The Cellular Location of Self-antigen Determines the Positive and Negative Selection of Autoreactive B Cells

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Abstract

Systemic autoimmune disease is frequently characterized by the production of autoantibodies against widely expressed intracellular self-antigens, whereas B cell tolerance to ubiquitous and highly expressed extracellular antigens is strictly enforced. To test for differences in the B cell response to intracellular and extracellular self-antigens, we sequestered a tolerogenic cell surface antigen intracellularly by addition of a two amino acid endoplasmic reticulum (ER) retention signal. In contrast to cell surface antigen, which causes the deletion of autoreactive B cells, the intracellularly sequestered self-antigen failed to induce B cell tolerance and was instead autoimmunogenic. The intracellular antigen positively selected antigen-binding B cells to differentiate into B1 cells and induced large numbers of IgM autoantibody-secreting plasma cells in a T-independent manner. By analyzing the impact of differences in subcellular distribution independently from other variables, such as B cell receptor affinity, antigen type, or tissue distribution, we have established that intracellular localization of autoantigen predisposes for autoantibody production. These findings help explain why intracellular antigens are targeted in systemic autoimmune diseases.

Key words: SLE • autoimmunity • self tolerance • B lymphocytes • hen egg lysozyme

Introduction

Our understanding of the mechanisms responsible for B cell tolerance has been largely established in a series of experiments using mice expressing Ig transgenes, encoding antibodies directed against naturally occurring or neo self-antigens. These studies have established that tolerance to abundant systemic extracellular antigens occurs by deleting (1–3), editing (4, 5), or inactivating (6–8) the autoreactive B cells, whereas B cells that bind low avidity or rare antigens may remain functionally ignorant (9, 10). This spectrum of tolerogenic or neutral responses is well described in transgenic mice expressing different forms of the foreign protein hen egg lysozyme (HEL) (11). Self-reactive B cells expressing transgenic Ig receptors for HEL (MD4 anti-HEL Ig [Ig^{HEL}]) undergo deletion or receptor editing in the BM when they encounter systemic membrane-bound HEL (mHEL) expressed on the cell surface under the class I promoter (1, 12). Ig^{HEL} B cells that encounter abundant but lower avidity

soluble HEL (sHEL) at a level in excess of 10–20 ng/ml enter the repertoire but are held in a potentially reversible state of functional inactivation called anergy (7, 8, 13, 14) and have a shortened life span which is largely due to their inability to compete with other B cells (15, 16). Ig^{HEL} B cells that encounter sHEL below the level of 10 ng/ml retain the functional characteristics of naive B cells and have therefore been described as immunologically ignorant (9). Deficiency in negative regulators of B cell receptor (BCR) signaling, such as the coreceptor CD22, src-kinase Lyn, or phosphatase SHP1, lowers the threshold for tolerance and causes Ig^{HEL} B cells to be deleted rather than anergized by abundant sHEL (17–19). In contrast, deficiency in positive regulators, such as coreceptors CD19 or CD45, raises the threshold for tolerogenic responses (20, 21). Together, the transgenic and genetic experiments have led to a widely

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Abbreviations used in this paper: BCR, B cell receptor; HEL, hen egg lysozyme; Ig^{HEL}, MD4 anti-HEL Ig; mHEL, cell surface membrane-bound HEL; mHEL-KK, ER-restricted mHEL; sHEL, soluble HEL; SLE, systemic lupus erythematosus; TBS, tris-buffered saline; TLR, Toll-like receptor.

accepted signal strength model of tolerance in which the fate of an autoreactive B cell is determined by the abundance and avidity of its target self-antigen and the affinity of its BCR (11).

When immunological tolerance to systemic antigens breaks down, as in systemic lupus erythematosus (SLE), autoantibodies are characteristically produced against a wide range of intracellular targets (22). Under these circumstances, factors other than signal strength may be critical in deciding the fate of autoreactive cells. Despite good evidence that a breakdown in tolerance to intracellular antigens is a key feature of systemic autoimmune disease, it has not been determined if the mechanisms maintaining B cell tolerance against intracellular and extracellular antigens are the same. Intracellular antigens targeted in different systemic autoimmune diseases are frequently grouped together in cell surface blebs on apoptotic cells, where they may perhaps give rare but high avidity stimulation to autoreactive B cells (23). Furthermore, several of the lupus susceptibility genes identified in animal models are involved in the clearance or sequestration of cellular debris and these intracellular targets (24). In familial human SLE due to C1q deficiency, the primary defect lesion seems likely to be due to reduced phagocytic clearance of apoptotic or necrotic cells (25, 26). These observations raise the possibility that there may be fundamental differences in B cell tolerance to intracellular self-antigens compared with those on the cell surface.

Because most of the autoantigens targeted in SLE are intracellular, we have set out to test the mechanisms acting to maintain self-tolerance to intracellular antigens. Using a newly generated panel of transgenic mice expressing an intracellularly sequestered variant of mHEL (ER–restricted mHEL [mHEL-KK]) we demonstrate that intracellular sequestration alone is sufficient to convert the tolerogenic self-antigen into a potent autoimmunogen. Whereas cell surface antigen causes the deletion or receptor editing of immature B cells, intracellular antigen induces autoantibodies and the differentiation of B1 B cells. These findings show that the cellular location of self-antigen is a critical factor in determining the positive and negative selection of autoreactive B cells, and they provide a basis for understanding the selective targeting of intracellular antigens by autoantibodies in SLE.

Materials and Methods

Generation of mHEL-KK Mice. The transgene was prepared by adapting the previously described mHEL-expressing KLK construct (1). A modified H-2K^b transmembrane region including a dilysine-retention motif was produced by PCR amplification of the original KLK construct. The forward primer (5′ to 3′: ccagggtcgcctggcgcaacc) encodes the junction between the 3′ sequence of the lysozyme gene and the 5′ H-2K^b transmembrane sequence. The reverse primer (5′ to 3′: ccatgtcggccgctgtcttcaatccctcttacccttttaccagtccttctcatcttcatcacaaaagcc) encodes a NotI site and the dilysine retention motif derived from human UDP-glucuronosyltransferase 2B4 as a 5′ tag (27), with the 3′

end priming off the H-2K^b transmembrane sequence. The 265-bp PCR product was digested with XhoI and NotI, subcloned into pBluescript, and checked for fidelity by sequencing. To allow directional cloning, the pKLK H-2K^b transmembrane 3′ XhoI restriction site was destroyed and a NotI site created via the introduction of an oligonucleotide linker (pKLK+linker). The final construct was obtained by digesting pKLK+linker with XhoI and NotI and ligating it to the 196-bp XhoI-NotI PCR fragment. The mHEL-KK transgene was excised by digestion with SalI and ClaI and prepared for microinjection into (C57BL/6 × CBA/CaJ)F1 oocytes as described previously (8). Transgenic founders were identified by PCR and Southern blotting. Animals were kept in SPF conditions. The animal experiments were approved by the Oxford University Ethical Review Committee and were performed under Home Office licence.

Immunohistochemistry. Organs were snap-frozen in liquid nitrogen and stored at −20°C until sectioning. Cryostat sections (7 μm) were cut, fixed, and stained as described previously (28) with the addition of a blocking step before the first primary antibody. Sections were blocked with 5% normal goat serum (Vector Laboratories) in tris-buffered saline (TBS) for 30 min at room temperature. Expression of HEL was detected by incubating with unpurified rabbit polyclonal anti-HEL serum followed by alkaline-phosphatase–conjugated anti–rabbit IgG (Sigma-Aldrich). Plasma cells and metallophillic macrophages were detected with syndecan-1 (BD Biosciences) and MOMA-1 (Serotec), respectively, and biotinylated goat anti–rat IgG (Serotec). Each was then followed with StreptABComplex/AP (Dako). Biotinylated AMS9.1 or B220 and StreptABComplex/HRP (Dako) were used to detect B cells. Enzyme reactions were developed with either DAB (Vector Laboratories) for peroxidase or Fast Red/Napthol AS-MX or Vector Blue (Vector Laboratories) for alkaline phosphatase. Sections were counterstained with hematoxylin.

Confocal Microscopy. All steps were performed at room temperature. Frozen sections were fixed with 4% paraformaldehyde/250 mM Hepes for 10 min and then 8% paraformaldehyde/Hepes for 50 min. After washing with PBS, sections were quenched with 50 mM NH$_4$Cl for 5 min and washed in PBS. Sections were then permeabilized with 0.1% Triton X-100/PBS for 10 min and washed in PBS and then in TBS. Sections were blocked with normal goat serum as for immunohistochemistry and washed with TBS. To stain for HEL expression, slides were incubated with rabbit anti-HEL serum for 30 min, washed with TBS, and incubated with goat anti–rabbit IgG-TRITC (Jackson ImmunoResearch Laboratories) for 30 min. Images were collected using the 568-nm line of a krypton–argon laser on a Bio-Rad Laboratories MRC1024 confocal scanning laser microscope. A 40× objective (NA 1.3) was used; images were collected using Lasersharp 2000 software with Kallman averaging and histogram stretched to fill an 8-bit intensity range. No further image processing was applied.

Flow Cytometry. BM and spleen suspensions were stained, as described previously (8), with the following mAbs: IgM^a, DS-1–phycoerythrin (PE; BD Biosciences); IgD^a, AMS9.1-fluorescein isothiocyanate (FITC); B220, 6B2-allophycocyanin (APC; Caltag), 6B2-PE (Caltag), and 6B2-FITC; class II, I-A^p–FITC (BD Biosciences); Mac-1–FITC (Caltag); CD21-biotin (Bi) followed by streptavidin-PE (Caltag); CD9-Bi, (BD Biosciences) followed by streptavidin-APC (Caltag); IgM^a-Bi, (BD Biosciences) followed by streptavidin-tricolor (Tc; Caltag); and CD21-FITC, CD23-PE, CD69-FITC, and CD86-PE (BD Biosciences). HEL binding cells were detected by incubating cells with 200 ng/ml unlabeled HEL

(Sigma-Aldrich) followed by the anti-HEL mAb HyHEL9-Tc or HyHEL9-FITC. All analysis was performed using a FACSCalibur flow cytometer and Cell Quest software (Becton Dickinson).

Quantification of Serum HEL Concentration. Splenocytes (10^5) from an IgHEL transgenic mouse (MD4; a gift from C. Goodnow, Australian National University, Canberra, Australia) were incubated on ice for 45 min with either HEL serially diluted in normal mouse serum or serum alone, followed by staining with HyHEL9-Tc and B220-FITC as for flow cytometry. A standard curve of the mean HyHEL9-Tc fluorescence of positively staining B cells versus the concentration of HEL was plotted. The concentration of serum HEL in the mHEL-KK transgenic lines was determined by incubating IgHEL cells with mHEL-KK serum and comparing the mean fluorescence with the standard curve.

Measurement of Receptor Occupancy. BM cells from IgHEL and IgHEL/mHEL-KK mice were incubated on ice for 20 min with either serially diluted concentrations of HEL in staining media containing 0.1% sodium azide or media alone, followed by staining with HyHEL9-Tc as for flow cytometry. Receptor occupancy was determined as described previously (9).

Measurement of Serum Anti-HEL IgM^a and Splenic IgM^a-secreting Plasma Cells. Anti-HEL IgM^a was measured from sera by ELISA as described previously (16). Anti-HEL IgM^a-secreting cells were measured in spot ELISA in 96-well plates coated with 1 mg/ml HEL in carbonate buffer, pH 9.8. Spots of bound antibody were revealed with biotinylated anti-IgM^a (DS-1; BD Biosciences) followed by avidin–alkaline phosphatase (Sigma-Aldrich).

In Vitro Culture. Splenocytes from IgHEL, IgHEL/mHEL-KK, or IgHEL/sHEL transgenic mice (sHEL ML5 mice; a gift from C. Goodnow, Australian National University) were harvested at room temperature in 10% complete medium (comprising RPMI supplemented with 10% FCS, 10 mM Hepes, 2 mM glutamine, 50 μM 2-mercaptoethanol) washed once and resuspended. 5×10^6 cells were incubated at 37°C in 5% CO_2 in 0.25 ml of complete medium with 0.5 μg/ml HEL. Control cells were held on ice without antigen. After 17 h, cells were analyzed by flow cytometry for surface expression of CD69 and CD86 (B7.2).

Cell Lysates and Western Blot Analysis. Splenocytes for stimulation from IgHEL, IgHEL/mHEL-KK, or IgHEL/sHEL transgenic mice were isolated in 10% complete medium at room temperature, washed, and resuspended in medium. Prior to stimulation, both cells and stimulant were warmed for 5 min at 37°C, and reactions were initiated by combining them in a ratio of 3:1, which gave instant mixing. Stimuli were medium alone for 3 min in the case of all unstimulated samples or HEL for 3 min (final concentration 1 μg/ml). Reactions were terminated by transferring cells into an equal volume of ice cold 2× NP-40 lysis buffer (composition of 1× lysis buffer: 1% NP-40, 50 mM Tris, pH 8.0, 150 mM sodium chloride, 5 mM EDTA, 10 mM sodium fluoride, 10 mM β-glycerol phosphate, 2 mM PMSF, and 10 μl/ml of the Sigma–Aldrich protease-inhibitor cocktail I and phosphatase-inhibitor cocktails I and II). After 15 min on ice, the samples were centrifuged at 13,000 g at 4°C for 20 min, the supernatants added to 2× SDS-PAGE reducing sample buffer, and boiled for 5 min before loading onto 10% SDS-polyacrylamide gels. Western blots were probed with antiphosphotyrosine (Mab 4G10; Upstate Biotechnology) followed by HRP–anti–mouse IgG (Upstate Biotechnology) or rabbit polyclonal anti-Lyn anti-CD79β or anti-CD79α (gifts from J. Cyster, University of California San Francisco, San Francisco, CA) followed by HRP–goat anti–rabbit (Zymed Laboratories). Detection was by enhanced chemiluminescence (Amersham Biosciences).

Fetal Liver Chimeras. Fetal liver cells were harvested at day 19 of gestation from embryos expressing the IgHEL transgene, and 0.5×10^7 cells were injected into the lateral tail vein of mHEL-KK and nontransgenic recipients that had been lethally irradiated with two doses of 5 Gy gamma irradiation separated by 3 h. The animals received antibiotics (amoxycillin 0.25 mg/ml in water bottles) for the first 3 wk of their reconstitution and were killed at 8 wk.

Results

Transgenic Mice Expressing HEL as a Ubiquitous Intracellular Self-antigen. To study the mechanisms involved in maintaining tolerance to intracellular self-antigens, we modified the tolerogenic cell surface mHEL construct by adding a dilysine ER retention motif to the COOH-terminal cytoplasmic tail (mHEL-KK; Fig. 1) (29). Proteins carrying dilysine or other dibasic motifs bind to cytosolic COP I proteins, which causes their continuous and avid retrieval from the golgi to the ER (30). Apart from the cytoplasmic tail, the mHEL and mHEL-KK constructs were identical and shared the same ubiquitous class I promoter, extracytoplasmic lysozyme domain, and transmembrane segment. Three lines of transgenic mice carrying the ER-restricted HEL were produced using fertilized eggs from (CBA×C57BL/6)F1 mice and backcrossed six generations to C57BL/6. The transgenic lines, designated mHEL-KK1, mHEL-KK2, and mHEL-KK3, were healthy and bred normally. Histological examination of fixed and permeabilized spleen and thymus showed expression in many cells with a typical ER distribution on confocal microscopy (Fig. 2, A and B). Flow cytometric analysis for cell surface and intracellular antigen in spleen and BM cells with anti-HEL antibodies confirmed that expression was intracellular (Fig. 2 C). In each organ sample preparation there were a minority of cells that were positive for surface-exposed HEL, ranging from 0.015% lymphocytes in BM to 0.4% in spleen samples (Fig. 2 D), but these were membrane-permeable necrotic or late stage apoptotic cells as determined by staining with propidium iodide (not depicted). In transgenic mice expressing mHEL on the cell surface, sHEL is present in serum at levels in excess of 200 ng/ml due to proteolytic cleavage from the intact protein (unpublished data). In the mHEL-KK lines, sHEL was present in a range of 2–10 ng/
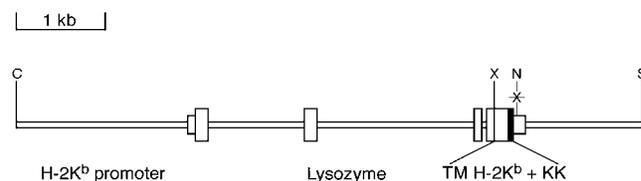

Figure 1. Generation of a transgene expressing a ubiquitous intracellular membrane-bound lysozyme. Derived from the construct used to express mHEL on the cell surface (KLK) (1), the sole modification is the addition of a dilysine ER reteintion motif to the cytoplasmic tail (black box). In other respects, the constructs are identical, including the lysozyme domain and the H-2K^b transmembrane region. Restriction sites: X, XhoI; N, NotI; S, SalI; C, ClaI.
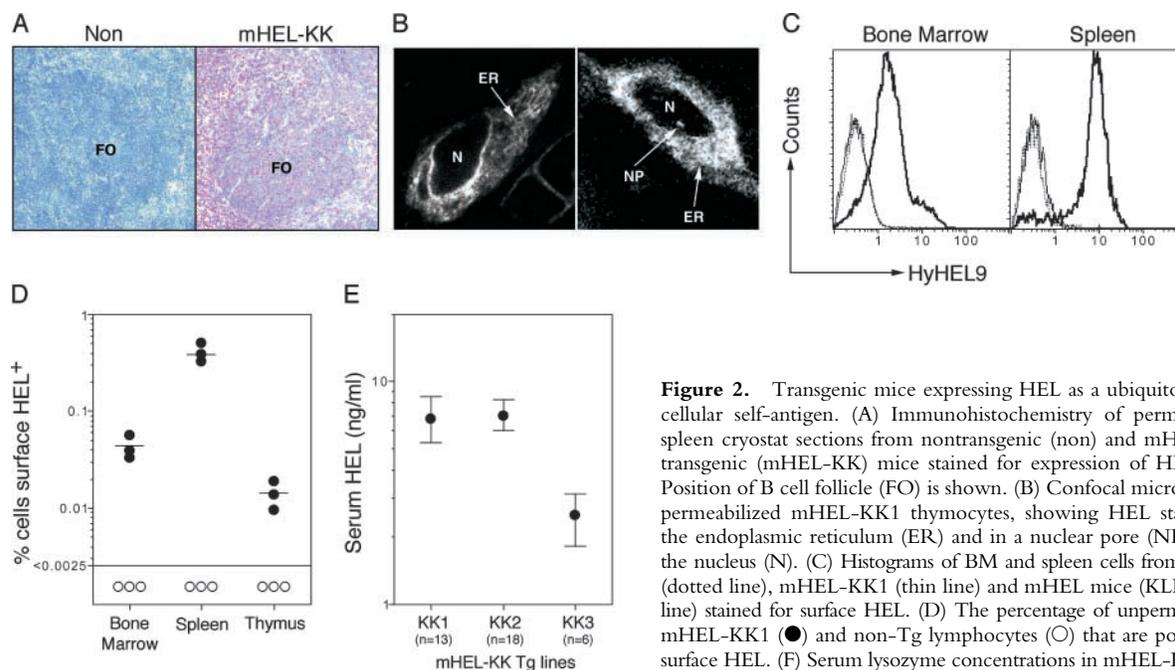
Figure 2. Transgenic mice expressing HEL as a ubiquitous intracellular self-antigen. (A) Immunohistochemistry of permeabilized spleen cryostat sections from nontransgenic (non) and mHEL-KK1 transgenic (mHEL-KK) mice stained for expression of HEL (red). Position of B cell follicle (FO) is shown. (B) Confocal microscopy of permeabilized mHEL-KK1 thymocytes, showing HEL staining in the endoplasmic reticulum (ER) and in a nuclear pore (NP) within the nucleus (N). (C) Histograms of BM and spleen cells from non-Tg (dotted line), mHEL-KK1 (thin line) and mHEL mice (KLK3; thick line) stained for surface HEL. (D) The percentage of unpermeabilized mHEL-KK1 (●) and non-Tg lymphocytes (○) that are positive for surface HEL. (F) Serum lysozyme concentrations in mHEL-KK lines.

ml, which would be compatible with release from cellular debris or rare surface expression (Fig. 2 E).

Intracellular HEL Antigen Fails To Induce Either Deletion or Anergy. To study the fate of B cells specific for the intracellular HEL autoantigen, mHEL-KK mice were crossed with MD4 Ig^{HEL} transgenic mice carrying Ig heavy and light chain transgenes encoding high affinity IgM^a and IgD^a specific for HEL (8). The resulting Ig^{HEL}/mHEL-KK double transgenic mice were of normal appearance, bred normally, and their survival up to 200 d was no different from single or nontransgenic littermates. Histological analysis of heart, lung, liver, LN, spleen, skin, kidney, and thymus showed no difference between transgenics and littermate controls. Mild peribronchial inflammation and focal lobular hepatitis, described previously as affecting the C57BL/6 strain (31), was neither affected by the presence of the transgene nor as-

sociated with known pathogens (unpublished data). Flow cytometric analysis showed similar numbers of immature HEL-binding B cells in the BM and mature HEL-binding B cells in the spleen of Ig^{HEL} single transgenic and Ig^{HEL}/mHEL-KK double transgenic mice (Table I). A small reduction of mature follicular cells was seen in the mesenteric LNs and BM of double transgenics (Table I). Importantly, there was no evidence of the extreme IgM antigen receptor down-regulation or deletion of immature B cells in the BM that characterizes acquisition of tolerance to surface-displayed mHEL (1) (Fig. 3 A and Table I).

By flow cytometry, only 7.19% (95% confidence: 5.63–8.75%) of antigen receptors on immature B cells were occupied with cleaved sHEL, values below that previously shown to be necessary for anergy induction in B cells from Ig^{HEL}/sHEL (ML5) double transgenics (Fig. 3 C) (7–9, 13).

Table I. *Effect of Intracellular Antigen on Central and Peripheral B Cell Numbers*

	Bone marrow		Spleen	Mesenteric lymph node
	Immature B cells	Mature B cells	HEL-binding B cells	
	10^5	10^5	10^5	
Ig	13.01 (*n* = 29)	9.71 (*n* = 29)	261.30 (*n* = 31)	50.83 (*n* = 25)
mHEL-KK Dbl	13.53 (*n* = 18)	3.72 (*n* = 18)	206.31 (*n* = 21)	28.07 (*n* = 15)
	P = 0.5749	P = 0.0021	P = 0.1748	P = 0.0257

Numbers shown are the means of the different populations, and *n* is the number of mice in each group. Immature and mature B cells in the BM are defined as HEL-binding IgM^{a+}/IgD^{a-} and IgM^{a+}/IgD^{a+}, respectively. The mean age of the Ig^{HEL} transgenic (Ig-Tg) mice was 77.66 d (SD 28.37) and Ig^{HEL}/mHEL-KK double transgenic (Dbl-Tg) 69.06 d (SD 23.29), with an overall range of 43–166 d. Statistical comparison was by an ANOVA test, with count as dependent variable, and genotype and each experiment as explanatory variables using STATA™ version 7.0. No significant effect of age or sex was detected.
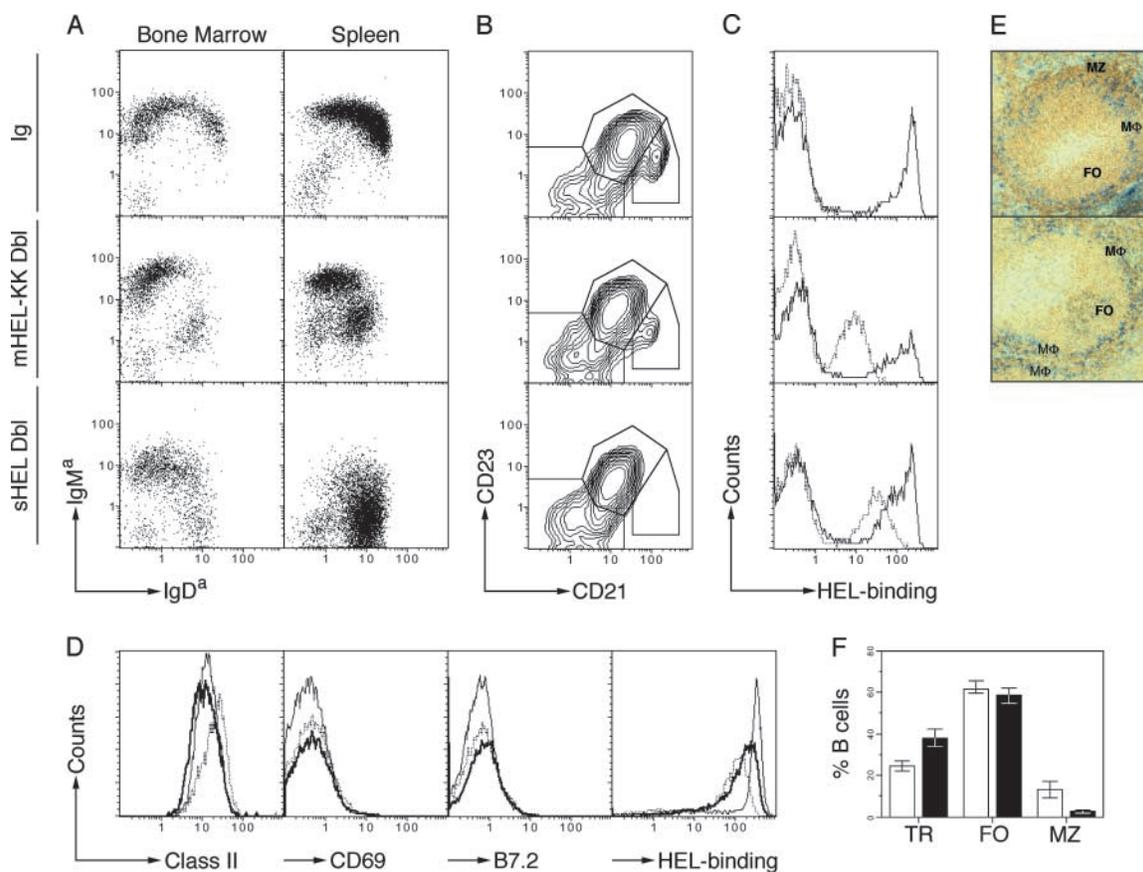
Figure 3. The development of conventional B cells in the presence of intracellular membrane-bound lysozyme. (A) Flow cytometry of BM and spleen B cells from Ig^{HEL} (Ig, top row A–C, and E), Ig^{HEL}/mHEL-KK (mHEL-KK Dbl, middle row A–C, and E) and Ig^{HEL}/sHEL (sHEL Dbl, bottom row A–C) transgenic mice gated on B220 and stained with antibodies to IgM^a and IgD^a (representative of all mice in Table I). (B) Flow cytometry of splenic B cells from Ig and mHEL-KK Dbl and sHEL Dbl transgenic mice gated on B220 and stained with antibodies to CD23 and CD21. (C) Flow cytometry of lymphocytes from Ig and mHEL-KK Dbl and sHEL Dbl transgenic BM stained with HEL/HyHEL9 (thick line) or Hy9Tc (thin line). (D) Flow cytometry of splenic B cells from Ig (thin line), mHEL-KK Dbl (thick line), and sHEL Dbl (dotted line) stained with antibodies to MHC class II, CD69, B7.2 (CD86), and HEL/HyHEL9. (E). Spleen sections from Ig and mHEL-KK Dbl mice stained with antibodies to B220 (brown) and metallophillic macrophages (blue). Positions of B cell follicles (FO), metallophillic macrophages (MΦ), and marginal zone B cells (MZ) are shown. (F). Percentage of splenic B cell populations from Ig ($n = 12$, white bars) and mHEL-KK Dbl transgenics ($n = 11$, black bars) gated as shown in B: transitional cells CD21^{lo}CD23^{lo} (TR), follicular CD21^{int}CD23^{hi} (FO), and marginal CD21^{hi}CD23^{int-lo} (MZ).

The phenotype of developing and mature B cells in the double transgenic mice was also consistent with subthreshold exposure to soluble self-antigen. In comparison to anergic B cells in Ig^{HEL}/sHEL (ML5) double transgenics, the B cells in Ig^{HEL}/mHEL-KK double trangenics showed only modest down-regulation of IgM and HEL binding (Fig. 3, A and D), little reduction in CD21 (Fig. 3 B), and no induction of MHC class II (Fig. 3 D). Levels of CD23, B7.2 (CD86), and B220 were normal (Fig. 3, B and D and not depicted). Transitional and follicular B cell populations were preserved in Ig^{HEL}/mHEL-KK double trangenics (Fig. 3, B and F), but there was a marked reduction in the CD21^{hi}, CD23$^{int/lo}$ marginal zone population, which was confirmed by histological analysis (Table I and Fig. 3 E). A similar but more marked reduction in B cells in the splenic marginal zones is also observed in Ig^{HEL}/sHEL double transgenic mice (Fig. 3 B) (28). In both cases, the loss of marginal zone cells may represent either developmental arrest or continuous depopulation of the marginal zone in response to antigen binding as described after immunization with soluble and particulate antigens (32, 33).

To demonstrate the absence of B cell anergy in Ig^{HEL}/mHEL-KK double transgenics, we performed a series of functional tests comparing B cells from the Ig^{HEL}/mHEL-KK mice with anergic B cells from Ig^{HEL}/sHEL double transgenic mice and naive B cells from Ig^{HEL} single transgenic mice. After overnight culture with antigen, B cells from Ig^{HEL}/mHEL-KK mice up-regulated both CD69 and CD86 (B7.2) to normal levels (Fig. 4 A) and showed no block in the proximal BCR signaling (Fig. 4 B), in contrast to anergic cells from Ig^{HEL}/sHEL double transgenics.

Intracellularly Derived HEL Autoantigen Triggers Autoantibody Production. In striking contrast to Ig^{HEL} mice expressing cell surface or soluble forms of HEL autoantigen (1, 7, 8, 34), mice expressing intracellular HEL autoantigen contained high autoantibodiy titers and large numbers of autoantibody-secreting plasma cells (Fig. 5, A and B). Compared with Ig^{HEL} single transgenic mice, Ig^{HEL}/mHEL-KK
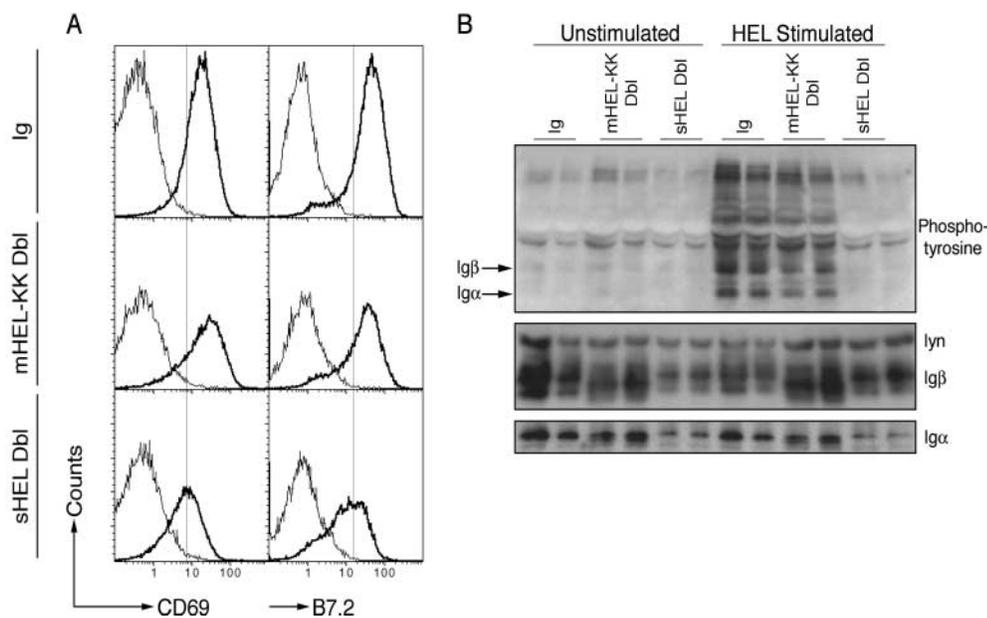
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Figure 4. Absence of functional inactivation in B cells from Ig[HEL]/mHEL-KK double transgenic mice. (A). Flow cytometry of splenocytes from Ig and mHEL-KK Dbl and sHEL Dbl transgenic mice incubated for 17 h with 0.5 µg/ml HEL (thick line) or kept on ice as controls (thin line) gated on B220 and stained with CD69 or B7.2 (CD86). (B) Western blot analysis of splenocytes from Ig and mHEL-KK Dbl and sHEL Dbl transgenic mice stimulated with media alone or 1 µg/ml HEL for 3 min, and stained with antibodies to phosphotyrosine (4G10), lyn, Igα (CD79α), and Igβ (CD79β). Results are representative of three separate experiments.

double transgenic mice contained 25-fold more plasma cells secreting anti-HEL IgM in the spleen and an equivalent increase in anti-HEL IgM antibodies in the serum. This corresponds to an elevation in serum antibody of >10,000 fold compared with mice expressing HEL as a cell surface autoantigen (1). Histological examination of Ig[HEL]/mHEL-KK double transgenic spleens showed numerous plasma cells confined to the red pulp cords (Fig. 5 C). Increased numbers of HEL-specific plasma cells were not found in the BM or LNs (Fig. 5 B). A similar increase in autoantibody titers was found in all three mHEL-KK lines. To test whether autoantibody production was T cell dependent, Ig[HEL]/mHEL-KK mice were crossed onto a $Rag2^{-/-}$ background. The mean number of plasma cells in the Ig[HEL] $Rag2^{-/-}$ was 12,223 per spleen (95% confidence: 0–33,930; $n = 4$) and in Ig[HEL]/mHEL-KK $Rag2^{-/-}$ was 297,267 per spleen (95% confidence: 111,577–482,956; $n = 5$). Therefore, in stark contrast to mHEL or sHEL (1, 7, 8) the sequestered intracellular

HEL is immunogenic, inducing autoreactive plasma cells and high titre autoantibodies in a T cell–independent manner.

Positive Selection of B1 Cells. The sequestered form of mHEL-KK autoantigen also stimulated the B cells to form a large population of partially activated cells in the peritoneal cavity that have the characteristics of B1 cells (35, 36). Whereas no cells of this type are formed in Ig[HEL] transgenic mice in the absence of antigen, nor in mice expressing tolerogenic forms of secreted or mHEL, flow cytometry of peritoneal cells from the Ig[HEL]/mHEL-KK double transgenic mice showed two populations of HEL-binding B cells: small B220^{hi}, IgD^{+}, IgM-modulated, Mac1^{-} cells typical of conventional B cells and large B220^{lo}, IgD^{-/lo}, IgM-bright, Mac1^{+} CD9^{+} cells characteristic of B1 cells (37) (Fig. 6, A and B). The total number of peritoneal IgD^{-/lo}, IgM-bright cells (gated as shown in Fig. 6 A) in Ig[HEL]/mHEL-KK mice was 6.4×10^4 cells (95% confidence: 2.86–9.96×10^4; $n = 13$), compared with 1.45×10^4 cells
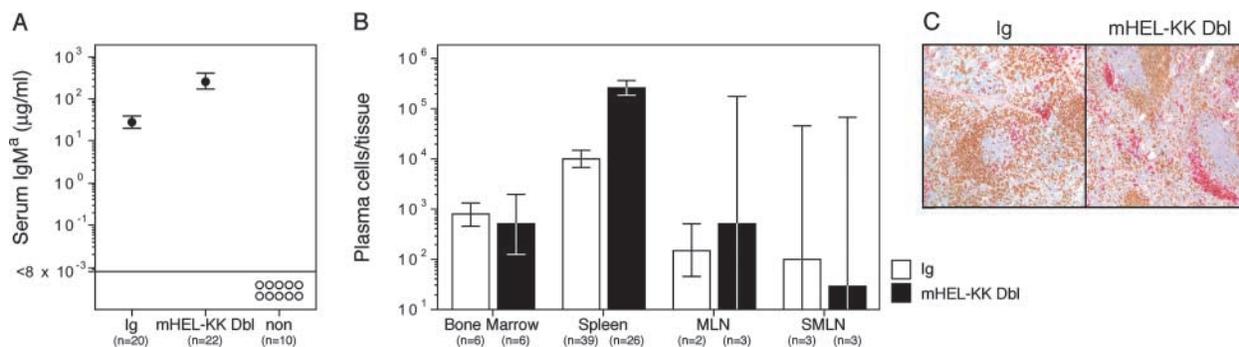

Figure 5. Intracellular HEL autoantigen triggers autoantibody production. (A) Serum anti-HEL IgM[a] titers in Ig, mHEL-KK Dbl, and nontransgenic (non) mice as determined by ELISA. Dots show geometric means, and bars represent the 95% confidence limits. The background is twice the level in blank wells. (B) Anti-HEL IgMa-secreting plasma cells in BM, spleen, mesenteric LN (MLN), and submandibular LN (SMLN) of Ig (white bars) and mHEL-KK Dbl mice (black bars). Columns show geometric means and bars represent the 95% confidence limits. (C) Spleen sections from Ig and mHEL-KK Dbl mice stained with antibodies to IgD[a] B cells (brown) and plasma cells (red).
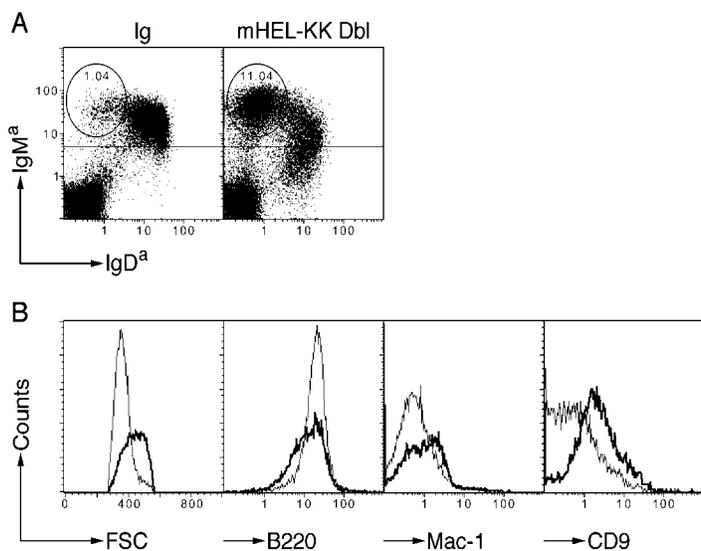
Figure 6. Positive selection of B1 cells in the peritoneum. (A) Flow cytometry of peritoneal lymphocytes from Ig and mHEL-KK Dbl mice stained with antibodies to IgM^a (IgM^a-PE) and IgD^a. The elliptical gate indicates B1 cells and the percentage of B1 cells in the lymphocyte gate. Plots are representative of >13 mice each. (B) Histograms gated on all IgM$^{a\text{-high}}$ peritoneal lymphocytes (rectangular gate shown in A) forward scatter (FSC), B220, Mac-1, and CD9 expression from Ig (thin lines) and mHEL-KK Dbl mice (thick lines).

(95% confidence: 0.82–2.08 × 10^4; $n = 18$) in the absence of antigen ($P = 0.0013$). The absence of CD5 expression on the peritoneal cells indicated that the B1 cells were of the B1b rather than B1a subtype (not depicted). Similar results were found in all three mHEL-KK transgenic lines. The activation and selection of B1 cells and the generation of natural antibodies is typically T independent (35), and we found that the absence of T cells has no effect on the selection of B1 cells induced by intracellular HEL (not depicted).

Autoimmunity and B1 Cell Selection Is Independent of Antigen Expression in the B Cells. Because the intracellular antigen is ubiquitous, it was a possibility that the observed phenotypes could be due to cis effects of antigen, either toxicity or by binding to the BCR, within the B cells. To exclude this possibility, we reconstituted lethally irradiated mHEL-KK and nontransgenic recipients with fetal liver from Ig^{HEL} single transgenic donors and showed that anti-HEL autoantibodies were still induced and large B220^{lo}, IgD$^{-/lo}$, IgM-bright, Mac1$^+$, CD9$^+$ Ig^{HEL} B1 cells were still positively selected in the mHEL-KK–expressing chi-

meras (Fig. 7; Table II; not depicted). This confirms that the immunogenic effect of intracellular antigen on B cells occurs in trans and that our findings will be applicable to other intracellular antigens, which may be abundant but not necessarily expressed in the lymphoid tissues.

Discussion

Our results establish that the fate of B cells reactive with membrane-bound autoantigens is profoundly different depending on whether the antigen is retained inside the cell or displayed on the cell surface. In contrast to the elimination or receptor editing of B cells reacting with surface autoantigens, B cells reactive with an intracellular autoantigen are not tolerized and are induced to form autoantibody-secreting plasma cells and B1 cells. Because the BCR specificity and the autoantigen are identical in the different model systems, we can state conclusively that differences in the location of membrane antigens in the cell are sufficient to cause these striking differences in B cell fate. These find-

Table II. *Numbers of HEL-binding B Cells in Lethally Irradiated Nontransgenic and mHEL-KK Transgenic Mice Reconstituted with Ig^{HEL} Fetal Liver*

	Bone marrow		Spleen	Mesenteric lymph node
Recipient	Immature B cells	Mature B cells	HEL-binding B cells	
	10^5	10^5	10^5	
Non ($n = 4$)	18.10	9.30	213.62	22.69
mHEL-KK ($n = 3$)	14.88	6.52	269.38	23.21
	$P = 0.2702$	$P = 0.0625$	$P = 0.0686$	$P = 0.9129$

Numbers of HEL-binding B cells in lethally irradiated nontransgenic (Non) and mHEL-KK transgenic mice reconstituted with Ig^{HEL} fetal liver. Numbers shown are the means of the different populations, and n is the number of mice in each group. Immature and mature B cells in the BM are defined as HEL-binding IgM^{a+}/IgD^{a-} and IgM^{a+}/IgD^{a+}, respectively. Mice were reconstituted for 42 d. Statistical comparison was by Student's t test using STATA™ version 7.0.
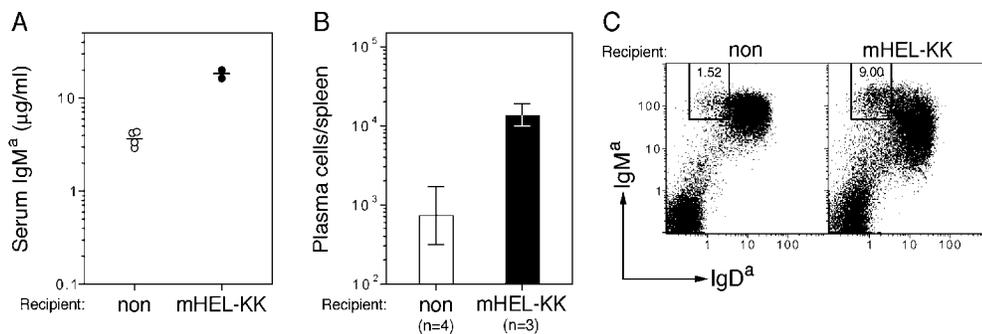
Figure 7. Autoimmunity and B1 cell selection is independent of antigen expression on the B cells. (A) Serum anti-HEL IgM[a] titers in lethally irradiated nontransgenic and mHEL-KK recipients reconstuted with Ig[HEL] expressing fetal liver. Dots show individual mice, and bars show the geometric means. (B) Anti-HEL IgM[a]-secreting plasma cells in the spleens of nontransgenic (white bars) and mHEL-KK recipient mice (black bars). Columns show geometric means, and bars represent the 95% confidence limits. (C) Flow cytometry of peritoneal lymphocytes from the chimeras stained with antibodies to IgM[a] (IgM[a]-Bi) and IgD[a]. The box gate indicates the location of Ig[HEL] B1 cells and the percentage of these cells in the lymphocyte gate.

ings demonstrate that intracellular localization is a predisposing factor for causing an antigen to be a target of autoantibodies. In addition, the results contradict the widely held view that the spectrum of B cell tolerance can be explained solely by the specificity, affinity, and avidity of interactions between autoreactive B cells and self-antigen.

It is instructive to compare our results with other immunoglobulin transgenic models, some of which are also characterized by the production of autoantibodies. B cells expressing μ and κ immunoglobulin transgenes against the thymocyte cell surface antigen CD90 (Thy-1) also have a conventional phenotype in the absence of self-antigen and generate B1 cells and autoantibodies in its presence (38). However, in contrast to our results the conventional anti-CD90 B cells developing alongside B1 cells in the presence of self-antigen are severely arrested, short lived, and functionally inactive, CD21-negative, HSA-bright cells that also express CD5, which may be a marker of anergy, and there are no plasma cells in the spleen. The late negative selection of the conventional anti-CD90 B cells may be due to the abundance of CD90^{+} T cells in the blood and their paucity in the BM, since a similar phenotype of developmental arrest and deletion is observed in immunoglobulin transgenic B cells reactive with the T cell surface antigen CD8 (39). The combination of peripheral deletion and B1 cell development also occurs in mice expressing an immunoglobulin transgene against an erythrocyte antigen. Autoreactive antierythrocyte B1 cells survive in the peritoneum where they produce autoantibodies that cause hemolytic anemia; however, B cells are eliminated from the blood, spleen, and LNs, where the antigen is abundantly expressed (3). The results of these models support the concept that B cell selection depends on the circumstances of antigen presentation and signal strength. However, the absence of antigen–negative controls or mice expressing different forms of the same antigen makes it difficult to know to what extent positive selection is independent of affinity, avidity, BCR, or unusual features of the particular antigen in question. Indeed, the function of CD90 is unknown and so it may play a specific role in B1 development. The critical advantage of the HEL model is that it allows comparisons to be made in a series of mice with well-characterized and carefully controlled differences, and it is this feature that has enabled us to identify the role of antigen location in B cell selection. On the basis of our findings, we think it is very likely that positive selection would be apparent in other Ig transgenic models directed against naturally occurring intracellular self-antigens, including DNA, if it were possible to compare affected mice with antigen-negative controls and thus distinguish this positive selection from immunological ignorance.

The selection of HEL-binding B1 B cells is a feature unique to HEL transgenic mice expressing the intracellular HEL autoantigen, since it does not occur in response to any of the extracellular forms of the same protein. It has been suggested that B1 cells represent a distinct lineage characterized by a particular and limited repertoire of BCRs (40, 41). Unlike conventional B cells, B1 cells appear to arise mainly but not exclusively in early ontogeny, and together with marginal zone B cells their development is IL-7 independent (42). They are capable of self-renewal, and they do not require expression of the TNF family ligand BAFF for continued survival (43, 44). The development of B1 cells is increased in mice with increased BCR signaling due to deficiencies in SHP1, CD22, and Lyn (18, 45–48) but absent from BCR signaling compromised CD19- and btk-deficient animals (20, 49). Transgenic B cells expressing higher levels of BCR are also more likely to develop into B1 cells in the presence of their antigen (50). These findings indicate a general requirement for increased BCR signaling in B1 development, which may be intrinsic to the receptor or generated by an interaction with antigen. Our results confirm that commitment to the conventional or B1 cell lineage is not necessarily predetermined by the expression of a particular BCR. Instead, our data suggests that the positive selection of the B1 cells is favored by rare encounters between immunogenic self-antigen and a minority of immature B cells.

The origin of autoreactive plasma cells in the Ig[HEL]/mHEL-KK double transgenic mice is uncertain, since it is possible that they arise from conventional B cells including marginal zone B cells, B1 B cells, or both (Fig. 8). Experiments using parabiotic mice have shown that B1 cells retain the capacity to recirculate through the secondary lymphoid organs (51), although they are Mac-1 negative and difficult to distinguish from large B220[lo], IgD^{−/lo}, IgM-bright tran-

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sitional B cells. Therefore, Ig^HEL-specific B1 cells may differentiate into autoreactive plasma cells in the spleen of the mHEL-KK double transgenic mice (Fig. 8 B). Increased splenic plasma cells and B1 cells are frequently found together in mice with exaggerated BCR signaling due to B cell–specific mutations as, for example, in SHP1 deficiency (18). The alternative possibility is that the anti-HEL plasma cells derive from follicular B cells, which undergo T-independent activation when they bind the high avidity multivalent intracellular self-antigen exposed on dying cells, or from marginal zone B cells, since rapid differentiation into plasma cells and relocation to the red pulp cords could in part account for the reduced size of the marginal zone in the Ig^HEL/mHEL-KK double transgenics (Fig. 8 A). Distinguishing between these possibilities is likely to require genetics experiments targeted at the different cell populations.

The fact that autoreactive B cells are so exquisitely susceptible to elimination by even low affinity interactions with surface-displayed membrane self-antigens (34, 52) implies that additional factors such as timing, frequency, and context must be critical in the autoimmune response to the intracellular antigens. One key factor may be in the presence or absence of costimulation during autoantigen encounter (Fig. 8). Previous experiments have shown that mHEL in combination with T cell costimuli (13) or LPS (12) is a potent activator of Ig^HEL transgenic B cells and inducer of antibody formation. In the mHEL-KK animals, the ER-sequestered membrane antigen is displayed on dying cells, where it may similarly activate antibody formation. Since we have shown that T cells are not involved in

development of anti-HEL autoantibody-producing plasma cells, another immunogenic costimulus may serve this role. Candidates include CpG DNA, which is displayed extracellularly on dying cells and activates B cells through Toll-like receptor (TLR)9 (53) and possibly heat shock proteins, which may also signal through TLRs (54, 55). In this context, it is interesting to note that the generation of autoantibodies and survival of peritoneal B1 cells in the anti-erythrocyte Ig transgenic model depended up LPS from pathogens or diet (56, 57). Therefore, it is possible that the endogenous Toll-like receptor ligands on dying cells could provide similar B cell costimulation in the absence of overt infections. Mechanisms of this type may account for the generation and targeting of autoantibodies against intracellular ER and nuclear antigens that are clustered on the surface of apoptotic cells, including histones and DNA (23). The absence of overt autoimmune disease in the Ig^HEL/mHEL-KK mice may be because the Ig^HEL MD4 transgene only encodes IgM and IgD, whereas the majority of pathogenic autoantibodies in SLE are of the IgG isotype; however, the appearance of disease may also require breakdown in other checkpoints in B cell self-tolerance. Under these circumstances, exaggerated positive selection by intracellular antigens would be the first step in a pathogenic process leading to systemic autoimmunity. This could explain why SLE occurs in the absence of factors such as c1q, Dnase1, c-mer, and serum amyloid P, which are normally required for the clearance of necrotic or apoptotic cells (25, 58–60).
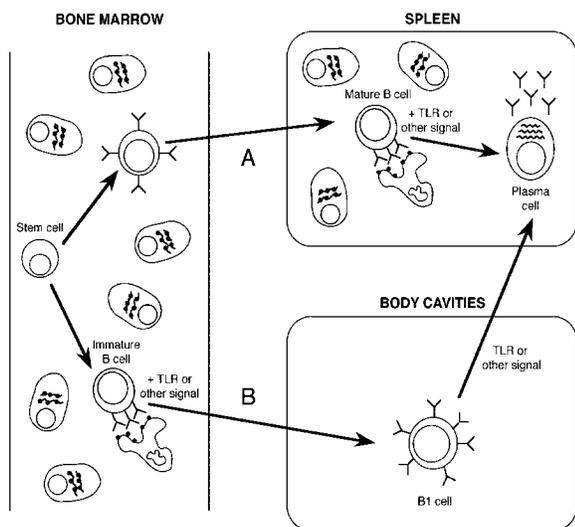
Figure 8. Models to account for autoantibody production in response to intracellular HEL self-antigen. The positive selection of autoantibody-producing plasma cells in the spleen may arise after rare encounters between self-reactive B cells and high avidity intracellular self-antigens exposed on apoptotic cells or debris, and immunogenic costimuli, signaling through TLRs or other pathways. The autoimmune response may arise from the activation and differentiation of either (A) mature conventional B cells that are hitherto functionally ignorant of antigen or (B) B1 cells that are first positively selected and then migrate to the spleen from the body cavities.

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