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Progressive Surface B Cell Antigen Receptor Down-Regulation Accompanies Efficient Development of Antinuclear Antigen B Cells to Mature, Follicular Phenotype

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Previous studies have suggested that B cell Ag receptor (BCR) down-regulation by potentially pathological autoreactive B cells is associated with pathways leading to developmental arrest and receptor editing, or anergy. In this study we compare the primary development of B cells in two strains of mice expressing transgenic BCRs that differ by a single amino acid substitution that substantially increases reactivity for nuclear autoantigens such as DNA. Surprisingly, we find that both BCRs promote efficient development to mature follicular phenotype, but the strongly autoreactive BCR fails to promote marginal zone B cell development. The follicular B cells expressing the strongly autoreactive BCR do not appear to be anergic, as they robustly respond to polyclonal stimuli in vitro, are not short-lived, and can participate in germinal center reactions. Strikingly however, substantial and progressive down-modulation of surface IgM and IgD takes place throughout their primary development in the BM and periphery. We propose that BCR-autoantigen interactions regulate this pathway, resulting in reduced cellular avidity for autoantigens. This process of “learned ignorance” could allow autoreactive B cells access to the foreign Ag-driven memory B cell response, during which their self-reactivity would be attenuated by somatic hypermutation and selection in the germinal center.


Although the expression of many autospecific BCRs by the primary B cell compartment might be benign, incorporation of such BCRs into the memory compartment could predispose to autoimmune disease. Memory B cells are high affinity, long-lived B cells that can rapidly differentiate into Ab-forming cells (AFC). They produce Abs of the stable IgG isotypes. Memory B cells are also not easily tolerized (21). In this regard, the etiology of many systemic autoimmune diseases resembles an autoantigen-driven memory B cell response (22, 23).

In A/J mice, anamnestic immune responses to p-azophenylarsonate (Ars) are dominated by Abs with a single type of V domain, encoded by a unique combination of V gene segments (termed canonical). Unmutated canonical Abs exhibit weak binding to intracellular autoantigens such as DNA (24). In contrast, canonical Abs expressed by memory B cells uniformly exhibit increased affinity for Ars and undetectable affinity for autoantigen (25). However, in strain A mice in which transgenic Bcl-2 expression is enforced in B cells, Ars immunization induces memory B cells with increased affinity for both Ars and DNA (26). These results inspired the specificity maturation hypothesis (25, 26). It contends that during their foreign Ag-driven development, memory B cell precursors are subjected to a concerted process of V gene hypermutation, positive selection for increased affinity for foreign Ag, and negative selection against unaltered or increased affinity for autoantigen. This hypothesis predicts that dual reactive primary B cells, whose BCRs possess functional affinity for a foreign Ag and potentially pathological autoreactivity, could serve as memory precursors in normal individuals, as this autoreactivity would be attenuated during the specificity maturation process.

To test this idea, we exploited the substantially increased autoreactivity conferred by an arginine (R) mutation at position 55 in the canonical VH (27) to create a VH knockin mouse line (termed HKIR) that expresses a BCR reactive with Ars as well as chromatin and dsDNA. Canonical R55 IgG mAbs exhibit the characteristics of pathological autoantibodies in that they are antinuclear Ag positive and deposit in kidney glomeruli. We reported that...
canonical HKIR B cells could access splenic follicles and participate in the germinal center (GC) response following Ars immunization (28).

Past studies of the developmental fate of anti-DNA B cells would predict that canonical autoreactive HKIR B cells would be either deleted in the bone marrow (BM) or anergic and short-lived in the periphery (9, 10, 14). As such, their participation in the GC response might have resulted from a break in tolerance caused by strong antigenic stimulation in adjuvant. Additionally, perturbations in primary development due to expression of the knockin locus might preclude the action of normal central or peripheral tolerance checkpoints. To address these issues, we created a knockin mouse line (HKI65) using the same VH gene in the HKIR line, but lacking the R55 mutation. Canonical B cells in HKI65 mice express a BCR equivalent to that expressed by anti-Ars memory precursors in strain A mice. This allowed a direct comparison of the development of canonical B cells whose expressed Ig loci are distinguished only by the presence or the absence of the VH R55 mutation, but whose BCRs have substantially dissimilar levels of autoreactivity.

Materials and Methods

Mice

The HKIR knockin mice expressing the R55V VH gene were previously described (28). HKI65 knockin mice were created in identical fashion by replacing the entire J H locus, but with a targeting vector differing only in its lack of the R55 canonical VH mutation (28). The resulting mice were screened by Southern blot or PCR for both the endogenous J H3 to J H4 region and the knockin VDJ gene region. Transgene-positive mice were bred to J H4 knockout mice (a gift from Dr. R. Hardy, Fox Chase Cancer Center, Philadelphia, PA). Mice were housed under specific pathogen-free conditions, and given autoclaved food and water. The use of mice in these studies was conducted in compliance with institute guidelines, and all protocols using animals were approved by the institutional animal care and use committee.

Flow cytometry

Single-cell suspensions were prepared from lymphoid organs of 8- to 12-wk-old, naive transgenic and age-matched transgene-negative littermates. Cells were stained with different combinations of the following Abs: α-IgM (Jackson Immunoresearch Laboratories, West Grove, PA), anti-IgD (11-26; Southern Biotechnology Associates, Birmingham, AL), anti-IgM* (DS-1), anti-IgM* (AF6-78), anti-κ (187.1; Southern Biotechnology Associates), anti-λ (JCS-1; Southern Biotechnology Associates), anti-BP-1 (6C3), anti-FAS (Jo2), anti-I-A/E (2G9), anti-CD1d-PE (1B1), anti-CD3 (145-2C11), anti-CD4 (H129.19), anti-CD5 (53-6.7) (eBiobioscience, San Diego, CA), anti-CD19 (1D3), anti-CD21/35 (7G6), anti-CD22 (Cy34.1), anti-CD23 (B3B4), anti-CD24 (M1/69, 30-F1), anti-CD25 (7D4), anti-CD43 (57), anti-CD44 (IM7), anti-CD45R (RA3-6B2; eBiobioscience), anti-CD69 (H1.2F3), anti-CD80 (16-10A1), anti-CD86 (GL-1), AA4.1 (gift from Dr. D. Allman, Abramson Family Cancer Center, University of Pennsylvania, Philadelphia, PA), or anti-idiotypic mAb E4 (prepared in-house). All Abs were obtained from BD PharMingen (San Diego, CA) unless otherwise indicated. SA-CyChome (BD PharMingen) was used to detect biotinylated Abs. Cells were assayed as described above.

Immunochemistry

Spleens from 8- to 12-wk-old naive mice were frozen, and cryosections were prepared as previously described (29). Sections were stained with the following reagents, biotin-E4, biotin-CD3 (BD PharMingen), biotin-B220, biotin-IgD, HPRT-pagglutinin (Sigma-Aldrich, St. Louis, MO), and HRP-I Fab′), of donkey anti-mouse IgM (Jackson Immunoresearch Laboratories). Branchied streptavidin–alkaline phosphatase (DAKO, Glostrup, Denmark) was used to detect biotinylated Abs. Detection was performed using the Vector Blue Alkaline Phosphatase Substrate Kit III and the Vector NovaRed Substrate Kit for Peroxidase (Vector Laboratories, Burlingame, CA).

Hybridomas and Southern blotting

Total spleen cells from adult HKIR mice were stimulated with LPS and dextran sulfate for 3 days, and hybridomas were constructed and screened for E4′ Ab production as previously described (26, 28). RT-PCR, nucleotide sequencing of PCR products, and Southern blot analysis of hybridoma DNA were all conducted as described previously (26, 28).

Autoreconstitution

Animals were exposed to a sublethal dose of whole body gamma irradiation (500 rad) and allowed to rest for 7 days as described by Cancro and colleagues (30). B cells were obtained from the BM, and cells were labeled for flow cytometry as described above.

5-Bromo-2′-deoxyuridine (BrdU) labeling analyses

Short and long term labeling was performed using the BrdU Labeling Kit from BD PharMingen. Briefly, for short term labeling, mice were injected i.p. with 0.6 mg of BrdU and rested for 2–4 h. Long term labeling was performed for 4 days with injections of 0.6 mg of BrdU every 12 h. Cells were removed and treated as described in the kit manual. Labeled cells were assayed as described above.

B cell purification

Single-cell spleen suspensions were prepared as described above. After removal of dead cells by centrifugation through Lympholyte-M (Cedarlane Laboratories, Hornby, Canada), cells were stained with either biotin-E4 or biotin-anti-IgM Ab, followed by incubation with magnetic SA-Beads (Miltenyi Biotech, Bergisch Gladbach, Germany). Following the manufacturer’s recommendations, labeled cells were passed over MiniMACS columns (Miltenyi Biotech) and eluted. Two column passes per sample were performed. The purity of the E4′ B cells as assessed by flow cytometry was always >85%, and the purity of IgM* B cells was always >95%.

In vitro proliferation assays

Purified E4′ HKIR, E4′ HKI65, and IgM* B cells were plated at 5 × 10^4 cells/well in 96-well dishes and stimulated with 2.5 μg/ml LPS (Difco, Detroit, MI), 25 μg/ml goat anti-mouse IgM (Fab′), (Pierce, Rockford, IL), 10 μg/ml anti-mouse CD40 (clone FGG45, a gift from Dr. A. Rolink, Biozentrum, University of Basel, Basel, Switzerland) plus 50 ng/ml recombinant mouse rIL-4 (PeproTech, Rocky Hill, NJ), or 10 ng/ml PMA (Sigma-Aldrich) plus 200 ng/ml ionomycin (Sigma-Aldrich). The concentrations of mitogens used were determined to induce maximum proliferation of C57BL/6 B cells. After 48 h, cells were pulsed for 12–16 h with [3H]thymidine (NEN, Boston, MA) and harvested, and [3H] incorporation was evaluated by scintillation counting.

Results

IgH allotypic differences between the HKIR- and HKI65-targeted loci (Igh^n) and the C57BL/6 Igh locus (Igh^n) were exploited to examine transgene expression and allelic exclusion. The use of an anti-Id mAb (E4) specific for the canonical BCR (31) allowed identification of canonical B cells. E4 reacts equally well with Abs encoded by the combination of V gene segments regardless of whether they contain or lack the R55 mutation. Therefore, we will refer to both types of Abs, BCRs and B cells expressing such BCRs, as canonical. Essentially all, and the majority of all canonical and total BM B cells, respectively, in both mouse lines expressed IgM* (Fig. 1A). In contrast, in the spleen approximately half the HKIR and HKI65 B cells expressed the endogenous μ allele (Fig. 1B). Few, if any, B cells expressed both H chain alleles, and the vast majority of the E4-gated B cells in both lines expressed only the Igh^n allelotype. Thus, the mice do not exhibit allelic inclusion. Transgene-mediated allelic exclusion is V H knockin mice is commonly lost due to apparent recombinae-activating gene-induced inactivation of the targeted allele (32, 33). To preclude the development of B cells that had inactivated the knockin allele, the HKIR and HKI65 lines were crossed to J H knockout mice (34). Splenic B cells from these lines expressed only the Igh^n allele (Fig. 1C).
Dramatically reduced surface levels of the canonical R55 BCR on peripheral B cells

Examination of allotype-specific IgM staining data such as those in Fig. 1 revealed that surface IgM\(^\text{a}\) (slgM\(^\text{a}\); knockin locus \(\mu\)-chain) levels were substantially lower on both B220\(^+\) and E4\(^+\) cells in the HKIR and the HKIR/JHD lines and appeared somewhat lower in the HKI65 and HKI65/JHD lines compared with controls. To evaluate this phenomenon more accurately, spleen cells from the HKI65/JHD and HKIR/JHD lines were stained with anti-B220, E4, and allotype-nonspecific anti-IgM and anti-IgD reagents, and the intensity of staining was evaluated by flow cytometry.

Consistent with the data in Fig. 1, the bulk B cell populations from both lines had reduced levels of slgM, with the HKIR/JHD B cells revealing a staining intensity at the low end of the normal distribution (Fig. 2A). This pattern of somewhat reduced and substantially reduced slgM staining on the HKI65/JHD and HKIR/JHD B cells, respectively, was also apparent in the E4\(^+\) subpopulation. Surface IgD levels in both lines appeared near normal in the bulk B cell compartment, but were dramatically reduced in the HKIR/JHD E4\(^+\) subpopulation. In the canonical subpopulation in HKI65/JHD mice, slgD staining was bimodal, with many of the cells having only slightly reduced slgD levels, but a subpopulation displaying levels similar to those in HKIR/JHD B E4\(^+\) cells. Fig. 2C presents these data as geometric mean fluorescence intensities of the various histogram distributions. Analogous results were obtained on slgD levels in the E4\(^+\) splenic B cell subpopulation of the HKI65 and HKIR lines (data not shown). As we observed analogous levels of canonical sBCR expression on splenic B cells in the \(V_\text{H}\) knockin and \(V_\text{H}\) knockin/JHD lines, we focused our subsequent studies on the latter, due to their higher frequency of E4\(^+\) B cells (see Fig. 1 for details).

In normal mice, lymph node (LN) B cells express lower and slightly higher average levels of slgM and slgD, respectively, than splenic B cells. As in spleen, the bulk population of B cells in HKIR/JHD LNs displayed a lower average level of slgM compared with those in HKI65/JHD mice (Fig. 2B) and controls, and these levels were decreased compared with those in spleen. Surface IgD was substantially reduced on total HKIR/JHD LN B cells and was somewhat reduced on HKI65/JHD LN B cells. Further, slgD levels were drastically reduced (\(>10\)-fold) on E4\(^+\) HKIR/JHD LN B cells compared with the bulk HKIR/JHD LN B cell populations. These levels were also lower than on canonical B cells in the spleen. The canonical B cells in the HKI65/JHD mice displayed a bimodal distribution of anti-IgD staining similar to the results obtained from spleen, although more slgD\(^\text{low}\) cells seemed to be present. Fig. 2D presents these LN data as geometric mean fluorescence intensities of the various histogram distributions.

Canonical R55 BCR expression promotes efficient development to follicular, but not marginal zone (MZ), B cells

Immunohistochemistry analyses showed that the overall microarchitecture of the adult splenic lymphoid compartments in the HKIR/JHD and HKI65/JHD mice was normal (Fig. 3A, upper panels). There was, however, a substantial decrease in size of the MZ B cell area in HKIR/JHD mice (Fig. 3A, right panels with IgM staining). As expected from the flow cytometric studies, B cells in
the HKIR/JHD sections stained weakly with anti-IgM. This staining was noticeably lower than that observed in HKI65/JHD spleens, which, in turn, was somewhat decreased compared with littermates (data not shown). Canonical B cells stained with E4 were dispersed throughout all B cell areas and in similar numbers in HKI65/JHD and HKIR/JHD mice (Fig. 3A).

Flow cytometry showed that the percentages of splenic B220⁺ B cells were indistinguishable in both knockin lines from controls (data not shown). As was seen in the histology studies, there was only a small MZ B cell population (CD21/35high CD23low) evident in the HKIR/JHD mice (Fig. 3B, lower panels), and this population appeared somewhat reduced in the HKI65/JHD mice. This was also true in the canonical B cell subpopulations. The use of CD1d, expressed at high levels on MZ B cells (35, 36), corroborated the reduction in the number of MZ B cells in these mice (Fig. 3C). In both HKI65/JHD and HKIR/JHD lines, the majority of total and E4⁺ B cells was found in the follicular population, and similar percentages of E4⁺ cells were found in this compartment in both lines.

As in spleen, the percentage of B220⁺ cells in LNs was indistinguishable in the two knockin lines from controls (data not shown). The bulk population and E4⁺ subpopulation of LN B cells appeared to be of a uniform follicular phenotype in both HKI65/JHD and HKIR/JHD mice (Fig. 3D and data not shown), and similar numbers of E4⁺ cells were found in the LNs of both knockin lines. Examination of B cells in the peritoneal cavity showed approximately normal numbers of B1 cells in both knockin lines, as indicated by anti-CD19, anti-CD43, and anti-CD5 staining. In addition, the bulk B cell compartment in the peritoneum displayed substantially and somewhat reduced levels of sBCR expression in the HKIR/JHD and HKI65/JHD lines, respectively. Analysis of a variety of activation and differentiation markers on splenic and LN B cells, including CD22, Fas, CD44, CD80, CD86, and MHC class II did not reveal differences in HKI65/JHD and HKIR/JHD mice from controls. However, both spleen and LN B cells in the HKIR/JHD mice contained major subpopulations (30% of B220⁺ cells and 30% of E4⁺ cells) expressing elevated levels of the early activation marker CD69 (data not shown). In addition, B cells in both knockin lines expressed equivalent, but slightly elevated (2-fold increase in mean fluorescence intensity), surface levels of CD19 compared with littermates. The significance of these latter two observations will require further studies.

L chain editing in HKIR/JHD mice

Inactivation of the canonical V_{H} gene, or somatic mutation of this gene or the knockin V_{H} gene might reduce the autoreactivity of canonical R55 B cells. To evaluate these possibilities, hybridoma panels were generated from polyclonally activated HKIR splenic B cells and screened for E4 reactivity. A high frequency of E4⁺ hybridomas was obtained, and the majority of these produced mAbs that bound strongly to Ars and chromatin in ELISAs (data not shown). Canonical V_{H} and V_{L} genes were successfully RT-PCR-amplified from each of eight randomly selected E4⁺ hybridomas. Sequencing of these PCR products revealed the low levels of
of mutation expected from PCR error and that the R55 codon was intact in all \( V_H \) genes (data not shown).

To further evaluate \( L \) chain editing, splenic B cells were stained with an anti-\( \kappa \) \( L \) chain mAb and analyzed by flow cytometry. In the bulk B cell compartment, \( HKI65/JHD \) mice contained percentages of \( B220^- \) cells present in the MZ vs follicular populations differed reproducibly between the two strains. The percentages (±SE) of the \( B220^- \) population made up by follicular and MZ B cells, respectively, were 74.7 ± 8.1 and 7.9 ± 1.4 for littermates, 75.7 ± 3.4 and 8.3 ± 2.3 for \( HKI65/JHD \) mice, and 84.3 ± 6.2 and 1.9 ± 0.6 for \( HKIR/JHD \) mice. Splenic \( B220^- \) cells were evaluated for the expression of \( CD1d \) by flow cytometry. Equivalent gates were set around the \( CD1d^- \) B cell subpopulations and represent the percentage of \( CD1d^- \) cells within the \( B220^- \) gate. LN \( B220^- \) or \( E4^+ \) cells were evaluated for the expression of \( CD21/35 \) and \( CD23 \) by flow cytometry. The cross-hairs are set to the center of the major littermate population to provide a point of reference. Data are representative of at least four mice of each genotype.

To assess whether multiple \( \kappa \) genes might be expressed by a subpopulation of \( HKIR \) B cells, DNA from the eight hybridomas described above were analyzed for rearrangements in the \( J_\kappa \) locus by Southern blotting. All hybridoma DNAs gave rise to bands expected from canonical \( V_\kappa \) genes. This combined with the results of the RT-PCR analysis rules out the possibility that a major fraction of canonical \( R55 \) B cells do not express a canonical \( V_\kappa \) gene due to editing. Nonetheless, half of \( E4^- \) hybridomas gave rise to single, additional bands consistent with
FIGURE 4. E4 and L chain expression on B cells from HKIR/JHD and HKI65/JHD mice. HKIR/JHD, HKI65/JHD, and littermate spleen cells were isolated; labeled with anti-B220 and E4, anti-\(\kappa\) L chain, or anti-\(\lambda\) L chain mAbs; and analyzed by flow cytometry. The littermate cells are indicated by the shaded distributions, the HKI65/JHD distributions are shown by a dashed line, and the HKIR/JHD distributions shown by a solid black line. Data are representative of at least four mice of each genotype.

**B cell development in the BM of HKIR/JHD and HKI65/JHD mice**

Flow cytometry was used to examine early stages of B cell development, where previous studies have demonstrated that deletion of and receptor editing in autoreactive B cells may take place (9, 10). Fig. 5A shows that the pre/preBI/early preBI (B220\(^{\text{low-int}}\); S7\(^{+}\)) BM subpopulation was present in comparable numbers in the both knockin and control mice. There was only a slight and similar reduction in the HKIR/JHD and HKI65/JHD total late preBI/immature/mature BM B cell population (B220\(^{\text{int-high}}\); S7\(^{-}\)) compared with littermates. In contrast, evaluation of the B220\(^{\text{int}}\), sIgM\(^{\text{high}}\), CD43\(^{-}\) (immature B) subpopulation indicated a modest and substantial reduction in this subpopulation in HKI65/JHD and HKIR/JHD mice, respectively (Fig. 5B). However, the experiments above indicate that the sIgM level may not be an appropriate measure of B cell maturity in these knockin mice. Indeed, as shown in Fig. 5C, when mature (recirculating) B cells (CD23\(^{+}\)) were excluded, the numbers of total sIgM\(^{+}\) BM B cells were similar in HKI65/JHD, HKIR/JHD, and littermate mice, but the IgM\(^{\text{high}}\) subpopulation was reduced in the knockin lines, most dramatically in HKIR/JHD mice.

To analyze BM development in the absence of mature B cells and in a situation where short-lived intermediate populations might be revealed, we used an autoreconstitution protocol (30). Fig. 5D illustrates that the percentage of B220\(^{+}\) HKIR/JHD BM cells that developed 7 days after sublethal irradiation was only slightly reduced compared with that in HKI65/JHD mice and littermates. Whether this mild reduction represents somewhat less efficient immature B cell development in HKIR/JHD mice will require further studies. Interestingly, however, whereas major fractions of cells in the preBI/early preBI subpopulations of both knockin lines expressed moderate levels of sIgM (Fig. 5E), at the late preBI/immature B stage there was an obvious reduction in these levels on HKIR/JHD B cells. Few littermate B cells at this stage expressed detectable sIgM, presumably because they had only recently completed V(D)J rearrangement at the H chain loci. Importantly however, neither expression of the transgene nor reduced levels of sIgM uniformly altered the kinetics of early B cell development after the preBI stage in the HKIR/JHD line, as all B cells in the late preBI/immature B cell gates (B220\(^{\text{int}}\), S7\(^{-}\)) stained positively for BP-1, but did not stain with anti-IgD or anti-CD23 (data not shown).

**Primary peripheral B cell development in the V\(\text{H}\) knockin mice**

To examine late primary developmental steps in the spleen, we evaluated levels of CD21/35 and CD24 (heat-stable Ag) via flow cytometry to identify transitional B cells. Others have also used sIgM and sIgD levels in such analyses (37, 38), but the down-regulation of sIg during BM development in HKIR mice complicates the use of this strategy. Indeed, as shown in Fig. 6A, HKI65/JHD and HKIR/JHD splenic B cells have reduced and nearly absent sIgM\(^{\text{high}}\), sIgD\(^{\text{low}}\) subpopulations, respectively. This subpopulation includes B cells undergoing peripheral primary development in normal mice. However, as shown in Fig. 6B, the number of CD21\(^{\text{low}}\); CD24\(^{\text{high}}\) transitional B cells (lower right gate) were similar in the bulk and E4\(^{+}\) B cell populations of the knockin lines and littermates. In contrast, the number of CD21\(^{\text{high}}\); CD24\(^{\text{int-high}}\) B cells was substantially reduced in the bulk B cell compartment of HKIR/JHD mice. Among E4\(^{+}\) cells, this subpopulation was slightly reduced in HKI65/JHD mice and was considerably reduced in HKIR/JHD mice. As this subpopulation includes both T2 transitional and MZ B cells, we labeled splenic B cells with AA-1, a marker expressed by all immature B cells (38). We found no noticeable differences in the percentage of AA4.1\(^{+}\) cells in either the bulk or E4\(^{+}\) B cell populations of the knockin lines compared with controls (data not shown). Therefore, the reduction in CD21\(^{\text{low}}\); CD24\(^{\text{int-high}}\) splenic B cells in HKIR mice appears largely due to the low frequency of MZ B cells (Fig. 3, B and C). Similar flow cytometric studies of B cells in the LNs revealed an absence of CD24\(^{\text{int-high}}\); AA4.1\(^{+}\) cells in both knockin lines, indicating that all such B cells were mature (data not shown).

**HKI65 and HKIR canonical B cells respond to in vitro stimulation and are not short-lived in vivo**

Reduced sBCR (particularly sIgM) levels have been previously viewed as a hallmark of anergic B cells in the periphery (9, 14). However, as shown in Fig. 7A, purified HKIR E4\(^{+}\) B cells proliferated vigorously when stimulated with LPS, anti-IgM, anti-CD40 plus IL-4, and PMA and ionomycin, to an extent comparable to that in HKI65 E4\(^{+}\) and littermate B cells (see Materials and Methods for details). The slightly reduced anti-IgM induced proliferative response exhibited by canonical HKIR splenic B cells might be expected given their very low levels of sIgM.

Many anergic B cells have displayed peripheral half-lives on the order of days (9, 14). To evaluate the in vivo life span of HKIR/JHD and HKI65/JHD B cells, BrdU incorporation analysis was performed. A pulse experiment demonstrated that E4\(^{+}\) splenic B cells were not rapidly proliferating in either knockin line (see Ma-
Materials and Methods; data not shown). As seen in Fig. 7B, BM B cells in both lines incorporated large amounts of BrdU that were comparable to control levels over 4 days of continuous labeling. In the periphery, the bulk and E4⁺ HKIR/JHD B cell populations had similar low levels of intermediate-term BrdU labeling to those in both the HKI65/JHD and littermate controls.

FIGURE 5. B cell development in the BM of HKIR/JHD and HKI65/JHD mice. A, BM cells were stained with a mixture of anti-B220, anti-CD43 (S7), and anti-IgM Abs and analyzed by flow cytometry. Gates were set around the major subpopulations present in the littermate plots. The gates represent Hardy fractions A–C (pro to preBI cells; right rectangle) and D–F (preBI, immature, and mature; left rectangle) (39). B, In this panel, gates represent Hardy fractions D (preBI), F (recirculating mature), and E (immature) from left to right. Note that in the HKIR/JHD plot, fractions E and F express similar, but intermediate to low, levels of sIgM compared with littermate and HKI65/JHD fractions E and F. C, CD23⁺ cells from the BM were analyzed using anti-B220 and anti-IgM. Gates were drawn on littermate data to represent sIgM high and sIgM int cells. D, HKIR/JHD, HKI65/JHD and littermate mice were subjected to sublethal irradiation (500 rad) and then rested, and BM cells were isolated 7 days later as described in A. E, Cells from irradiated mice treated as described in B were also analyzed for sIgM levels. Data from autoreconstitution experiments are representative of two mice, and all other figures are representative of at least four mice of each genotype.
Discussion

We previously demonstrated that canonical HKIR B cells participate in the GC response following Ars immunization (28), suggesting that potentially pathological autoreactive primary B cells can serve as memory precursors in normal mice. However, this participation could have resulted from a break in tolerance due to immunization in inflammatory adjuvant, transgene-altered primary development that prevented the action of a tolerance checkpoint, or both. By directly comparing the development and functional status of canonical HKIR and HKI65 B cells to those of nontransgenic mice, these possibilities were rendered unlikely. In contrast, these studies revealed a novel mechanism for allowing autoreactive B cells to enter the mature, follicular, foreign Ag-responsive compartment: active sBCR down-regulation during primary development. This mechanism operates progressively, influencing the level of expression of sIgM during immature B cell development in the BM and the level of sIgD during peripheral stages of maturation. Our data support the idea that the regulation of quantity of sIgM is due to down-regulation, but further studies will be required to determine whether regulation of sIgD levels in the periphery is mediated by down-regulation or inhibition of induction.

We propose that during this process of “learned ignorance,” autoantigen-BCR interactions induce a reduction of sBCR levels, and this lowers the avidity for autoantigen of individual B cells. Modulation of surface levels of expression of IgM and IgD are hallmarks of various stages of primary and secondary B cell development that, in turn are regulated by BCR engagement (7, 37, 39, 40). Therefore, it seems reasonable that the extent of such engagement could influence the degree of sIgM and sIgD modulation, creating a feedback loop that allows B cells expressing a wide range of specificities to develop along similar paths. Presumably, this feedback mechanism results in adjustment of BCR signaling to amounts compatible with survival and developmental progression, but not clonal deletion or anergy.

Past studies are compatible with the idea that developing B cells can adjust the level of surface expression of their BCRs to adapt to alterations in signaling levels through this receptor. For example, mice deficient in CD19, CD81, or CD45, proteins that enhance BCR signaling, contain B cells with elevated levels of sIgM (41–43). In addition, B cells in mice with targeted inactivation of the Ig immunoreceptor tyrosine-based activation motifs express increased amounts of sIgM as well as sIgD (44). Conversely, mice deficient in the negative regulator of BCR signaling CD22 display lower than normal levels of sIgM on B cells (41, 45, 46). Finally, Finkelman and colleagues (47) discovered a minor population of B cells in the well-studied soluble hen egg lysosome-Ig anti-hen egg lysosome transgenic mice that is not anergic and is long-lived. These B cells express reduced levels of sBCR.

Earlier experiments on the fate of autoreactive B cells have shown that those with high avidity for certain autoantigens undergo either apoptosis or receptor editing in the BM (9, 10). Our experiments provide no evidence for clonal deletion of R55 B cells, but indicate that at least a subpopulation have undergone L chain editing, resulting in an increased frequency of λ L chain-expressing cells. Our hybridoma studies also leave open the possibility that some canonical R55 B cells express two κ L chains. These observations are consistent with previous studies indicating that sBCR down-modulation at the immature B cell stage in the BM is often accompanied by protracted L chain rearrangement (48, 49).

However, receptor editing alone cannot explain why canonical R55 B cells develop normally. Canonical κ genes are composed of the Vκ10A, Jκ1, and, sometimes, Jκ2 gene segments, and so could be deleted or inactivated by rearrangement of other Vκ segments to

FIGURE 6. Transitional B cells in the HKIR/JHD and HKI65/JHD mice. A, Splenic B220⁺ and E4⁺ cells were analyzed using anti-IgM and anti-IgD and flow cytometry. The cross-hairs are set to the middle of the littermate population to provide a point of reference. B, B220⁺ or E4⁺ splenocytes from HKIR/JHD, HKI65/JHD, and littermate mice were evaluated for expression of CD21/35 and CD24. Equivalent gates were set around the T1 (lower right), MZ/T2 (upper right), and follicular (middle left) B cell subpopulations. Data are representative of at least four mice of each genotype.
Determining whether such cells have life spans identical with those of the majority of follicular cells will require long term BrdU labeling, but our intermediate term labeling studies demonstrated that these cells are not short-lived. Surface BCR levels are reduced on most HKIR/JHD B cells, not just the canonical subpopulation, indicating that many such cells may be autoreactive. Therefore, HKIR/JHD mice could lack the competitor B cells necessary for the follicular exclusion mechanism to function. However, canonical R55 B cells are also found in abundance in the follicles of HKIR mice with a functional, endogenous Igh allele (data not shown). Approximately half the B cells in these mice express only endogenous BCRs (Fig. 1), and these probably constitute a normal competitor population.

A more general issue must be considered when comparing our results to those of studies of B cell tolerance that have employed conventional Ig transgenic approaches. Such comparisons may be confounded by differences in the regulation of conventional Igh transgenic arrays vs \( V_\mu \) knockin loci. Indeed, we previously found that B cells expressing canonical BCRs partially encoded by a conventional \( \mu \delta \) transgene construct showed no evidence of sBCR down-regulation (6). In fact, examination of several lines of mice containing this construct revealed a correlation among transgene copy number, levels of slgM and slgD expression that were sometimes supranormal, and degree of differentiation to an unusual T2-MZ phenotype. Moreover, a very high copy number of this transgene led to massive B cell deletion in the BM. In this context, normal regulation of slgD levels may be particularly relevant, as lack of this isotype has been found to affect both positive and negative selection of developing B cells (50, 51).

Canonical B cells in nontransgenic strain A mice serve as efficient memory precursors during the anti-Ars response, but do not mount early primary AFC responses. We previously speculated that this might be due to the low levels of autoreactivity of their BCRs (29). The data we present in this study are compatible with this idea, as the sBCR phenotype of peripheral canonical HKI65 B cells is consistent with autoantigen engagement. These cells have slightly down-regulated levels of slgM. They also display bimodal expression levels of slgD that may result from slowed progression to the slgD\(^{high}\) follicular phenotype. It is tempting to speculate that the reduced sBCR levels on canonical B cells precludes the efficient capture, processing, and presentation of Ag to T cells required for induction of Ab secretion, but does not prevent them from receiving the low levels of T cell help known to be sufficient to allow GC formation (52). Consistent with this idea are preliminary data suggesting that this GC only behavior is also displayed by canonical R55 B cells (L. Heltemes-Harris and T. Manser, unpublished observations). In general, if potentially pathological B cells that have undergone the process of learned ignorance are prohibited from differentiation to AFC phenotype before the specificity maturation of their BCRs in the GC, they would pose little threat for the development of autoimmune disease.

Interestingly, the expression of the canonical R55 BCR does not support the development of MZ B cells. Although the origin of this subpopulation remains controversial, past studies have suggested that the expression of certain autoreactive BCRs, including some with antinuclear Ag reactivity (53), promotes positive selection into this compartment (7). As the canonical R55 BCR is clearly autoreactive, our results would seem to be in contradiction of such past studies. In contrast, our observations are compatible with the idea that highly autoreactive B cells may be excluded from the MZ population due to strong constitutive signaling through their BCRs (54). However, the fact that MZ B cells are characterized not only by their anatomical locale, but also by their preactivated state, as well as the expression of high levels of slgM and low levels of
slgD (7) further complicates this issue. In addition, many deficiencies that compromise BCR signaling pathways result in a reduction in MZ B cells (7). We hypothesize that the early and substantial down-regulation of slgM levels on developing canonical R55 B cells precludes their development to the MZ phenotype, perhaps by denying them the level of autoantigen- mediated signaling necessary for positive selection into this subpopulation.

Elucidation of the mechanism(s) involved in the pathway of sBCR down-regulation that we describe in this study is an important area for future studies. A starting point may be provided by the previous work of Goodnow and colleagues (55), who suggested that the reduced levels of slgM characteristic of anergic B cells result from a slowing of egress of the IgM BCR complex from the endoplasmic reticulum to the Golgi. It would also seem of value to determine whether an analogous mechanism operative on the expression of TCR contributes to peripheral T cell tolerance. One past study provided evidence supporting this idea (56), but subsequent research has not addressed this possibility in detail (57). Finally, it will be crucial to determine whether this mechanism is operative in GC B cells, as the specificity maturation hypothesis predicts that if such cells are “ignorant” of self-Ags, they could not be subjected to the negative selection required to create a tolerant, antigen-specific memory compartment.

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