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Autoantigen-B Cell Antigen Receptor Interactions That Regulate Expression of B Cell Antigen Receptor Loci1

Xiaohé Liu,* Lawrence J. Wysocki,† and Tim Manser2∗∗

Levels of AgR (BCR) expression are regulated during B cell development, activation, and induction of tolerance. The mechanisms responsible for and consequences of this regulation are poorly understood. We have described a class of DNA-based autoantigen-reactive B cell that down-regulates BCR expression during development to mature follicular phenotype. In this study, we show that at immature stages of primary differentiation, individual B cells of this type can dynamically modulate levels of expression of BCR in inverse proportion to degree of autoantigen engagement and induced BCR signaling. These adjustments in BCR expression are not associated with cell death, BCR revision, or altered development, and do not require TLR 9. Strikingly, modulation of BCR subunit gene RNA levels and transcription parallels these changes in BCR expression, indicating a direct link between autoantigen-BCR interactions of this type and regulation of transcription of BCR-encoding loci. We propose that this adaptive process allows this class of autoreactive B cell to avoid conventional tolerance pathways and promotes development to mature phenotype. The Journal of Immunology, 2007, 178: 5035–5047.

The expression of an AgR (BCR) or its signaling surrogate is absolutely required for B cell survival and development starting at the pre-B cell stage (1–5). However, levels of expression of the various isoforms of the BCR change during B cell development (6–8). For example, immature B cells express low levels of surface IgM (sIgM)3 which increase during later, transitional, stages of development (7, 8). Conversely, levels of BCR expression are reduced on germinal center (GC) and memory B cells (9, 10). Moreover, autoantigen engagement of the BCR can lower levels of BCR expression, particularly of sIgM, and this is often associated with induction of tolerance (11). The mechanisms responsible for these quantitative changes in levels of BCR expression are not well understood.

Three mechanisms have been described that contribute to B cell tolerance: clonal deletion, receptor editing, and anergy (11, 12). These result in either physical (deletion, editing) or functional (anergy) elimination of autoreactivity from the mature B cell compartment. Such purging is consistent with the forbidden clone corollary to the clonal selection hypothesis (13). However, removal of B cells with any degree of autoreactivity would severely limit the size of the anti-foreign Ag repertoire, as BCRs cannot be monospecific (14, 15). In fact, many members of the primary B cell compartment display “multi” or “polyreactivity,” including autospecificity (16, 17). Such reactivity may ensure that a protective Ab response can be mounted to any infectious agent in a reasonable period of time (18, 19).

Moreover, some self Ags promote the positive selection of B cells into the mature, peripheral pool (20–22), and these B cells can serve useful physiological functions (16, 23). In addition, evidence is accumulating to suggest that a significant percentage of autoreactive B cells that enter the periphery and are exposed to autoantigen is not anergic. Such B cells are said to be “ignorant” or “indifferent” to self Ags (11, 24).

Why particular autoreactive B cells are selected to clonal deletion, receptor editing, anergy, or ignorance is unclear. Even more puzzling is why certain autoantigen-BCR interactions lead to positive selection. Models invoking thresholds of BCR signal strength (22, 25) and changes in the topology and membrane organization of BCRs (26) have been forwarded, but arguably none of these satisfactorily accounts for the observations discussed above. Adding to the confusion concerning the role of autoantigens in B cell development are studies showing that BCR signaling surrogates lacking Ag-binding domains can promote B cell maturation (4, 5).

We have described two lines of gene-targeted mice in which different Ab H chain V (VH) region genes are inserted into the endogenous H chain locus. These VH genes differ only by a mutation to arginine (R) at position 55 in CDR 2 (27, 28). We term these lines HKIR (with the R mutation) and HKI65, respectively. These H chain loci, in combination with a single, unmutated, endogenous κ L chain gene, encode Abs termed “canonical” that bind the hapten arsonate (Ars) with indistinguishable affinity. The HKI65 and HKIR forms also have weak and high avidity for DNA-based autoantigens, respectively.

B cells expressing either canonical HKIR or HKI65 BCRs develop to mature follicular (FO) phenotype, reside in splenic and lymph node follicles, and are not short-lived (28). However, canonical HKIR B cells express very low levels of both sIgM and sIgD isoatypes of the BCR (14), indicating that they are not “ignorant” of self Ag(s). Taken together, these observations led us to

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3 Abbreviations used in this paper: sIgM, surface IgM; GC, germinal center; Ars, arsonate; sIgD, surface IgD; FO, follicular; BM, bone marrow; HEL, hen egg lysozyme; MFI, mean fluorescence intensity; QPCR, quantitative PCR; HPRT, hypoxanthine phosphoribosyltransferase; MjCD, methyl-β-cyclodextrin; PABA, para-aminobenzoic acid.

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suggest that developing canonical HKIR B cells down-regulate levels of expression of BCR in response to autoantigen engagement, but this does not perturb developmental progression, or result in induction of conventional tolerance pathways (28). In this study, we provide support for this idea and show that modulation of BCR levels is regulated by both endocytosis and a feedback loop in which BCR engagement by a DNA-based autoantigen(s) is linked to transcriptional control of BCR-encoding loci.

Materials and Methods

Mice
HKIR and HKI65 V \( \gamma \) knockin, V\( x \)10-Jx1-transgenic mice, and TLR9 knockout mice have been described previously (27–31). The construct used to generate the V\( x \)10-Jx1 mice was identical with that previously described to generate Ars/Al mice (29), except that the V\( x \)10-Jx1 region lacked somatic mutations. C57BL/6, MD4 HEL-Ig, and ML5 soluble hen egg lysozyme (HEL) transgenic mice were obtained from The Jackson Laboratory. Mice were maintained in specific pathogen-free isolation and given autoclaved food and water and used at 6–12 wk of age. The use of mice was conducted in compliance with institute guidelines and all protocols using animals were approved by the Institutional Animal Care and Use Committee.

Reagents
p-Azophenylarsonate-t-tirosine (Ars-Tyr) and p-aminobenzoic acid-t-tyrosine were conjugated and purified in-house as described (29). Goat anti-mouse \( \kappa \) Ab was obtained from GeneTex. Actinomycin D, cycloheximide, methyl-\( \beta \)-cyclodextrin and mimosin were obtained from Sigma-Aldrich and the NFAT inhibitory peptide VIVIT was obtained from Calbiochem. Plasmids containing Ig\( \mu \) constant region exons and mouse \( \beta \)-actin cDNA were gifts from Dr. W. Dunnick (University of Michigan, Ann Arbor, MI) and Dr. L. Vercouly (The Scripps Research Institute, La Jolla, CA), respectively. The following Abs were used (from BD Pharmingen, unless otherwise noted): anti-CD45R (RA3-6B2; eBioscience), Fab goat anti-monomeric IgM or anti-IgM (Jackson ImmunoResearch Laboratories), anti-IgG (11-26; Southern Biotechnology Associates), anti-\( \alpha \) (187.1; Southern Biotechnology Associates), anti-ClqR (AA4.1; eBioscience), anti-CD21/CD35 (766), anti-CD23 (B384), anti-CD24 (HAS, M1/69), anti-CD19 (1D3), anti-CD22 (C34.1), anti-\( \alpha \) (JC5-1), anti-CD43 (57, Ly48), anti-BPI (6C3), and anti-idiotypic mAb E4 (prepared in-house). SA-R670 was used to detect biotinylated Abs.

Bone marrow (BM) cultures
The S17 stromal cell line (a gift from Dr. R. Hardy, Fox Chase Cancer Center, Philadelphia, PA with permission from Dr. K. Dorshkind, University of California, Los Angeles, CA) was used to generate BM cultures as described (32). Medium was supplemented with 16 ng/ml recombinant murine IL-7 (R&D Systems) or 10–100 U/ml IL-7 (derived from culture supernatants of J558L cells transfected with an \( \alpha \)-IL-7 expression vector, a gift from Dr. F. Melchers, University of Basel, Basel, Switzerland). These cultures were free of mature recirculating B cells as determined by flow cytometry. For enrichment of more mature IgD\( ^{+} \) B cells, immature cells were further cultured on irradiated S17 cells in medium lacking IL-7 for 48–72 h.

Flow cytometry and cell sorting
Cells (10\(^6\)/sample) were stained and analyzed as previously described (28) using the reagents described above. For measuring total levels of IgM, cells were first stained with anti-B220 and anti-IgM Abs, then washed, and fixed with 3.7% formaldehyde and permeabilized with 0.5% saponin, 0.2% FBS, and 0.05% Tween 20, 0.01% NaN\(_3\) for 24 h before restaining with the same anti-IgM reagent. Flow cytometric analyses were performed on EPICS Elite (Coulter) or FACSCalibur (BD Biosciences) flow cytometers and data were analyzed using the FlowJo software (Tree Star). In some experiments, B cell subpopulations were stained and purified using a MoFlo high-performance cell sorter (DakoCytomation).

Surface autoantigen-binding assay
BM culture cells grown in low concentrations (10\(^{-6}\) M) of Ars-Tyr were harvested, washed, and stained with DNase-I-FITC (Molecular Probes) and anti-B220. Steady state surface DNase-I-FITC binding was measured by flow cytometry (Coulter) gating on B220\(^{+}\) cells. Next, a higher concentration (10\(^{-4}\)) of Ars-Tyr or para-aminobenzoic acid (PABA)-Tyr was added to the samples and kinetic data were collected and analyzed using the FlowJo software (Tree Star).

Immunohistochemistry
Spleens from 8- to 12-wk-old naive mice were frozen and cryosections were prepared and processed as previously described (28, 33). Sections were stained with combinations of various mAbs (described above) and imaged via fluorescence microscopy.

CFSE labeling
Day five BM culture cells were harvested, washed, and loaded with CFSE (Molecular Probes) as described (34). Labeled cells were quenched, washed with cold RPMI 1640 medium, and placed in culture as described above for another 48–72 h. Cells were then harvested and surface stained with various Abs and analyzed by flow cytometry as described above.

slgM internalization
To measure endocytosis, BM-cultured B cells were pre-equilibrated at 4°C in RPMI 1640 0.5% FBS and incubated with biotin goat anti-mouse IgM Fab. Cells were then either chilled on ice (T\(_0\)) or incubated at 37°C for the indicated times (T\(_n\)) before termination at low temperature on ice. Cells were then stained with FITC-B220 and streptavidin R670, and surface BCR expression was determined by flow cytometry. Percent internalization was calculated by the formula (mean fluorescence intensity (MFI) slgM (T\(_n\)) – MFI slgM (T\(_0\)))/MFI IgM (T\(_0\)) \times 100 (35).

Cell viability assay
Apoptotic and viable cell frequency was determined using an Annexin V FITC/PI Apoptosis Detection kit (BD Pharmingen) following the protocol supplied by the manufacturer.

Real-time RT-PCR
The TaqMan real-time PCR system was used as described previously (30). RNA was extracted and purified using the RNeasy mini kit (Qiagen). All RNA templates were subjected to DNase I digestion following the protocol in the kit. After reverse transcription, cDNA was subjected to PCR using designed primers and TaqMan probes (Custom Oligonucleotide Factory) for C\(_\mu\) or a premade one-tube TaqMan Gene Expression Assay (Applied Biosystems) for C\(_\kappa\), RAG-1, IgB, GAPDH, hypoxanthine phosphoribosyltransferase 1 (HPRT1), and CD19. The reaction was performed on an ABI Prism 7000 sequence detection system (PerkinElmer). Relative RNA concentrations were determined and the data were analyzed with the ABI prism 7000 SDS software (Applied Biosystems). All results were normalized to those obtained from GAPDH RNA.

\( \kappa \) joint PCR
BM cells were lysed in RNase A containing buffer and used directly for PCR, as described (30). The primers used in these experiments were the V\( \kappa \) FW, degenerate primer described (36), paired with J\( \kappa \)1, J\( \kappa \)2, J\( \kappa \)3, or J\( \kappa \)4 intron-specific primers.

Nuclear run-on assay
Nuclei from 2 \times 10\(^5\) BM culture cells per condition were used in these experiments as described (37), with minor modifications. \( ^{32}\)P-labeled nuclear transcripts were hybridized to 5 \( \mu \)g of denatured plasmid DNA per spot, immobilized on nitrocellulose membranes (Schleicher & Schuell). Hybridization was conducted in PerfectHyb Plus hybridization buffer (Sigma-Aldrich) for 72 h at 42°C. Quantitation of signals was performed on a PhosphoImager (Molecular Dynamics) with the ImageQuant software package provided by the manufacturer.

Intracellular [Ca\(^{2+}\)] measurements
BM culture B cells were harvested, washed, and loaded with 3 \( \mu \)M fura-2/AM (Molecular Probes) as described (38). Intracellular [Ca\(^{2+}\)] was then analyzed in a quartz cuvette using excitation at 340 and 380 nm and a fluorescence spectrometer (LS55; PerkinElmer Life Sciences). After the baseline [Ca\(^{2+}\)] was established (50 s), cells were stimulated with (Fab\(_{\gamma}\)) anti-IgM (Jackson ImmunoResearch Laboratories). Calibration was performed using 0.1% Triton X-100 for total fluorophore release and 10 mM EGTA to chelate free Ca\(^{2+}\). Intracellular Ca\(^{2+}\) concentrations were calculated using a fluorescence spectrometer measurement program as described previously (38).

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Statistical analysis

MFI means were compared by Student’s t test and a significant difference was accepted at \( p < 0.05 \).

Results

To facilitate the mechanistic analysis of BCR down-regulation on canonical HKIR B cells, we increased their frequency by crossing the HKIR and HKI65 lines to a line expressing a conventional, unmutated, canonical V\(_{\mu}\)J\(_{\lambda}\) L chain transgene (33). Canonical B cells, detected by the anti-clonotype mAb E4, were indistinguishable in their FO locale (Fig. 1A) and phenotype in the spleens and lymph node of the resulting double transgenic mice (termed HKIR/V\(_{\mu}\)k10 and HKI65/V\(_{\mu}\)k10) as compared with HKIR and HKI65 mice (data not shown). Levels of sIgM and sIgD on HKI65/V\(_{\mu}\)k10 and HKIR/V\(_{\mu}\)k10 B cells overlap the lower third and end of the distribution displayed by B cells in C57BL/6 mice, respectively (Fig. 1B), analogous to E4\(^+\) B cells in HKIR and HKI65 mice (data not shown). Thus, transgenic expression of the canonical L chain has no overt effect on the development of canonical HKIR B cells and accompanying BCR down-regulation.

**BCR down-regulation takes place early during BM development of canonical HKIR B cells and is recapitulated in BM cultures**

Shortly after expression of IgM (the only BCR isotype expressed at early stages of development), surface levels of this isotype are comparable on HKIR and HKI65 BM B cells, but by the immature B cell stage (B220\(^{low}\),CD43\(^+\)) HKIR B cells display substantially lower sIgM levels than their HKI65 counterparts (28). This is also the case in HKIR/V\(_{\mu}\)k10 as compared with HKI65/V\(_{\mu}\)k10 BM (Fig. 1C). In all other respects, primary BM B cell development in these lines appears similar (data not shown).
Given the asynchrony of development and low frequency of B cell precursors in BM in vivo (39), and the difficulties in experimentally manipulating this development, we turned to stromal cell- and IL-7-supported BM cultures (32, 40). After 5 days, nearly all B cells arising in HKIR/V\textsubscript{k10}/H9260 and HKI65/V\textsubscript{k10}/H9260 BM cultures express E4+H11001 sIgM (Fig. 1D). These cells are phenotypically immature (see below). As was observed among peripheral B cells in these mice, sIgM expression is uniformly 6- to 7-fold lower on immature HKIR/V\textsubscript{k10} B cells as compared with HKI65/V\textsubscript{k10} and control B cells in BM cultures (Fig. 1E). The mean level of sIgM on HKI65/V\textsubscript{k10} B cells is 3- to 4-fold lower than on the sIgM\textsuperscript{+} B cells in C57BL/6 BM cultures (Fig. 1E). Analogous differences are observed between mature canonical HKI65 B cells and control B cells in vivo (Ref. 14 and Fig. 1B). Therefore, HKI65/V\textsubscript{k10} clonotypes apparently also undergo BCR down-regulation, but to a lesser degree than HKIR/V\textsubscript{k10} B cells.

A DNA-based autoantigen is bound to the sIgM of HKIR/V\textsubscript{k10} B cells in BM cultures and can be displaced by a monovalent form of Ars. The DNA-based autoantigen reactivity of HKIR/V\textsubscript{k10} mAbs (27) suggested that BCR down-regulation on B cells expressing this type of BCR was due to engagement of such an autoantigen(s) in the BM. Indeed, Fig. 2A shows that FITC-labeled DNase I bound extensively to the surface of HKIR/V\textsubscript{k10} BM B cells, but not to control B cells. This binding was reduced over a short time if a monovalent form of Ars (conjugated to L-tyrosine: Ars-Tyr) was added to established HKIR/V\textsubscript{k10} BM cultures and levels of sIgM monitored 12 h later by flow cytometry. The data are representative of three independent experiments.

**FIGURE 2.** Ars-Tyr-mediated alteration of interaction of a DNA-based autoantigen with the BCR on HKIR/V\textsubscript{k10} BM B cells results in modulation of sIgM levels and this does not require cell cycle progression. A, B cells of the indicated genotypes in BM cultures were stained with DNase-I-FITC, and the MFI of the entire population was monitored before and after Ars-Tyr or PABA-Tyr were added (arrows) to 0.1 mM. B, BM cells from HKIR/V\textsubscript{k10} mice were cultured for 5 days in the presence of 0.1 mM Ars-Tyr or PABA-Tyr and the cells were stained and analyzed for levels of sIgM by flow cytometry. Values of mean MFI ± SE from four independent experiments showed sIgM levels increased ~6-fold (5.8 ± 1.4, p = 0.026) in Ars-Tyr cultures as compared with control PABA-Tyr cultures. C, sIg\textbeta levels were analyzed using the same method as described in B. Values of mean MFI ± SE from three independent experiments were 0.79 ± 0.1, 2.93 ± 0.12, and 2.16 ± 0.12 in PABA-Tyr, Ars-Tyr, and C57BL/6 cultures, respectively (nine mice of each genotype). D, B cells from day 5 HKIR/V\textsubscript{k10} BM cultures were loaded with CFSE and recultured for 72 h in the presence of the indicated reagents (hapten at 0.1 mM, mimosine at 300 μM). Surface IgM and CFSE levels were then evaluated by flow cytometry. E, Mimosine (300 μM) and Ars-Tyr (0.1 mM), or PABA-Tyr (0.1 mM), were added to established HKIR/V\textsubscript{k10} BM cultures and levels of sIgM monitored 12 h later by flow cytometry. The data are representative of three independent experiments.
Addition of the structurally related hapten PABA conjugated to l-tyrosine (PABA-Tyr), for which canonical Abs have no measurable affinity, had no effect. Neither of these haptens induces detectable BCR signaling in mature HKR/V/H9260 B cells (data not shown). Thus, the BCR Ag-combining sites on HKIR/V/H9260 B cells in BM cultures are continually engaging a DNA-based autoantigen(s).

**Ars-Tyr modulates levels of sIgM expression on HKIR B cells in BM cultures**

To determine whether inhibition of BCR-autoantigen interactions on developing HKR/V/H9260 B cells would influence sIgM levels, Ars-Tyr or PABA-Tyr were added at the time of initiation of BM cultures. Five days later, sIgM levels on HKR/V/H9260 B cells were uniformly 5- to 6-fold higher than those seen in PABA-Tyr cultures (Fig. 2B). Moreover, levels of sIgM were correlated with concentration of Ars-Tyr (data not shown), maximum levels being achieved at 0.1 mM. This modulation of expression levels appeared to involve the entire BCR complex, as surface expression of Ig-β was low in PABA-Tyr-containing HKR/V/H9260 BM cultures, but not in Ars-Tyr-treated cultures (Fig. 2C).

**Up-regulation of sIgM induced by autoantigen disengagement is not due to cellular selection**

The rate of B cell proliferation in BM cultures is rapid and levels of apoptosis high. To test the possibility that increases in sIgM levels were due to preferential outgrowth of pre-existing or rapidly emerging subpopulations of immature B cells expressing different levels of sIgM, nonadherent B cells from HKR/V/H9260 BM cultures were labeled with CFSE and replated in cultures containing either PABA-Tyr or Ars-Tyr. All cell division subpopulations showed equivalent increases in expression of sIgM 3 days after reculture with Ars-Tyr but not PABA-Tyr (Fig. 2D). Moreover, addition of the cytostatic drug mimosine to HKR/V/H9260 BM cultures to block proliferation did not alter up-regulation of sIgM in response to addition of Ars-Tyr (Fig. 2E). Finally, addition of Ars-Tyr or PABA-Tyr to HKR/V/H9260 BM cultures or removal of Ars-Tyr from such cultures did not alter the frequency of apoptotic cells in these cultures (Fig. 3A).

**Modulation of sIgM expression is not accompanied by altered development**

Progressive increases in levels of sIgM are characteristic of primary B cell development. As such, we next examined whether modulations of sIgM expression in BM cultures was accompanied by changes in the expression of a variety of cell surface Ags that are up- or down-regulated during the developmental progression of immature B cells. Fig. 3B shows that while levels of expression of sIgM and E4 differ substantially in HKR/V/H9260 BM cultures containing Ars-Tyr or PABA-Tyr, expression of the developmental markers CD21, CD23, CD43, L-selectin, and AA4.1 do not. Additional studies revealed that levels of expression of B220, CD19,
and CD22 were also comparable in these two types of cultures (data not shown).

Autoantigen engagement results in increased rates of endocytosis of sIgM

Detailed kinetic studies showed that when BM cultures initially contained PABA-Tyr that was subsequently replaced with Ars-Tyr, sIgM levels on HKIR/Vκ10 and HKI65/Vκ10 B cells rose gradually initially and then rapidly and homogeneously over a period of 8 h, subsequently stabilizing at levels similar to C57BL/6 control cultures (Fig. 4A). Conversely, when Ars-Tyr was replaced with PABA-Tyr in such cultures (washout), sIgM levels dropped homogeneously and precipitously on HKIR/Vκ10 and HKI65/Vκ10 B cells, but not C57BL/6 B cells over a period of several hours, and then stabilized at the lower levels characteristic of these cells in long-term PABA-Tyr-containing cultures (Fig. 4B).

The results of the Ars-Tyr washout studies were consistent with rapid endocytosis of a major fraction of sIgM due to sudden restoration of autoantigen engagement. To determine whether this was taking place in BM cultures lacking Ars-Tyr, rates of sIgM internalization were measured on cells from such cultures. Fig. 4C, left panel, shows that the rate of sIgM internalization was highest for HKIR/Vκ10, lowest for control B cells, and intermediate for HKI65/Vκ10 B cells. That the elevated rate for HKIR/Vκ10 B cells was due to autoantigen engagement was supported by the finding that cultures containing Ars-Tyr gave rise to rates similar to control B cells (Fig. 4C, right panel). These data suggest that differences in rate of BCR internalization contribute to the different levels of BCR on HKIR/Vκ10 and HKI65/Vκ10 B cells. This raised the possibility that changes in BCR internalization and membrane recycling alone might account for the different levels of sIgM expression.

FIGURE 4. Kinetics of sIgM modulation, changes in rates of endocytosis, and differences in total IgM levels induced by alterations in autoantigen engagement on HKIR/Vκ10 and HKI65/Vκ10 BM B cells. A. Ars-Tyr (0.1 mM) was added to day 5 C57BL/6 (green line, left panel), HKI65/Vκ10 (pink line, left panel), or HKIR/Vκ10 (blue line, left panel) BM cultures and at the indicated times aliquots were stained with anti-IgM and evaluated by flow cytometry. The individual points in the left panel represent the sIgM MFI of the entire B cell population at these different times. The right panels present some of these data in histogram form. B. Ars-Tyr (0.1 mM) was added to HKIR/Vκ10 (blue line), HKI65/Vκ10 (pink line), and C57BL/6 (green line) BM cultures for 36 h, and then the cells were washed free of Ars-Tyr and replated in cultures containing the same concentration of PABA-Tyr. At the indicated time points, aliquots were analyzed for levels of sIgM by flow cytometry, and MFI values at these times are indicated. Singlet “X” points at time zero indicate sIgM levels in cultures in which PABA-Tyr was continually present. C, Left panel, Relative rates of BCR internalization by HKIR/Vκ10, HKI65/Vκ10, and wild-type (C57BL/6) B cells in BM cultures at 37°C as measured with a biotinylated Fab anti-IgM Ab. Right panel, Results obtained when HKIR/Vκ10 BM B cells were cultured with either Ars-Tyr or PABA-Tyr (both at 0.1 mM) and endocytosis was measured using the same method as in the left panel. D, HKIR/Vκ10 and C57BL/6 BM cultures containing the indicated haptens (0.1 mM) were stained with anti-IgM or anti-Igβ and then fixed, permeabilized, and restained with the same reagent and analyzed by flow cytometry. Values of mean MFI ± SE from three independent experiments (nine mice of each genotype) showed that total IgM levels and total Igβ levels were -2-fold (2.2 ± 0.8, p = 0.006) and 3-fold (2.8 ± 0.20, p = 0.002) higher in Ars-Tyr cultures as compared with control PABA-Tyr cultures, respectively. All data are representative of at least three independent experiments.
on these B cells. However, flow cytometric analysis of permeabilized B cells showed that amounts of total cellular IgM and Igκ/H9252 were down-modulated in HKIR/Vκ/H926010 PABA-Tyr cultures (Fig. 4D). Moreover, simultaneous addition of cycloheximide and Ars-Tyr to these cultures substantially inhibited up-regulation after 1 h and completely blocked up-regulation at 12 h (data not shown). Thus, increases in slgM require de novo protein synthesis.

FIGURE 5. TLR 9 is not required for development of or alteration of slgM levels on canonical HKIR B cells. BM cultures containing the indicated haptens at 0.1 mM were generated from HKIR/TLR9−/− mice and, after 5 days, levels of surface E4 and slgM levels were evaluated by flow cytometry. The diagonal gates indicate that E4+ cells are present at similar frequencies in the two types of culture and express substantially reduced slgM levels in PABA-Tyr cultures as compared with Ars-Tyr cultures. All data are representative of at least two independent experiments.

FIGURE 6. Modulation of surface IgM levels on HKIR/Vκ10 BM B cells is not associated with receptor editing. A, RAG1 RNA levels were evaluated by real-time RT-PCR in B cells of the indicated genotypes in BM cultures containing either Ars-Tyr or PABA-Tyr at 0.1 mM. B, Expression of surface λ L chains on HKIR/Vκ10 B cells in BM cultures was evaluated by flow cytometry 72 h after the indicated hapten additions (0.1 mM); washout: cultures in which Ars-Tyr had been replaced with PABA-Tyr 12 h earlier. C, Upper panel, The ratio of E4 and slgM staining MFIs of HKIR/Vκ10 (solid lines) or HKI65/Vκ10 (dashed lines) on B cells in BM cultures containing the indicated haptens at 0.1 mM; washout: cultures in which Ars-Tyr had been replaced with PABA-Tyr 12 h earlier. Lower panel, Mean MFI ± SD values for slgM on B cells in BM cultures under the same conditions. D, PCR was used to detect Vκ segment joints to various Jκ segments in B cells of the indicated genotypes in BM cultures containing either Ars-Tyr (0.1 mM, +), PABA-Tyr (0.1 mM, −) or in which Ars-Tyr had been replaced by PABA-Tyr 12 h earlier (±). E, Frequencies of apoptotic cells in HKIR/Vκ10 BM cultures were evaluated by annexin V/PI staining and flow cytometry 48 h after addition of the indicated reagents (haptens at 0.1 mM). F, HKIR/Vκ10 BM cultures containing or lacking 1 μg/ml anti-κ were evaluated for levels of surface λ L chain expression by flow cytometry (upper panel) and for levels of RAG1 RNA by real-time RT-PCR (lower panel). All data are representative of at least two independent experiments.
Regulation of sIgM on and development of canonical HKIR B cells does not require TLR 9

Previous studies have indicated that the BCR on autoreactive B cells can deliver extracellular DNA to the endosomal compartment, resulting in activation of TLR 9 (41). Because the above data indicated that the sIgM on canonical HKIR B cells in BM cultures was continually engaging and internalizing an apparently multivalent DNA-based autoantigen, we determined whether TLR9 might play a role in the development of or modulation of BCR levels on canonical HKIR B cells by crossing HKIR mice to TLR9-deficient mice.

Despite their lower frequency as compared with HKIR/Vx10 mice, canonical splenic B cells can readily be detected in HKIR single transgenic mice using the E4 mAb. Such B cells in HKIR/TLR9−/− mice were of FO phenotype, and were present in numbers similar to TLR9-sufficient controls. These canonical, HKIR/TLR9−/− B cells also expressed levels of sIgM and sIgD indistinguishable from such B cells in TLR9-sufficient HKIR mice (data not shown). Likewise, immature B cells that developed in HKIR/TLR9−/− BM cultures containing PABA-Tyr or Ars-Tyr expressed the low and high levels of sIgM, respectively, characteristic of canonical, TLR9-sufficient HKIR immature B cells in such cultures (Fig. 5).

Receptor editing does not contribute to or result from modulation of sIgM levels on immature canonical B cells in response to changes in autoantigen engagement

Receptor editing can be induced in immature B cells by BCR engagement of certain autoantigens, or by lack of expression of amounts of BCR sufficient to promote survival and development (42–45). B cells in HKIR/Vx10 and HKI65/Vx10 BM cultures expressing endogenous H chains were undetectable under all of the experimental conditions described above (data not shown), ruling out loss of H chain allelic exclusion as an explanation for modulation of sIgM levels.

To determine whether H or L chain editing might contribute to or have been induced by modulation of sIgM expression in HKIR/Vx10 and HKI65/Vx10 BM cultures, four assays were performed. Real-time RT-PCR (QPCR) showed that levels of RAG1 RNA were similarly low in Ars-Tyr and PABA-Tyr HKIR/Vx10 and HKI65/Vx10 cultures (Fig. 6A). In both cultures, RAG1 RNA was substantially lower than in control C57BL/6 cultures, consistent with normal functioning of HKIR/Vx10 and HKI65/Vx10 sIgM in down-regulation of RAG1 transcription (11, 31). Levels of surface expression of λ L chains, indicative of L chain editing (32), in these culture conditions, as well as after Ars-Tyr had been replaced by PABA-Tyr, were indistinguishable (Fig. 6B). The ratio of levels of BCR staining with the E4 mAb (Fig. 6C, upper panel), the reactivity of which depends on association of the canonical H and L chains, relative to total sIgM staining (Fig. 6C, lower panel), remained relatively constant in all of these conditions, arguing against multiple L chain expression by single HKIR and HKI65 B cells, and H chain editing. Finally, DNA PCR assays for the products of endogenous Vκ-Jκ gene segment joining showed that in none of these cultures were Vκ-Jκ joins other than that in the Vκ10 transgene detected (Fig. 6D).

Nonetheless, cross-linking of the BCR by adding 5–10 μg/ml anti-κ L chain Ab to HKIR/Vx10 BM cultures led to extensive apoptosis (Fig. 6E). At 1 μg/ml, this Ab induced receptor editing in these cultures, as indicated by dramatically increased RAG1 RNA levels and frequency of B cells expressing λ L chains (Fig. 6F). Also, addition of preparations of Ars-conjugated sheep RBC to these cultures induced detectable editing (data not shown). Finally, Ars-Tyr could induced parallel increases in E4 and sIgM levels on the canonical subpopulation of B cells (expressing endogenous L chains) in HKIR single-transgenic BM cultures (data not shown). In total, these data demonstrate that while immature canonical HKIR B cells are fully competent to undergo clonal deletion and receptor editing, the BCR-signaling pathways activated
by engagement or displacement of the DNA-based autoantigen do not induce these processes.

Modulation of sIgM levels on immature HKIR/Vκ10 B cells in BM cultures directly correlates with changes in levels of BCR gene expression

The data above clearly indicate that BCR endocytosis contributes to changes in sIgM levels on HKIR (and HKI65) Vκ10 B cells. To determine whether alteration of rates of synthesis of sIgM might also be involved, levels of RNA encoding various BCR components were measured in B cells under various conditions. Levels of μ H chain RNA were comparable, if not higher, in B cells in HKIR/Vκ10 BM cultures containing Ars-Tyr, but were significantly lower in PABA-Tyr-containing cultures as compared with levels in analogous C57BL/6 cultures (Fig. 7A). Consistent with their less severe sIgM down-regulation, HKI65/Vκ10 B cells in PABA-Tyr-containing BM cultures displayed an intermediate level of μ H chain RNA as compared with Ars-Tyr-treated cultures (14.8 ± 1.1 and 17.3 ± 1.1, respectively). Data were obtained from three independent experiments (six mice of each genotype).

FIGURE 8. Transcriptional control of sIgM levels on HKIR/Vκ10 BM B cells. A, Measurement of Cμ RNA stability in HKIR/Vκ10 BM cultures containing either Ars-Tyr or PABA-Tyr (both at 0.1 mM added 12 h earlier). Levels of this RNA were evaluated by QPCR in B cells purified from BM cultures at the indicated times after addition of actinomycin D (20 μg/ml). B, Nuclear run-on analysis of Cμ and β-actin gene transcription rates in HKIR/Vκ10 BM B cells in cultures either containing Ars-Tyr (0.1 mM), or in cultures in which Ars-Tyr had been replaced by PABA-Tyr (0.1 mM) 12 h earlier (washout). The panel shows the relative differences in Cμ relative to β-actin transcription in two independent experiments (indicated by □ and ■). C, HKIR/Vκ10 B cells from day 5 BM cultures were further cultured with either Ars-Tyr or PABA-Tyr (both at 0.1 mM) in the presence or absence of actinomycin D (20 μg/ml) for the indicated times. Levels of surface IgM and B220 were then evaluated by flow cytometry. At 2- and 4-h time points, the values of sIgM mean MFI ± SE in actinomycin D (Act.D) plus Ars-Tyr-treated cultures were reduced to 10.5 ± 0.9 (p = 0.01) and 6.1 ± 0.5 (p = 0.001), respectively, as compared with Ars-Tyr-treated cultures (14.8 ± 1.1 and 17.3 ± 1.1, respectively). Data were obtained from three independent experiments (six mice of each genotype).

FIGURE 9. Levels of IgM-encoding RNA correlate with levels of sIgM expression on HKIR/Vκ10 and wild-type FO B cells in vivo. A, QPCR quantification of Cμ RNA expression levels in ex vivo BM and splenic B cells of the indicated phenotypes. B, Left panel, sIgM low and high subpopulations. CD23highCD21low splenic B cells were sorted from C57BL/6 mice by FACS and reanalyzed for sIgM expression by flow cytometry (black line, IgMhigh; gray line IgMlow). Values of sIgM mean MFI ± SE from three independent isolations of these two populations were 34.5 ± 2.3 and 3.8 ± 0.5, respectively. Right panels, Levels of μ H chain and Igκ chain RNA in the fractions analyzed in the left panel were quantitated by QPCR. All data in this figure are representative of at least two independent experiments.
levels were increased compared with those in the BM but were substantially and somewhat reduced in HKIR/Vκ10 and HKI65/Vκ10 B cells, respectively, relative to controls. In contrast, levels of Cμ RNA in anti-HEL-transgenic splenic B cells were slightly higher than controls either in the presence (ML5/MD4) or absence (MD4) of anergy-inducing levels of HEL (data not shown).

To determine whether this correlation extended to normal B cells, sIgM low and high subpopulations of mature, FO B cells from C57BL/6 mice were purified by FACS, reanalyzed for sIgM expression, and levels of IgM H chain and Igκ chain RNA quantitated by QPCR. Fig. 9B, left panel, shows the ~10-fold different levels of sIgM expression displayed by these two subpopulations. Fig. 9B, right panels, illustrates that both IgM H chain and Igκ L chain encoding RNA were significantly lower in the sIgM<sup>low</sup> subpopulation of normal splenic B cells.

Ca<sup>2+</sup>-signaling pathways are involved in autoantigen-mediated regulation of canonical HKIR BCR levels

A major signaling pathway downstream of the BCR results in intracellular Ca<sup>2+</sup> flux and B cells that chronically engage autoantigen often reveal elevated basal levels of intracellular Ca<sup>2+</sup> (29, 46, 47). Fig. 10A shows that such levels are indeed raised in HKIR/Vκ10 BM cells cultured in PABA-Tyr but not Ars-Tyr, or in cultures where Ars-Tyr had recently been removed. Nonetheless, addition of anti-IgM to both types of culture (arrow) induced robust and sustained B cell Ca<sup>2+</sup> responses, inconsistent with a state of anergy. Interestingly, addition of the raft inhibitor methyl-β-cyclodextrin (MβCD) to HKIR/Vκ10 BM cultures did not result in reduction of basal cell intracellular Ca<sup>2+</sup> levels (Fig. 9A), in agreement with the idea that sIgM clustering in rafts is not required for autoantigen-driven elevation of basal Ca<sup>2+</sup> levels (47). Moreover, addition of a peptide inhibitor of the Ca<sup>2+</sup> responsive NFAT transcription activation factors (termed VIVIT, Ref. 48) to HKIR/Vκ10 BM cultures at the same time as Ars-Tyr significantly blocked sIgM up-regulation, but did not noticeably alter sIgM down-regulation when Ars-Tyr was removed from such cultures (Fig. 10B).

Discussion

Past studies are compatible with the idea that developing B cells can alter BCR levels in response to changes in coreceptor or BCR signaling (reviewed in Ref. 49). However, these studies did not address whether engagement of the BCR played a role in these...
“adjustments” or whether cellular selection or cell autonomous adaptive processes were responsible. We previously suggested that an instructive feedback loop that alters surface levels of BCR in response to degree of autoantigen engagement might account for the down-regulation of BCR expression characteristic of canonical HKIR B cells and allow them to avoid conventional tolerance pathways and be positively selected, a mechanism we termed “learned ignorance” (28). Our results strongly support this idea. Perhaps unexpectedly, these data demonstrate that BCR endocytosis plays a central role in this process (Fig. 4C). More interestingly, an active mechanism linking BCR engagement of autoantigen and control of transcription of BCR-encoding loci is also a major component of this pathway (Figs. 7 and 8).

Our data are consistent with the idea that BCR levels on HKIR/Vk10 and HKI65/Vk10 BM B cells are determined by a dynamic balance between autoantigen-induced BCR cross-linking and endocytosis and the transcriptional regulatory process. HKIR/Vk10 BM B cells display increased rates of sIgM endocytosis relative to control B cells in the absence, but not presence, of Ars-Tyr (Fig. 4C). Conversely, BCR component RNA levels in these cells are low when autoantigen is constitutively engaging the BCR, but are rapidly increased when this engagement is blocked by addition of Ars-Tyr (Fig. 7). Moreover, the transcription rate of the μ H chain gene (Fig. 8B), and μ, κ L chain and Igβ RNA levels (Fig. 7C) drop when Ars-Tyr is removed from HKIR/Vk10 BM cultures, and complete up-regulation of sIgM levels in HKIR/Vk10 cultures to which Ars-Tyr has been added is blocked by inhibition of transcription using actinomycin D (Fig. 8C). This inverse relationship between levels of BCR autoantigen engagement and levels of BCR component transcription suggest a mechanistic link between the two processes.

However, the complexity of BCR-signaling pathways, combined with our incomplete understanding of how stage of development, nature of the BCR ligand, and influence of coreceptor signaling influence the outcome of BCR engagement, limit constructive speculation on the detailed mechanisms operative in this adaptive process. Nonetheless, our results indicate that coordinated and rapid alteration of expression of Igκ, Igκ, and Igβ loci is its end result. In addition, our studies demonstrate that TLR9 is not required for this regulation (Fig. 5). Past studies have indicated that transcription factor activity differs qualitatively during activating vs tolerogenic BCR and TCR signaling (47, 50–52), providing precedent for the idea that these signaling pathways can be distinguished by the transcriptional regulatory apparatus of lymphocytes. However, while the signaling pathway resulting in the dynamic changes in BCR expression we describe here may overlap with those mediating B cell activation and anergy, it is likely to have unique characteristics and, perhaps, components. This is exemplified by our finding that up-regulation of BCR levels on immature HKIR/Vκ10 B cells due to reduced autoantigen engagement is associated with lowered basal intracellular Ca2+ levels but is inhibited by blocking NFAT activation (Fig. 10).

Perhaps not unexpectedly, the endocytic and gene regulatory contributions to this mechanism appear to impact BCR levels on HKIR/Vκ10 and HKI65/Vκ10 BM B cells with very different kinetcs. In the time frame of minutes, alterations in sIgM levels due to changes in autoantigen engagement seem to primarily result from variations in rate of BCR endocytosis and, apparently, degradation (Fig. 4). In contrast, more long-term changes in sIgM levels require the superimposition of adjustments of BCR component gene transcription on BCR endocytosis (Figs. 4, 7, and 8). This logic is compatible with a simple model in which endocytosis primarily determines the rate of disappearance of BCR from the B cell surface, while transcription primarily controls the rate of appearance of newly synthesized BCR there. In the steady state, these two processes are balanced such that a stable level of BCR surface expression results and is maintained.

The regulation of sIgM levels by this mechanism appears to continue to operate during more mature stages of HKIR/Vκ10 and HKI65/Vκ10 development, as levels of Cμ RNA are substantially and somewhat reduced in HKIR/Vκ10 and HKI65/Vκ10 splenic B cells, respectively, as compared with controls (Fig. 9A). However, the relative contributions of BCR endocytosis and transcriptional regulation to maintenance of BCR levels in these more mature cells remains to be determined. Importantly, we found that Cμ and Cκ RNA levels in C57BL/6 FO B cells correlate with sIgM levels (Fig. 9B), indicating that regulation of BCR component transcription may play a general role in control of BCR levels on mature B cells. Further studies will be required to determine whether surface IgD levels on mature HKIR/Vκ10 and HKI65/Vκ10 B cells are regulated by a similar mechanism. This is an important issue, as the majority of BCR on the surface of FO B cells is composed of this isotype.

Our data indicate that both the macromolecular form and BCR-binding affinity of a ubiquitous DNA-based autoantigen recognized by canonical HKIR (and HKI65) BCRs in the BM in vivo and in BM cultures (e.g., Figs. 2A and 9A) are important parameters in driving adaptive adjustment of BCR levels. The presumed linear distribution of repeating autoepitopes on this DNA-based complex is clearly sufficient to induce BCR signaling and endocytosis. Estimation of the in vitro and in vivo concentration and affinity of HKIR and HKI65 BCRs for the DNA-based autoantigen(s) responsible for driving BCR down-regulation will require the identification of this autoantigen(s). Because low concentrations of Ars-Tyr, for which canonical HKIR and HKI65 BCRs have a Kd of 2 × 107, can displace a large portion of this autoantigen, these parameters may have small values, perhaps contributing to the inability of this autoantigen(s) to induce clonal deletion or receptor editing.

Our findings are consistent with “tunable activation threshold” and “adaptive” models for lymphocyte tolerance and positive selection (53, 54). However, while evidence supporting these models in the case of T cells has been obtained, adaptation to changes in TCR signal strength is usually attributed to alteration in levels of expression or activity of coreceptors such as CD5 and CD8 (54). A single previous report correlated persistence of autoreactive T cells to reduction in transgene-encoded TCR surface expression (55). Past studies are also compatible with the idea that developing B cells can alter BCR levels in response to changes in coreceptor or BCR signaling (49), and that subpopulations of autoreactive B cells expressing low BCR can persist in the periphery (56, 57). However, these studies did not address whether autoantigen engagement of the BCR played a role in these “adjustments” or whether cellular selection or cell autonomous adaptive processes were responsible.

Whether B cells with other Ag and autoantigen specificities may use this adaptive “learned ignorance” pathway to modulate levels of BCR expression, resulting in avoidance of tolerance and induction of positive selection, remains to be determined. It is likely that the macromolecular form, concentration, BCR affinity for, and temporal availability of, cognate Ag(s) are important parameters in this regard. The time that Ag is first encountered may be critical, as BCR-signaling pathways differ qualitatively at early vs late stages of primary development and this affects the functional outcome of Ag-BCR interactions (58–60). For example, even low-affinity BCR interactions with membrane-bound autoantigens seem to predominantly induce receptor editing in early immature B cells (61, 62), but induce
clonal deletion at later stages of development (32). Autoantigens present in monomeric, soluble form seem to predominately induce anergy in immature B cells, but mature B cells may be “indifferent” to such autoantigens encountered in the periphery (11). It is important to note that while autoantigen-mediated BCR down-regulation is also associated with clonal deletion, receptor editing, and anergy (11, 32), the above discussion points out that many factors impinge upon the kinetics and magnitude of this down-modulation, as well as its functional outcome.

Also relevant to this issue is the fact that BCR levels on mature, FO B cells in normal mice are heterogeneous (11). The learned ignorance hypothesis would predict that a significant fraction of the BCRlow subpopulation of FO B cells in normal mice has undergone adaptive BCR down-regulation during its development. Indeed, we have obtained preliminary data suggesting that the frequency of anti-DNA B cells resident in the BCRlow subpopulation of mature, FO B cells in normal mice is 3- to 4-fold higher than in the BCRhigh subpopulation. However, future studies are required to determine the percentage of B cells in the former subpopulation that may be anergic.

The recruitment of mature, multireactive B cells with autoreactivity into a foreign Ag-driven immune response would appear to represent a significant risk factor for development of autoimmune. However, during foreign Ag-driven responses in nonautoimmune prone mice many B cells transiently produce IgM, a short-lived Ab isotype (63, 64). This would minimize the pathological impact of multireactive Abs secreted by B cells. In fact, temporary activation and production of serum IgM by such B cells might facilitate clearance of autoantigens during periods of infection or tissue damage, thus promoting tolerance (16, 23). In contrast, a subset of responding B cells enters the memory B cell pathway, leading to the production of long-lived IgG Abs. However, we (65) and others (66–69) previously proposed that such cells must transit a foreign Ag-driven tolerance checkpoint operative during GC/memory B cell development. This checkpoint may operate by allowing only GC B cells expressing BCRs whose level of autoreactivity has been substantially reduced due to alteration of BCR structure and specificity by V region somatic hypermutation to developmentally progress. Scenarios in which perturbations of the learned ignorance or GC/memory tolerance mechanisms would contribute to the etiology of systemic autoimmune diseases could be easily envisioned.

Past studies, including our own (29, 70), may have failed to reveal the adaptive pathway described here due to their extensive use of conventional Ab H chain transgenic approaches. Analysis of mice expressing conventional µ,κ H chain transgenes encoding the HKI65 form of canonical BCRs did not reveal evidence for BCR down-regulation and BCR levels were observed to be supernormal when the transgenes were present in multiple copies (70). Moreover, B cells expressing conventional Ig transgenes encoding a canonical H chain and a heavily somatically mutated canonical L chain were observed to have near normal levels of sIgM, elevated levels of sIgD, and a phenotype and functional status consistent with autoantigen-mediated induction of abortive activation and anergy (29). Taken together, these findings suggest that due to chromosomal context effects, lack of requisite cis-acting elements, or both, conventional H chain transgenes are not responsive to the transcriptional regulatory controls required for operation of the adaptive process we describe here. Interestingly, our data suggest that conventional Igκ transgenes are sensitive to such regulation, providing an experimental avenue to define the cis- and perhaps trans-acting factors involved.

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Disclosures

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References


