B Cells Are Exquisitely Sensitive to Central Tolerance and Receptor Editing Induced by Ultralow Affinity, Membrane-bound Antigen

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Summary

To assess the sensitivity of B cell tolerance with respect to receptor/autoantigen affinity, we identified low affinity ligands to the 3-83 (anti-major histocompatibility complex class I) antibody and tested the ability of these ligands to induce central and peripheral tolerance in 3-83 transgenic mice. Several class I protein alloforms, including K^bm3 and D^k, showed remarkably low, but detectable, affinity to 3-83. The 3-83 antibody bound K^k with \( K_A \approx 2 \times 10^5 \text{ M}^{-1} \) and bound 10-fold more weakly to the K^bm3 (\( K_A \approx 2 \times 10^4 \text{ M}^{-1} \)) and D^k antigens. Breeding 3-83 immunoglobulin transgenic mice with mice expressing these ultralow affinity K^bm3 and D^k ligands resulted in virtually complete deletion of the autoreactive B cells from the peripheral lymphoid tissues. These low affinity antigens also induced receptor editing, as measured by elevated RAG mRNA levels in the bone marrow and excess levels of id^− variant B cells bearing λ light chains in the spleen. Reactive class I antigens were also able to mediate deletion of mature B cells when injected into the peritoneal cavity of 3-83 transgenic mice. Although the highest affinity ligand, K^k, was consistently able to induce elimination of the 3-83 peritoneal B cells, the lower affinity ligands were only partially effective. These results demonstrate the remarkable sensitivity of the deletion and receptor-editing mechanisms in immature B cells, and may suggest a higher affinity threshold for deletion of peripheral, mature B cells.

Self/nonself discrimination is initiated by antigen receptor engagement, but, unlike other receptor/ligand systems, the antigen receptors do not have predefined ligands. It is therefore important to understand how antigen form, affinity, and avidity affect activation and subsequent biological consequences, including tolerance. In B cells, multiple tolerance mechanisms appear to work together to maintain control of autoreactivity (reviewed in 1–6). These mechanisms, including central deletion (7–11), receptor editing (12–16), energy (17–19), follicular exclusion (19, 20), peripheral deletion of resting B cells (21–26), and antigen and Fas-mediated death of germinal center B cells (27–30), have been described. Importantly, the affinity and avidity of the antigen/B cell receptor (BCR)^6 interaction may determine extent of tolerance induction, as well as tolerance phenotype (1, 31–36).

Tolerance susceptibility and mechanisms are also affected by the developmental stage at which the autoreactive B cell encounters antigen. In adult mice, the initial antigen-specific negative selection step in B cell development occurs in the bone marrow through a process of developmental arrest (37) associated with ongoing light chain gene rearrangement that can alter BCR specificity, a process referred to as receptor editing (12–16). B cells encountering self-antigen later in development can be controlled by a number of other mechanisms, all of which accelerate B cell death (21–30).

To evaluate the relative significance of the various B cell tolerance mechanisms in controlling autoreactive B cells, it is important to determine the sensitivity of these mechanisms to BCR/antigen affinity. Here, we test the hypothesis that cell-surface antigens with very low affinity for BCR might fail to mediate central or peripheral tolerance.

Materials and Methods

Mice. The 3-83 μ/β transgenic mice (21), which express IgM and IgD forms of the 3-83 antibody, were bred and maintained in the animal care facility at the National Jewish Center for Immunology and Respiratory Medicine (NJICRM). 3-83 μ/β mice were backcrossed a minimum of 10 times onto a B10.D2 back-
ground and then bred one generation with C57Bl/6 J-H-2bm3/EaAoEg (H-2bm3), C57Bl/6 bm6 (H-2bm6), C57B1/6 Kh-
H-2bm6/KrEg (H-2bm6), B6.C-H-2bm6/KrEg (H-2bm6), C3H-
H-2bm6/SsEg (C3H.OH: Kd4b), B10.M/Sn (H-2d), B10.BR/Sg-
Snj (H-2d), C57Bl/6 (H-2d), and B10.D2/NsEg (H-2d) mice (all
purchased from Jackson Laboratory, Bar Harbor, ME). The F1
mice were directly analyzed. Initial screening for affinity ligands
was performed on H-2bm1,4,9,10,11 mice, kindly provided by
Dr. Roger Melvold (Northwestern University Medical School,
Chicago, IL). β2-microglobulin-deficient mice were a generous
gift from Beverly Koller (University of North Carolina, Chapel
Hill, NC) (38). These mice were crossed with B10.D2 3-83 trans-
genics. The F1 mice were interbred to obtain 3-83 μ/δ trans-
genic, β2-microglobulin−/− mice on a H-2d homozygous back-
ground. These resulting mice were analyzed as negative controls.

Cell Lines. Sp2/0-derived H-2d and H-2d mutant cell lines (bm3, bm6, bm8, and bm11) were a generous gift from Janko
Nikolic-Zugic and M.J. Bevan (formerly at The Scripps Research
Institute, La Jolla, CA) (39). The 4549 (H-2d) cell line was
obtained from L. Wysocki (NJICIRM). The Sp2/0 (H-2d) cell
line (40) was purchased from American Type Tissue Collection
(Rockville, MD).

Antibodies and Immunofluorescence. Lymphoid cells were
prepared as described previously (7), and were stained with the fol-
lowing mAbs: anti-3-83 clone 54.1/biotin (7), anti-B220/
FITC, RA3-3A1 (41); anti-IgM/biotin, AM5 (42); anti-
H-2Kk,b,mn,t (43); and 3-83P (44); anti-IgD (45), AMS 9.1/biotin
(46); and goat anti-mouse IgM, JR3-6A3 (47), (Zymed Laboratories, South San Francisco, CA); and goat
anti-A/FITC (Fisher Scientific, Pittsburgh, PA). Biotin-conju-
gated antibodies were revealed with PE- streptavidin (Becton
Dickinson & Co, Mountain View, CA). Samples were analyzed on
a FACScan® (Becton Dickinson), FACS® Profile, or Profile
ELITE ( Coulter Electronics Inc., Hialeah, FL). Purification and
fluorochrome conjugation of the antibodies were performed as
described previously (48). Except for the cell-staining binding as-
says, histogram analysis data were gated by side scatter to include
the predominant lymphocyte population.

Class I Molecules. Soluble Kβ and Kbm3 were purified as de-
scribed previously (49). Briefly, Drosophila S2/M3 cells were
cotransfected with a soluble form of H-2Kβ or Kbm3 cDNA and a
full-length cDNA encoding murine β2m. Stable transfecants
grown in serum-free medium were induced by cuspic sulfate
(0.7 mM). Culture supernatants were collected using Centrifug-
ate, washed twice, and resuspended in 10-mM Na-acetate, pH 5.0, to a concentration of ~50 μg/ml for soluble
Kβ, and 20 μg/ml for all antibodies. The Kbm3 molecules were
resuspended in 10 mM morpholino propane sulfonic acid buffer,
pH 6.0, to a concentration of 20 μg/ml. Ligands were covalently
bound to the sensor chips using the BIACore® Amine Coupling
Kit (Pharmacia Biosensor), according to the manufacturer's in-
suctions. The amount of protein bound to the CM5 sensor chips
was as follows: chip 1: Kβ (3421 resonance units [RU]); chip 2:
Y3 (6854 RU), 3-83 (7236 RU), and Hopc-1 (50) (purchased
from American Type Culture Collection; 6123 RU); chip 3: 54.1
(3671 RU), Kβ (2821 RU), and 33.18.12 (42) (anti-mouse k;
2458 RU); chip 4: Kbm3/κ (3246 RU), Kbm3 (3864 RU), and
54.1 (2188 RU); chip 5: 3-83 (6667 RU) and Hopc-1 (3364
RU). Analyte solutions were diluted in running buffer (PBS, 1
mM EDTA, 0.05% P20 BIACore™ or 1:100 Triton-X 100 sur-
factant) to the following concentration ranges representing two-
fold dilutions: run 1 (chip 1): 3-83 (25–800 nM) over Kβ; run 2
(chip 2): soluble Kβ over Y3 (62–500 nM), 3-83 (62–1,000 nM),
and Hopc-1 (62–1,000 nM); run 3 (chip 3): 3-83 IgG (25–800
nM), 3-83 Fab (250–8,000 nM), Y3 IgG (62–200 nM), and
Hopc-1 (25–800 nM) over Kβ; run 4 (chip 4): soluble Kbm3
(39–625 nM), Kbm3/m7 (125–500 nM) over Y3 and Hopc-1;
run 5 (chip 5): soluble Kbm3 (32–4,140 nM) and Kbm3/m7
(143–9,140 nM) over 3-83 and Hopc-1; run 6 (chip 4): 3-83 IgG (25–800
nM) over 33.18.12; run 7 (chip 4): 3-83 IgG (59–1,875 nM); 3-83 Fab
96–24,450 RU), and Hopc-1 (109–3500 RU) over Kbm3/m7.
Since initial experiments showed a probability of aggregated
antibodies, the antibody preps were spun at 100,000 g for 45 min im-
mediately before dilution and passage over the Kbm3 bound sensor
chips. Each analyte preparation was injected over the covalently
bound ligand on the BIASensor chip at a flow rate of 15–25 μl/
min for 54 min. The analyte was allowed to dissociate with buffer
for adequate time to remove analyte from the chip (5–60 min).
For binding of IgG Y3 antibody to Kβ, 3-83 IgG to 54.1, and
3-83 Fab to 33.18.12, the dissociation rate was too slow to com-
pletely remove all analyte. For this reason, kinetic data was limited
to the first injection only. The experiments with soluble Kβ
were performed on the BIACore 1000, which allows passage of
analyte over a single flowcell (single protein bound to chip),
while the Kbm3 experiments used the BIACore 2000, which al-
 lows flow of analyte over all four flowcells on a single chip in se-
neries. The BIACore 2000 has the added advantage of lower signal/
noise ratio and higher sensitivity, measuring affinities as low as
10^6 M^-1, while the BIACore 1000 has limitations in the 10^9 M^-1
range.

Data sensograms (showing relative RU changes caused by in-
jection, buffer changes, and binding of ligand) were analyzed to
calculate association and dissociation rate constants using the BIA-
Evaluation 2.0 program and time points relevant to the associa-
tion and dissociation phases, excluding the first few seconds of
each phase from analysis to avoid buffer and mass transport ef-
tects. The dissociation rate constant (kd) was determined initially
by the AB = A + B regression model to fit slope of ln(Ru/Rt) vs.
time. Plot of because of steepness of the slope and the short length
of dissociation period, this method proved inaccurate for instances
of very fast off rates. Therefore, the half-life of bound protein was
determined by subtracting the initial time of dissociation phase
from the time at which half of the bound protein was dissociated.
The amount of protein bound was calculated as the maximal
RU of sensogram over experimental chip minus maximal RU of same
analyte over a blank or irrelevant protein chip. The k4 was then
calculated as 0.693/t 1/2 (s^-1) and averaged over several concen-
trations. For each experiment, the association rate constant \( (k_a) \) was calculated by both the \( \Delta A = AB \) type 1 and 4 options of the BIAEvaluation program. The first model is a regression analysis for first-order kinetic reactions and assumes a known \( k_p \). The data from this analysis showed large variations, with the \( k_p \) value decreasing with concentration. The second method, referred to as the \( k_v \) vs. concentration method, does not assume \( k_v \) values, and calculates \( k_v \) based on several concentrations of analyte. For each concentration of analyte, the program determines the slope \( (k_v) \) of \( dk/k \ vs. k \). The association constant was then calculated as the negative slope of \( k \ vs. \) concentration. The \( K_a \) value determined by the \( k_v \) vs. concentration method correlated well with the \( K_a \) calculated by the regression analysis for the highest analyte concentration. BIACore kinetic analysis is considered most accurate in cases where the least free ligand is available for rebinding (51). This occurs at high concentrations of analyte. Because of the very low affinity binding, the data were difficult to interpret in some experiments. The \( k_v \) vs. concentration method did not always generate a straight line, and in these cases, analysis for \( k_v \) was restricted to the regression analysis. Also in instances where analyte dissociated very slowly, analyses of both \( k_v \) and \( k_d \) were restricted to the regression models for the first concentration of analyte tested. The equilibrium association constant \( (K_a) \) was calculated as the dividend of \( k_v \) over \( k_d \).

Detection of mRNA. The levels of \( G_{\alpha} \), RAG-1, and RAG-2 mRNA expression in the bone marrow of PI mice were detected using reverse transcription-PCR and Southern analysis, and the quantification of radioactive signals was performed as described previously (13).

Intraperitoneal Injection Assays. Sp2/0 hybrids derived from bm mutant spleen cells (39), in addition to sp2/o (H-2\(^d\)) antigen-negative control cells, were washed twice in sterile PBS and resuspended at \( 5.0 \times 10^7 \) cells/ml. Using 26-gauge needles, 100 \( \mu l \)/mouse of hybridoma cells were injected into the peritoneums of age-matched, nondeleting \( (H-2d) \) background 3-83 hemizygous transgenic mice. One mouse from each group received 100 \( \mu l \) of PBS alone. Approximately 16 h later, the mice were killed and the peritoneal cells were removed by flushing with 10 ml of either HBSS or PBS. In some experiments, the lymph nodes, spleen, and/or bone marrow were also analyzed. The peritoneal cells were centrifuged, treated with either Gey’s solution or buffered ammonium chloride to remove red blood cells, washed twice in staining buffer, counted, and stained. Samples were analyzed on either the Profile or ELITE flow cytometers (Coulter Electronics). To exclude the larger, injected tumor cells, detection of idiotypic-positive B cells was limited to the lymphocyte population, as defined by forward and side scatter. Detection of class I molecules by Y3 antibodies was analyzed for both the small lymphocytic and larger, tumor-containing myeloid populations.

Results

3-83 Antibody Cross-reacts Weakly to K\(^b\) and Natural Mutations in the K\(^b\) Molecule Further Lower the Relative Binding Affinity. The 3-83 antibody was raised in a BALB/c (H-2\(^d\)) mouse by immunization with C3H (H-2\(^d\)) spleen cells, and has specificity for K\(^b\), but also binds very weakly to D\(^k\) and to other allotypes of H-2\(^d\) (44). Relative rankings of 3-83 affinities to various class I molecules were determined by flow cytometry (Fig. 1). Cross-reactive binding of 3-83 to K\(^b\) cells required far higher concentrations of antibody relative to that required for comparable binding to K\(^a\) cells (reference 44 and Fig. 1A). Despite the relatively low affinity for K\(^b\), 3-83 \( \mu l/5 \) Ig transgenic mice bred onto a H-2\(^b\) background completely deleted the autoreactive B cells from secondary lymphoid organs (13, 21, 34), indicating that this weak binding is physiologically relevant.

Natural mutants of the K\(^b\) molecule, the so-called “bm” series (52), had further reduced affinity to the 3-83 antibody, relative to K\(^b\). Spleen and lymph node cells of H-2\(^{km3}\) strains were analyzed directly for relative binding to 3-83 in complement-mediated cytotoxicity and flow cytometry assays. All target cells tested, except bm1, had lower binding to 3-83 than to the natural K\(^b\) molecule (not shown). Hybridomas derived from bm mutant spleen cells (39) were stained with 3-83 (Fig. 1A) or the mAb Y3 (Fig. 1B), which recognizes the same epitope on H-2K\(^d\) as that seen by 3-83 (53). All antigen-bearing cells showed similar levels of class I expression, as detected by Y3 binding. Mutations in K\(^{km3}\) reduced 3-83 binding (Fig. 1A), but not Y3 binding (Fig. 1B). Staining of lymphocytes from mice homozygous at the H-2 locus for the K\(^b\) mutations confirmed these results (Fig. 1, C and D). When reacted with 3-83 antibody, K\(^{km3}\) was bound less well than K\(^b\), whereas both were bound similarly by the Y3 antibody. The reactivity of 3-83 to the D\(^k\) molecule was also much weaker than its cross-reactivity to the K\(^b\) molecule. When the data are corrected for nonspecific binding, it is apparent that the very low affinity ligands K\(^{km3}\) and D\(^k\) required >10-fold higher concentrations of 3-83 to achieve a level of binding comparable to that observed with K\(^b\) cells (Fig. 1, A and D). Thus, flow cytometry was useful in identifying and ranking the relative binding strength of ligands that were very weakly reactive to the 3-83 antibody.

The 3-83 Antibody Binds the Intermediate Affinity Ligand K\(^b\) with an Association Constant in the 10\(^s\) M\(^{-1}\) Range. Using the technique of surface plasmon resonance (54, 55), the equilibrium association constant \( (K_a) \) for the interaction between K\(^b\) and 3-83 antibody was measured (Table 1). Appropriate control antibodies were studied in parallel to assure the specificity of the interaction and the activity of the antibodies. 3-83 F\(^\alpha\) binding to immobilized K\(^b\) demonstrated a very fast off rate \( (t_{1/2} \sim 7.4 s) \) largely responsible for the low \( K_a \) value of \( 1.7 \times 10^5 \) M\(^{-1}\). Soluble K\(^b\) binding to immobilized 3-83 was consistent with this result, yielding a \( K_a \) value of \( 3.3 \times 10^5 \) M\(^{-1}\). The bivalent, IgG form of 3-83 bound to immobilized K\(^b\) \( (K_a \sim 5.5 \times 10^6 \) M\(^{-1}\) \) with only 10-fold higher affinity than did F\(^\alpha\) 3-83, whereas bivalency improved the binding of the Y3 antibody by >500-fold (Table 1). Consistent with the binding studies (Fig. 1), the monoclonal affinity of 3-83 for the putative very low affinity ligand K\(^{km3}\) was \( \sim 10\)-fold lower: \( K_a \sim 2 \times 10^4 \) M\(^{-1}\), and the bivalent affinity was \( K_a \sim 5 \times 10^4 \) M\(^{-1}\). This binding appeared to be peptide independent, since K\(^{km3}\) molecules were bound with similar affinities whether or not the groove-binding peptide was present. We refer to these weak interactions as ultralow affinities.
Table 1. Measurement of Equilibrium Association Constants for Anti-class I Antibodies Using Real-time Kinetic Analysis with BIAcore

| Ligand | Analyte | Valency | Concentration | \( k_\alpha (\text{M}^{-1} \text{s}^{-1}) \) | \( R^2 \) | Half-life | SD \( k_\alpha (\text{s}^{-1}) \) | SD | \( K_a (\text{M}^{-1}) \) |
|--------|---------|---------|---------------|----------------|----------|------------|----------------|---|------------------|
| \( K^b \) | Y3 IgG | Bi | 62 nM | 4.11E + 05 | 4.21* | >3,600 | 2.43E-04 | 4.77* | 1.7E + 09 |
| \( K^b \-vov \) | 3-83 IgG | Bi | 800 nM | 2.90E + 04 | 0.991 | 135.3 | 28.1 | 5.29E-03 | 1.09E-03 | 5.5E + 06 |
| \( K^b_{3-83} \) | 3-83 IgG | Bi | 1,875 nM | 1.42E + 04 | 0.999 | 26.5 | 1.5 | 2.63E-02 | 1.61E-03 | 5.4E + 05 |
| \( K^b_{3-83-vov} \) | 3-83 IgG | Bi | 1,875 nM | 1.25E + 04 | 0.984 | 50.8 | 5.8 | 1.38E-02 | 1.73E-03 | 9.1E + 05 |
| \( K^b \-vov \) | Hopc IgG | Bi | 800 nM | No binding | | | | | | |
| \( K^b_{3-83} \) | Hopc IgG | Bi | 3,500 nM | No binding | | | | | | |
| \( K^b_{3-83-vov} \) | Hopc IgG | Bi | 3,500 nM | No binding | | | | | | |
| \( K^b \-vov \) | 3-83 Fab | Mono | 8,000 nM | 1.67E + 04 | 0.93 | 7.4 | 2 | 9.98E-02 | 2.93E-02 | 1.7E + 05 |
| \( K^b_{3-83} \) | 3-83 Fab | Mono | 2,4450 nM | 3.74E + 03 | 0.977 | 3.2 | 0.8 | 2.27E-01 | 6.05E-02 | 1.6E + 04 |
| \( K^b_{3-83-vov} \) | 3-83 Fab | Mono | 2,4450 nM | 3.82E + 03 | 0.979 | 5.1 | 1 | 1.40E-01 | 2.89E-02 | 2.7E + 04 |
| 54.1 | 3-83 Fab | Mono | 2,000 nM | 4.12E + 04 | 0.997 | 79.5 | 0 | 8.72E-03 | 0.00E + 00 | 4.7E + 06 |
| 54.1 | Y3 IgG | Poly | 25 nM | 6.57E + 05 | 0.01* | NA | 3.49E-04 | 0.07* | 1.9E + 10 |
| 54.1 | Y3 IgG | Poly | 200 nM | 83.18.12 | 3-83Fab | Mono | 1.40E + 05 | 6.49* | NA | 3.39E-06 | 2.77* | 4.1E + 10 |
| 3-83 | \( K^b \-vov \) | Mono | 1,000 nM | 1.42E + 04 | 0.898 | 18.6 | 7.6 | 4.27E-02 | 1.83E-02 | 3.3E + 05 |
| 3-83 | \( K^b_{3-83} \) | Mono | 4,140 nM | 3.44E + 05 | 0.996 | 3.5 | 2.1 | 2.43E-01 | 1.47E-01 | 1.4E + 04 |
| 3-83 | \( K^b_{3-83-vov} \) | Mono | 9,140 nM | 3.44E + 03 | 0.996 | 13.1 | 1.2 | 5.31E-02 | 4.80E-03 | 6.5E + 04 |
| Y3 | \( K^b \-vov \) | Mono | 500 nM | 7.41E + 04 | 0.966 | 30.4 | 3.1 | 2.30E-02 | 2.27E-03 | 3.2E + 06 |
| Y3 | \( K^b_{3-83} \) | Mono | 625 nM | 2.03E + 04 | 0.25* | 25.9 | 4.3 | 2.74E-02 | 4.71E-03 | 7.4E + 04 |
| Y3 | \( K^b_{3-83-vov} \) | Mono | 500 nM | 3.52E + 04 | 0.22* | 16.8 | 2.2 | 4.19E-02 | 5.80E-03 | 8.4E + 05 |
| Hopc | \( K^b \-vov \) | Mono | 1,000 nM | No binding | | | | | | |
| Hopc | \( K^b_{3-83} \) | Mono | 4,140 nM | No binding | | | | | | |
| Hopc | \( K^b_{3-83-vov} \) | Mono | 9,140 nM | No binding | | | | | | |

*The dissociation rate was very slow disabling regeneration of the chip. Kinetic analysis is limited to one concentration of analyte. The statistical analysis is the \( \chi^2 \) value for the regression analysis of the on and off rates.

†The \( K_\alpha \) vs. concentration plot did not generate a linear slope, therefore the A + B = AB regression model was used to calculate an on rate. The statistical analysis is the \( \chi^2 \) value for the regression analysis of the highest concentration measured.

‡Association rate analysis for this experiment was uninterpretable, therefore the on rate for the \( K^b_{3-83} \) experiment was assumed to be comparable. As described in Materials and Methods, the BIAcore was used to measure association constants for divalent and monovalent binding of Y3 and 3-83 antibodies to soluble \( K^b \) and \( K^b_{3-83} \) with and without peptide. The ligand refers to the protein bound to the sensor chip and the analyte the protein passed over the chip. The method for association constant calculation is derived in Matherials and Methods. The \( R^2 \) and SD columns represent statistical analysis for \( k_\alpha \) and \( k_d \) calculations, respectively. The concentration column shows the highest concentration of analyte evaluated. Several rungs of two fold dilutions from this concentration were also evaluated. The irrelevant IgG2a Hopc-1 antibody was used to control for specificity. Binding of the 3-83 antibody to its antigenotype 54.1 antibody was included as a positive control. Blank cells of the sensor chip were also used as negative controls.

Immature B Cells Bearing Ultralow Affinity Autoreactive Receptors for Membrane-bound Self-antigens Are "Deleted" from Peripheral Lymph Organs. To examine the effect of the ultralow affinity ligands on B cell tolerance, 3-83 T/8 transgenic mice were bred with mice that expressed these weakly reactive class I haplotypes (Fig. 2). Transgenic mice bred on the non-3-83-reactive H-2a or H-2f backgrounds had a nearly monoclonal population of 3-83 idiotype-positive B cells in the peripheral lymph organs, as expected, whereas idiotype-positive B cells were not found in the lymph nodes or spleens of F1 transgenic mice in the peripheral lymph organs, as expected, whereas idiotype-positive B cells were not found in the lymph nodes or spleens of F1 transgenic mice. Transgenic mice heterozygous for \( K^b_{3-83} \) alleles, regardless of the affinity for the 3-83 antibody (Fig. 2 and Table 2). Virtually no B220-positive, IgM-negative cells were present (Table 2), arguing against the possibility that the autoreactive B cells had downregulated surface Ig receptor expression. These data demonstrate that even ultralow affinity, membrane-bound self-antigens are capable of "deleting" autoreactive B cells from peripheral lymph organs.

Ultralow Affinity Ligands to Autoreactive Ig Receptors Are Capable of Mediating Receptor Editing in Immature B Cells. To determine the mechanism by which 3-83-bearing B cells were absent from the spleens of F1 transgenic mice that expressed ultralow affinity ligands, we used two inde-
Figure 1. Relative binding of 3-83 antibody to various class I molecules in a flow cytometry assay. Binding of (A) 3-83 and (B) Y3 anti-class I antibodies to H-2 disparate, Sp2/0-derived hybridomas, or (C) binding of 3-83 antibody to spleen cells from MHC disparate mice. (D) Expands the lower range of the ordinate of C to differentiate poorly bound class I antigens. The data shown are representative of four independent experiments.

dependent assays based on previous work (13) to measure the extent of receptor editing. Lymphocytes from 3-83 transgene-positive, H-2 heterozygous mice were double stained with antibodies specific for the IgD\(^\beta\) (transgene-encoded) heavy chain and \(\lambda\) light chain. This assay identifies transgene-bearing B cells that have undergone endogenous light chain gene rearrangements because the endogenous heavy chain antibodies are of the \(\beta\) allotype and the 3-83 transgenic light chain is \(\kappa\). In transgenic mice of the H-2\(^d\) or H-2\(^\beta\) (nondeleting) background, the vast majority of B cells expressed the IgD\(^\beta\) allotype (Fig. 3 A and summarized in Fig. 3 B), indicating expression of the transgenic heavy chain. These cells were not \(\lambda\)-positive, and antidiotypic staining indicated that the transgenic heavy chain paired with the 3-83 \(\kappa\) light chain. But in the presence of autoantigen-expressing bone marrow, regardless of the antigen affinities examined, the percentage of cells double-positive for the transgenic heavy chain and \(\lambda\) light chain was significantly increased (Fig. 3). Because the total spleen cell numbers among H-2 disparate mice were comparable (data not shown), the increased percentage of idiootype-negative B cells in the presence of antigen represented an increase in the absolute number of cells bearing these altered receptors. This increase in IgD\(^\beta\), \(\lambda\) double-positive cells was not observed in 3-83 mice expressing a K\(^\beta\) transgene only in the periphery (liver, kidney; 21; Fig. 3 B column marked Per and reference 13), demonstrating that the abundant \(\lambda\)-positive B cells found in the centrally deleting mice were antigen induced and not merely the outgrowth of preexisting variants.

Evidence for receptor editing was also apparent in assays that measured expression levels in the bone marrow of the V(D)J recombinase genes RAG-1 (56) and RAG-2 (57). As observed previously (13), H-2\(^d\) 3-83 \(\mu/\delta\) transgenic mice expressed a low level of RAG-2, consistent with the observation that endogenous Ig rearrangements are suppressed by expression of the transgenic receptor (Fig. 4). Breeding of the 3-83 \(\mu/\delta\) transgenic mice onto class I haplotypes that were even weakly reactive with the 3-83 antibody resulted in significant upregulation of the RAG-2 gene expression, relative to that observed on the nonreactive H-2\(^\beta\) backgrounds (Fig. 4), again suggesting that these
low affinity ligands can induce receptor editing. Similar results were obtained in assays of RAG-1 levels (data not shown). In contrast, little or no increase in RAG-2 expression was observed in mice bearing the MT-Kb transgene, which restricts Kb antigen expression to extra bone marrow tissues and demonstrated little endogenous light chain rearrangement (Fig. 3B). Taken together with the changes in levels of spleen IgDVX/λ double-positive cells, these data

Table 2. Deletion of Idiotype-positive B Cells from Peripheral Lymph Organs of (3-83 × H-2\(\text{a}\)) F\(_1\) Transgenic Mice

| 3-83 transgenic mice | Percent of 54.1\(^+\) cells | Percent of B220\(^+\) cells |
|----------------------|-----------------------------|-----------------------------|
| H-2 haplotype (F\(_1\)) | Lymph node | Spleen | Bone marrow | Lymph node | Spleen | Bone marrow |
| d | 16.6 ± 1.2\(^*\) (18) | 39.3 ± 1.3 (19) | 27.0 ± 1.8 (11) | 13.4 ± 1.2 (17) | 36.1 ± 1.5 (18) | 26.7 ± 2.0 (8) |
| k | 0.2 ± 0.2 (3) | 0.3 ± 0.2 (3) | 0.1 ± 0.1 (2) | 5.4 ± 3.4 (2) | 10.0 ± 3.9 (2) | 26.5 ± 7.5 (2) |
| b | 0.1 ± 0.1 (8) | 1.1 ± 0.5 (10) | 2.7 ± 2.3 (8) | 2.2 ± 0.8 (7) | 7.9 ± 0.8 (9) | 36.9 ± 2.0 (5) |
| bm3 | 0.1 ± 0.0 (7) | 0.4 ± 0.2 (7) | 2.1 ± 1.9 (4) | 2.4 ± 0.5 (6) | 8.6 ± 1.7 (6) | 35.9 ± 5.6 (3) |
| bm6 | 0 ± 0 (4) | 1.2 ± 0.3 (4) | ND | 1.9 ± 0.3 (4) | 17.3 ± 2.8 (3) | 31.7 (1) |
| bm8 | 0.2 ± 0.1 (6) | 1.4 ± 0.6 (6) | 2.8 ± 2.7 (2) | 2.8 ± 0.4 (6) | 14.6 ± 2.0 (6) | ND |
| bm11 | 0 ± 0 (4) | 0.9 ± 0.9 (4) | 3.1 ± 0.9 (3) | 3.9 ± 0.5 (4) | 13.9 ± 3.0 (4) | 35.4 ± 0.7 (2) |
| K\(\text{D}\)K | 0 ± 0 (6) | 0.5 ± 0.2 (5) | 2.4 ± 2.1 (2) | 0.9 ± 0.1 (6) | 7.4 ± 0.6 (6) | 41.0 (1) |
| Nontransgenic mice | 0.3 ± 0.1 (15) | 0.7 ± 0.2 (15) | 0.3 ± 0.1 (9) | 23.1 ± 2.0 (15) | 40.8 ± 2.8 (15) | 52.8 ± 4.9 (6) |

\(^*\)Data are mean ± SEM of three to six experiments.

\(^1\)Numbers in parentheses indicate number of mice examined.

\(^2\)The lower percentage of id-positive cells is primarily the result of receptor downmodulation, which prevented detection at the concentration of 54.1 used.

\(^3\)These cells coexpressed IgM receptors and are presumably the result of receptor editing.
Figure 3. Increased percentage of B cells coexpressing IgDα (transgene heavy chain) and λ light chain (endogeneous) in spleens of autoantigen-bearing transgenic mice. (A) Spleen cells from 3-83 transgenic mice bred with non-autoantigen bearing mice (H-2d) or antigen-expressing mice (Kb', bm3' bm11 or Dk) were stained with FITC anti-λ and biotinylated anti-IgDα antibodies, followed by PE-streptavidin. On these genetic backgrounds, only the transgenic heavy chain is of the 'α' allotype. A nontransgenic control is included to show specificity of the 'α' allotype reagent for transgene. 50,000 cells from each sample were collected in a gate defined by forward and side scatter to include the predominant lymphocyte population. Each row represents an individual experiment and is representative of a minimum of three experiments for each H-2 haplotype. (B) The combined data are shown as the mean ± SEM (error bars) percent of IgDα, λ double-positive cells in transgenic mice of the indicated H-2 haplotype. The number above each bar indicates the number of mice per group. Per, peripherally deleting, MT-Kb/3-83 bt/8 double-transgenic mice; Non Tg, nontransgenic littermates.

A

| H-2d x 3-83 Tg | H-2b x 3-83 Tg | H-2bm3 x 3-83 Tg |
|----------------|----------------|-----------------|
| 43.3% .4%     | 57% 7.8%      | 7.5% 10.2%     |

Anti-IgDα

Anti-λ

| H-2d x 3-83 Tg | H-2bm11 x 3-83 Tg | K'bKk x 3-83 Tg | NonTg |
|----------------|--------------------|----------------|--------|
| 46.5% .2%      | 7.8% 8.6%          | 2.6% 2.1%      | 2.2% .1% |

B

| H-2 (xd) of F1 Transgenic Mice |
|-------------------------------|
| % IgDα, λ, Double-Positive Cells |
| d | k | b | bm3 | bm6 | bm8 | bm11 | Dk | Per | NonTg |
|---|---|---|-----|-----|-----|------|----|-----|-------|
| 0 | 2 | 17 | 10  | 7   | 5   | 5    | 7  | 13  | 2     |

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demonstrate the ability of even ultralow affinity ligands to fully induce central tolerance and receptor editing in autoreactive B cells.

In Acute Challenge with Antigen, Ultralow Affinity Ligands Are Only Moderately Effective at Deleting Mature B Cells as Compared to the "High" Affinity Kα Ligand. To test the effect of the receptor/antigen interaction on tolerization of mature B cells, we took advantage of a system originally described by Murakami et al. in which it was shown that ligands of surface Ig could induce rapid apoptosis of peritoneal B cells (9, 22, 58). We injected mice intraperitoneally with Sp2/0 (H-2d)-derived cell lines coexpressing the following class I molecules: Kk, Kb, Kb'bm11, and Kb'bm3, which bind 3-83 with decreasing affinities. Approximately 16 h later, the peritoneal cavity cells were isolated and deletion of the id-positive B cells was scored. The Sp2/0 (H-2d control)-injected mice generally showed a modest, non–antigen-specific reduction in transgenic B cells compared to PBS-injected controls (Fig. 5, A and B). In contrast, injection of the high affinity Kα-expressing tumors led to a consistent 5–10-fold decrease in the percentage of id-positive B cells over eight experiments (Fig. 5 and Table 3).
...was less consistent over several experiments, and the ability of B-cells (Fig. 5 and Table 3). Compared to Kk-injected lines resulted in only twofold reduction of the 3-83-bearing cells, the amount of deletion with the lower affinity antibody. Intraperitoneal injection of the low affinity ligand, we tested the sensitivity of splenic transgenic B cells was observed (Table 3). tematically, since no change in the percentage (or number) of peripheral B cell deficiency (13, 21). Results with nontransgenic mice represent the mean ± SEM RAG-2/Gas values for each haplotype.

The remaining id-positive B cells usually showed down-regulation of IgM receptor expression, indicating that these cells had encountered antigen (Fig. 5 A). To exclude the possibility that the B cells completely lost their receptor expression and thus were not actually deleted, cells were double stained with anti-IgM and anti-B220 antibodies. As can be seen in Table 3, no increase in the percentage of IgM-negative, B220-positive cells accompanied the decrease in id-positive B cells. Based on the previous work, we presume the id-positive cells that disappeared had undergone antigen-induced apoptosis and had not merely left the peritoneal cavity (9, 22, 58). This deletion effect induced by the intraperitoneal injection of antigen was not seen systematically, since no change in the percentage (or number) of splenic transgenic B cells was observed (Table 3).

Having shown deletion of reactive, mature B cells upon injection of high affinity K\textsuperscript{b} ligand, we tested the sensitivity of this tolerance mechanism to the antibody/antigen-binding affinity. Intraperitoneal injection of the low affinity (K\textsuperscript{b}) and ultralow affinity (K\textsuperscript{bm3,11}) ligand-expressing cell lines resulted in only twofold reduction of the 3-83-bearing B-cells (Fig. 5 and Table 3). Compared to K\textsuperscript{b} injected cells, the amount of deletion with the lower affinity antigens was less consistent over several experiments, and downregulation of the IgM receptor was rarely seen. Overall, a difference in the extent of deletion of mature B cells upon acute antigenic challenge was clearly noted between the high affinity K\textsuperscript{b} molecule and the lower affinity K\textsuperscript{bm3,11} ligands, whereas even these weakly reactive molecules were capable of inducing complete deletion of immature, autoreactive B cells, as was clear from analysis of F\textsubscript{1} crosses (Fig. 2 and Table 2).

Discussion

In concordance with earlier studies investigating the sensitivity of B cell tolerance with respect to BCR/soluble antigen affinity (31, 32, 36), we have verified tolerance induction in immature B cells upon interaction with very low affinity, membrane-bound self-antigens. We were able to follow the fate of the immature, self-reactive B cells and to show that deletion of this cell population was indeed the mechanism of censorship. Our data also indicate that extremely low affinity membrane ligands are sufficient to signal the immature B cell to alter receptor specificity ("receptor editing").

In this study, the affinity between MHC class I autoantigen and Ig receptor that was sufficient to stimulate receptor editing/deletion of the immature B cell was so low that we were unable to place a lower limit on its value. In two independent studies, we measured the affinity constant of the monovalent interaction between K\textsuperscript{b} and 3-83 to be in the range of 1-3 × 10\textsuperscript{5} M\textsuperscript{-1}. K\textsuperscript{bm3} and D\textsuperscript{k} were bound by 3-83 more weakly than K\textsuperscript{b}, requiring more than 10-fold more IgG to achieve similar binding. Because these ultralow affinity ligands were fully as effective as high affinity ligands in mediating central deletion, an affinity constant of 4 × 10\textsuperscript{5} M\textsuperscript{-1} between BCR and membrane self-antigen represents a very conservative value sufficient to induce tolerance in the immature B cell population, this range of affinity was in fact observed in the case of K\textsuperscript{bm3} (1-6 × 10\textsuperscript{6} M\textsuperscript{-1}). This affinity is lower than many reported for TCR/class I peptide agonist interactions (59), suggesting that central deletion of autoreactive B cells in the bone marrow can be as sensitive as central T cell tolerance and indeed is in the affinity range for positively selecting ligands (60).

An important implication of a low affinity threshold for self-tolerance is that it may have profound effects on the B cell repertoire and directly influence the fate of a large proportion of generated B cells. Theoretical models predict that self-reactivity should be common in the preselected lymphocyte repertoire and that negative selection may play a powerful role in the evolution of lymphocyte specificity (61).

While the multivalent nature of the cell/cell interactions between the B cell and antigen-bearing cells is important in permitting weak interactions to lead to biologically significant avidities, such multimerization does not make receptor/ligand affinity irrelevant. Analysis of peripheral B cell tolerance using the same panel of ligands that led to central tolerance revealed clear differences between high and low affinity ligands in the efficiency of peripheral deletion.

Figure 4. Elevated expression of RAG-2 in the presence of bone marrow autoantigen, including low affinity ligands. Reverse transcription PCR analysis of bone marrow RNA of (3-83p68 Tg/H-2\textsuperscript{b}) F\textsubscript{1} mice, where \( x \) includes H-2 haplotypes both reactive and nonreactive to the 3-83 antibody. cDNA was amplified with 17–22 cycles of PCR using oligos specific for RAG-2 and, as a normalization control, the ubiquitously expressed GTP-binding protein, Gts. Southern blot analysis revealed specific fragments that were quantitated using a Phospho−\textgamma−Imager. The data represent the mean ± SEM RAG-2/Gas values for each haplotype. Numbers of mice per group are shown above each bar. 3-83p68/MT-K\textsuperscript{b} peripherally deleting mice (Per Tg), in which K\textsuperscript{b} antigen driven by the metallothionein promoter is expressed in the periphery, but not in the bone marrow, are included as negative controls for the nonspecific effects of peripheral B cell deficiency (13, 21). Results with nontransgenic mice (Non Tg) define normal levels of bone marrow RAG-2 expression.

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Discussion

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While the multivalent nature of the cell/cell interactions between the B cell and antigen-bearing cells is important in permitting weak interactions to lead to biologically significant avidities, such multimerization does not make receptor/ligand affinity irrelevant. Analysis of peripheral B cell tolerance using the same panel of ligands that led to central tolerance revealed clear differences between high and low affinity ligands in the efficiency of peripheral deletion.

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An unexpected finding in the present study was that the IgG form of the 3-83 antibody had only a slightly higher overall avidity for immobilized ligand than the corresponding F\textsubscript{ab} fragment. The comparative increase seen in the bivalent vs. monovalent binding of the Y3 antibody to the same class I ligand was more than 500-fold and was consistent with results from previous studies on affinity constants for anti-class I antibodies to their ligands (62), whereas the affinity increase resulting from bivalent binding in the 3-83/K\textsubscript{b} interaction was unusually small. Since Y3 and 3-83 were passed over the same K\textsuperscript{b}-containing sensor chip, a density difference resulting in spatial limitations could not account for this difference, particularly because Y3 and 3-83 reportedly see the same epitope (53). Importantly, there was excellent agreement between measured affinities of the IgG antibodies and the relative affinities observed in the flow cytometry analysis of binding to K\textsubscript{b}-expressing cells: \( \sim 1,000 \) fold higher concentration of 3-83 was required to achieve levels of binding equivalent to that observed with Y3 (Fig. 1). These findings lend credulity to the extreme sensitivity of B cell tolerance induction with respect to affinity, since the polyvalency appeared to play a limited role in this particular Ig transgenic system.

Because of the peptide dependency of some anti-class I antibodies (63), it is difficult to formally exclude the possibility that the weak binding of D\textsuperscript{k} and K\textsuperscript{bm3} ligands by 3-83 actually represents expression of a lower density of higher affinity ligands rather than a comparable density of low af-
finity ligands. We do not favor this notion, however, because it is inconsistent with the observation that binding to putative low affinity ligands fails to plateau at high 3-83 concentrations (Fig. 1 D) and the similar binding affinities of 3-83 to peptide-bearing and peptide-free Kbm3. Nonetheless, if the weak binding to certain class I molecules such as Dk does represent a low density of high affinity antigens, the interpretation of our data would be that tolerance can be induced by higher affinity membrane antigens present at very low epitope density, as opposed to the interpretation that we favor, namely that tolerance is induced by very low affinity self-antigens with moderate to high cell surface abundance. We have attempted to address the issue of antigen density by breeding B2-microglobulin–deficient mice with 3-83 transgenic mice bearing H-2bm3. The F1 mice expressed one half the normal class I molecules because of heterozygosity of the B2-microglobulin locus deficiency, and only one half of the H-2k molecules were antigenic (Kbm3), since the mice were heterozygous at the MHC locus (bm3/d). With one quarter the normal number of class I molecules, the resulting transgenic mice were still capable of fully deleting the autoreactive B cells and inducing receptor editing (data not shown), suggesting that only a few thousand molecules per cell of a low affinity membrane self-antigen, when present in the bone marrow, can efficiently induce B cell tolerance.

The possible peptide dependency of anti-class I antibodies also presents limitations to our affinity measurements, since the recombinant soluble H-2k molecules contained only one or a limited number of peptides. However, the binding affinity measurements correlated well with binding of these antibodies to spleen or hybridoma cell–derived Kb molecules, which bear diverse peptides.

Do weakly self-reactive B cells contribute to the emergence of low affinity autoantibodies? In contrast to the affinity studies in the HEL radiation chimera system (36), we do not see the emergence of 3-83–bearing cells in F1 transgenic mice on any low affinity Dk, Kbm3, or Kbm11 background in mice tested out to 18 mo of age (data not shown). Nevertheless, B cells with low affinity receptors may be more sensitive to a breakdown in tolerance. Although all of the ligands of 3-83 that we tested induced central deletion, it is likely that there exists an affinity threshold for membrane antigen at which the reactive B cell is no longer tolerized or the tolerance phenotype is altered, perhaps leading to an anergic state rather than to cell death. We have evidence for this change in tolerance phenotype to a membrane-bound antigen in cases of limiting antigen dose (our unpublished data), implying that the strength of the tolerance signal is dependent on the number or duration of B cell/antigen interactions. It will be important to determine if a low affinity ligand requires longer duration or more antigenic “hits” for a tolerance signal to satisfy a minimum threshold for tolerance.

Developing B cells are generally thought to be more sensitive to tolerance induction than mature B cells (1, 64), but this is by no means proven (32). Our data are consistent with, but do not prove, this notion, since deletion of the autoreactive mature B cells occurred consistently only in the presence of the high affinity Kk ligand. The intermediate affinity, Kk, and low affinity Kbm3,11 ligands were only able to induce partial deletion, suggesting that low affinities (<106 M–1) limit the potential to induce deletion. A difference in total H-2k density could not account for this difference in deletion induction, since staining of the hybridomas showed equivalent surface expression. In contrast to the partial deletion seen upon intraperitoneal injection of Kk cells into 3-83 µδ mice, transgene–driven, tissue-specific expression of the intermediate affinity ligand Kk on the liver and kidney results in complete deletion of the 3-83–bearing B cells from the lymph node in MT-Kk/3-83 µδ double-transgenic mice (21). The inability of Kk-bearing cells to achieve the same level of deletion upon intraperitoneal injection could be caused by the more mature devel-

### Table 3. Degree of Reduction in the Number of Idiotype-positive B Cells from the Peritoneal Fluid of Antigenically Challenged Transgenic Mice Correlates with Affinity of 3-83 Antibody for Antigen

| H-2 (X × d) of injected cells | Peritoneal exudate cell |
|------------------------------|------------------------|
|                              | %54.1⁺ | %IgM⁺⁺ | %B220⁺⁺ | %B220⁺, IgM⁻ |
| d                            | 11.2 ± 1.2⁺ (5) | 23.2 ± 6.7 (6) | 15.4 ± 2.5 (6) | 2.2 ± 0.5 (4) | 48.6 ± 4.7 (5) | 53.1 ± 2.9 (4) | 49.1 ± 3.7 (4) |
| k                            | 1.8 ± 0.2 (8) | 7.8 ± 1.1 (8) | 8.4 ± 1.0 (8) | 2.2 ± 0.3 (5) | 48.3 ± 6.3 (5) | 52.3 ± 5.9 (4) | 49.9 ± 6.0 (4) |
| b                            | 6.1 ± 1.6 (6) | 14.2 ± 5.2 (5) | 11.7 ± 3.5 (1) | 2.6 ± 0.6 (4) | 41.5 ± 2.4 (5) | 50.2 ± 3.2 (4) | 48.5 ± 3.2 (4) |
| bm3                          | 5.4 ± 1.0 (6) | 14.6 ± 2.6 (6) | 10.7 ± 1.9 (6) | 2.0 ± 0.5 (5) | 44.9 ± 4.1 (4) | 54.3 ± 0.5 (3) | 52.8 ± 2.0 (3) |
| bm11                         | 7.0 ± 1.1 (6) | 16.4 ± 4.3 (6) | 11.4 ± 2.4 (6) | 1.5 ± 0.3 (5) | 34.8 ± 7.9 (3) | 48.1 ± 2.7 (3) | 48.4 ± 2.4 (3) |
| pbs                          | 15.2 ± 2.1 (6) | 26.0 ± 3.8 (5) | 18.3 ± 2.4 (5) | 1.3 ± 0.4 (3) | 38.3 ± 3.3 (5) | 42.1 ± 0.5 (3) | 41.2 ± 2.0 (3) |

*The peritoneal cells consist of a variable population of IgM⁺, B220⁻/κc cells, which are not gated as B220⁺ cells, and account for the discrepancy between the percentage of IgM⁺ and B220⁺ cells.

Numbers in parentheses represent number of mice examined.
opment of the B cells that encounter autoantigen in this case, or because of a longer duration of exposure to antigen in the double-transgenic mice, compared to the short-term peritoneal injection assay. No increase in the deletion of transgenic B cells was observed 2 d after peritoneal injection of Kb-hybridomas, suggesting that a longer period of antigen exposure did not affect the phenotype, but further experiments are required to establish this point.

An important conclusion to be drawn from our study is that tolerance to membrane self-antigens can be extraordinarily efficient, and can eliminate precursors of antibody-forming cells that have very low affinity for self. This tolerance may be essential to the survival of the organism because low affinity IgM antibodies to cell-surface proteins are potentially toxic, as has been observed in tissue rejection by isoantibodies (65, 66). In view of these efficient tolerance mechanisms, one might ask how autoantibodies arise. One clue from the present study is the apparently higher affinity threshold for the deletion of mature B cells, at least in response to transiently expressed antigen. Perhaps failure in these mechanisms resulting from stricter requirements for tolerance induction in mature B cells contribute to the autoimmune process for peripheral antigens.

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