The Journal of Immunology

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J Immunol 2006; 176:7402-7411; ;
doi: 10.4049/jimmunol.176.12.7402
http://www.jimmunol.org/content/176/12/7402

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Signals from a Self-Antigen Induce Positive Selection in Early B Cell Ontogeny but Are Tolerogenic in Adults[1]

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Positive and negative signals from self-Ags shape the B cell repertoire and the development of distinct B cell subsets, but little is known about what distinguishes these signals. To address this question, we have studied the development of anti-hen egg lysozyme MD4 Ig transgene B cells while systematically varying the level, distribution, and timing of exposure to different forms of hen egg lysozyme as a self-Ag. This process has allowed us to explore the effects of Ag independent of BCR specificity. Our findings show how the selection of autoreactive B cells is a competitive process involving immunogenic and tolerogenic forms of self-Ags. Due to a developmental switch during B cell ontogeny, autoreactive anti-hen egg lysozyme MD4 Ig transgene B cells are negatively selected by self-Ags in adult bone marrow but susceptible to positive selection by some of the same self-Ags in fetal and neonatal life. However, the persistence of B1 cells and IgM autoantibodies from early ontogeny enables autoreactive B cells from the adult bone marrow to escape negative selection. Our data suggest that this rescue may be due to the clearance or masking of self-Ag by IgM autoantibody. We discuss the implications of these findings in terms of B cell selection and the maintenance of self-tolerance during early and adult life. *The Journal of Immunology,* **2006, 176: 7402–7411.**

The preimmune repertoire of humans and mice contains three distinct subsets of mature B cells: B1, follicular (also called B2), and marginal zone B cells. B1 cells appear to arise mainly, but not exclusively, in early ontogeny, self-renew, and give rise to "natural" Abs (1). They are readily detected in pleural and peritoneal cavities, but have also been found to recirculate freely through the secondary lymphoid organs in adult mice (2). The B1 cell repertoire is skewed toward reactivity with self-Ags and common bacterial epitopes, which has led in part to the suggestion that some Ag recognition is required for the their selection and survival (3–5). In contrast, follicular B cells are generated continuously from the bone marrow (BM),[3] and most are subject to negative selection (6, 7). Those follicular B cells that survive recirculate for a few weeks through the secondary lymphoid organs, where they are typically involved in T cell-dependent Ab responses. Finally, marginal zone B cells are fixed in the spleen, where they are involved in rapid Ab responses against blood borne particulate Ags (8, 9).

The development of the different B cell subsets is now a matter of considerable interest, particularly because greater understanding may hold the key to the selection of autoreactive B cells (10, 11). The prevailing theory is that BCR signal strength is the critical determinant in guiding B cells into B1, follicular, and marginal zone compartments. This theory is based on experiments with mice lacking or overexpressing components of the BCR, where increased BCR signaling leads to increased selection of B1 cells, as well as transgenic mice expressing variable levels of self-Ags such as CD90 (Thy-1) (12). Mice lacking negative regulators of BCR signaling, such as the phosphatase SHP1, tyrosine kinase Lyn, and coreceptor CD22, or transgenic for positive regulators, such as CD19, develop increased numbers of B1 cells (13–19). B1 cell selection still occurs in naive Lyn and SHP1-deficient B cells co-expressing a transgenic BCR against a foreign protein hen egg lysozyme (HEL), in the absence of Ag (H. Ferry and R. J. Cornall, unpublished observations) (20). In mice expressing variable levels of Ig transgenes against self-Ags, the BCR receptor density has also correlated with the level of positive selection presumably due to increased signaling (21–24). Moreover, it has recently been shown that low expression of a constitutively active surrogate BCR, with no Ag specificity, can substitute for the native receptor in follicular and marginal zone B cell development, and, at high expression, drives the generation of B1 cells (25).

However, without postulating additional variables, it is not easy to reconcile the positive selection of B1 cells by increased BCR signaling with the negative selection of B cells by self-Ags. This is because the degree of negative selection also appears to depend on the strength of BCR signaling, which is determined by the concentration and avidity of the self-Ag. The spectrum of tolerogenic responses corresponding to signal strength is well described in transgenic mice expressing different forms of HEL as a neo self-Ag (26). Self-reactive B cells expressing transgenic Ig receptors for HEL (Ig^{HEL}) undergo deletion or receptor editing in the BM when they encounter systemic high-avidity membrane-bound HEL (mHEL) (27, 28), but they are held in a potentially reversible state of functional inactivation or anergy when they encounter lower avidity soluble HEL (sHEL) at a high dose (29–32). Deficiency in the same negative regulators of BCR signaling that cause the positive selection of B1 cells in the absence of Ag, such as SHP1 and Lyn, lowers the threshold for tolerance and causes Ig^{HEL} B cells to be deleted rather than anergized by abundant sHEL (20, 33, 34). This result suggests that other factors must be important in both positive and negative selection of B cells, such as timing of Ag presentation or the presence of costimulation (11).

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Received for publication March 14, 2005. Accepted for publication March 29, 2006.

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[1] This work was supported by the Wellcome Trust. R.J.C. is a Wellcome Trust Senior Clinical Fellow. J.C.H.L. was supported by a Wellcome Trust Clinical Training Fellowship.

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[3] Abbreviations used in this paper: BM, bone marrow; HEL, hen egg lysozyme; Ig^{HEL}, anti-HEL MD4 Ig transgene; mHEL, membrane-bound HEL; sHEL, soluble HEL; mHEL-KK, ER-restricted membrane HEL; PEC, peritoneal cavity; NMS, normal mouse serum; int, intermediate.

0022-1767/06/$02.00

Recently, we have shown that the cellular location of self-Ags is also a determinant of the positive and negative selection of autoreactive B cells (35). By sequestering the tolerogenic self-Ag mHEL intracellularly in the endoplasmic reticulum, we converted it into an identical but immunogenic self-Ag (endoplasmic reticulum-restricted membrane HEL; mHEL-KK) that positively selects Ig^{HEL} B1 cells and a 50-fold T-independent increase in IgM autoantibody-secreting plasma cells. This result also suggests that negative vs positive selection may be due to the key differences in the presentation of self-Ags as well as signal strength. To address this further, we have now studied the development of Ig^{HEL} B cells while systematically varying the level, distribution, and timing of exposure to different forms of self-Ag. Our results show that the selection of autoreactive B cells is a competitive process, involving immunogenic and tolerogenic forms of self-Ags. During early life, B cells are susceptible to positive and negative selection, but, due to a developmental switch, only negative selection is triggered in adults. However, neonatally selected B1 cells can rescue autoreactive conventional B2 cells from deletion in adult BM. Our data suggest that this *trans* effect may be due to the masking or clearance of self-Ag by B1-derived autoantibody. These findings help to reconcile several previous observations in a single model of positive and negative selection, dependent upon signal strength, Ag specificity, timing, and context of Ag presentation.

Materials and Methods

Mice

Transgenic mice expressing anti-HEL IgH and L chains (Ig^{HEL}; MD4) were determined by PCR as described previously (27). Mice expressing intracellular membrane HEL (mHEL-KK, mHEL-KK1), extracellular membrane HEL (mHEL, KLK4), and sHEL (ML5) were distinguished using the primers HEL-DISCF (5′-CTCCGCCCAGGGTCGCCTGG-3′) and HEL-DISCR (5′-TATAGTCGTTTTAATGAGGGATGCG-3′) to give product sizes 417 bp for mHEL-KK and 180 bp for sHEL transgenes. All mice were backcrossed at least seven generations to C57BL/6 and maintained in specific pathogen-free conditions. All experiments were approved by the Oxford University Ethical Review Committee and performed under Home Office license.

Flow cytometry

BM, spleen, mesenteric lymph node, and peritoneal lavage cell suspensions were stained as previously described with the following mAbs: IgM^a, DS-1-PE (BD Pharmingen) and DS-1-biotin (Bi; BD Pharmingen) followed by streptavidin-tricolor (SA-Tc; Caltag Laboratories); IgD^a, AMS9.1-FITC; B220, 6B2-allophycocyanin (Caltag Laboratories), 6B2-PE (Caltag Laboratories), 6B2-FITC (Caltag Laboratories), and 6B2-Bi (BD Pharmingen); CD45.1, A20-FITC, A20-PE and A20-Bi; class II, $I\text{-}A^p$-FITC (BD Pharmingen); CD21-FITC and CD21-Bi followed by SA-PE (Caltag Laboratories); CD23-FITC, CD23-PE, CD24-FITC. 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 CellQuest Pro software (BD Biosciences).

Measurement of serum anti-HEL IgM^a and splenic IgM^a-secreting plasma cells

Anti-HEL IgM^a serum titer was measured by ELISA as described previously. 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 Ab were revealed with biotinylated anti-IgM^a (DS-1; BD Pharmingen) followed by avidin-alkaline phosphatase (Sigma-Aldrich) using the substrate 5-bromo-4-chloro-3-indolyl phosphate disodium salt.

Neonatal liver/mHEL mixed chimeras

Nontransgenic recipients were lethally irradiated with two doses of 5-Gy gamma irradiation separated by 3 h. Neonatal liver cells were harvested at day 4 postpartum from pups expressing either the Ig^{HEL} or mHEL transgene. Irradiated recipients were injected via the lateral tail vain with 4×10^6 Ig^{HEL} cells mixed with mHEL cells in the ratios 19:1, 199:1, 1999:1, and 1:0 in a total volume of 200 μl. The animals received antibiotics (Amoxycillin; 0.25 mg/ml in water bottles) for the first 3 wk of their reconstitution and were sacrificed at 6 wk.

Fetal liver and adult BM chimeras

$CD45.1^+$ Ig^{HEL} cells were obtained from either adult BM, or fetal liver at day 19 of gestation, from $CD45.1^+$ animals expressing the Ig^{HEL} transgene. Lethally irradiated $CD45.2^+$ mHEL-KK and nontransgenic recipients were injected with $0.7–1.0 \times 10^7$ Ig^{HEL}-expressing cells via the lateral tail vein. The animals received antibiotics (Amoxycillin; 0.25 mg/ml in water bottles) for the first 3 wk of their reconstitution and were sacrificed at 6–7 wk.

Adult BM/peritoneal mixed chimeras

$CD45.2^+$ nontransgenic and mHEL-KK mice were lethally irradiated with two doses of 5-Gy gamma irradiation separated by 3 h. Adult BM cells were harvested from $CD45.1^+$ mice expressing the Ig^{HEL} transgene (Ig BM), and peritoneal cells were obtained by peritoneal lavage of $CD45.2^+$ mice expressing both the Ig^{HEL} and mHEL-KK transgenes (mHEL-KK Dbl peritoneal cavity (PEC)). BM cells (1×10^6 in 200 μl) were injected i.v., and peritoneal cells (1×10^6 in 100 μl) were injected i.p. When only Ig BM was used to reconstitute the recipients, an intraperitoneal injection was performed using medium alone. Four nontransgenic and three mHEL-KK mice received Ig BM only, and seven mHEL-KK mice received Ig BM and Dbl PEC. The animals received antibiotics (amoxicillin; 0.25 mg/ml in water bottles) for the first 3 wk of their reconstitution and were sacrificed at 8–10 wk

Ab transfer to adult BM chimeras

$CD45.2^+$ nontransgenic and mHEL-KK mice were lethally irradiated as described before and reconstituted with a single BM sample from $CD45.1^+$ mice expressing the Ig^{HEL} transgene (Ig BM). Chimeras were then left alone or injected with pooled serum containing anti-HEL IgM^a collected from Ig^{HEL}/mHEL-KK double transgenic mice or normal B6 mouse serum (both stored frozen). The animals received antibiotics for 3 wk as described before but were sacrificed at 35 days. The titer of pooled anti-HEL IgM^a serum was 350 μg/ml. In tests, an i.v. injection of 200 μl of serum resulted in a serum anti-HEL titer of 17.28 μg/ml after 2 days ($n = 2$; range, 16.13–18.43 μg/ml); while, an i.p. injection of 200 μl for the same interval resulted in a serum anti-HEL titer of 10.37 μg/ml ($n = 2$; range, 7.30–13.44 μg/ml). The timing of injections was decided after serial assay of IgM^a Ab titers in nontransgenic recipients of Ig^{HEL} BM. This showed an early burst of anti-HEL IgM^a production at 11 days ($n = 3$; mean 8.00 μg/ml, SD 1.44), followed by a decline to a level normally seen in fully reconstituted mice (see Fig. 5*C*) at 21 days ($n = 3$; mean 3.01 μg/ml, SD 0.96). This level was maintained at 32 days ($n = 3$; mean 2.56 μg/ml, SD 0.20). Recirculating conventional Ig^{HEL} B cells also began to appear at 21 days (data not shown). This data suggested to us that rescue by anti-HEL-secreting plasma cells should start between 11 and 21 days. Accordingly, serum was injected i.v. on days 15 and 22 (200 μl), followed by an i.p. injection on day 28 (250 μl), which was given because not all mice had patent veins at this stage.

Results

The B cell repertoire is shaped by competition between tolerogenic and immunogenic forms of self-Ags

To look for qualitative differences between immunogenic and tolerogenic signals from self-Ags, we asked first whether the positively selecting intracellular Ag mHEL-KK could rescue the development of autoreactive B cells undergoing negative selection. Despite the positive selection of B1 cells and autoantibody-secreting plasma cells, follicular B cell development and function is normal in Ig^{HEL}/mHEL-KK double transgenic mice. This is in part because the serum sHEL released by cleavage from mHEL-KK mice is below the 10–20 ng/ml threshold required to induce B cell anergy in Ig^{HEL}/sHEL(ML5) double transgenic mice (35, 36). By generating Ig^{HEL}/mHEL-KK/sHEL triple transgenics, in which the level of sHEL is above this threshold, we could study the combined effects of positive and negative selection on B cell development and compare them with positive (Ig^{HEL}/mHEL-KK) and negative (Ig^{HEL}/sHEL) selection in littermate controls.

Interestingly, our analysis of B cell development showed that expression mHEL-KK with sHEL exaggerates the negative selection of all B cell subsets compared with sHEL alone. Despite normal numbers of immature B cells in the BM, few mature cells survived in the lymph nodes or peritoneum of Ig^{HEL}/mHEL-KK/sHEL triple transgenics (Fig. 1*A* and data not shown). Analysis of B cell development showed lower CD21 expression on HEL-binding B cells in BM and spleen, and a greater proportion of $B220^{low}HSA^{bright}$, $CD23^{-}CD21^{-}$ transitional cells in the spleen (Fig. 1, *B* and *C*). Splenic marginal B cells were absent, and the remaining B cells showed a phenotype intermediate between that of anergy alone and deletion due to mHEL with up-regulation of MHC class II similar to anergic cells but greater modulation of both IgM and IgD (Fig. 1, *B* and *C*). These effects would be compatible with an interaction between the autoreactive B cells and an Ag, such as mHEL-KK, which has higher avidity than sHEL. However, despite the evidence of negative selection, the Ig^{HEL}/mHEL-KK/sHEL triple transgenic mice also showed positive selection with higher plasma cell numbers than Ig^{HEL}/sHEL mice, in whom tolerance remained intact. In the presence of sHEL, mHEL-KK was sufficient to restore plasma cell numbers and circulating IgM to levels seen in naive Ig^{HEL} mice, but not to levels normally seen in Ig^{HEL}/mHEL-KK double transgenics (Fig. 1*A*). This experiment leads to two conclusions. The first is that some self-Ags, such as mHEL-KK, must have both positive and negative effects on B cell selection. The second is that selection can be a competitive process triggered by exposure to immunogenic and tolerogenic forms of a single self-Ag.

One aspect of Ag expression that might distinguish positively and negatively selecting Ags and account for the effects of competition is rarity. The intracellular Ag mHEL-KK is infrequently expressed on the surface of dead or dying cells (35). Because of this, we considered the possibility that positive selection might simply be due to a rare encounter with any high-avidity self-Ag. To test this hypothesis, we used fetal liver from Ig^{HEL} and cell surface-expressing mHEL (KLK4) transgenics (27) to generate a series of radiation chimeras containing $CD45.1^{+}$ Ig^{HEL} B cells and 20- to 2000-fold dilutions of $CD45.2^{+}$ cells expressing surface mHEL. In chimeras containing the highest frequency of mHEL B cells (ratio 1:19), the autoreactive Ig^{HEL} B cells underwent developmental arrest and deletion in the BM that was indistinguishable from findings in nonchimeric Ig^{HEL}/mHEL double transgenics (Fig. 2*A*). There were few or no HEL-binding B cells in the spleen, lymph node, or PEC, and no detectable plasma cells or anti-HEL

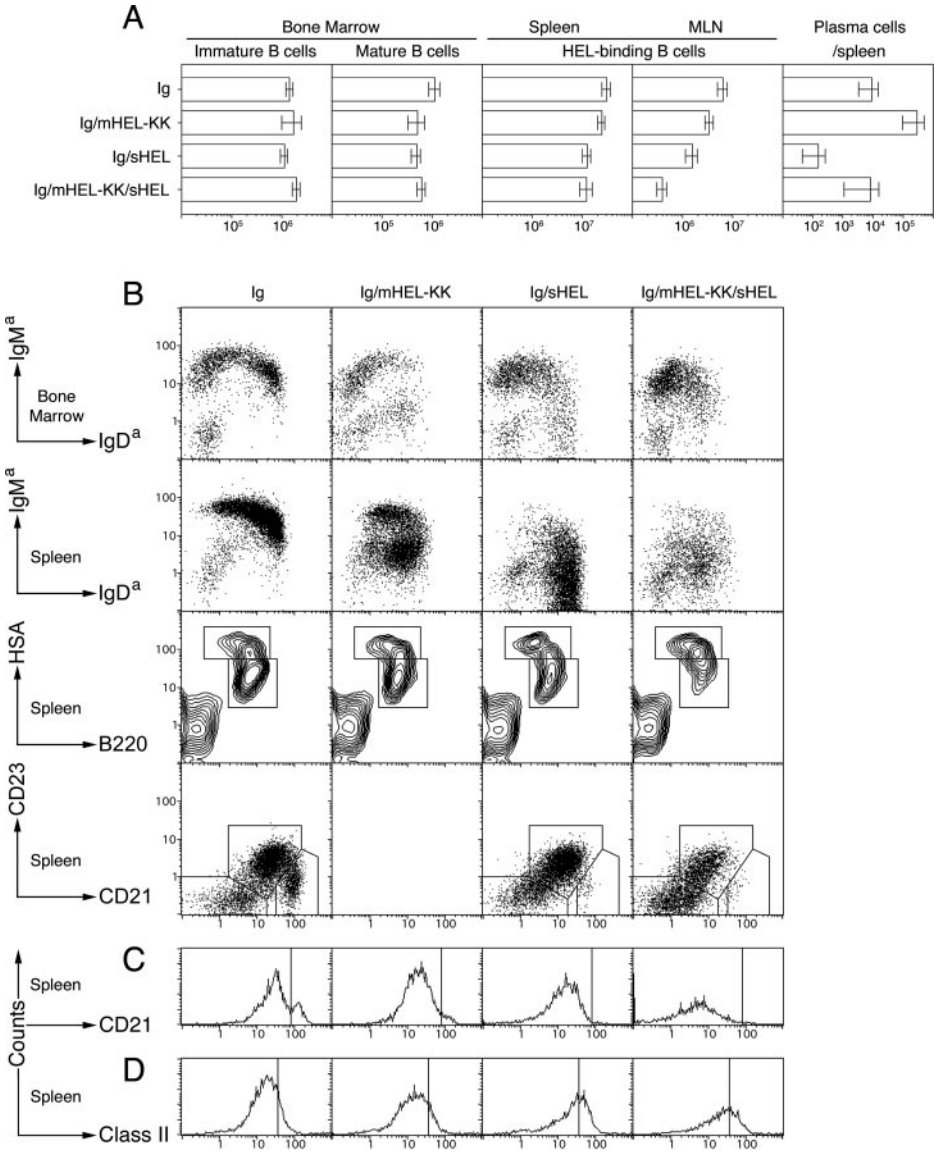

FIGURE 1. Self-Ags provide opposing tolerogenic and immunogenic signals that shape the B cell repertoire. *A*, Numbers of immature (IgM^{a+}/IgD^{a-}) and mature (IgM^{a+}/IgD^{a+}) $B220^{+}$ lymphocytes in the BM, HEL-binding $B220^{+}$ lymphocytes in the spleen and mesenteric lymph node (MLN), and anti-HEL IgM^{a}-secreting plasma cells in the spleen of single (Ig^{HEL}; $n = 13$), double (Ig^{HEL}/mHEL-KK; $n = 4$, or Ig^{HEL}/sHEL; $n = 13$), and triple (Ig^{HEL}/mHEL-KK/sHEL; $n = 13$) transgenics. Columns show mean, and bars represent the 95% confidence limits. *B*, Flow cytometry of BM and splenic B cells, gated on B220 and stained with Abs to IgM^{a} and IgD^{a}, splenic lymphocytes stained with heat-stable Ag (HSA; CD24) and B220, and splenic B cells, gated on B220 and stained with CD21 and CD23 (no sample for Ig/mHEL-KK, but see Ref. 35). *C*, Flow cytometric histograms of HEL-binding $B220^{+}$ splenocytes stained with Abs to CD21. *D*, Flow cytometric histograms of $B220^{+}$ splenocytes stained with Abs to MHC class II.

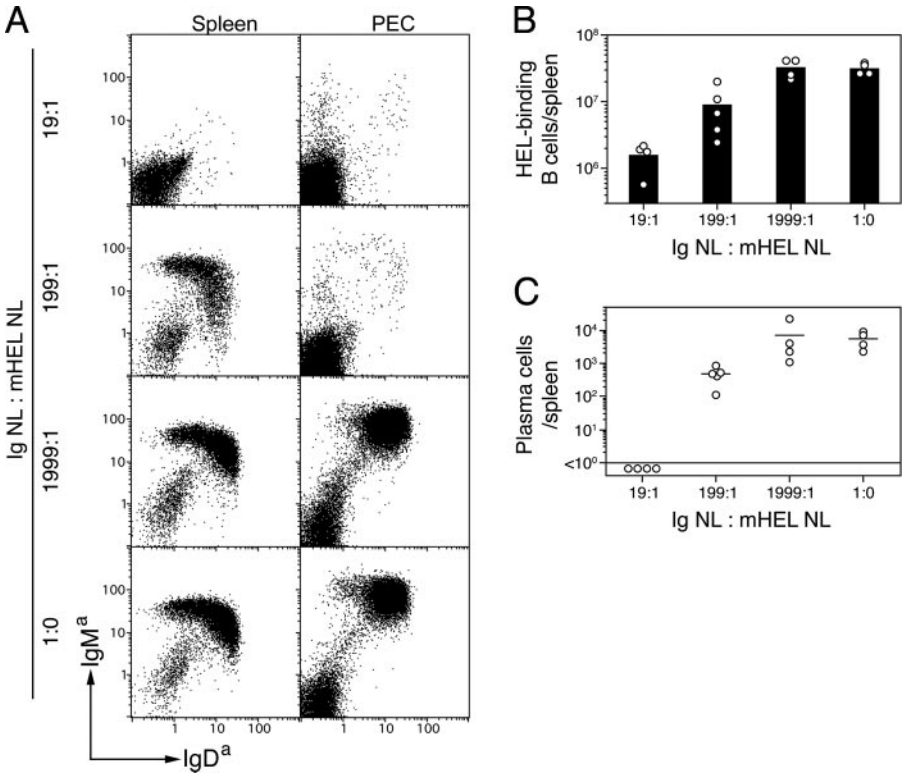

FIGURE 2. Avidity and rarity does not account for positive selection by intracellular Ag. *A*, Flow cytometry of splenic B cells (gated on B220) and PEC lymphocytes from lethally irradiated nontransgenic recipients reconstituted with neonatal liver-expressing Ig^{HEL} (Ig NL), and extracellular membrane-bound HEL (mHEL NL) in varying ratios (19:1, 199:1, 1999:1. and 1:0). Cells stained with Abs to IgM^a (anti-IgM^a-PE, *left*; and anti-IgM^a-Bi/SA-Tc, *right*) and IgD^a. *B* and *C*, Number of HEL-binding B cells (*B*) and anti-HEL IgM^a-secreting plasma cells (*C*) in the spleen of mixed chimeras. Dots represent individual mice, and columns/bars represent the mean.

Ab (Fig. 2, *B* and *C*). In contrast, Ag had no discernible effect on B cell numbers or tolerance in chimeras containing a low frequency of mHEL cells (ratio 1:1999) when it was indistinguishable from effects of no Ag (Fig. 2, *A–C*). The middle frequency of mHEL-expressing cells (1:199) induced an intermediate level of B cell deletion and tolerance and characteristic IgM modulation of follicular B cells in the spleen (Fig. 2, *A–C*). However, no frequency of mHEL induced any positive selection: neither B1 cell selection nor plasma cell differentiation (Fig. 2, *A–C*). There was no positive selection of marginal zone B cells at any dilution of mHEL (data not shown). This data suggests that other qualitatively distinct aspects of the Ag presentation must distinguish tolerogenic from immunogenic signals.

Positive selection of autoreactive B cells is restricted to early ontogeny

Because previous experiments have shown that B1 cells arise predominantly during early ontogeny (1), two features of Ag presentation that may be necessary for positive selection of autoreactive cells are the timing of the encounter in fetal or adult development and the state of receptiveness of the B cell. To explore this further, we compared B cell development and B1 cell selection and IgM autoantibody production in Ig^{HEL} and Ig^{HEL}/mHEL-KK double transgenic mice aged 1–4 wk of age. A distinct population of mature splenic IgD bright follicular B cells was present at 18 days of age, but not at 7 days (Fig. 3*A*). As described previously (35), the emerging follicular B cells in Ig^{HEL}/mHEL-KK mice showed increased IgM modulation compared with Ig^{HEL}, which was characteristic of binding to sHEL Ag (Fig. 3*A*). Adult Ig^{HEL}/mHEL-KK double transgenic mice are characterized by a 50-fold increase in IgM^a-secreting plasma cells (35). We observed that the selection of IgM^a-secreting plasma cells by mHEL-KK began just after weaning at 23 days (Fig. 3*D*). From 23–26 days, plasma cell numbers were constant and lower in Ig^{HEL} mice (mean, 1,029/spleen; $n = 8$; 95% confidence interval, 276–1,783), compared with Ig^{HEL}/mHEL-KK mice (mean, 17,971/spleen; $n = 11$; confidence interval, 4,096–13,586). By this time, Ag-induced $IgM^{bright}IgD^{low}$ peritoneal B1 cells had yet to accumulate in the PEC (Fig. 3*A*), and $CD23^+CD21^{bright}$ splenic marginal zone B cells were only beginning to appear in Ig^{HEL} controls (Fig. 3, *B* and *C*). The observation that $CD21^{bright}$ marginal zone B cells are reduced in Ig^{HEL}/mHEL-KK double transgenics (Fig. 3*B* and data not shown) has also been made before in adult mice (35). This experiment demonstrates that the positive selection of autoreactive B cells first occurs before 4 wk of age and just after weaning.

To discover whether there was a fetal or neonatal window for B cell selection by intracellular HEL, we reconstituted lethally irradiated mHEL-KK and nontransgenic mice with cells from Ig^{HEL} transgenic fetal liver or adult BM. In the presence of mHEL-KK, only fetal liver-derived precursors could regenerate the phenotype of Ig^{HEL}/mHEL-KK transgenics, with positive selection to B1 cells and plasma cells (Fig. 4, *A–C*). In these fetally derived chimeras the follicular B cells developed normally, and only marginal zone B cell numbers were slightly reduced compared with controls (Fig. 4*B* and data not shown). In contrast, adult BM-derived Ig^{HEL} B cells were largely deleted in mHEL-KK recipients (Fig. 4, *A* and *B*), with B cell tolerance similar to that caused by encounter with extracellular mHEL (Fig. 4*C*). In the absence of Ag, both fetal and adult BM reconstituted similar numbers of follicular and marginal zone B cells, which were phenotypically identical with nonchimeric Ig^{HEL} single transgenic mice (Fig. 4, *A* and *B*). This demonstrates a developmental switch between fetal/neonatal and adult periods, presumably after 4 wk, when positive selection turns to negative selection.

To discover whether the developmental switch existed in B cells or other cells associated with Ag presentation, we next reconstituted irradiated mHEL-KK recipients with 50:50 mixtures of fetal liver and adult BM from Ig^{HEL} mice expressing either IgM and IgD isotypes (MD) or IgD only (DD). By ensuring that all the B cells expressed the same HEL BCR, we avoided effects due to

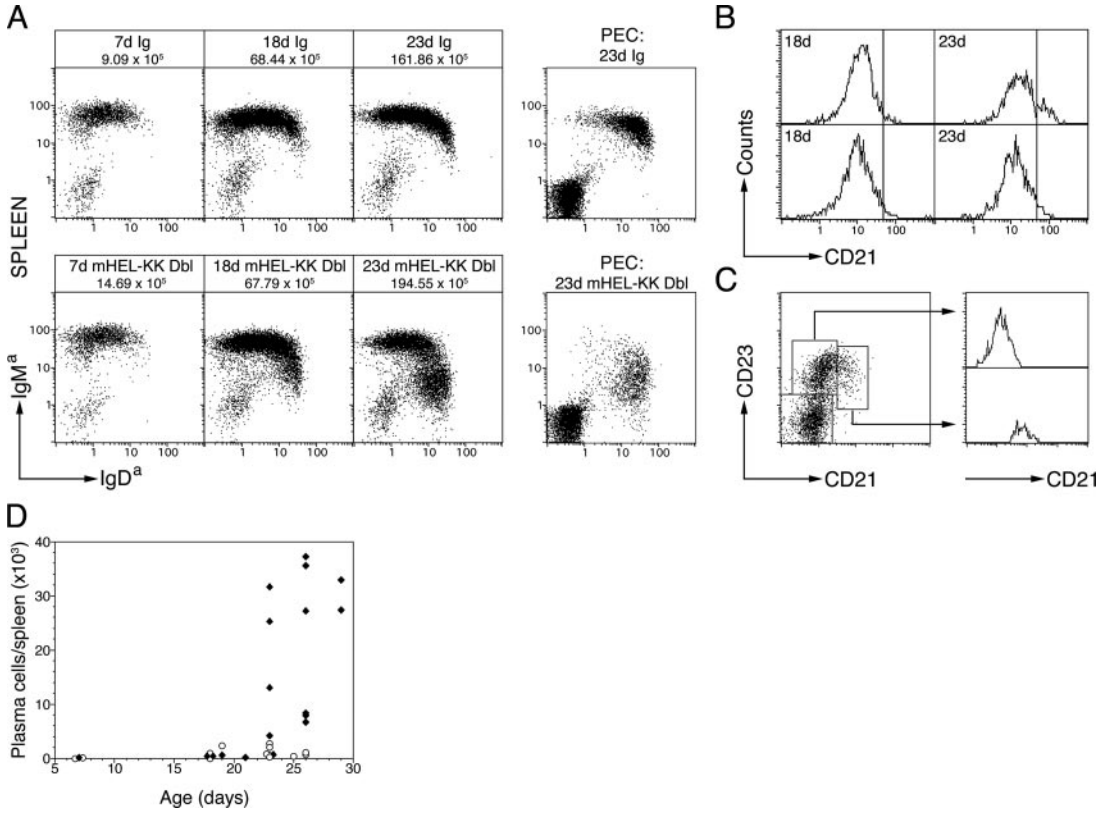

FIGURE 3. Positive selection occurs between 3 and 4 wk of age. *A*, Flow cytometry of B220$^+$ splenocytes and peritoneal lymphocytes (PEC), from Ig^{HEL} transgenic (Ig, *upper panel*) and Ig^{HEL}/mHEL-KK double transgenic (mHEL-KK Dbl, *lower panel*) mice, stained with Abs to IgM^a and IgD^a, at 7, 18, and 23 days postpartum. The mean number of HEL-binding splenic B cells are shown above the plots. The 95% confidence limits: Ig at 7 days (n = 8), 2.10–16.07 $\times$ 10^5; at 18 days (n = 3), 59.96–79.93 $\times$ 10^5; at 23 days (n = 8), 90.30–233.41 $\times$ 10^5; mHEL-KK Dbl at 7 days (n = 4), 3.56–25.82 $\times$ 10^5; at 18 days (n = 3), 42.26–93.31 $\times$ 10^5; at 23 days (n = 8), 121.14–267.97 $\times$ 10^5. *B*, Flow cytometric histograms of HEL-binding B220$^+$ splenocytes stained with Abs to CD21 from Ig and mHEL-KK Dbl mice (Ig, *top panel*; mHEL-KK Dbl, *lower panel*) at days 18 and 23 postpartum. Line illustrates the division between the follicular (CD21^{int}) and marginal zone (CD21^{high}) B cell populations. *C*, Flow cytometry of HEL-binding Ig splenic B cells at 23 days stained with Abs to CD21 and CD23, with gates to show CD21 expression on follicular (*top*) and marginal zone B cells (*bottom*), in comparison to *B*. *D*, Anti-HEL IgM^a-secreting plasma cell in the spleen from Ig (○) and mHEL-KK Dbl mice (♦) during early development. Each circle represents a single animal.

competition between B cells and Ag-induced follicular exclusion. However, in none of these experiments were fetal DD liver-derived cells able to rescue the positive selection of IgM-secreting plasma cells or B1 cells from adult MD BM precursors in *trans* (data not shown). Similar negative results were also found with mixtures of fetal liver from B cell-deficient μMT$^{-/-}$ mice and adult Ig^{HEL} marrow. These results suggest, but cannot prove, that there is an intrinsic susceptibility to positive selection in fetal and neonatal B cells. This susceptibility would be independent of BCR specificity and reduced or absent in adult BM-derived cells.

Peritoneal cells rescue follicular and marginal zone B cells from negative selection in adult mice

We next considered the possibility that the normal development of follicular and marginal zone B cells in mHEL-KK chimeras reconstituted with fetal liver, but not adult BM, might be due to the selection of autoreactive B1 cells. To test the ability of B1 cells to rescue adult-derived follicular and marginal zone B cells from negative selection by mHEL-KK, we reconstituted lethally irradiated mHEL-KK transgenic recipients with adult CD45.1$^+$ Ig^{HEL} BM and simultaneously injected them i.p. with either medium alone or CD45.2$^+$ peritoneal cells, including B1 cells, from adult Ig^{HEL}/mHEL-KK double transgenic mice. As described previously, adult BM-derived cells were deleted by mHEL-KK in recipients that were coinjected with medium (Fig. 5, *A* and *B*). However, mHEL-KK recipients that received peritoneal cells from Ig^{HEL}/mHEL-KK donors showed normal development of adult BM-derived CD45.1$^+$ follicular and marginal zone B cells (Fig. 5, *A* and *B*), and positive selection of plasma cells and autoantibodies compared with nontransgenic recipients (Fig. 5, *A* and *C*). Although the small numbers of transferred CD45.2$^+$ peritoneal cells were undetectable by 8 wk, the effect of the mixed peritoneal cells in rescuing the BM-derived cells was highly specific. Additional experiments showed that these effects were B cell specific because μMT$^{-/-}$ peritoneal cells also failed to rescue BM development (data not shown).

These findings suggest that IgM autoantibody from peritoneal-derived plasma cells neutralizes the tolerogenic effects of mHEL-KK, either by clearance of Ag or masking. To test this hypothesis, we asked whether injected serum containing anti-HEL IgM^a autoantibody or normal mouse serum (NMS) could rescue conventional B cell development in mHEL-KK recipients of adult Ig^{HEL} BM. Animals were injected on day 15, 21, and 28 following reconstitution. Analysis at 35 days showed rescue of conventional B cell development in 3 of 5 mHEL-KK recipients injected with anti-HEL IgM^a serum. These mice had normal numbers of mature Ig^{HEL} conventional B cells in the spleen, mesenteric lymph node, and PEC (Fig. 5*D*). Some variation in the efficiency of rescue was not surprising given the technical issues surrounding Ab delivery as well as possible differences in the rate of reconstitution. As

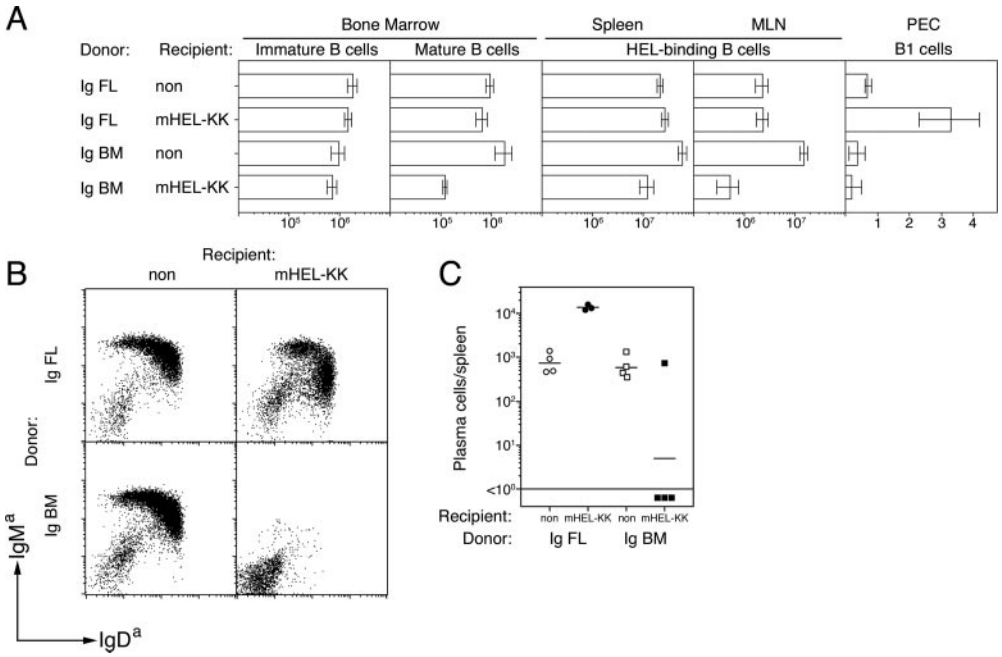

FIGURE 4. Positive and negative selection by self-Ag depends on timing in B cell ontogeny. *A*, CD45.1$^+$ cell numbers from lethally irradiated CD45.2$^+$ nontransgenic (non) and mHEL-KK transgenic recipients, reconstituted with CD45.1$^+$ Ig^{HEL}-transgenic fetal liver (Ig FL) or BM (Ig BM). Immature and mature B cells in the BM are defined as IgM^{a+}/IgD^{a-} and IgM^{a+}/IgD^{a+}, respectively. Absolute numbers are shown for all populations except peritoneal B1 B cells (PEC B1), which are shown as a percentage of the IgM^{a+}/IgD$^{a-/+}$ peritoneal lymphocytes. *B*, Flow cytometry of CD45.1$^+$ splenic B cells, gated on B220 and stained with Abs to IgM^a and IgD^a. *C*, Anti-HEL IgM^a-secreting plasma cells in the spleen. Bars show the mean, and circles/squares represent individual samples.

expected, there was no development of B1 cells, although the PEC was repopulated by conventional cells (Fig. 5*D*). By 35 days, anti-HEL plasma cell numbers were elevated to a mean of 17,172/spleen ($n = 5$; SD 23,551) compared with 2,549/spleen ($n = 4$; SD 2,495) in nontransgenic recipients. Plasma cell numbers were also slightly higher in mice injected with NMS (6,682/spleen; $n = 5$; SD 7,300) compared with uninjected controls (1,096/spleen; $n = 4$; SD 1,227). However, there was no rescue of conventional B cell development in the absence of injected Ab or in mice that received NMS, even allowing for a 2-fold reduction in immature B cells in these mice (Fig. 5*D*). These observations suggest that anti-HEL-specific serum can rescue conventional B cells from negative selection in adult BM.

Discussion

In this study, we explore how self-Ag presentation affects the selection of autoreactive B cells and B cell subsets. This leads us to conclude that positive and negative selection are affected by 1) qualitative differences in the form of Ag, 2) the timing of Ag exposure during ontogeny, and 3) the level of exposure, allowing for possible effects of masking by autoantibody. Using Ig^{HEL} transgenic mice and the single Ag HEL, we show that these effects are independent of receptor specificity. The selection of autoreactive B cells is a competitive process, involving immunogenic and tolerogenic forms of self-Ags. During early life, B cells are susceptible to positive and negative selection; but, due to a developmental switch, negative selection is dominant in adults.

Our study agrees with earlier findings showing that fetal liver-derived stems cells are more effective at generating B1 cells (37, 38) and extends this observation to autoreactive cells with a single specificity. Significantly, this allows us to show that the increased selection of B1 cells in fetal and neonatal animals is not just a function of the distinct repertoire of BCR specificities that are expressed in early B cell ontogeny (39, 40). In our mice, the positive selection of B1 B cells occurs in Ig^{HEL}/mHEL-KK double transgenics at ~23–28 days and coincides with the appearance of high-titer IgM autoantibodies. Radiation chimeras show that Ig^{HEL} B1 B cells mainly arise by positive selection from fetal or neonatal progenitors because the same mHEL-KK Ag is tolerogenic for adult progenitors. Our findings suggest that there is a developmentally regulated switch in B cell susceptibility to positive selection by self-Ags. The suggestion that this switch is B cell intrinsic is supported by a previous experiment using mixed fetal liver and adult BM chimeras from mice with normal repertoires (38). In this experiment, the development of B1 cells from adult BM was not increased by the presence of fetal liver-derived cells.

The coincidence of B1 cell selection and autoantibody generation at 3–4 wk of age suggests that B1 cells are responsible for the high level of autoantibodies at this time. These B1 cells are able to recirculate to the spleen, where they give rise to natural Abs (41). Our data now give support to the idea that these autoantibodies mask self-Ag, either directly or through its clearance. The clearance or masking of self-Ag would account for the rescue of adult follicular and marginal zone B cells from deletion by peritoneal B cells from Ig^{HEL}/mHEL-KK double transgenics. This would also explain why mHEL-KK is partially tolerogenic in Ig^{HEL}/sHEL/mHEL-KK triple transgenics, if greater competitive negative selection in early development and 50-fold lower autoantibody levels compared with Ig^{HEL}/mHEL-KK double transgenics unmasked Ag. The fact that these effects are titratable suggests that natural IgM autoantibodies may have measurable effects in nontransgenic mice, especially when the level of Ab is high or when the negatively selecting Ag is rare. Pentameric IgM may also be uniquely capable of masking multiple epitopes, because IgM but not IgG against the HyHEL10 epitope on mHEL prevents the interaction of IgG with the adjacent HyHEL9 binding site (H. Ferry and R. J. Cornall, unpublished observations). The postulated link between B1 cells and autoimmune disease (42, 43) could now be explained

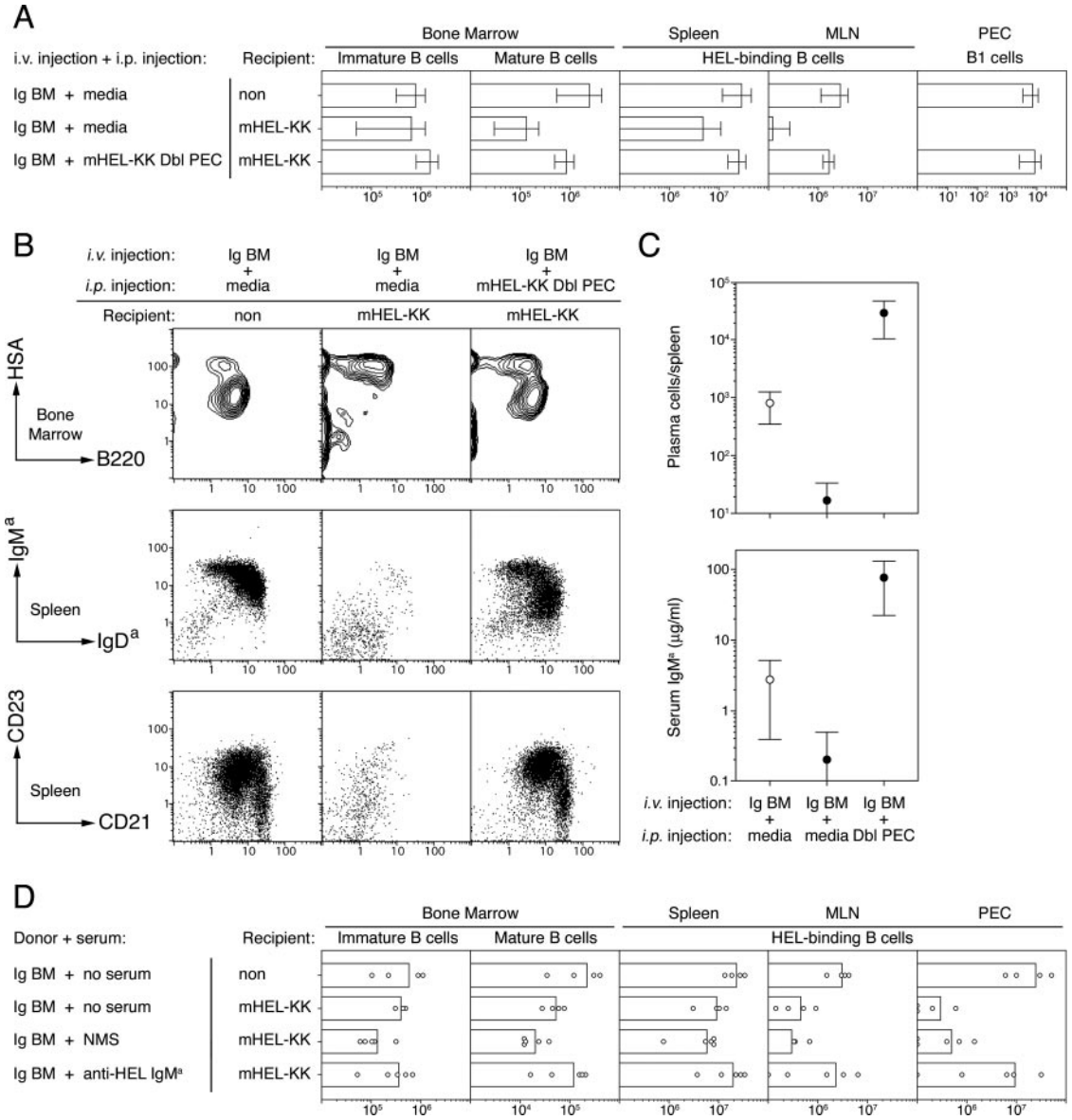

FIGURE 5. Peritoneal cells rescue the negative selection of follicular and marginal B cells in adult mice. *A*, CD45.1$^+$ Ig^{HEL} B cell numbers from lethally irradiated CD45.2$^+$ nontransgenic (non) and mHEL-KK transgenic recipients reconstituted with CD45.1$^+$ Ig^{HEL} transgenic BM (Ig BM) with or without peritoneal cells from CD45.1$^+$ Ig^{HEL}/mHEL-KK Dbl (mHEL-KK Dbl PEC) mice. BM cells were injected i.v. (i.v. injection), and peritoneal cells were injected i.p. (i.p. injection). Immature and mature B cells in the BM are defined as IgM^{a+}/IgD^{a-} and IgM^{a+}/IgD^{a+}, respectively, and B1 cells were gated on IgM^{a+}/IgD^{a-} using a Ig^{HEL}/mHEL-KK Dbl control. Columns show the mean, and bars represent the 95% confidence limits. *B*, Flow cytometry of CD45.1$^+$ BM lymphocytes stained with Abs to B220 and heat-stable Ag (HSA; CD24) (*top row*), and CD45.1$^+$/B220$^+$ splenocytes stained with Abs to IgM^a and IgD^a or CD21 and CD23 (*middle row* and *bottom row*, respectively). *C*, Anti-HEL IgM^a-secreting plasma cells in the spleen, and anti-HEL IgM^a-serum titers, in nonrecipients (○) and mHEL-KK recipients (●). Circles show the mean, and the bars represent the 95% confidence limits. *D*, CD45.1$^+$ Ig^{HEL} B cell numbers from lethally irradiated CD45.2$^+$ nontransgenic (non) and mHEL-KK transgenic recipients reconstituted for 35 days with CD45.1$^+$ Ig^{HEL} transgenic BM (Ig BM) and either left alone (no serum) or injected with NMS or anti-HEL IgM^a containing serum on days 15, 21, and 28 postreconstitution. Immature and mature B cells in the BM are defined as in *A*, and other cells gated as HEL-binding and B220$^+$.

by the escape of autoreactive B2 cells that are capable of undergoing T-dependent activation, switching, and affinity maturation. In diseases such as systemic lupus erythematosus, the cycle of B2 escape, activation and high Ab titers, and masking could become a self-sustaining pathological process.

Although the ability to generate B1 cells declines during ontogeny, there is good evidence that this decline is relative rather than absolute. This is because adult BM can give rise to B1 cells in chimeras reconstituted with cells from a mixed repertoire (44–47) and expressing Ig transgenes that have been preselected from B1 cells (48). This implies that the inability to generate any B1 cells from adult BM in mHEL-KK chimeras is due to negative selection and declining susceptibility to positive selection. It seems likely that negative selection to Ags like mHEL-KK would also be dominant in the BM of unmanipulated wild-type adults, because Ag-specific B1 cells and masking IgM autoantibodies would be much rarer than in the transgenics. These processes would increase self-tolerance in adults, but could leave the selection of B1 cells against foreign Ags or more highly sequestered self-Ag intact.

It is useful to compare our results with two other Ig transgenic models, which are also characterized by the selection of B1 cells directed against self-Ags. B cells expressing μ and κ Ig transgenes

against the thymocyte cell surface Ag CD90 (Thy-1) also have a conventional phenotype in the absence of self-Ag and only generate B1 cells and autoantibodies in its presence (49). However, in contrast to Ig^{HEL}/mHEL-KK double transgenics, the follicular anti-CD90 B cells developing alongside B1 cells in the presence of self-Ag are severely arrested, short-lived, and functionally inactive. This would be consistent with greater exposure of autoreactive B cells to unmasked CD90 compared with mHEL-KK. In support of this idea, the pattern of B cell development in anti-CD90 transgenics is similar to that in Ig^{HEL}/sHEL/mHEL-KK triple transgenics. The same combination of positive and negative selection also occurs in an anti-erythrocyte model of tolerance (50). Autoreactive anti-erythrocyte B1 cells are positively selected in the peritoneum, where they produce autoantibodies that cause hemolytic anemia, but are eliminated from the blood, spleen, and lymph nodes, where the self-Ag is abundantly expressed. These observations are also consistent with the idea that competition between positive and negative signals in early development is largely superseded by negative selection in later life.

Our data provide further encouragement to explore the long-standing question of why fetal liver-derived cells are more favored to become B1 cells. Although we highlight B cell intrinsic effects, it also remains possible that other fetal and neonatal cells play an important part in this selection. One possibility is that there are hard-wired differences between early and late B cells in the threshold for BCR signaling, perhaps due to variation in BCR expression or downstream signaling molecules. The increased sensitivity of neonatal B cells to tolerogenic stimulation in vitro may be one example of such differences (51). The survival of Ig^{HEL} B cells with reduced signaling due to CD45 deficiency requires positive selection by sHEL (52), and it has been suggested that similar requirements for Ag stimulation may occur in adult follicular B cells expressing dual specificity receptors due to incomplete allelic exclusion (53). However, neither modulation of the BCR expression nor failure of allelic exclusion seem to be causative in our model because we see no change in Ig^{HEL} BCR expression on immature B cells during development, and mHEL-KK positively selects B1 cells on a rag-deficient background, where only the transgenic BCR can be expressed (35). Indeed, it is not easy to see how the level of BCR-signaling could alone account for the differences in signals from identical multivalent Ags like mHEL and mHEL-KK, unless the pattern of Ag array is critically different. Instead, there may be other qualitative differences between competing tolerogenic and immunogenic self-Ags.

Rarity and avidity alone are not sufficient to explain the positive selection of B1 cells because the high-avidity surface Ag mHEL is not able to induce positive selection at any frequency. In contrast to mHEL-KK, the mHEL cell surface Ag causes negative selection or has no effect on B cell development. These results agree with previous experiments showing the extreme sensitivity of H-chain only anti-HEL transgenic B cells to deletion by mHEL (54). Recently, a similar titration of mHEL-expressing cells with adult BM donors on a rag-deficient background showed the same effect on deletion of titrating mHEL (55). However, the interpretation of a 3-fold increase in IgM induced by infrequent mHEL-expressing cells in this experiment requires caution, given that the level of Ab in anergic mice was the same as in the naive controls. The 10-fold reduction in B cells in the absence of Ag in these mice is also likely to be due to the rag-deficient background. Our results show that in mice with normal secondary lymphoid organs, rarity alone does not account for the positive selection of Ig^{HEL} cells by high-avidity Ag.

Although the dilution experiment did not test for the effects of rare Ag expressed outside the hemopoietic system, the other chimera experiments show that Ag expression in these tissues is sufficient to induce positive selection. The expression of self-Ag outside the hemopoietic system is clearly unnecessary for positive selection in the anti-CD90 and probably anti-erythrocyte transgenic models (12, 49, 50). Furthermore, mHEL expression in thyroid, pancreas, and skin does not on its own lead to positive selection (56)(R. J. Cornall, unpublished data). Differences in the mode of positive selection by different self-Ag are likely to depend on their structure, BCR affinity, and the context of self-Ag presentation. Self-Ag like CD90 interact at lower affinity but are repetitively arrayed even on single molecules (12). Differences in the way in which intracellular and extracellular Ags are arrayed may also distinguish between positive vs negative selection in a way that cannot be mimicked by mHEL at any level. In addition, the Ig transgenes against CD90 and erythrocyte Ag may be biased in more subtle ways toward positive selection by their native self-Ag, because they were originally cloned from autoantibody-producing B1 cells. This contrasts with the Ig^{HEL} transgene, which was generated by immunization with a foreign Ag. None of these facts, however, alters the conclusion that rarity alone is not sufficient to explain the difference between positively and negatively selecting Ag in our model.

Another important possibility is that adult and fetal/neonatal B cells differ in their sensitivity to accessory signals, including lineage-specific factors. This could also account for the ability of fetal and neonatal B cells to discriminate between positively and negatively selecting self-Ags. Molecules that play a role as accessory factors in the selection of autoimmune cells from the established repertoire include CpG DNA, which is displayed extracellularly on dying cells and activates B cells through TLR9 (57), and LPS from pathogens or diet that is required for the generation of autoantibodies and survival of peritoneal B1 cells in the anti-erythrocyte Ig transgenic model (58, 59). Similar factors, such as endogenous Toll receptor ligands displayed on dying cells with mHEL-KK, could provide the necessary accessory signals to select B1 cells in young animals. Additional experiments to explore the differences between early and late Ig^{HEL} B cells should help to distinguish between these possibilities.

Our findings suggest a model of positive and negative selection of B cells in which selection varies with the timing, form, and the level of different self-Ag exposure. During early life, autoreactive immature B cells are responsive to competing signals from both tolerogenic and immunogenic self-Ags. Signals from self-Ags such as mHEL-KK positively select autoreactive B1 cells and plasma cells that generate IgM autoantibodies, whereas signals from other abundant self-Ags, including mHEL, sHEL, and mHEL-KK itself, negatively select conventional B cells to varying degrees. Later in life, the ability to generate new autoreactive B1 cells is reduced and negative selection dominates. However, B1 cells persist from early life and continue to give rise to IgM autoantibodies, which can rescue the development of follicular and marginal zone B cells by masking tolerogenic self-Ags when they are highly abundant. For these reasons high levels of natural IgM Ab may predispose to autoimmunity in the conventional B2 cell compartment.

Under normal circumstances, however, the generation of natural autoantibodies in early life is more likely to have a role in immune regulation, particularly in the clearance of self-Ags. This hypothesis is supported by finding germline autoantibody binding specificities that are evolutionarily conserved and expressed without mutation by B1 cells in early ontogeny (60) (61), and the fact that deficiency in IgM is itself autoimmunogenic (62, 63). Alternatively, the generation of some weakly autoreactive IgM autoantibodies may be the price to be paid for development of an early

adaptive immunity against dangerous but not highly immunogenic gut and respiratory pathogens (64, 65). In this scenario, autoreactive B cells could be the byproduct of a process of selection that discriminates less easily between self and foreign proteins in the early stages of life. The selection of autoreactive B1 cells would not be harmful in later life because T cell tolerance to systemic self-Abs remains intact and, in the absence of high-titer autoantibody, positively selecting self-Ags are tolerogenic for follicular and marginal zone B cells.

Acknowledgments

We thank J. G. Cyster for helpful discussions and the staff of Biomedical Services (Oxford, U.K.) for excellent animal husbandry.

Disclosures

The authors have no financial conflict of interest.

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