and L chain V gene rearrangements. Chimeric mice were obtained through blastocyst injection of one representative IgA ntES cell line. Southern blotting analysis and PCR amplification of tail-tip genomic DNA of chimeric offspring confirmed germ-line transmission of cloned Ig V gene rearrangements (Fig. 1*A* and Fig. S1 *A* and *B*). These data indicate that PCs can undergo nuclear reprogramming to become pluripotent stem cells.

IgA Can Replace IgM to Drive B-Cell Development. IgA transnuclear mice allowed us to test whether an IgA BCR selected by an intestinal PC could replace IgM to drive B-cell development. IgA monoclonal mice inherited a productive, unmutated, V_H rearrangement consisting of the D_H-proximal *$V_HQ52.a27.79$* gene joined to *DFL16.1* and *J_H3* segments. The V_L gene rearrangement consisted of Vκ*gr32* joined to *Jκ4* (Fig. 1*B* and Fig. S1*B*). Mice inheriting prerearranged V_H and V_L genes were called, respectively, V_HQ52^{NT} and $V\kappa gr32^{NT}$.

V_HQ52^{NT};$V\kappa gr32^{NT}$ heterozygous (HT) mice were analyzed on the *Rag1*-deficient genetic background to study B-cell development under conditions of Ig monospecificity. V_HQ52^{NT}; $V\kappa gr32^{NT}$; $Rag1^{-/-}$ mice showed normal numbers of CD19$^+$ B cells, all expressing surface IgA (sIgA), in spleen (SP) and lymph nodes (LNs) (Fig. 1 *C* and *D*). Immunophenotypic analysis revealed the presence in the SP of mature follicular/B-2 (FO; CD19$^+$CD21$^+$CD23$^+$) and marginal zone (MZ; CD19$^+$CD21^{hi}CD23^{lo}) B cells (Fig. 1*E*). In contrast, B-1 B cells (CD19^{hi}CD23^{lo}) were largely missing in the peritoneal cavity of IgA monoclonal mice (Fig. 1*E*). These results indicate that a BCR specificity selected by an intestinal PC and expressed in the form of IgA can drive early B-cell development and promote differentiation into mature FO and MZ B cells.

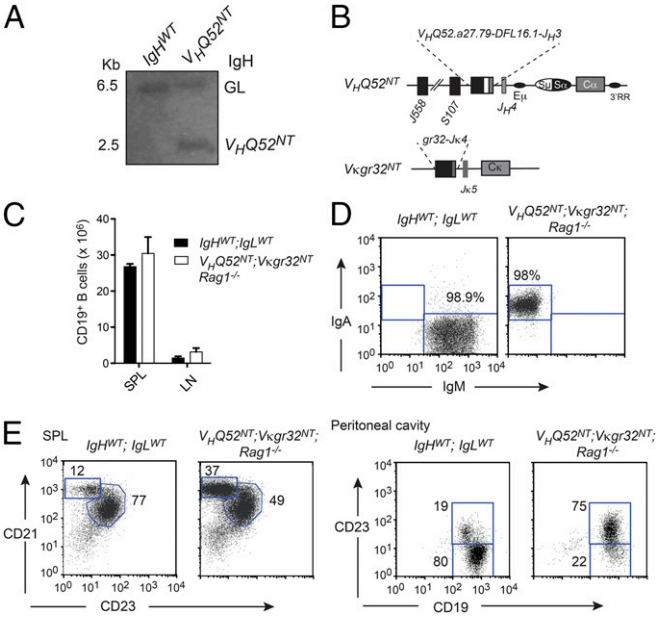

Fig. 1. B-cell development in V_HQ52^{NT}; $V\kappa gr32^{NT}$ monoclonal mice. (*A*) IgH Southern blotting analysis of wild-type and V_HQ52^{NT} HT mice. Bands corresponding to IgH germ line (GL) and V_HQ52^{NT} alleles are indicated. (*B*) Structure of rearranged IgH and Igκ loci in IgA transnuclear mice. (*C*) Numbers of CD19$^+$ B cells in SP and inguinal LNs of control (IgH^{WT};IgL^{WT}) and IgA monoclonal mice (V_HQ52^{NT}; $V\kappa gr32^{NT}$; $Rag1^{-/-}$), determined by flow cytometric analysis. (*D*) Representative flow cytometric analysis of splenic CD19$^+$-gated B cells in control ($n = 2$) and IgA monoclonal mice ($n = 2$). (*E*) Representative flow cytometric analysis of splenic CD19$^+$ B cells in controls and IgA monoclonal mice ($n = 2$). Peritoneal cavity B cells were analyzed after gating, respectively, on IgM$^+$ (IgH^{WT};IgL^{WT}) or IgA$^+$ (V_HQ52^{NT}; $V\kappa gr32^{NT}$; $Rag1^{-/-}$) cells ($n = 2$). Numbers indicate percentage of boxed B-cell subsets.

The V_HQ52^{NT}; $V\kappa gr32^{NT}$ BCR Is Neither Autoreactive Nor Signals Autonomously. Derivation of transnuclear mice from an intestinal PC predicts that the cloned BCR recognized a gut luminal antigen. Despite multiple attempts, we failed, thus far, to identify the selecting antigen. The polyreactive nature of IgA antibodies may lead to the recognition of self-antigens (20). To determine whether the BCR of V_HQ52^{NT}; $V\kappa gr32^{NT}$ B cells recognized common self-antigens represented by single- and double-stranded DNA, we measured anti-DNA antibody reactivity in the serum of V_HQ52^{NT};$V\kappa gr32^{NT}$;$Rag1^{-/-}$ monoclonal mice. ELISAs revealed minimal anti-DNA reactivity in the serum of IgA monoclonal mice, which was comparable to that of wild-type littermate controls and significantly lower than that of autoimmune-prone MRL-*lpr*/*lpr* mice (Fig. 2*A*). We also tested whether the cloned IgA BCR displayed spontaneous (antigen-independent) self-aggregation, possibly resulting from the binding to an internal epitope (21, 22). For this, we measured spontaneous Ca$^+$ mobilization in *Rag-2; λ5; SLP65* triple knockout (TKO) pro-B cells that were reconstituted with a BCR (in the form of IgM or IgA) carrying V_HQ52^{NT} and Vκgr32^{NT} specificities (Fig. 2*B* and Fig. S2*A*). As controls, TKO progenitors were reconstituted with either an autonomously active BCR, cloned from an autoreactive TCL1 transgenic B cell or a nonautonomously active hen egg lysozyme-specific BCR (HEL) (Fig. 2*A*). Expression of the TCL1-derived BCR induced a robust autonomous intracellular Ca$^+$ flux

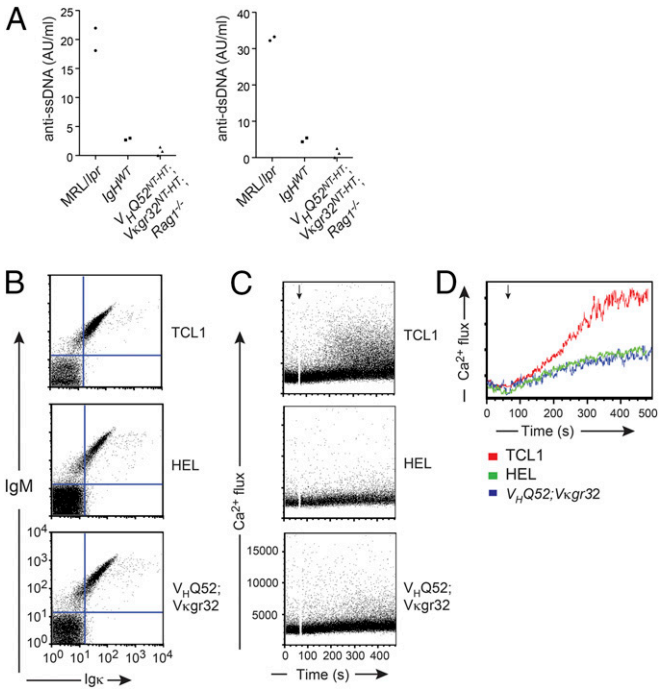

Fig. 2. Transnuclear IgA neither recognizes DNA nor signals spontaneously. (*A*) Serum titers of antibodies reactive against, respectively, single- (ss) and double (ds)-stranded DNA in IgA monoclonal mice (n = 3), age-matched littermate controls (IgH^{WT}; n = 2), and MRL/LPR (n = 2) animals. Each dot represents one animal. (*B*) Surface expression of IgH (IgM isotype) and IgL chains isolated from IgA monoclonal B cells reconstituted in *Rag-2; Slp65; λ5* TKO pro-B cells. Autonomously active TCL1-derived and nonautonomously active HEL-specific BCRs served as controls. (*C*) Flow cytometric measurement of spontaneous Ca^{2+} flux upon treatment with 4-OHT (black arrow) of BCR-complemented TKO pro-B cells to induce the activity of an ERT2–SLP65 transgene. (*D*) Overlay of spontaneous Ca^{+} fluxes shown in *C*. Data in *B–D* are representative of two experiments.

in TKO progenitors upon tamoxifen-dependent activation of an ERT2–SLP65 fusion protein (21). In sharp contrast, BCR specificities from both IgA transnuclear and HEL-specific B cells failed to trigger spontaneous Ca^{+} mobilization in response to SLP65 activation (Fig. 2 *C* and *D* and Fig. S2 *B* and *C*). These results, together with the observations that IgA monoclonal mice (both $V_HQ52^{NT\text{-}HT}$ and $V_HQ52^{NT\text{-}HT}$; $Vκgr32^{NT\text{-}HT}$ animals) aged in a comparable fashion to wild-type littermate controls lacked signs of systemic autoimmunity and displayed a normal (or, at most, lower) proportion of sIgλ^{+} B cells (Fig. S2*D*), render it unlikely that the IgA BCR expressed by transnuclear B cells is self-reactive.

V_HQ52^{NT} HT Mice Have a Substantial Number of IgM^{+} B Cells. Next, we analyzed B-cell development in V_HQ52^{NT} HT mice ($V_HQ52^{NT\text{-}HT}$) on a Rag-proficient background. CD19^{+} B cells in primary and secondary lymphoid organs were only modestly reduced in $V_HQ52^{NT\text{-}HT}$ mice compared with age-matched littermate controls (Fig. 3*A*). FACS analysis of SP and LN cell suspensions from $V_HQ52^{NT\text{-}HT}$ animals revealed that most B cells expressed sIgA (Fig. 3*B*). Analysis of Vκ gene rearrangements in sorted splenic IgA^{+} B cells revealed a highly diversified repertoire of IgL chains (Fig. 3*C*), thus excluding major restrictions in the pairing of the V_HQ52^{NT} IgA H chain. Remarkably, around 20% of B cells in the SP and LNs of $V_HQ52^{NT\text{-}HT}$ mice lacked sIgA and expressed instead IgM (Fig. 3 *D* and *E*). A similar fraction of sIgM^{+} B cells was found in the bone marrow (BM; Fig. 3*E*). Both IgA^{+} and IgM^{+} single-producer B cells matured into FO and MZ B cells (Fig. 3*F*), with the latter subset enriched among IgA^{+} B cells (Fig. 3*F*). In contrast, peritoneal cavity B-1 B cells consisted mainly of IgM^{+} B cells (Fig. 3*F*), in agreement with data obtained from V_HQ52^{NT};$Vκgr32^{NT}$;$Rag1^{-/-}$ mice. V_H gene rearrangement analysis revealed a highly diversified IgH repertoire expressed by IgM^{+} B cells of $V_HQ52^{NT\text{-}HT}$ mice (Fig. S3*A*). All together, these results indicate that the antibody repertoire of IgA monoclonal mice undergoes diversification through IgH editing.

V_HQ52^{NT} Mice Diversify the IgH Repertoire Through VH Replacement. The substantial number of IgM^{+} B cells found in lymphoid organs of $V_HQ52^{NT\text{-}HT}$ mice pointed to the silencing/inactivation of the V_HQ52^{NT} allele in these cells. Because IgM^{+} B cells were not identified in V_HQ52^{NT};$Vκgr32^{NT}$;$Rag\text{-}1^{-/-}$ animals, we hypothesized that Rag-dependent VH replacement was responsible for V_HQ52^{NT} inactivation. To this end, we performed genomic PCR on sorted IgM^{+} splenic B cells, using a pool of forward primers complementary to members of the main V_H families, except Q52. As a reverse primer, we used an oligonucleotide annealing to a footprint (FP) sequence of the CDR3 of V_HQ52^{NT} (Fig. 4 *A* and *B*). An RT-PCR approach using cDNA from sorted IgM^{+} B cells complemented the DNA analysis (Fig. S4*A*). Both strategies generated

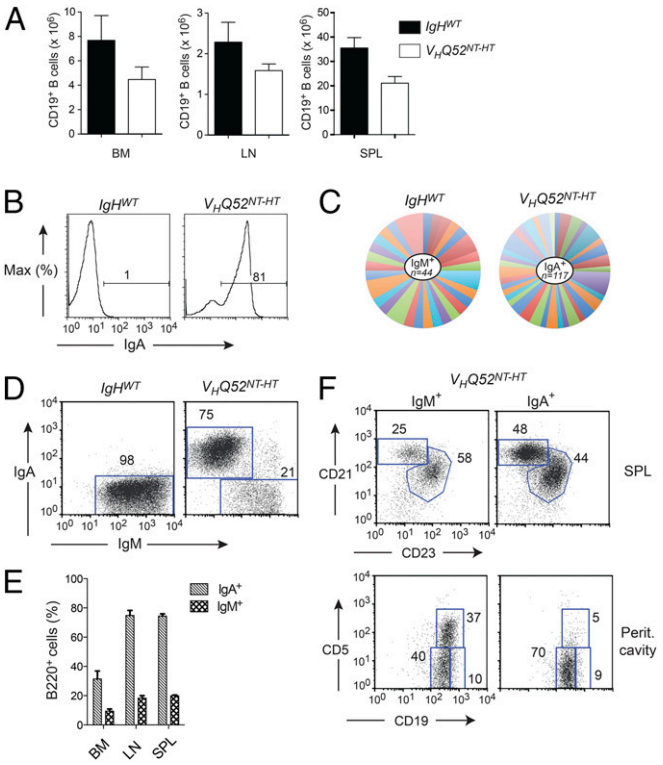

Fig. 3. A substantial number of B cells lose sIgA expression in V_HQ52^{NT} mice. (*A*) Absolute number of CD19^{+} B cells in BM, inguinal LNs and SP of controls (n = 6), and V_HQ52^{NT} HT (n = 7) mice. (*B*) Frequency of splenic CD19^{+}-gated sIgA^{+} B cells in representative $V_HQ52^{NT\text{-}HT}$ HT animals and littermate controls, as determined by flow cytometric analysis. Numbers indicate percentage of sIgA^{+} B cells. (*C*) Pie chart representation of Vκ gene rearrangement analysis obtained from splenic IgM^{+} B cells of controls (n = 2) and IgA^{+} B cells of $V_HQ52^{NT\text{-}HT}$ mice (n = 6). Each colored segment represents a unique rearrangement. Segment size defines frequency of individual Vκ rearrangements. (*D*) Representative flow cytometric analysis of SP B cells in controls (n = 6) and $V_HQ52^{NT\text{-}HT}$ mice (n = 7) stained for sIgM and sIgA, respectively. Numbers indicate frequencies of CD19^{+}-gated boxed B cells. (*E*) Frequency of sIgM^{+} and sIgA^{+} cells among B220^{+} B cells in lymphoid organs of $V_HQ52^{NT\text{-}HT}$ mice (n = 7). (*F*) Representative flow cytometric analysis of cell suspensions from SP and peritoneal cavity lavages of $V_HQ52^{NT\text{-}HT}$ mice, gating, respectively, on sIgM^{+} and sIgA^{+} B cells. Numbers indicate frequency of boxed B-cell subsets.

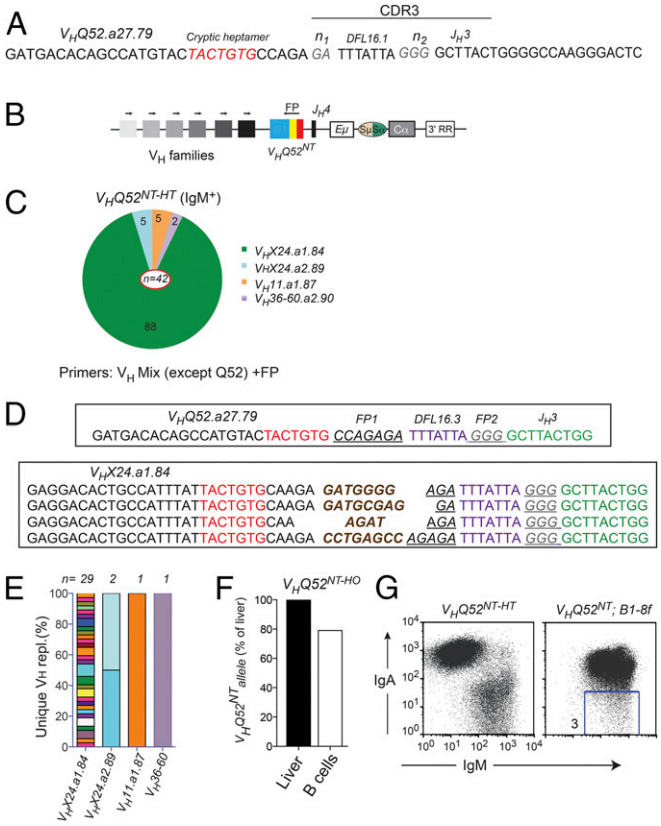

Fig. 4. V_HQ52^{NT} is edited through VH replacement. (*A*) CDR3 nucleotide sequence of V_HQ52^{NT}. Cryptic heptamer and *n*-nucleotides are indicated, respectively, in red and gray. (*B*) PCR strategy to identify VH replacements. Arrows indicate PCR primers. Colored blocks symbolize V_H families. An oligonucleotide annealing to a unique sequence (FP) within the CDR3 of V_HQ52^{NT} was used as a reverse primer. (*C*) Pie representation of V_H gene use in VH replacements cloned from splenic IgM$^+$ B cells of V_HQ52^{NT-HT} mice (n = 4). Numbers indicate frequency of rearrangements carrying the indicated V_H genes. PCR primers used for the analysis are indicated. (*D*) CDR3 nucleotide sequence of representative VH replacements cloned from IgM$^+$ B cells of V_HQ52^{NT-HT} mice using the $V_HX24.a1.84$ donor V_H gene. First line shows CDR3 sequence of the original V_HQ52^{NT} rearrangement. Underlined are V_HQ52^{NT} FP sequences indicated, respectively, as *FP1* and *FP2*. De novo added *n*-nucleotides are labeled in brown. cRSSs are indicated in red. (*E*) Frequency of unique VH replacements cloned from IgM$^+$ splenic B cells of V_HQ52^{NT-HT} mice, as assessed through CDR3 sequence analysis. Replacements using the same V_H germ-line gene were grouped. (*F*) Southern blotting quantification of V_HQ52^{NT} gene copy number in splenic IgA$^+$ B cells of V_HQ52^{NT} homozygous (HO) mice. Data were normalized for DNA input and represented as relative to V_HQ52^{NT} copy number in liver cells of transnuclear mice. (*G*) Representative flow cytometric analysis of splenic CD19$^+$ B cells of V_HQ52^{NT};*B1-8f* double IgH insertion mice (n = 4). Number within dot plot refers to frequency of IgM-only B cells.

PCR products that were subsequently cloned and sequenced. Sequence analyses revealed the consistent replacement of the original $V_HQ52.a27.79$ gene of V_HQ52^{NT} with other germ-line V_H genes (Fig. 4*C*, Fig. S4*B*, and Table S1). All VH replacements shared with the original V_HQ52^{NT} rearrangement part of the CDR3 FP sequence and the J_H3 segment (Fig. 4*D*). VH replacements were further diversified by de novo *n*-nucleotide addition (Fig. 4*D* and Table S1). Importantly, CDR3 sequence analysis indicated that most VH replacements in V_HQ52^{NT-HT} mice were unique, thus underscoring the polyclonal origin of IgM$^+$ B cells (Fig. 4*E*, Fig. S4*C*, and Table S1). In accordance with the sIgA-negative phenotype, VH replacements in IgM$^+$ B cells of V_HQ52^{NT-HT} mice were predominantly OF (Table S1). This result is compatible with a scenario whereby the occurrence of OF VH replacements caused the inactivation of V_HQ52^{NT}, which in turn prompted further VDJ recombination on the second IgH chromosome favoring the generation of IgM$^+$ B cells. To estimate the frequency of secondary V_H rearrangements in an unbiased fashion, we performed IgH Southern blotting analysis on genomic DNA from V_HQ52^{NT} homozygous splenic B cells. We compared the intensity of the bands corresponding to the V_HQ52^{NT} allele between B cells and liver cells that were used as negative control (Fig. S4*D*). Quantification of the data revealed that around 22% of splenic IgA$^+$ B cells disrupted one V_HQ52^{NT} allele (Fig. 4*F*).

To validate our results, we used V_HQ52^{NT} as a reporter allele to monitor VH replacement in *B1-8f* IgH knock-in mice (23). Flow cytometric analysis of splenocytes of double IgH knock-in mice revealed a major population of B cells expressing concomitantly sIgM and sIgA (Fig. 4*G*). Importantly, we also detected a distinct subset of IgM-only B cells that ranged between 3% and 5% of splenic B cells (Fig. 4*G* and Fig. S4*E*). V_H rearrangement analysis in sorted IgM-only B cells identified OF VH replacements that disrupted the V_HQ52^{NT} allele (Table S1). We rarely identified sIgA-only B cells, possibly because inactivation of *B1-8f* through VH replacement is rendered unlikely by its chromosomal location (J_H locus) and the lack of a cRSS (23). In summary, using two independent mouse models starting with a highly restricted repertoire of productive IgH specificities, we found that 3–20% of their mature B cells diversified antibody specificity through VH replacement.

VH Replacements Are Already Detected in Pro-B Cells. To investigate the temporal onset of V_H replacement, we first evaluated early B-cell development in V_HQ52^{NT-HT} mice. Flow cytometric analysis revealed reduced numbers of Igκ$^-$B220^{lo} BM B-cell progenitors in V_HQ52^{NT} mice in comparison with controls (Fig. 5*A*). The impairment was mainly caused by fewer CD25$^+$ pre-B cells, whereas CD43$^+$ pro-B cells were largely comparable in number to controls (Fig. 5*B*). To test whether VH replacement had already occurred in early B-cell progenitors, we sorted B220^{lo}Igκ$^-$ CD43$^+$ pro-B cells from V_HQ52^{NT-HT} mice and performed VH replacement analysis. Sequencing of PCR products revealed a considerable number of unique VH replacements, which were also detected in their CD25$^+$ pre–B-cell progeny (Fig. S5*A*). V gene use in VH replacements cloned from pro-B cells indicated a similar pattern to that of SP IgM$^+$ B cells (Fig. 5 *C* and *D*, Fig. S5 *A* and *C*, and Table S1). Ninety-seven percent of unique VH replacements sequenced from pro-B cells were nonproductive (Fig. 5*E*). In contrast, close to 10% of VH replacements in pre-B cells were IF (Fig. S5*B*). These results suggest that OF VH replacements block the development of pro-B cells unless rescued by functional secondary IgH rearrangements.

VH Replacement Occurs Independently of Pre-BCR Expression/Signaling. The lower number of pre-B cells in V_HQ52^{NT} mice may result from impaired expression and/or signaling of a pre-BCR composed of an IgA H chain (24). Altered pre-BCR function may, in turn, prolong RAG-mediated recombination at the IgH locus, hence facilitating VH replacement. To test this hypothesis, we analyzed V_HQ52^{NT-HT};$V\kappa gr32^{NT-HT}$ double Ig insertion mice. The latter animals displayed a significant reduction in both pro-B cells and pre-B cells, as a result of the premature expression of pairing IgH/IgL chains (Fig. 6 *A* and *B*). Remarkably, flow cytometric analysis of splenic cell suspensions from IgH/L insertion mice revealed a fraction of sIgM$^+$ B cells comparable to that of V_HQ52^{NT}-only animals (Fig. 6 *C* and *D*). Molecular analysis confirmed the inactivation of V_HQ52^{NT} through OF VH replacements in IgM$^+$ B cells of V_HQ52^{NT-HT};$V\kappa gr32^{NT-HT}$ mice (Fig. 6 *E* and *F* and Table S1). These results indicate that pre-BCR expression and/or signaling is not required to initiate VH replacement of a productive IgH rearrangement.

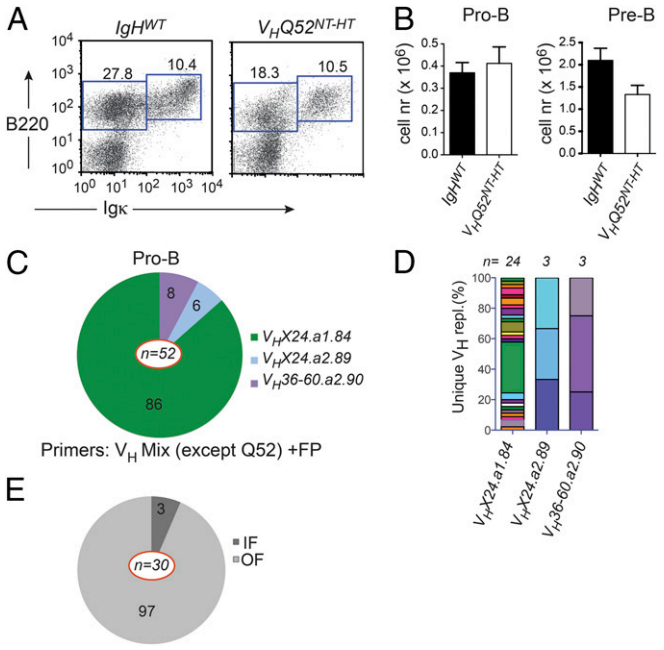

Fig. 5. VH replacement is detected in pro-B cells. (*A*) Representative flow cytometric analysis of BM cells in $V_HQ52^{NT\text{-}HT}$ mice and age-matched littermate controls (IgH^{WT}). Numbers indicate frequencies of gated progenitor ($B220^+Ig\kappa^-$) and sIg^+ ($B220^+Ig\kappa^+$) B cells. (*B*) Absolute number of BM pro-B ($B220^+Ig\kappa^-CD43^+CD25^-$) and pre-B ($B220^+Ig\kappa^-CD43^-CD25^+$) cells in representative cases of controls (IgH^{WT}) ($n = 5$) and $V_HQ52^{NT\text{-}HT}$ mice ($n = 4$) as assessed by flow cytometric analysis. (*C*) V_H gene use in VH replacements cloned from pro-B cells of $V_HQ52^{NT\text{-}HT}$ mice. Numbers within pie segments indicate frequency of germ-line V_H genes involved in VH replacements ($n = 52$). (*D*) Frequency of unique VH replacements cloned from BM pro-B cells of $V_HQ52^{NT\text{-}HT}$ mice, assessed by CDR3 sequence analysis. VH replacements carrying the same V_H gene were grouped. (*E*) Frequency, respectively, of IF and OF VH replacements cloned from pro-B cells of $V_HQ52^{NT\text{-}HT}$ mice. Numbers refer to percentage of unique VH replacements ($n = 30$).

V_HQ52^{NT} Is Preferentially Replaced by One Neighboring Germ-Line V Gene. Progenitor B cells inheriting an OF V_H rearrangement recruit an unbiased set of donor germ-line V_H genes to promote VH replacement (4). In sharp contrast, our data show that pro-B cells carrying a productive IgH rearrangement use a highly restricted set of V_H germ-line genes for VH replacement. In particular, V_HX24 was identified in over 80% of VH replacements. Alignment to the *Igh* locus indicated that V genes involved in VH replacement in V_HQ52^{NT} mice mapped within a 160-Kb genomic interval proximal to V_HQ52^{NT} (Fig. 7*A*). We tested whether preferential V_H gene use correlated with higher expression of their corresponding germ-line transcripts (GLTs). For this, we established IL-7–dependent progenitor B-cell cultures. Quantitative RT-PCR (qRT-PCR) analysis revealed that GLTs for V_HX24 *and S107* members, which mapped closely to V_HQ52^{NT} and were frequently recruited in VH replacement, were substantially increased in V_HQ52^{NT} progenitor B cells compared with *Rag1* mutant pro-B cells that were used as control. Conversely, GLTs for members of the distal J558 V_H family were markedly higher in *Rag-1* mutant pro-B cells compared with V_HQ52^{NT} progenitors (Fig. 7*B*). These results indicate that pro-B cells edit a productive V_H rearrangement using preferentially neighboring germ-line V genes as recombination substrates.

Partial Impairment of IgH Allelic Exclusion in V_HQ52^{NT} Mice. The substantial differences between IgA and IgM cytoplasmic domains may impact the ability of IgA to enforce IgH allelic exclusion. For this reason, we determined the IgH isotype status expressed by peripheral B cells of $V_HQ52^{NT\text{-}HT}$ mice. Over 80% of transnuclear B cells expressed IgA only on the surface. The remaining fraction consisted of IgM-expressing B cells. Among them, a small, yet consistent, population of $sIgA^+/sIgM^+$ double-positive B cells was identified in lymphoid organs of $V_HQ52^{NT\text{-}HT}$ mice. In particular, whereas B-2 B cells included 2–5% of $sIgA^+/sIgM^+$ cells, B-1 B cells consisted of close to 10% of IgH double producers (Fig. 8 *A* and *B*). Next, we investigated whether sIgM-only B cells included cells that retained the original V_H rearrangement but were unable to express it on the surface in the form of IgA due to a failure to pair with the IgL chain. To address this, we performed V_H gene rearrangement analysis in sorted IgM^+ B cells including in the PCR primer mixture a V_H primer to amplify Q52 family members. Sequencing analysis revealed that a substantial number of PCR products represented the original V_HQ52^{NT} rearrangement (Fig. 8*C*). In support of this result, we found that around 20% of IgM^+ B cells in $V_HQ52^{NT\text{-}HT}$ mice expressed IgA H in the cytoplasm (Fig. 8*D*). These results indicate that signaling through the cytoplasmic tail of the IgA H chain can enforce IgH allelic exclusion, although with lower efficiency than IgM. The observation that B cells, retaining the V_HQ52^{NT} allele, can undergo V-to-DJ recombination on the second IgH chromosome suggests that the latter process can be uncoupled from VH replacement.

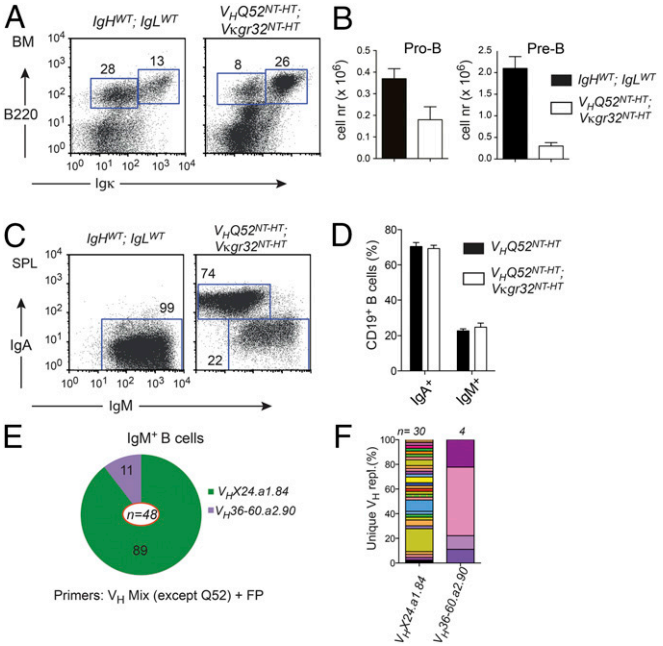

Fig. 6. VH replacement occurs before pre-BCR expression. (*A*) Representative flow cytometric analysis of BM cells from $V_HQ52^{NT\text{-}HT}$; $V\kappa gr32^{NT\text{-}HT}$ double Ig insertion mice and littermate controls (IgH^{wt}; IgL^{wt}). Numbers indicate frequency of boxed progenitors ($B220^{lo}Ig\kappa^-$) and sIg^+ B cells. (*B*) Average number of BM pro-B ($B220^{lo}Ig\kappa^-CD43^+CD25^-$) and pre-B ($B220^{lo}Ig\kappa^-CD43^-CD25^+$) cells in $V_HQ52^{NT\text{-}HT}$; $V\kappa gr32^{NT\text{-}HT}$ ($n = 4$) and control ($n = 7$) mice. (*C*) Representative flow cytometric analysis of gated $CD19^+$ B cells in SP of IgH^{wt}; IgL^{wt} controls and $V_HQ52^{NT\text{-}HT}$; $V\kappa gr32^{NT\text{-}HT}$ mice ($n = 6$). Numbers indicate frequency of B cells in boxed gates. (*D*) Comparison of IgA^+ and IgM^+ SP B-cell frequencies between $V_HQ52^{NT\text{-}HT}$ ($n = 8$) and $V_HQ52^{NT\text{-}HT}$; $V\kappa gr32^{NT\text{-}HT}$ double-insertion mice ($n = 6$). (*E*) V_H gene use in VH replacements ($n = 48$) cloned from splenic IgM^+ B cells of $V_HQ52^{NT\text{-}HT}$; $V\kappa gr32^{NT\text{-}HT}$ animals ($n = 3$). Numbers within pie segments indicate frequency of VH replacements using the indicated germ-line V_H genes. (*F*) Frequency of unique VH replacements ($n = 34$) cloned from IgM^+ B cells of $V_HQ52^{NT\text{-}HT}$; $V\kappa gr32^{NT\text{-}HT}$ mice, assessed by CDR3 sequence analysis. VH replacements carrying the same V_H germ-line gene were grouped.

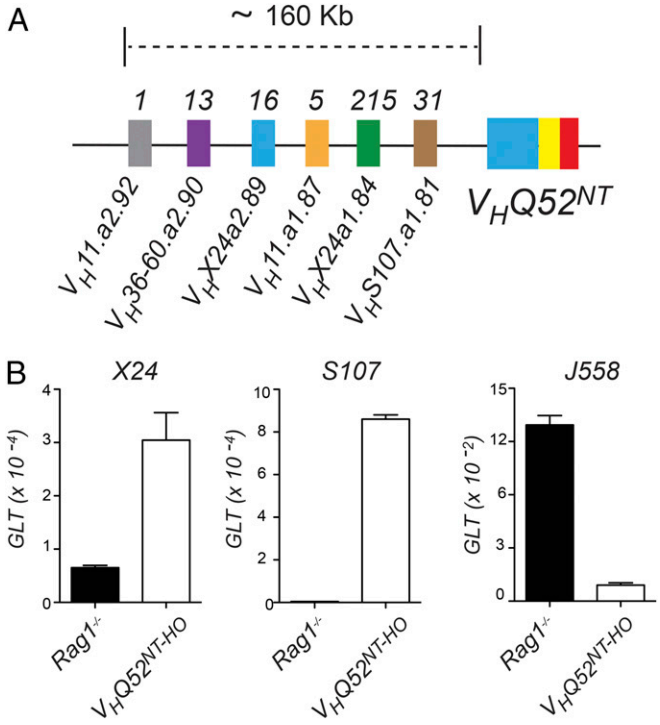

Fig. 7. VH replacement of V_HQ52^{NT} employs adjacent V_H donor genes. (*A*) Schematic view of the D_H-proximal portion of the IgH locus mapping upstream of the V_HQ52^{NT} rearrangement. Colored blocks indicate functional germ-line V_H genes ordered based on their physical distance from V_HQ52^{NT}. Numbers above blocks indicate unique VH replacements using the indicated V_H genes. (*B*) qRT-PCR determination of V_H GLTs for the indicated V_H families in pro–B-cell cultures established from *Rag1*-mutant and V_HQ52^{NT-HO} mice. Levels of GLTs are relative to those of the housekeeping gene *Rplp0*.

Discussion

Here, we describe the cloning of mice from the nucleus of an IgA-secreting PC. The derivation of pluripotent ES cells through reprogramming of a PC unravels the epigenetic plasticity of terminally differentiated B cells, extending previous work (19).

IgA monoclonal mice were established from a PC residing in the LP of the small intestine, possibly as a result of the recognition of a luminal antigen (25). Although the selecting antigen remains yet undefined, several evidences exclude that the cloned IgA BCR is autoreactive (20). First, serum antibodies in V_HQ52^{NT}; $V\kappa gr32^{NT}$ monoclonal mice lacked reactivity to common self-antigens represented by single- and double-stranded DNA. Second, *Rag2; λ5; SLP65* TKO pro-B cells reconstituted with a BCR carrying V_HQ52^{NT}; $V\kappa gr32^{NT}$ specificities lacked autonomous calcium mobilization, hence excluding spontaneous self-aggregation (26). Third, Igκ/λ light chain ratio was comparable between IgA transnuclear and littermate controls. Fourth, IgA cloned mice lacked signs of systemic autoimmunity and aged similarly to littermate controls.

Mice expressing a prerearranged V_H rearrangement from an IgA class-switched locus generated close-to-normal numbers of mature B cells, the majority of which expressed IgA. In monoclonal V_HQ52^{NT}; $V\kappa gr32^{NT}$ mice, splenic mature B cells consisted of both FO and MZ B cells. Instead, peritoneal cavity B-1 B cells were largely missing. These results are in accordance with, and extend, previous data (24) indicating that IgA can replace IgM to drive early B-cell development and instruct peripheral B-cell maturation. Whereas V_H gene use may influence the FO versus MZ B-cell fate (27), our data reveal that BCR extrinsic factors critically contribute to this decision. Also, the development of FO and MZ B cells at the expense of B-1 B cells may reflect the expression by transnuclear B cells of a BCR specificity enabling selectively the development of a B2/MZ B-cell progenitor (28, 29). Weak tonic BCR signaling may also contribute to the preferential development of FO and MZ B cells in IgA transnuclear mice (30).

We anticipated that transnuclear mice on the Rag-proficient background retained expression of the V_HQ52^{NT} specificity among mature B cells. Remarkably, instead, a substantial fraction of SP B cells in V_HQ52^{NT} mice lost expression of the original V_H rearrangement. In particular, in V_HQ52^{NT} HT mice, over 20% of peripheral B cells expressed IgM instead of IgA. An allele-specific PCR strategy revealed that in the latter cells the V_HQ52^{NT} allele was consistently inactivated through OF VH replacements. These results are compatible with a scenario whereby an initial OF VH replacement, disrupting V_HQ52^{NT}, prompts further VDJ recombination on the second IgH chromosome, allowing the rescue of those B cells that acquire a productive V_H rearrangement. IF VH replacements were also identified, hence contributing to the diversification of the IgA$^+$ B-cell pool. CDR3 sequence analysis of VH replacements revealed a highly diversified pool of secondary IgH rearrangements. Within the yet-limited coverage of the IgH repertoire of V_HQ52^{NT} mice, these results suggest that transnuclear B cells undergoing VH replacement do not represent a rare population that has undergone substantial clonal expansion. Southern blotting analysis performed on V_HQ52^{NT} homozygous B cells revealed that ~20% of peripheral IgA$^+$ B cells inactivated one V_HQ52^{NT} allele. Because V_H-to-J_H direct joining events were rarely found in V_HQ52^{NT} B cells, we conclude that VH replacement represents the preferred mechanism for the diversification of the productive, preimmune, IgH V gene repertoire.

VH replacement was proposed to occur in pro-B as well as in immature B cells, where it may contribute to neutralize BCR autoreactivity (4, 12, 15, 31). In V_HQ52^{NT} mice, VH replacements were already found in Ig$^-$B220^{lo}CD43$^+$ BM pro-B cells. Because VH replacement was accompanied by de novo addition of *n*-nucleotides, our data indicate that this process likely occurs in an early pro-B cell that expresses Tdt (32). The high frequency of VH replacements seen in V_HQ52^{NT} mice could result from

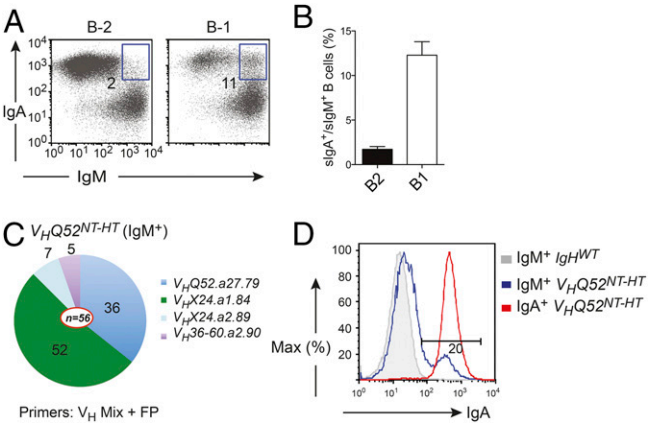

Fig. 8. IgH allelic inclusion is observed in a small fraction of V_HQ52^{NT} mature B cells. (*A*) Representative flow cytometric determination of sIgA/IgM double producers within, respectively, gated B2 (CD19$^+$B220$^+$) and B1 (B220^{lo}CD19^{hi}) B cells in the peritoneal cavity of V_HQ52^{NT-HT} mice (n = 5). Numbers indicate frequency of boxed sIgA/sIgM double producers. (*B*) Average frequency of sIgA/sIgM double producers among the indicated B-cell subsets in the peritoneal cavity of V_HQ52^{NT-HT} mice (n = 5). (*C*) V_H gene use in purified IgM$^+$ splenic B cells of V_HQ52^{NT-HT} mice, assessed by genomic PCR using primers annealing to V genes of the main V_H families including Q52. Numbers indicate frequency of IgH rearrangements (n = 56) consisting of the indicated V_H genes. (*D*) Intracellular flow cytometric determination of IgA H expression in the indicated subset of SP B cells in controls (IgH^{WT}) and V_HQ52^{NT-HT} mice.

impaired pro-B to pre-B cell transition. However, the analysis of V_HQ52^{NT}; $V\kappa gr32^{NT}$ double Ig insertion mice, largely lacking the progenitor B-cell compartment, showed frequencies of IgM$^+$ B cells that were similar to those of V_HQ52^{NT}-only animals. Hence, whereas a contribution of the pre-BCR to the induction of IgH editing cannot be formally excluded, our results, in accordance with previous evidences (4), support a scenario whereby VH replacement of a productive IgH rearrangement starts in an early pro-B cell before pre-BCR expression/signaling.

The loss of a large genomic segment of the C_H locus resulting from IgA class switch recombination could contribute to the high rate of VH replacements observed in V_HQ52^{NT} mice, through the loss of putative, yet unidentified, negative *cis* regulatory elements. To address this question, we crossed V_HQ52^{NT} mice to B1-8f IgH knock-in mice. In Ig double-insertion mice, V_HQ52^{NT} served as a reporter allele to monitor VH replacement in B cells transiting through early B-cell development as a result of B1-8f expression. Whereas most B cells were IgH allelically included, a distinct subset of them ranging between 3% and 5% lost the V_HQ52^{NT} allele through VH replacement. The different frequency of B cells undergoing VH replacement in V_HQ52^{NT}; *B1-8f* mice compared with V_HQ52^{NT}-only animals renders unlikely the existence of regulatory elements embedded in the C_H locus that influence VH replacement. Instead, these results point to differences between IgA and IgM H chains, or levels of their corresponding transcripts (33), in the regulation of IgH locus accessibility to the recombination machinery.

Analysis of V-gene use in VH replacements of V_HQ52^{NT} B cells revealed a different scenario from the one described for mice carrying OF V_H rearrangements (4). Indeed, IgA transnuclear mice showed a highly restricted set of V_H genes engaged in VH replacement. In particular, $V_HX24.a1.84$ contributed to over 80% of all VH replacements. Notably, all V_H germ-line genes recruited in VH replacement mapped in close proximity to the V_HQ52^{NT} rearrangement. The biased use of V_H genes in VH replacement could reflect the limited time to which the productive V_HQ52^{NT} rearrangement is exposed to the VDJ recombination machinery. This condition may facilitate the targeting of the RAG proteins to neighboring V genes, as previously suggested (34). The evidence that GLTs for V_H genes proximal to V_HQ52^{NT} were more abundant in transnuclear pro-B cells compared with controls supports this hypothesis. Higher germ-line transcription of D_H-proximal V genes coupled to their preferential recruitment in VH replacement may result from the lack of IGCR1 on the V_HQ52^{NT} allele (18). VH replacement may hence represent a mechanism that acquires relevance for the editing of productive V_H rearrangements carrying D_H-proximal V genes. The failure of V_HQ52^{NT} B cells to recruit V genes of distal V_H families may depend on VH replacement occurring in a developmental window that precedes IgH locus contraction (35). The identification of a small subset (1–2%) of IgM$^+$ B cells in ovalbumin-specific IgG1 and hematoagglutinin-specific IgG2b transnuclear mice (both using distal J558 V_H genes) suggests that secondary IgH rearrangements may also occur in these animals (36, 37).

The IgA H chain cytoplasmic domain is longer and differs substantially in the primary sequence from that of IgM (38). We wondered whether such differences affected the ability of the IgA H chain to signal IgH allelic exclusion. Flow cytometric data revealed that over 80% of B cells in V_HQ52^{NT} mice expressed IgA only. The remaining fraction consisted of sIgM$^+$ B cells. Around 20% of the latter cells (accounting for 4% of total peripheral B cells) expressed cytoplasmic IgA H, which was confirmed by successful PCR amplification of the V_HQ52^{NT} rearrangement. Moreover, around 2% of B2 B cells and over 10% of peritoneal cavity B-1 B cells expressed both IgA and IgM on the surface. From these results, we conclude that the IgA cytoplasmic tail is able to instruct IgH allelic exclusion, although with lower efficiency than IgM. Similar data were reported for the IgG1 H chain (36).

In summary, this study unravels the contribution of VH replacement to the diversification of the productive preimmune IgH repertoire of mice starting with a restricted pool of antibody specificities. Using two independent Ig transgenic models, we show that 3% or more (reaching a maximum of 20%) of mature B cells edited their initially productive IgH rearrangement through VH replacement. Our results are in agreement and complement data presented in the accompanying paper by Sun et al. (39).

The concomitant presence in HT V_HQ52^{NT} mice of diversified pools of, respectively, IgM- and IgA-only B cells offers the unprecedented opportunity to study in a competitive setup the properties of IgA and IgM BCRs to control homeostasis of B cells and their recruitment into T-cell–dependent and –independent immune responses, especially in mucosa-associated lymphoid tissues.

Materials and Methods

Animal Care. Animals were housed under specific pathogen-free conditions at the IFOM-IEO Campus and maintained according to protocols approved by the IFOM Institutional Animal Care and Use Committee and the Italian Ministry of Health. *Rag1*$^{-/-}$ mice were purchased from Jackson Laboratories. B1-8f mice were previously described (23). Experiments were performed with 8–16-wk-old animals.

Generation of IgA Transnuclear Mice. Small intestine LP cells of C57BL/6 X B10D2 F1 mice were prepared as previously described (40) and stained with fluorescent-labeled anti-B220, anti–MHC-II, and anti-IgA antibodies to isolate B220$^-$MHC-II$^-$IgA$^+$ PCs using a MoFlo (Beckman Coulter) cell sorter. SCNT procedures have been previously published (41) and are summarized in *SI Materials and Methods*.

Flow Cytometry and Cell Sorting. Single-cell suspensions from lymphoid organs were stained using fluorescent- or biotin-conjugated monoclonal antibodies against mouse CD19 (ID3), CD21 (8D9), CD23 (B3B4), CD45R/B220 (R3A3-6B2), CD25 (7D4), TCRβ (H57-59), CD5 (53-7.3), and IgA (mA-6E1), all from eBioscience. Anti-CD43 (S7) was from BD Biosciences. Anti-IgM (R33.24.12) and anti-Igκ (R33-18-10) antibodies were conjugated in house. For intracellular staining, B cells were fixed, permeabilized in Cytofix/Cytoperm (BD Biosciences), and stained with IgA-specific antibody. Samples were acquired on a FACSCalibur (BD Biosciences) and data analyzed with FlowJo software (Tree Star). Cell sorting was performed on FACSAria (BD Biosciences) after size exclusion of dead cells.

Analysis of Ig V Gene Rearrangements. Genomic DNA or total RNA was extracted using the All Prep DNA/RNA mini kit according to the manufacturer's protocol (Qiagen). For VH replacement analysis, genomic DNA was PCR amplified using a mixture of V_H family-specific primers (42) in combination with a V_HQ52^{NT} FP-specific primer. PCR conditions are listed in *SI Materials and Methods*. qRT-PCR analysis of VH replacements was performed using cDNA prepared from purified IgM$^+$ B cells using a mixture of V_H family-specific primers (42) combined with an oligonucleotide complementary to Cα. VH rearrangements occurring on the second IgH chromosome in IgM$^+$ $V_HQ52^{NT\text{-}HT}$ B cells were amplified by RT-PCR using V_H family-specific primers together with an oligonucleotide annealing to Cμ. Igκ V gene rearrangements were amplified by RT-PCR using a mixture of degenerate forward primers annealing to most Vκ genes (30), in combination with a Cκ reverse primer. PCR products were cloned and subjected to Sanger sequencing. V gene analyses were performed using IGBLAST. Primer sequences are listed in Table S2.

Quantification of V_H GLTs. qRT-PCR was performed in triplicate with SYBR Green-I Master Mix using primer combinations for GLTs listed in Table S2. Values were calculated using the comparative CT method. To normalize for cDNA input, a segment of the housekeeping *Rplp0* gene was amplified with primers listed in Table S2.

Southern Blotting Analysis. Southern blotting was performed on genomic DNA isolated from CD19$^+$ splenic B cells, as previously described (43) and summarized in *SI Materials and Methods*.

BCR Complementation of *Rag2*; *λ5*; *SLP65* TKO Pro-B Cells. *Rag2; λ5; SLP65* TKO pro-B cells expressing a tamoxifen-inducible ERT2–SLP65 fusion protein were previously described (26). SLP65 signaling function was restored incubating

TKO pro-B cells with 1 μM of 4-Hydroxytamoxifen (4-OHT, Sigma-Aldrich). V_HQ52^{NT} and $V\kappa gr32^{NT}$ specificities were cloned, respectively, into pMIZCC and pMIZYN retroviral vectors, as previously described (26). Retroviral vectors for TCL1-derived IgH and IgL chains were previously described (22). The HEL-specific HyHEL 10 antibody was described in ref. 44. Primers used for cloning of V_HQ52^{NT} and $V\kappa gr32^{NT}$ IgH and IgL chains are listed in Table S2. Retrovirus production and transduction of TKO pro-B cells was performed as previously described (26).

Measurement of Intracellular Calcium Flux. BCR reconstitution of *Rag2*; *λ5; SLP65* TKO pro-B cells was revealed by staining cells with goat anti-mouse IgM (Jackson ImmunoResearch), anti-mouse Igκ (Southern Biotech), or rat anti-mouse IgA (e-Bioscience) antibodies, respectively. $Ca2^+$ mobilization was measured as described before (26). Briefly, 5×10^6 transduced pro-B cells expressing ERT2–SLP65 were incubated with 5 μg/mL of Indo1 (Molecular probes) and 0.5 μg/mL of Pluronic F-127 (Molecular Probes) in Iscove medium supplemented with 1% serum (Vitromex) at 37 °C for 45 min. After loading, cells were centrifuged, resuspended in Iscove medium with 1% FCS, and stimulated with 1 mM of 4-OHT. Calcium flux was measured on an LSR II flow cytometer (Becton Dickinson).

Enzyme Linked Immunosorbent Assay. Titration of serum anti-DNA antibodies was performed by ELISA, as previously described (45). Procedures are summarized in *SI Materials and Methods*.

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