Influence of B Cell Antigen Receptor Expression Level on Pathways of B Cell Tolerance Induction

Xiaohe Liu, Shixue Shen and Tim Manser

*J Immunol* 2009; 182:398-407; doi: 10.4049/jimmunol.182.1.398

http://www.jimmunol.org/content/182/1/398

---

**References**

This article cites 35 articles, 20 of which you can access for free at:
http://www.jimmunol.org/content/182/1/398.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Influence of B Cell Antigen Receptor Expression Level on Pathways of B Cell Tolerance Induction

Xiaohu Liu,2,3 Shixue Shen,2 and Tim Manser4

We have described an Ig-transgenic, autoreactive B cell clonotype that undergoes a novel tolerance pathway. Early in development this clonotype expresses average BCR levels, but these levels are progressively down-regulated as development proceeds efficiently to the mature, follicular compartment. This clonotype does not display conventional features of anergy and can be induced to undergo apoptosis and receptor editing in in vitro bone marrow cultures, but these pathways are not taken in vivo. These data suggested that autoantigen-driven down-regulation of BCR levels and, hence, avidity for autoantigen allows this clonotype to bypass conventional tolerance mechanisms. To test this idea, we enforced elevated levels of expression of BCR in this clonotype by making the transgenic Igh locus homozgyous. This resulted in retarded clonotype development and L chain receptor editing in vivo. These data support a pivotal role for adaptive, autoantigen-induced adjustment of BCR expression levels in the regulation of primary B cell development and tolerance. The Journal of Immunology, 2009, 182: 398–407.

Three mechanisms of B lymphocyte tolerance induction have been extensively described: clonal deletion, receptor editing, and anergy (1–5). These result in either physical or functional elimination of autoreactivity from the mature B cell population. This is consistent with the forbidden clone corollary to the clonal selection hypothesis (6). However, removal of all autoreactive B cells from the functional pool would severely limit the size of the anti-foreign Ag repertoire, as BCRs cannot be monospecific (7, 8). In fact, many mature B cells display “multi” or “polyreactivity” (9–11), including autospecificities (11, 12). Also, a subset of autoreactive B cells that enter the periphery may not encounter sufficient quantities of their cognate autoantigen to induce tolerance pathways. Such B cells are said to be “ignorant” of or “indifferent” to self-Ags (13, 14). Nonetheless, some self-Ags clearly promote the positive selection of autoreactive B cells into the mature pool (15–18) and these B cells can serve useful functions (19). Collectively, these observations suggest that developing B cells expressing only certain types of autospecificity are subsumed (19). The studies were supported by National Institutes of Health Grant AI038965 (to T.M.).

Received for publication August 21, 2008. Accepted for publication November 3, 2008.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 The studies were supported by National Institutes of Health Grant AI038965 (to T.M.).

2 X.L. and S.S. contributed equally to this work.

3 Current address: Renal Electrolyte & Hypertension Division, School of Medicine, University of Pennsylvania, 415 Curie Boulevard, 115 Clinical Research Building, Philadelphia, PA 19104.

4 Address correspondence and reprint requests to Dr. Tim Manser, Department of Microbiology and Immunology, Jefferson Medical College, 469 JAH, 1020 Locust Street, Philadelphia, PA 19107. E-mail address: manser@mail.jci.tju.edu

Copyright © 2008 by The American Association of Immunologists, Inc. 0022-1767/08/$2.00

www.jimmunol.org
BM cells from day 5 cultures of HKIR+/+ mouse were analyzed by flow cytometry using allotype-nonspecific anti-IgM reagent, this value was obtained by PCR for both the endogenous JH3 and VDJ gene region. The right panel shows the mean fluorescence intensity (MFI) of sIgM levels on sIgM cells illustrated in the left panels. These data were obtained using a fluorochrome-labeled intact allotype-nonspecific anti-IgM reagent and indicated that HKIR+/+ BM cells in BM cultures containing Ars-Tyr express 1.5-1.7-fold more sBCRs than HKIR+/+ B cells in analogous cultures. When the analysis was done with a Fab anti-IgM reagent, this value was ~1.8-fold (data not shown). B, Splenic B220+ B cells from the indicated mice were analyzed for surface levels of BCR via flow cytometry using allotype-nonspecific anti-κ, anti-IgM, and anti-IgD Abs. Overlays of staining intensity on splenic B cells from these mice are shown. The right panel shows the mean fluorescence intensities of the staining patterns illustrated in the left panel. Figures are representative of data obtained from at least four mice of each genotype in multiple experiments.

of the learned ignorance pathway leads to activation of alternative central tolerance mechanisms. These results strongly support the hypothesis that adaptive down-regulation of BCR levels by autoantigen(s) of the type recognized by canonical B cells must result in reduction in the avidity of the B cell-autoantigen interaction below a certain threshold if retarded developmental progression and receptor editing are to be avoided.

**Materials and Methods**

**Mice**

The HKIR Vκ transgenic mice, Vκ10-Jκ1-transgenic mice, and double-transgenic HKIR/Vκ10 mice were previously described (20, 25). Mice expressing homozygous and heterozygous knockin H chain loci were genotyped by PCR for both the endogenous Jκ3 to Jκ4 region (absent in the HKIR line) and the knockin VDJ gene region. C57BL/6 (CD45.2+) and C57BL/6J (CD45.1+) mice were purchased from The Jackson Laboratory. Animals were approved by the Institutional Animal Care and Use Committee. The use of mice in these studies was conducted in compliance with institute guidelines and all protocols using autoclaved food and water. All mice were 8–12 wk of age at the time of initiation of the experiments. 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 and cell sorting**

Single-cell suspensions were prepared from lymphoid organs of 8- to 12-wk-old mice. Cells were stained with different combinations of the following Abs: anti-IgM (Jackson ImmunoResearch Laboratories), anti-IgD (11-26; Southern Biotechnology Associates), anti-κ (187.1; Southern Biotechnology Associates), anti-λ (goat anti-mouse PE and FITC; Southern Biotechnology Associates), anti-CD1d-PE (1B1), anti-CD3 (145-2C11), anti-CD21/35 (7G6), anti-CD22.2 (Cy34.1), anti-CD23 (B3B4), anti-CD45R (RA3-6B2; eBioscience), anti-CD45.2 (clone 104), anti-ClqRp (AA4.1; b Bioscience), or anti-idiotypic mAb E4 (prepared in-house). In some experiments monovalent Fab of anti-IgM (Jackson ImmunoResearch Laboratories,) were used for flow cytometric analysis of surface IgM levels. All Abs were obtained from BD Pharmingen unless otherwise indicated. Streptavidin-CyChrome (BD Pharmingen) was used to detect biotinylated Abs. Peanut agglutinin-FITC was from Vector Laboratories. Cells were assayed on an EPICS Elite flow cytometer (Coulter) and data were analyzed using FlowJo software (Tree Star). In some experiments, B cell subpopulations were stained and purified using a MoFlo high-performance cell sorter (DakoCytomation).

**BM cultures**

The S17 stromal cell line and IL-7 were used to generate BM cultures as previously described (25). Medium was supplemented with 16 ng/ml recombinant mouse IL-7 (R&D Systems). To block autoantigen binding to the BCR, monomeric p-azophenylarsonate (Ars)-Tyr was added to the cultures at 0.1 mM for at least 12 h as described before (25). The hapten p-azobenzoic acid-Tyr, for which canonical Abs have no measurable affinity, was used as a control.

**Immunohistochemistry and immunofluorescence**

Spleens from 8- to 12-wk-old naive mice were frozen and cryosections were prepared and processed as previously described (26). Sections were stained with combinations of various mAbs (described above), as well as MOMA-1-FITC (SeroTech), analyzed on a fluorescence microscope, and digital images were captured with a confocal laser-scanning microscope (Zeiss 510 Meta).

**Hybridomas and V region nucleotide sequencing**

FACS purified B220+ splenocytes from both HKIR+/+ and HKIR+/+ mice were stimulated with LPS (20 μg/ml) and IL-4 (50 ng/ml) for 3 days. Hybridomas were constructed using the SP2/0 fusion partner as previously described (27). Hybridomas were screened for λ, κ, and E4 Ab production by ELISA. Hybridomas were subcloned by limiting dilution and supernatants from IgM-secreting hybridomas were collected and used for ELISA and anti-nuclear Ab (ANA) staining. V region RT-PCR and nucleotide sequencing of PCR products were conducted as described previously (20).
Autoreconstitution

Adult mice were exposed to a sublethal dose of whole-body gamma irradiation (550 rad) as described before (26) and were allowed to rest for 4 wk. Cells were then obtained from lymphoid organs, labeled, and analyzed by flow cytometry as described above.

Adoptive transfer and immunization

After FACS purification of $\lambda^+$ and $\lambda^-$ splenic B cells from 8- to 12-wk-old mice, $0.5 \times 10^6$ cells of each population were injected into the tail vein of syngeneic B6.CD45.1 recipients that were immunized (i.p.) 12 h later with 100 $\mu$g of Ars-keyhole limpet hemocyanin (KLH) in alum. Germinal center responses were analyzed on day 6 as described before (24, 28).

ELISA

Total and dsDNA-binding IgM was measured by ELISA on 96-well plates (Immulon-4; Dynatech Laboratories) as previously described (22). Bound Abs were also elaborated using biotin-conjugated goat anti-mouse $\lambda$ Ab or alkaline phosphatase-conjugated goat anti-mouse $\kappa$ Ab (Southern Biotechnology Associates).

ANA staining

Slides coated with human epithelioid Hep-2 cells (Antibodies) were used to determine the ANA reactivity of hybridoma supernatants according to the manufacturer’s instructions and as previously described (20). Fluorescein-labeled (FITC) affinity pure donkey anti-mouse IgM Ab, $\mu$-chain specific (Jackson ImmunoResearch Laboratories), was used to detect the bound Abs.

Results

Increased expression of the canonical BCR in HKIR homozygous mice

We intercrossed the HKIR line to generate mice expressing homozygous H chain knockin loci. For many experiments, these mice were then crossed to a line of conventional $V_{kJ10A}$-Jk1-transgenic mice, resulting in mice homozygous or hemizygous for the HKIR knockin $Igh$ locus and hemizygous for the canonical

FIGURE 2. Retarded B cell autoreconstitution in HKIR+/+/VkJ10 mice. Mice of the indicated genotypes were given 550 rad whole-body irradiation, allowed to autoreconstitute for 4 wk, and then spleen cells were isolated, stained with mAbs specific for the indicated markers, and analyzed by flow cytometry. A. Percentages of B cells in these mice in the indicated gates are shown. B. The absolute number of mature and transitional B cells in each spleen sample were calculated from the data shown in A. C. Percentages of B220$^+$ $\lambda^+$ B cells in the spleens of the indicated types of mice are shown. Data in A and C are representative of those obtained from two mice of each genotype. All of these data are illustrated in B. The data in A were used to generate the values illustrated in B.
Vκ10A-Jκ1-transgenic L chain locus. The former mice are termed HKIR1+/+/Vκ10 and the latter mice HKIR1+/−/Vκ10.

To compare levels of canonical BCR expression on developing HKIR1+/+/Vκ10 B cells under conditions when this BCR was engaging autoantigen(s) and when it was not, we used monomeric Ars-tyrosine to block canonical BCR-autoantigen interactions in BM cultures as described before (25). After 5 days, sIgM levels on immature B cells arising in HKIR1+/+/Vκ10 BM cultures containing Ars-tyrosine were on average 1.7-fold higher than those on B cells in analogous HKIR1+/−/Vκ10 cultures (Fig. 1A and legend). In addition, sIgM levels on both types of B cells in these cultures were ~10-fold elevated as compared with the levels observed in cultures containing p-aminobenzoic acid-tyrosine, a control hapten for which canonical Abs have no measurable affinity (Fig. 1A).

These levels approached those on nontransgenic control B cells. Analysis of the peripheral B cell compartment of HKIR1+/+/Vκ10 mice revealed that the majority of canonical B cells, as detected by the anti-clonotypic mAb E4, were indistinguishable in their FO locale in the spleens and lymph nodes as compared with HKIR1+/−/Vκ10 mice (data not shown). However, although slgM levels on splenic B cells were only slightly higher, slgD and total sBCR (slgκ) levels were ~1.5-fold higher in HKIR1+/+/Vκ10 mice as compared with HKIR1+/−/Vκ10 mice (Fig. 1B). These data indicate that most peripheral B cells in HKIR1+/+/Vκ10 mice have not down-regulated surface BCR to levels characteristic of HKIR1+/−/Vκ10 mice. Because we do not know whether either type of B cell expresses normal levels of slgD at some point during primary development, an alternative explanation for these observations is that developing HKIR1+/−/Vκ10 and HKIR1+/+/Vκ10 B cells fail to up-regulate IgD expression, but levels of slgD are higher on HKIR1+/+/Vκ10 B cells since they express two copies of the transgenic Igκ locus.

Retarded B cell development and λ+ B cells in autoreconstituting HKIR1+/+/Vκ10 mice

To determine whether the elevated levels of autoreactive BCR expression in HKIR1+/+/Vκ10 mice influenced the kinetics of B cell developmental progression, we used an autoreconstitution strategy. Four weeks after sublethal irradiation, the percentages of B220+, slgM+,slgD+, and B220+/AA4.1+ splenic lymphocytes were reduced ~2-fold in HKIR1+/−/Vκ10 as compared with HKIR1+/+/Vκ10 mice. Also, lower percentages of CD23+ splenic B cells were observed in autoreconstituting HKIR1+/+/Vκ10, as compared with HKIR1+/−/Vκ10 mice (Fig. 2A). The absolute numbers of both immature (AA4.1+) B cells and mature B cells in the spleens of autoreconstituting HKIR1+/+/Vκ10 mice were substantially lower than in HKIR1+/−/Vκ10 mice (Fig. 2B). All of these observations are consistent with slowed B cell developmental progression in HKIR1+/+/Vκ10 mice. Surprisingly, λ+ B cells were also detected in autoreconstituting HKIR1+/+/Vκ10 mice (Fig. 2C), an indication that L chain receptor editing was taking place (4). These λ+ cells could not have been derived from fetal precursors, indicating that the processes that produce them take place during BM B cell development.

**FIGURE 3.** Reduction of T1 and T2 transitional B cells in HKIR1+/+/Vκ10 mice. Splenic B cells from mice of the indicated genotypes were stained with Abs specific for the indicated markers and analyzed by four-color flow cytometry. The percentage of cells in each fraction are indicated next to the gates. The data are representative of at least three mice per experiment and multiple experiments.
Reduction of T1 and T2 transitional B cells in HKIR<sup>+/+</sup>/V<sub>κ10</sub> mice

To determine whether the efficiency of B cell development was also abnormal in unmanipulated HKIR<sup>+/+</sup>/V<sub>κ10</sub> mice, we next examined the BM and splenic transitional B cell compartments in these mice via flow cytometry. Comparison of the various BM B cell developmental subsets in HKIR<sup>+/+</sup>/V<sub>κ10</sub> and HKIR<sup>+/−</sup>/V<sub>κ10</sub> mice did not reveal any obvious qualitative or quantitative differences (data not shown). However, analyses of splenic B cells showed that the overall percentage of AA4.1<sup>+</sup> transitional B cells was lower in HKIR<sup>+/+</sup>/V<sub>κ10</sub> mice as compared with HKIR<sup>+/−</sup>/V<sub>κ10</sub> mice (Fig. 3, left panels). This was specifically due to a reduction in the representation of sIgM<sup>high</sup> T1/T2 stage B cells (Fig. 3, middle and right panels). As such, the data presented in Figs. 2 and 3 suggest that the early stages of developmental progression in the periphery are slowed in the HKIR<sup>+/+</sup>/V<sub>κ10</sub> B cells. Whether this is accompanied by clonal deletion and can account for the elevated percentage of T3 transitional B cells in the spleens of these mice will require further investigation.

Elevated frequency of λ<sup>+</sup> B cells in the spleens of HKIR<sup>+/+</sup>/V<sub>κ10</sub> and HKIR<sup>+/−</sup> mice

Due to enforced expression of the transgenic V<sub>κ10</sub> gene, HKIR<sup>+/−</sup>/V<sub>κ10</sub> mice contain very few λ<sup>+</sup> L chain-expressing B cells (Ref. 25 and see below). In contrast, Fig. 2A shows that there is a distinct subpopulation of λ<sup>+</sup> B cells in the spleens of autoreconstituting HKIR<sup>+/+</sup>/V<sub>κ10</sub> mice. To determine whether this subpopulation was present in unmanipulated HKIR<sup>+/+</sup>/V<sub>κ10</sub> mice, we performed flow cytometric analysis on spleen cells from these mice as well as HKIR<sup>+/−</sup>/V<sub>κ10</sub>, HKIR<sup>+/+</sup>, and HKIR<sup>+/−</sup> mice.
Fig. 4 shows that HKIR +/+ mice indeed contain a distinct subpopulation of λ⁺ cells, comprising >1% of splenocytes that is essentially absent in HKIR +/+ λκ10 mice. Interestingly, HKIR +/+ mice also contain levels of λ⁺ cells similar to HKIR +/+ λκ10 mice. HKIR +/+ mice also contain a subpopulation of λ⁺ cells that is less prevalent than that observed in HKIR +/+ mice. However, further studies showed that these mice largely express an endogenous H chain rather than the HKIR H chain (data not shown).

L chain editing and isotypic inclusion in λ⁺ B cells in HKIR +/+ mice

As mentioned above, the appearance of λ⁺ B cells in mice expressing an Igκ transgene is an indication of L chain receptor editing and isotypic inclusion. The λ⁺ cells in HKIR +/+ mice have MZ-like phenotypes, as shown in Fig. 6. The λ⁺ double-positive B cells in HKIR +/+ mice have MZ-like phenotypes. A, Spleen cells from mice of the indicated genotypes were stained with an anti-λ Ab as well as Abs specific for IgM and IgD and analyzed by flow cytometry. Panels show data obtained for cells gated on λ staining. Gates are set around areas expected to contain FO (left) or MZ (right) B cells, although the gates set for HKIR +/+ λκ10 and HKIR +/+ mice do not exactly correspond to those in the B6 panel due to the unusual BCR expression levels of the former B cells. B, Upper panels, spleen cells from mice of the indicated genotypes were stained with Abs specific for B220, CD1d, and λ and analyzed by flow cytometry. Gates are set around areas expected to contain largely MZ (upper gates) or FO (lower gate) B cells. B, Lower panels, splenic B cells from the indicated mice were stained with an anti-λ Ab as well as Abs specific for CD21 and CD23 and analyzed by flow cytometry. Panels show data obtained for cells gated on λ staining. Gates are set around areas expected to contain FO (left) or MZ (right) B cells, although the MZ-like cells in HKIR +/+ λκ10 and HKIR +/+ mice express higher levels of CD23 than those in B6 mice. All data are representative of those obtained from at least two experiments using three or more mice per experiment.

FIGURE 6. The λ⁺ double-positive B cells in HKIR +/+ mice have MZ-like phenotypes. A, Spleen cells from mice of the indicated genotypes were stained with an anti-λ Ab as well as Abs specific for IgM and IgD and analyzed by flow cytometry. Panels show data obtained for cells gated on λ staining. Gates are set around areas expected to contain FO (left) or MZ (right) B cells, although the gates set for HKIR +/+ λκ10 and HKIR +/+ mice do not exactly correspond to those in the B6 panel due to the unusual BCR expression levels of the former B cells. B, Upper panels, spleen cells from mice of the indicated genotypes were stained with Abs specific for B220, CD1d, and λ and analyzed by flow cytometry. Gates are set around areas expected to contain largely MZ (upper gates) or FO (lower gate) B cells. B, Lower panels, splenic B cells from the indicated mice were stained with an anti-λ Ab as well as Abs specific for CD21 and CD23 and analyzed by flow cytometry. Panels show data obtained for cells gated on λ staining. Gates are set around areas expected to contain FO (left) or MZ (right) B cells, although the MZ-like cells in HKIR +/+ λκ10 and HKIR +/+ mice express higher levels of CD23 than those in B6 mice. All data are representative of those obtained from at least two experiments using three or more mice per experiment.
whether the H9261 inantly develop to FO phenotype and locale (20, 21, 25). To test with Abs to
Therefore, we costained splenic B cells in the various transgenic lines
B cells in both HKIR Figs. 2 and 4, these results suggest that a subset of developing
and appears to be plasma cells located in the red pulp. We currently have
no explanation for the presence of these cells in this mouse line. The data are
representative of those obtained from at least three mice per genotype.

FIGURE 7. Most λ+ splenic B cells in HKIR+/+/Vk10 mice reside in the MZ. Spleen sections from mice of the indicated genotypes were stained with MOMA-1-FITC and anti-λ-PE and digital images were captured by immunofluorescence microscopy. MOMA-1 staining defines the border of the B cell follicle and the MZ. Original magnification, ×200. The intensely staining λ+ cells in HKIR+/+/Vk10 spleens was reproducibly observed and appears to be plasma cells located in the red pulp. We currently have no explanation for the presence of these cells in this mouse line. The data are representative of those obtained from at least three mice per genotype.

FIGURE 8. λ+ B cells from HKIR+/+/Vk10 mice respond to Ars-KLH immunization in vivo. FACS-purified λ+ and λ+ splenic B cells from HKIR+/+/Vk10 mice were transferred to recipient C57BL/6.CD45.1 mice that were immunized (i.p.) 12 h later with 100 μg of Ars-KLH in alum. Mice were sacrificed on day 6 after transfer and immunization, and splenocytes were stained with Abs specific for the indicated markers and analyzed by three-color flow cytometry. Gated percentages represent peanut agglutinin-positive (PNA+), CD45.2+ GC B cells. The data are representative of two experiments.

editing (4). Indeed, previous studies showed that κ+, λ+ dual L chain-expressing B cells present in the marginal zone (MZ) in another line of IgH antinuclear Ag transgenic mice usually are the product of receptor editing and L chain isotypic inclusion (29). Therefore, we costained splenic B cells in the various transgenic lines with Abs to κ and λ L chains. Whereas good L chain isotypic exclusion was seen in HKIR+/−/Vk10 and HKIR+/− B cells (data not shown), nearly all λ+ B cells in HKIR+/+/Vk10 mice stained at low intensity with anti-κ as well (Fig. 5, upper left panel). The isotypically included κ-chain on these cells is very likely the canonical Vk10-Jk1, since λ+ HKIR+/+/Vk10 B cells also showed low levels of clonotype-specific E4 staining (Fig. 5, lower left panel).

In contrast, the λ+ B cells in HKIR+/+ mice do not stain with anti-κ and are E4− (Fig. 5, middle panels). Interestingly, the λ+ subset in HKIR+/+/Vk10 mice give rise to E4 staining intensities higher than “bulk” E4+ cells when costained with anti-λ-PE and E4-R670 (data not shown), but lower than bulk E4+ B cells when costained with anti-κ-FITC and E4-R670 (Fig. 5). This difference is likely due to fluorescence energy transfer between PE and R670 when these two fluorochromes are in close juxtaposition, as would be the case when anti-λ-PE and E4-R670 were staining the same BCR. Taken together with the data presented in Figs. 2 and 4, these results suggest that a subset of developing B cells in both HKIR+/++/Vk10 and HKIR+/++ mice undergo L chain receptor editing, resulting in expression of high levels of λ-chain containing BCRs on their mature progeny.

λ+expressing B cells in HKIR+/+/Vk10 mice have an unusual cell surface phenotype and reside largely in the MZ.

Canonical B cells in HKIR+/− and HKIR+/−/Vk10 mice predominantly develop to FO phenotype and locale (20, 21, 25). To test whether the λ+expressing B cells in HKIR+/+ mice developed to
FO phenotype, we first costained splenic cells with Abs to λ L chains as well as IgM and IgD. As shown in Fig. 6A, left panel, λ+ B cells in HKIR+/+/Vk10 mice are largely IgMhighIgDhigh. In contrast, the λ+ B cells in HKIR+/+ are mainly IgMlowIgDhigh (Fig. 6A, middle panel), as expected of FO B cells.

Given these results, we further analyzed these λ+ expressing subpopulations with markers routinely used to distinguish FO from MZ B cells. Fig. 6B shows that the λ+ subpopulation in HKIR+/+/Vk10 mice is CD21high and CD1dhigh as expected of MZ B cells, but expresses higher levels of CD23 than most MZ B cells in B6 mice. The λ+ subpopulation in HKIR+/+ mice revealed a phenotype intermediate between FO and MZ B cells in B6 mice. It is CD1dhigh and bimodal with respect to CD21 and CD23 expression, indicating it is composed of both FO and MZ-like B cells.

To expand on these flow cytometric studies, immunohistology was performed on the spleens of HKIR+/+/Vk10, HKIR+/+/Vk10, HKIR+/−/Vk10, HKIR+/− and HKIR+/− mice. Fig. 7 shows that the few λ+ cells observed in white pulp and MZ regions in HKIR+/−/Vk10 spleens are located in both of these areas (the inner perimeter of the MZ is defined by MOMA-1 (green) staining). A substantially elevated frequency of λ+ cells is seen in HKIR+/+ spleens, but as in the other mice these are found in both

Moma-1 Lambda

FIGURE 8. λ+ B cells from HKIR+/−/Vk10 mice respond to Ars-KLH immunization in vivo. FACS-purified λ+ and λ+ splenic B cells from HKIR+/−/Vk10 mice were transferred to recipient C57BL/6.CD45.1 mice that were immunized (i.p.) 12 h later with 100 μg of Ars-KLH in alum. Mice were sacrificed on day 6 after transfer and immunization, and splenocytes were stained with Abs specific for the indicated markers and analyzed by three-color flow cytometry. Gated percentages represent peanut agglutinin-positive (PNA+), CD45.2+ GC B cells. The data are representative of two experiments.
The receptor editing hypothesis proposes that secondary L (and perhaps H) chain V(D)J rearrangements induced by autoantigen-BCR interactions take place until a new BCR is expressed that is less autoreactive (2). To evaluate whether this prediction was fulfilled by λ+ B cells in HKIR+/+Vκ10 and HKIR++/ mice, hybridomas were produced from the splenic B cells of these mice after polyclonal activation in vitro. As expected from the flow cytometric studies illustrated in Fig. 5, the majority of λ+ hybridomas derived from HKIR+/+Vκ10 B cells expressed mAbs containing both κ and λ L chains and were E4+, indicating that the expressed λ L chain was derived from the Vκ10-Jκ1 transgene. In contrast, all of the λ+ hybridomas from HKIR+/+ mice expressed only A L chains and were E4+. HKIR+/+Vκ10 hybridomas had not lost expression of the canonical VH gene, since all were positive by ELISA for expression of several VH-dependent idiotypes in addition to E4, and two of these assayed were positive for expression of HKIR RNA as assessed by RT-PCR and nucleotide sequencing (data not shown). Although the mAbs produced by the HKIR+/+ λ+ hybridomas did not express detectable levels of VH-dependent idiotypes, two of these were shown to express an intact HKIR locus via RT-PCR and nucleotide sequencing (data not shown).

Because the autoantigen(s) with which canonical BCRs interact in vivo has not been biochemically defined, the resulting hybridomas were tested for general levels of autoreactivity using whole cell staining (ANA) assays. Fig. 9 shows that although all of the mAbs produced by representative hybridomas stained elements in the cytoplasm, the nuclear staining intensity of λ+ mAbs from HKIR+/+Vκ10 mice was less than the levels obtained with mAbs obtained from HKIR+/+Vκ10 mice, while such staining with λ+ mAbs obtained from HKIR++/ mice appeared to be even lower.

**Discussion**

The data we present here show that when the learned ignorance pathway is inhibited in developing canonical clonotypes in vivo, B cell development is retarded and receptor editing is induced. These data illustrate the pivotal influence of adaptive regulation of BCR expression levels on the pathway(s) of development and tolerance taken by an immature B cell. They also add further weight to the conclusion that BCR-autoantigen interactions of the same specificity can lead to induction of different central tolerance mechanisms in vivo, depending on the avidity of these interactions (1). Interestingly, BCR-transgenic B cells expressing another version of the canonical BCR, in which the H chain is encoded by a conventional transgenic locus and lacks the position 55 R mutation and the L chain contains numerous amino acid substitutions as compared with the Vκ10 gene (termed Ars/A1), appear not to undergo adaptive BCR down-regulation and become anergic (30). Although differences in the autoantigen specificities of Ars/A1 and HKIR/Vκ10 BCRs may contribute to the distinct fates of B cells expressing these two types of Ag receptors, we speculate that the inability of conventional transgenic H chain loci to undergo adaptive, autoantigen-driven down-regulation of expression of IgM and IgD in the early stages of B cell development may also be a factor in this regard.

Enforced expression of two copies of the canonical HKIR H chain locus resulted in increased expression levels of the canonical BCR on immature HKIR+/+Vκ10 B cells developing in vitro.
under conditions where autoantigen engagement was blocked, as compared with HKIR+/+Vκ10 B cells. This was also the case for peripheral HKIR+/+Vκ10 B cells in vivo and a subpopulation of these B cells underwent receptor editing, leading to expression of high levels of HKIR BCRs that contained both κ and λ L chains. Such BCRs displayed reduced autoreactivity for nuclear Ags as compared with canonical HKIR/Vκ10 BCRs.

Previous analyses of another line of site-directed IgH-transgenic mice expressing antichromatin BCRs revealed the presence of κ-λ L chain isotypically included B cells in the MZ compartment (29). Because these B cells expressed an autoreactive IgH-A BCR, it was argued that tolerance was maintained in this population due to a combination of a reduced level of autoreactivity due to dual L chain expression, as well as “sequestration” of these B cells in the MZ. Our data suggest that the κ-λ isotypically included population of MZ-like B cells present in HKIR+/+Vκ10 mice may have followed a similar pathway of tolerance induction, although the relevance of B cell localization to the MZ for maintenance of tolerance in both of these cases is unclear at present. The data we illustrate in Fig. 8 show that the + B cells in HKIR+/+Vκ10 mice are able to respond to Ag in vivo and thus might be capable of responding to autoantigen if located in a supportive microenvironment such as the follicle.

In HKIR+/+ mice, receptor editing also appears to be taking place. However, the + B cells found in HKIR+/+ mice do not coexpress a κ L chain. In addition, they are observed in both FO and MZ regions of the spleen and have a “mixed” FO and MZ phenotype. The phenotypic differences of these cells as compared with those found in HKIR+/+Vκ10 mice may be due to the difficulty in editing or otherwise altering expression levels of the transgenic Vκ10 locus. These data argue that preferential selection into the MZ compartment of κ-λ isotypically included B cells in HKIR+/+Vκ10 mice is due to their elevated levels of BCR expression, higher avidity for autoantigen, or both.

Clearly, the bulk of the developing B cells in both HKIR+/+Vκ10 and HKIR+/+ mice do not underexpress a λ L chain, as only 3–5% of peripheral B cells in these mice express a λ L chain. Whether a major subpopulation of developing B cells in these mice follows pathways of receptor editing leading to expression of noncanonical κ L chains was not examined. If this is taking place, however, it apparently does not alleviate the retarded developmental progression displayed by HKIR+/+Vκ10 B cells. This suggests that most κ L chains are not “good editors” of the autoreactivity characteristic of canonical HKIR BCRs. This may explain why the T1 and T2 stages of splenic B cell development are underrepresented in HKIR+/+Vκ10 mice, and the T3 subset, which may be largely composed of anergic B cells (31), is overrepresented. This could also account for the fact that the frequency of + B cells is similar in HKIR+/+Vκ10 and HKIR+/+ mice. Further studies will be required to more directly address these issues. Finally, in agreement with a previous report in which B cell development was assessed in mice expressing two copies of the 3H9/56R antichromatin BCR (32), H chain editing does not seem to play a predominant role in the development of either HKIR+/+Vκ10 or HKIR+/+ B cells, since hybridomas derived from such B cells express mAbs with canonical VH-dependent idiotopes, RNA derived from the intact HKIR locus, or both.

Although the data we present here demonstrate that B cells expressing a canonical HKIR BCR are competent to undergo L chain receptor editing in vivo, as we previously demonstrated was the case in vitro (25), the question of why developing canonical HKIR+/+ B cells undergo learned ignorance as opposed to receptor editing remains incompletely resolved. Current data indicate that three parameters predominantly influence the tolerance pathway taken by a developing autoreactive B cell: the stage and locale of development when autoantigen is first encountered, the avidity of the BCR-autoantigen interaction, and the nature of the autoantigen itself. With regard to the latter factor, we considered that since the cognate autoantigen for canonical HKIR B cells in vivo is most likely nucleic acid-based, that TLR 3, 7, or 9 signaling might be increasing the efficiency of the learned ignorance process. However, we observed no differences in B cell development and BCR down-regulation in TLR9 or MyD88-deficient and sufficient versions of HKIR+/+ and HKIR+/+Vκ10 mice (Ref. 33 and F. Coffey and T. Manser, unpublished results).

With respect to the nature of the first two parameters, our in vitro studies suggest that the cognate autoantigen(s) for the canonical HKIR BCR is ubiquitous and likely present in the BM and can be derived from developing B cells themselves (25). This is consistent with past studies showing that intracellular autoantigens are externalized by apoptotic or postapoptotic necrotic cells (34, 35). However, when normal apoptotic cell scavenging and phagocytic pathways are operative, such Ags are probably present at only low concentrations and predominantly in soluble form in the milieu of developing B cells. This could favor the induction of learned ignorance over receptor editing early in B cell development, as our data show that the latter process requires higher avidity BCR-autoantigen interactions than the former.

Acknowledgments

We thank Dr. Larry Wysocki for p-aminobenzoic acid-tryosine and rabbit anti-CRI antiserum, the Kimmel Cancer Center Flow Cytometry and Bio-imaging Facilities, and all members of the Manser laboratory for their indirect contributions to this work.

Disclosures

The authors have no financial conflict of interest.

References


