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*J Immunol* 2004; 172:4700-4708; doi: 10.4049/jimmunol.172.8.4700

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Requirements for Follicular Exclusion and Competitive Elimination of Autoantigen-Binding B Cells

Eric H. Ekland,* Reinhold Forster,† Martin Lipp,‡ and Jason G. Cyster²*

Results from several mouse tolerance models indicate that autoreactive B cells in peripheral lymphoid organs develop an anergic phenotype, migrate to the boundary between the T cell zone and the B cell follicle (T/B boundary), and undergo rapid cell death. We have used B cells from mice that are double-transgenic for soluble hen egg lysozyme (HEL) and an Ig that recognizes HEL with a high affinity to characterize the mechanisms underlying the migration and elimination of autoreactive B cells. In contrast to the situation for acutely activated B cells, we find that anergic B cells have reduced levels of CXCR5, the receptor for the follicular chemokine, CXCL13, and this contributes to their exclusion from follicles. CCR7 expression is required for follicular exclusion of anergic cells, although up-regulation of the receptor does not appear to be necessary. By TUNEL analysis, we observe that excluded anergic cells die in situ at the T/B boundary. We also show that this elimination occurs via a Fas-independent mechanism. Using CCR7−/− IgHEL-transgenic B cells we find that localization to the T/B boundary is not a necessary event to achieve the competitive elimination of autoregulatory B cells. These findings characterize the mechanism for follicular exclusion of autoreactive B cells and they indicate that B cells compete for survival by mechanisms that are separate from competition for the follicular niche. The Journal of Immunology, 2004, 172: 4700–4708.

As they mature in the bone marrow (BM), B cells transit through developmental checkpoints that help ensure that they maintain tolerance to self. Those that recognize self Ags with high affinity undergo receptor editing or they are eliminated (1, 2). However, several Ig-transgenic models demonstrate that autoreactive B cells recognizing Ags as diverse as soluble hen egg lysozyme (sHEL), dsDNA, and MHC class II molecules can mature and emigrate to the periphery where additional mechanisms are required for maintaining tolerance (3–5). Autoreactive B cells in the periphery demonstrate an anergic phenotype, characterized by down-modulation of surface IgM, decreased responsiveness to B cell receptor (BCR) stimulation, and a shortened lifespan (4–8). Anergic B cells are sensitive to Fas-mediated killing when they interact with activated T cells (9). In the presence of a normal repertoire of competitor B cells, anergic B cells are unable to access B cell follicles. Instead, they accumulate at the boundary between the T cell zone and the B cell follicles (T/B boundary) and they have a highly truncated lifespan (7, 10, 11).

In this study, we have used the hen egg lysozyme (HEL) system of mice that are double-transgenic for sHEL and for an Ig that recognizes HEL with a high affinity (IgHEL). Some of the first evidence suggesting that competitors B cells affected the survival of peripheral autoreactive B cells came from the initial analyses of mice that were transgenic only for the H chain of a HEL-binding Ab. In these mice, ~1% of the B cells express an endogenous L chain that pairs with the transgenic H chain to produce a HEL-binding Ag receptor. When the H chain-transgenic mice were crossed to mice that express sHEL, the population of HEL-binding B cells disappeared from the periphery (7). This stood in contrast to the situation when mice transgenic for both the HEL-binding H chain and L chain were crossed to sHEL-expressing mice. In these mice nearly all of the B cells bind HEL and the total number of B cells is only about 2-fold lower than in non-HEL-expressing mice (6).

Further studies in mixed BM chimeras and in transfer experiments also indicated that anergic B cells had an acutely abbreviated lifespan when they had to compete with a wild-type population of B cells. When irradiated sHEL-transgenic recipients were reconstituted with a mixture of wild-type and IgHEL-transgenic BM, the anergic B cells had a lifespan of only 2–3 days, whereas when the recipients received only IgHEL-transgenic BM they had a lifespan of over a week (7, 10). Analysis of the localization of the autoreactive B cells in both the mixed chimera experiments and in transfer experiments indicated that the autoreactive B cells were unable to access follicular niches and instead accumulated at the T/B interface (7, 10). These data were consistent with a model where the localization of the autoreactive B cells itself contributed to their reduced lifespan. Migration to the T/B boundary might preclude autoreactive B cells from receiving survival factors that are present within the B cell follicle. Alternatively, it might expose them to death-inducing signals produced by some cell type in the T cell zone. The experiments presented in this study set out to characterize the molecular mechanisms regulating the migration of anergic B cells, as well as to test the relationship between the localization and elimination of autoreactive B cells.

During an immune response to foreign agents, Ag-specific B cells migrate to the boundary of the T and B cell zones. Within 6 h of Ag exposure, naive B cells up-regulate their surface expression

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Received for publication October 10, 2003. Accepted for publication February 2, 2004.

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1 This work was supported by National Institutes of Health Grant AI40098. E.E.K. was supported by a Howard Hughes Medical Institute predoctoral fellowship and J.G.C. is an assistant investigator of the Howard Hughes Medical Institute.

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3 Abbreviations used in this paper: BM, bone marrow; HEL, hen egg lysozyme; sHEL, soluble HEL; DEL, duck egg lysozyme; T/B boundary, the boundary between the T cell zone and the B cell follicle; MLN, mesenteric lymph node; BCR, B cell receptor; RGS, regulator of G protein signaling; BAFF, B cell activating factor belonging to the TNF family.
levels of CCR7 about 3-fold and demonstrate an increased migratory potential to the CCR7 ligands, CCL19/ECL and CCL21/SLC (12). These acutely activated B cells do not show any changes in their receptor levels of CXCR5 or in their migratory response to its ligand CXCL13/BLC, which is expressed in the B cell follicle. These studies indicated that for activated B cells the change in CCR7 responsiveness appears to be sufficient to tip the balance toward migration out of the B cell follicle to the outer T cell zone (12).

In addition to studies with acutely activated B cells, several reports using the VH3H9 anti-dsDNA-transgenic system have suggested that chemokines and their receptors play a role in guiding the migration of anergic B cells to the T/B boundary. In one study, it was observed that signaling through TNFR-1 caused alterations in the CXCL13/CCL19/CCL21 ratios in the spleen and these changes correlated with changes in the migratory patterns of anergic B cells (13). In another study, anti-dsDNA B cells were found to express lower levels of CXCR5 (14). However, CCR7 levels on these cells were not determined and it was not clear whether the changes in CXCR5 were sufficient to account for the localization pattern of these cells.

In the present study we find that, as for acutely activated B cells, CCR7 expression on anergic B cells is required for proper migration to the T/B boundary. However, in contrast to the situation for acutely activated B cells, up-regulation of CCR7 does not play a critical role in directing the migration of anergic B cells. Instead, anergic B cells shift the balance of chemokine responsiveness by down-modulating their surface CXCR5 levels. Once anergic B cells are excluded from the follicle, we show that they die in situ at the T/B boundary in a Fas-independent manner. Furthermore, using chemokine receptor-deficient donor cells, we find that migration to the T/B boundary is not a prerequisite for the competitive elimination of anergic or acutely activated B cells.

**Materials and Methods**

**Mice**

C57BL/6 IgHEL-transgenic mice were of the MD4 line that carries a HEL-specific IgM and IgD H chain gene and a HEL-specific L chain gene and these together encode IgM and IgD that bind HEL with high affinity (3). The MD4 line had previously been crossed to a congenic C57BL/6J CD45.1 line so that all hematopoietic cells could be tracked by CD45.1 staining. CXCR5-/- mice (15) were backcrossed to C57BL/6J mice for 10 generations and CCR7-/- mice (16) for 8. Each line was then intercrossed with the MD4 line to generate IgHEL-transgenic, Ly5.2-/-, chemokine receptor-deficient offspring. HEL-transgenic mice were of the ML5 line (3, 17), which carries a transgene, encoding a soluble form of HEL under the zinc-sensitive metallothionein promoter. Fas-deficient lpr/lpr mice on a C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME), and intercrossed with the MD4 line. Colonies were maintained at the University of California (San Francisco, CA).

**BM chimeras**

Non- or metallothionein-HEL (ML5 line)-transgenic recipients were lethally irradiated with 1200 rad from a cesium irradiator (Mark I, Model 68A; J. L. Shepherd & Associates, San Fernando, CA). They were then rescued by injecting ~4 million donor BM cells via tail vein injection. To generate mixed BM chimeras, recipients were reconstituted with 80% IgHEL-CXCR5-/- BM, IgHEL-CXCR7-/- BM, or IgHEL wild-type BM and 20% nontransgenic wild-type C57BL/6 BM. Although a greater proportion of Ig-transgenic than nontransgenic BM is used, the Ig-transgenic B cells proliferate less than nontransgenic B cells during development in the BM and this mixing ratio was previously shown to generate an immature B cell compartment composed of 5–30% Ig-transgenic B cells (7). The chimeric mice were maintained on water containing 25 mM zinc chloride to ensure high expression of HEL from the metallothionein promoter (17) as well as the antibiotics polymyxin-B (100 mg/L; Sigma-Aldrich, St. Louis, MO) and neomycin (1 g/L; Sigma-Aldrich). BM reconstitution was allowed to proceed for 6–9 wk before experiments were conducted.

**Adaptive transfer**

Donor lymphoid organs were harvested and suspended in RPMI 1640 containing 5% FCS, HEPEs, penicillin, and streptomycin, by mashing through a 70-μm strainer. For histological examinations, 4 × 10^6 donor cells were injected into recipients via the lateral tail vein. For survival studies, 2 × 10^7 anergic B cells or 3 × 10^5 naive B cells were transferred.

For the duck egg lysozyme (DEL) experiments, recipients were injected with 0.3 mL of PBS or a 3.2 mg/mL DEL preparation prepared as previously described (18). Sixteen hours later, spleen cells from IgHEL-,transgenic CXCR5-/- or CXCR5+/+ donors were transferred by tail vein injection. Recipient spleens were harvested after 7 h and frozen in OCT compound (Bayer, Elkhart, IN) for sectioning and histology.

For localization and survival studies using the HEL Ag, recipients were injected with 1 mg of HEL (Sigma-Aldrich) suspended in 0.3 mL RPMI 1640 or with RPMI 1640 alone 2 h before transfer of the donor cells. Anergic donor cells were harvested from the spleen and mesenteric lymph nodes (MLN) of HEL-transgenic BM chimeras reconstituted with IgHEL-transgenic BM that was CXCR5-/-, CCR7-/-, or wild-type. Naïve donor cells were harvested from the spleen of IgHEL-transgenic CXCR5-/-, CCR7-/-, lpr/lpr, or wild-type mice. For localization studies, the recipient spleens were harvested and frozen at 18 h. B cell survival was assessed at 48 h for anergic B cells and at 72 h for naïve B cells. For survival studies, donor cells were labeled for 20 min at 37°C with CFSE (Molecular Probes, Eugene, OR) before transfer. Blood, spleen, and MLN were collected from IgHEL-transgenic CXCR5-/- or lpr/lpr recipients and their controls. For CCR7-/- experiments, peripheral lymph nodes (axillary, brachial, and inguinal) were also collected.

**TUNEL experiments**

For each experiment, spleens from eight donors were harvested in PBS plus 0.5% BSA and anergic B cells were enriched by magnetic bead depletion of CD43(S7) and CD11c-positive cells on a MACS column (Miltenyi BioTech, Auburn, CA) according to the manufacturer's protocols. Eight donors typically yielded a 95–99% pure population of ~1 × 10^7 anergic B cells. A total of 4 × 10^6 anergic B cells were injected by tail vein into either B6 or ML5 recipients. After 12–18 h, spleens were harvested and frozen. TUNEL labeling was performed as previously described (19). Dig-labeled DNA was detected with sheep anti-dig (Boehringer Mannheim, Indianapolis, IN) followed by HRP-conjugated anti-sheep IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), and HEL-binding B cells were stained as described below.

**Immunohistochemistry and flow cytometry**

Cryostat sections (7–8 μm) were fixed and stained using standard techniques. Briefly, HEL-binding cells were detected by incubating the sections in 500 ng/mL HEL followed by incubation with a biotinylated polyclonal rabbit anti-HEL serum (Rockland, Gilbertsville, PA). The biotinylated Abs were visualized with alkaline phosphatase-conjugated streptavidin (Jackson ImmunoResearch Laboratories) followed by staining with Fast Red TR (Bayer, Elkhart, IN) for sectioning and histology.

For flow cytometry, single-cell suspensions were stained using standard techniques. Abs used for flow cytometry were: FITC-conjugated IgD (AMS15.1) and HSA (BD Pharmingen); PE-conjugated CD4, CD8, and IgM (BD Pharmingen); PerCP-conjugated B20 (BD Pharmingen); tricolor-conjugated Hy9 anti-HEL, biotinylated IgD (AMS15.1), CD21 (7G6), rabbit anti-HEL (Rockland), donkey anti-rat IgG, and donkey anti-human Fc (Jackson ImmunoResearch Laboratories); APC-conjugated streptavidin (Molecular Probes); and unconjugated rat anti-CXC4, CXCR5, and keyhole limpet hemocyanin (BD Pharmingen). CCR7 levels were detected using the previously described CCL19-Fc recombinant protein, which is a fusion of mouse CCL19 and the human IgG1 constant region (20). All flow cytometry was conducted on a FACSCalibur (BD Biosciences, San Diego, CA) and analyzed using Flowjo software (Tree Star, San Carlos, CA). Naive and anergic B cells did not differ in forward or side scatter characteristics and had identical levels of surface CD19.
Results

Anergic B cells demonstrate a distinct profile of chemokine receptor surface expression from either naive or acutely activated B cells

A comparison of anergic B cells from sHEL/\textit{IgHEL} double-transgenic mice with naive B cells from \textit{IgHEL} single-transgenic mice by flow cytometry indicates that the anergic B cells express less CXCR5 on their surface (Fig. 1A). In contrast, they demonstrate no difference from naive cells in their CCR7 levels (Fig. 1A). To determine whether CXCR5 or CCR7 levels change when anergic B cells are a minor B cell population competing for access to follicles, anergic B cells were transferred into HEL injected recipient mice with a wild-type B cell repertoire. We have previously demonstrated that mice injected with HEL can make suitable substitutes for HEL-transgenic mice as they maintain comparable serum levels of HEL for extended periods. Also the behavior of HEL-specific B cells transferred into HEL-injected recipients is indistinguishable from the behavior of such cells transferred into HEL-transgenic recipients with respect to follicular localization and elimination (21). Under these conditions, anergic B cells still had reduced levels of CXCR5. CCR7 levels were now elevated, although not to the extent of acutely activated B cells (Fig. 1B). To see whether the elevation in CCR7 levels was a general property of excluded anergic B cells or was somehow associated with the adoptive transfer of mature B cells, we also examined chemokine receptor levels on anergic B cells in mixed BM chimeras. \textit{IgHEL} double-transgenic BM was mixed with nontransgenic BM at a ratio previously shown to generate a minor (5–30%) population of \textit{IgHEL} transgenic B cells relative to nontransgenic B cells (7), and the mixture was transferred into non-HEL- and HEL-transgenic (ML5) recipients. When \textit{IgHEL}-transgenic B cells develop as a minor population in mixed BM chimeras expressing HEL autoantigen, the anergic cells are excluded from follicles to a similar extent as occurs following adoptive transfers (Ref. 7 and see also Fig. 3B). The anergic B cells in HEL-transgenic mixed BM chimeras had levels of CXCR5 expression that were similar to or somewhat lower than observed for anergic B cells developing in HEL/IgHEL double-transgenic mice (Fig. 1C), and they showed no increase in CCR7 expression levels (Fig. 1C). Quantitation of CXCR5 levels on HEL-binding vs naive \textit{IgHEL}-transgenic B cells in mixed BM chimeras indicated a 2.31 ± 0.18-fold reduction ($n = 4$, $p < 0.0003$, Student’s $t$ test) of CXCR5 on the anergic cells. Measurements of BCR saturation by HEL in the various model systems indicated that occupancy was similar for \textit{IgHEL}-transgenic cells in

![FIGURE 1. Anergic B cells demonstrate a distinct profile of chemokine receptor surface expression from either naive or acutely activated B cells. A, Chemokine receptor expression levels on B220$^{+}$IgD$^{a}$$^{+}$IgD$^{b}$$^{-}$-gated IgHEL-transgenic naive (gray line) or anergic (black line) splenic B cells. B, Chemokine receptor expression levels on naive (filled line), acutely activated (gray line), or anergic (black line) B cells 18 h after transfer into saline or HEL-injected recipients. C, Chemokine receptor levels on naive (gray line) or excluded anergic (black line) B cells in mixed BM chimeras. The presented histograms are representative of data collected from at least four experimental animals of each type. CXCR5 levels were detected with a rat anti-CXCR5 mAb. CCR7 levels were measured with a ELC (CCL19)-Fc fusion protein. Ctrl, Background staining of cells in the absence of primary Ab. D, BCR occupancy by HEL Ag. Spleen cells were stained with HyHEL9 (Hy9) alone, to detect the amount of HEL already bound to the B cell, or with HEL followed by Hy9 to determine the total amount of HEL that could be bound. In addition the samples were stained to detect CD45.1, CD4, and CD8. The profiles shown are gated on lymphocyte size CD45.1$^{+}$CD4$^{+}$CD8$^{-}$ cells. The numbers presented in each histogram represent the percentage of in vivo HEL saturation of the B cells and are calculated by dividing the mean fluorescence intensity of the Hy9-stained cells by that of the HEL/Hy9-stained cells. Data are representative of a minimum of four animals of each type.](http://www.jimmunol.org/)

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the adoptive transfer system and in the mixed BM chimeras and both showed higher occupancy than in HEL/Ig<sup>HEL</sup> double-transgenic mice (Fig. 1D). This is consistent with recent findings indicating that HEL concentrations are reduced in mice where all the B cells are HEL-binding (11). Taken together, these findings imply reduced CXCR5 expression in follicular exclusion of anergic B cells and they indicate that CCR7 up-regulation is not required.

**Decreased CXCR5 expression predisposes B cells in favor of migration to the T/B boundary**

To test whether decreased CXCR5 expression levels alone might suffice to account for the altered localization of anergic B cells, we examined the behavior of B cells from CXCR5 heterozygous mice. CXCR5<sup>+/−</sup> B cells express significantly less CXCR5 (1.8 ± 0.2-fold, n = 6, p < 0.0005) on their surface than do CXCR5<sup>+/+</sup> B cells (Fig. 2A). When CXCR5<sup>+/−</sup> B cells were transferred into CXCR5<sup>+/+</sup> recipients, they homed into the B cell follicles similarly to transferred CXCR5<sup>+/+</sup> B cells indicating that the 1.8-fold reduced level of CXCR5 expression is not sufficient to lead to follicular exclusion (Fig. 2B). To further test whether reduced CXCR5 expression would predispose cells for migration to the T/B boundary, we injected recipient mice i.p. with 1 mg of DEL 16 h before the transfer of CXCR5<sup>+/+</sup> or CXCR5<sup>+/−</sup>Ig<sup>HHEL</sup> B cells. DEL binds to the Ig<sup>HHEL</sup> transgene with a 3400-fold lower affinity than HEL (22) and, in contrast to HEL, it does not provide enough stimulation to cause efficient redistribution of CXCR5<sup>+/+</sup>Ig<sup>HHEL</sup> B cells (Fig. 2B). However, when provided with this suboptimal stimulus the CXCR5<sup>+/−</sup> B cells did relocalize to the T/B boundary (Fig. 2B), indicating that 1.8-fold reduced CXCR5 levels leave B cells poised to migrate to the T/B boundary.

To further test the importance of CXCR5 for follicular exclusion, CXCR5-deficient anergic B cells were generated by reconstituting irradiated HEL-transgenic recipients with CXCR5<sup>−/−</sup>Ig<sup>HHEL</sup>-transgenic BM. When CXCR5<sup>−/−</sup> anergic B cells from these reconstituted mice were transferred into HEL-transgenic recipients, they did migrate to the T cell zone but their localization was abnormal. They tended to cluster near bridging channels between follicles or they scattered throughout the T cell zone rather than localizing to the T/B boundary (Fig. 3A). These observations indicate that although CXCR5 expression is reduced on anergic HEL-binding B cells, this receptor continues to function on these cells, helping to promote their localization at the T/B boundary.

**CCR7 expression is necessary to achieve a normal distribution of anergic B cells at the T/B boundary**

Although increased CCR7 expression did not appear to be required for the follicular exclusion of anergic B cells, we speculated that this receptor would be needed for anergic B cells to position at the T/B boundary. To test this, we generated anergic CCR7<sup>−/−</sup>Ig<sup>HHEL</sup>-transgenic B cells and studied their localization in adoptive transfer and mixed chimera experiments. When anergic CCR7<sup>−/−</sup> B cells were transferred into wild-type recipients in the presence of HEL Ag, they failed to localize properly at the T/B boundary and instead they frequently moved to the outer boundary of the follicle (Fig. 3A). Similar findings were made for anergic B cells developing in CCR7<sup>−/−</sup>Ig<sup>HHEL</sup>-transgenic/wild-type mixed BM chimeras (Fig. 3B). It is also notable that in the absence of Ag stimulation, CCR7 affects the distribution of naive B cells. In the nontransgenic recipients that received Ig<sup>HHEL</sup>-transgenic CCR7<sup>−/−</sup> BM, few of the CCR7<sup>−/−</sup>Ig<sup>HHEL</sup>-transgenic B cells are found in the portion of the follicle immediately adjacent to the T cell zone (Fig. 3B, upper right panel). This contrasts with the situation for naive CCR7<sup>+/+</sup>Ig<sup>HHEL</sup>-transgenic B cells, which distribute uniformly throughout the follicles (Fig. 3B, upper left panel).

**Autoreactive B cells die at the T/B boundary**

Previous studies in the HEL transgenic system and in the anti-dsDNA system have failed to detect apoptotic anergic B cells at the T/B boundary. The results in the dsDNA system led to speculation that autoreactive B cells might migrate to a different location to die (23). However, apoptotic cells are rapidly engulfed and cleared by scavenger cells and even at sites where it is known that a large amount of cell turnover is occurring, such as in germinal centers and in the thymus, the percentage of TUNEL-positive apoptotic cells that can be detected at any given time is low (19). In an effort to improve the chances of detecting dying autoantigen-binding cells, we transferred large numbers of purified anergic B cells, 4 × 10<sup>7</sup> per recipient, from HEL/Ig<sup>HHEL</sup> double-transgenic donors into HEL-transgenic recipients or, as a control, into non-HEL-transgenic recipients. After 18 h we looked for HEL-binding

**FIGURE 2.** B cells expressing reduced levels of CXCR5 are predisposed to migrate to the T/B boundary. A. Flow cytometric analysis of CXCR5 expression on CXCR5<sup>+/+</sup> (gray line) or CXCR5<sup>+/−</sup> (black line) Ig<sup>HHEL</sup>-transgenic splenic B cells (B220<sup>+</sup>Ig<sup>D−</sup>CD4<sup>+</sup>CD8<sup>−</sup>). Ctrl. Background staining with isotype control Ab (dashed line). B. Immunohistochemical analysis of spleen sections from recipients harvested 7 h after receiving CXCR5<sup>+/+</sup> (left panels) or CXCR5<sup>+/−</sup> (right panels) Ig<sup>HHEL</sup>-transgenic B cells. Recipients were injected with PBS (top panels) or with 1 mg of DEL (bottom panels) 16 h before receiving transferred cells. Sections were stained with anti-B220 or CD3 (brown) to identify the B cell follicles or T zones, respectively, and for HEL-binding (red or blue). Objective magnification, ×5, F, follicle; T, T zone. Data are representative of three different experiments.
cells that were TUNEL positive. In the HEL transgenic recipients significantly more (7.6 ± 3.4%) of the HEL-binding B cells were TUNEL-positive apoptotic cells compared with only 1.4 ± 0.8% in the recipients lacking HEL autoantigen (p < 0.006, paired t test) (Fig. 4). Furthermore, all the TUNEL-positive anergic B cells detected in the HEL autoantigen-expressing recipients were IgHEL-transgenic, the B cell follicles stained strongly for HEL-binding B cells, causing the blue and brown colors to almost fully overlap. Spleen sections are representative of results from at least six different mice of each type. Objective magnification: A, ×5; B, ×10.

Homing to the T/B boundary is not required for the competitive elimination of autoantigen-engaged B cells

The finding that CCR7 is required for the relocation of autoantigen-engaged B cells to the T/B boundary allowed us to perform experiments to test whether the rapid death of autoantigen-binding B cells is due to their exclusion from the follicle. For these experiments, we tested the fate of both anergic B cells and of naive B cells acutely exposed to HEL autoantigen, as previous experiments have indicated that both cell types undergo competitive elimination, although with a 1 day delay in the case of acutely activated B cells (10). In these experiments, CCR7−/− B cells underwent the same extent of deletion as wild-type cells (Fig. 6A).

Similar experiments were performed with anergic and naive CXCR5−/− B cells, and these cells were also eliminated at a similar efficiency to wild-type cells (Fig. 6A).

As a further test of the relationship between follicular exclusion and elimination, the extent of peripheral deletion of CCR7−/− cells was measured in mixed BM chimeras. Under these steady-state conditions...
we still saw no difference in the extent of elimination of CCR7−/− autoreactive B cells relative to CCR7+/+ autoreactive B cells (Fig. 6B). IgHEL-transgenic immature B cell numbers in the BM were similar in both groups, demonstrating that the changes in peripheral B cell number did not reflect a defect in B cell precursor numbers in the BM (Fig. 6B).

Discussion

The above findings establish that follicular exclusion of autoantigen-binding B cells occurs at least in part due to reduced expression of CXCR5. CCR7 is required for exclusion of the anergic B cells but up-regulation of this receptor is not necessary. Under competitive conditions, autoantigen-binding B cells are shown to undergo Fas-independent cell death at the T/B boundary. Taking advantage of these new findings regarding the mechanism for anergic B cell exclusion from follicles, we have tested the relationship between follicular exclusion and elimination of autoantigen-binding B cells. We demonstrate that localization at the T/B boundary is not required for competitive elimination of HEL autoantigen-binding B cells.

Previously we demonstrated that the rapid relocalization of acutely Ag-activated B cells from follicles to the outer T zone involved an up-regulation of CCR7 that shifted the balance of responsiveness in favor of the T zone chemokines relative to B zone chemokines (12). Positioning of anergic B cells at the T/B boundary also appears to occur by a shift in the balance of chemokine responsiveness, but rather than depending on changes in CCR7 expression, the mechanism involves reduced expression of CXCR5. The explanation for the different chemokine receptor profile of anergic B cells in HEL/IgHEL double-transgenic mice compared with acutely activated B cells may relate to differences in BCR signaling in these cells. In anergic B cells, BCR signaling is partially uncoupled and the quality of the transmitted signal is altered, with impairment of NF-κB and c-Jun N-terminal kinase activation, and continued extracellular signal-regulated kinase signaling (24). Alternatively, as B cells in HEL/IgHEL double-transgenic mice encounter HEL autoantigen as immature cells during development in the BM, the cells may be blocked from fully up-regulating CXCR5 during maturation. Our finding that CXCR5−/− cells have 1.8 ± 0.2-fold reduction in CXCR5 compared with wild-type cells and are poised for migration to the T/B boundary indicates that the 2.3 ± 0.2-fold reduction of CXCR5 on anergic B cells developing in mixed BM chimeras may be sufficient to cause follicular exclusion. Consistent with the notion that changes in CXCR5 expression may be sufficient to cause follicular exclusion, it is notable that excluded dsDNA-reactive B cells show reduced CXCR5 levels (14) and they do not have increased in vitro responsiveness to the T zone chemokine CCL19/ELC (25). In preliminary experiments analyzing the nontransgenic B cell repertoire, we find that mature IgDhigh IgMlow B cells have slightly lower CXCR5 levels than mature IgDhigh IgMhigh B cells (data not shown). Because it has been proposed that mature IgDhigh IgMlow B cells correspond to cells experiencing chronic autoantigen engagement (6), this observation suggests that reduced CXCR5 expression may be a common property of peripheral autoantigen-binding B cells. In addition to reduced CXCR5 expression, it is

FIGURE 4. Anergic B cells die in situ at the T/B boundary. A–C, TUNEL analysis of spleen sections from HEL (ML5)- or nontransgenic mice that received anergic B cells 18 h before. Spleen sections are stained in blue to detect HEL-binding transferred anergic B cells and in brown for TUNEL-labeled DNA to detect apoptotic cells. Green letters denote: T, T cell zone; B, B cell zone; CA, central arteriole. Numbers in top right corner of each image denote objective magnification. Note that anergic B cells transferred into an ML5 recipient cluster at the T/B boundary (A) while cells transferred into a B6 recipient home to the B cell zone (C). The boxed region in A is shown at higher magnification in B, and D shows quantification of HEL/TUNEL double-positive cells. At a ×20 magnification, 5 white-pulp fields containing large numbers of HEL-binding cells were selected from different spleen sections of each recipient mouse. The objective was then changed to ×40 and the total number of HEL-binding cells and the number of double-positive cells within the visible field was counted. Values graphed are the averages with SD from four different experiments. Only TUNEL-positive cells that were surrounded by a uniform halo of blue HEL staining were counted as double positive. The green arrow in B indicates a cluster of HEL/TUNEL double-positive apoptotic anergic B cells whereas the black arrow denotes a TUNEL-positive HEL-negative cell that would not have been counted. A total of 722 HEL− cells was counted in the nontransgenic recipients, and 907 HEL− cells in the HEL-transgenic recipients.
CXCR5

However, access to this region is CXCR5 dependent, as we fail to see distributed in a pattern that extends several cell layers into the follicle through the region of the follicle near the T zone. Consistent with such region of the follicle distal to the T zone, indicating that expression of /H11001

/HEL

hel/B220+/IgD+/CD4+/CD8+ T cells expressing Fas ligand. In the VH3H9 anti-dsDNA system, Fas deficiency leads to a breakdown in B cell tolerance, an outcome that is secondary to the receipt of T cell help from autoreactive T cells that develop in this system (32, 33). By contrast with these findings, our results assessing the survival of /prpr

IgHHEL

transgenic B cells demonstrate that Fas signaling does not play a role in the competitive elimination of HEL autotigengenerated B cells. An important distinction between the studies described in this article and the previous work in the HEL-system by Rathmell et al. (9, 31) is that the latter studies were performed with anergic cells in the absence of competitor B cells and under conditions where autotigeng-specific T cells were provided. Anergic B cells were shown to present HEL-peptides to the autoreactive T cells but due to the blunted BCR signals, the anergic cells were not able to resist the killing effect of the Fas ligand induced on the T cell. We have previously shown that competitive elimination of anergic B cells does not involve T cells (21).

Studies in the membrane HEL system, in anti-H-2kβ Ig-transgenic mice and in rheumatoid factor-transgenic mice demonstrated that the elimination of autoreactive B cells could occur via a Fas-independent mechanism (34–36). However, in each of these systems rapid elimination of peripheral Ig-transgenic B cells is nearly complete even in the absence of competitor B cells. Instead, it appears that the strong BCR signal may be sufficient to promote rapid B cell death (in the absence of sufficient rescuing signals from Th cells). By contrast, many soluble HEL-binding anergic B cells can survive for >1 wk, and their rapid elimination only occurs when they are forced to compete with a larger compartment of non-HEL-binding B cells, indicating that BCR signaling alone is not sufficient to promote their elimination and that some extrinsic factor must be involved. Our experiments exclude the possibility that competitor B cells cause anergic cells to die due to increased exposure to Fas ligand.

In the original studies describing the fate of anergic HEL-binding B cells under conditions of competition, a strong correlation was observed between exclusion of the cells from follicles and rapid elimination of the cells (7, 10). Both the exclusion and elimination depended on BCR engagement and the presence of competitor non-autotigengenerated B cells. These observations led to a model suggesting that B cells compete for access to trophic factor-containing niches within the follicle (7). A long-term goal of our studies has been to follow up on those initial observations and test the relationship between follicular exclusion and competitive elimination. By characterizing the mechanism for follicular exclusion, we have now been able to perform an experiment to directly test the model, asking whether CCR7-deficient anergic cells that are not excluded from follicles are still rapidly eliminated. These experiments have demonstrated that follicular exclusion is not a prerequisite for competitive elimination of anergic HEL-binding B cells, indicating that these are separable processes.

One model consistent with our data is that B cells compete for a widely expressed or secreted factor. A potential candidate for such a factor is the TNF family ligand, BAFF (B cell activating factor belonging to the TNF family), which has been demonstrated to play a critical role as a survival factor for mature B cells (37). BAFF is a widely expressed molecule, produced in both soluble and membrane bound forms and its availability in the animal appears to be limiting as manipulations that increase or decrease BAFF levels lead to increases or decreases in B cell numbers, respectively (37). We are currently testing whether anergic B cells have a reduction in their ability to respond to BAFF. However, we must also consider the possibility that counterbalancing survival and death-inducing signals masked our ability to detect the effects of localization on anergic B cell survival. For example, it has been likely that other changes occur within anergic cells that contribute to shifting the balance of chemokine responsiveness. Members of the regulator of G protein-signaling (RGS) protein family, including RGS-1, RGS-3, and RGS-14, negatively regulate Gi protein signaling and antagonize chemokine responses (26, 27). The finding that anergic HEL-binding B cells have altered RGS-1, RGS-3, and RGS-14 expression provides an indication of the types of additional change that may contribute to anergic B cell localization (27, 28).

Although reduced expression of CXCR5 contributes to follicular exclusion of autotigengenerated B cells, the continued expression of CXCR5 on these cells contributes to their positioning at the T/B zone interface as CXCR5+/− anergic B cells become more uniformly distributed throughout the T zone than CXCR5+/+ cells. Reciprocally, although up-regulation of CCR7 is not required, the presence of this receptor is necessary for anergic B cells to move to the T/B boundary. Rather than moving to this boundary, naive CXR7−/−IgHEL+ B cells that are acutely activated by exposure to Ag relocate to the outer rim of the follicle, near the marginal zone (12). This was also seen with anergic CXR7−/−IgHEL+ B cells (Fig. 3) although not as prominently as for acutely activated B cells, suggesting that BCR signal strength determines the propensity for this effect. We speculate that Ag-engaged B cells have increased expression of a receptor for an attractant emanating from the marginal zone area. It is also of note that in mice containing a mixed population of naive wildtype and CXCR5−/− B cells, the CXR7−/− cells tended to occupy the region of the follicle distal to the T zone, indicating that expression of CXCR7 contributes to the propensity of naive B cells to migrate through the region of the follicle near the T zone. Consistent with such a requirement, we have routinely observed that CCL21/SLC protein is distributed in a pattern that extends several cell layers into the follicle (Ref. 29 and S. A. Luther and J. G. Cyster, unpublished observations). However, access to this region is CXCR5 dependent, as we fail to see CXCR5−/− B cells traveling into this region. Therefore, B cell migration in the region of the follicle closest to the T zone appears to involve B cells responding to both CCR7 and CXCR5 ligands. Signaling induced by the Fas/Fas ligand interaction plays an important role in maintaining B cell tolerance either directly or indirectly because when the lpr mutation is crossed onto numerous different strains of mice it results in the induction of autoantibodies (30). Studies by Rathmell et al. (9, 31) demonstrated that anergic HEL-binding B cells are sensitive to Fas-mediated killing and under some circumstances they can be induced to die by activated CD4+ T cells expressing Fas ligand.
observed that excluded autoreactive B cells have an enhanced survival due to the presence of T cells (21). Bruton’s tyrosine kinase-deficient B cells also show a strong dependence on T cells for survival (38). These effects appear to be independent of T cell Ag recognition but the T cell-derived signals involved are not defined. Thus, although CCR7−/− anergic B cells may be relieved of some of the selective pressures associated with exclusion from the B cell follicle, they may simultaneously be deprived of survival signals that are available at the T/B boundary. These signals may include the CCR7 ligands themselves. Further research will be required to address these possibilities.

It is notable that, although exclusion from follicles and localization at the T/B boundary is not a prerequisite for rapid elimination of anergic B cells, the cells undergo apoptotic death in this location. This is consistent with the previous finding that anergic B cells rescued by a Bcl-2 transgene remained at the T/B boundary, and that anergic B cells may be relieved of some constraint in this location. A study tracing the fate of anergic B cells revealed that anergic B cells did not undergo apoptosis at the T/B boundary (39).

In summary, although follicular exclusion and rapid elimination of anergic B cells both depend on competition with non-autoantigen-binding B cells, the two processes are at least partially separable. We propose that the requirement for competitor B cells to cause follicular exclusion of autoantigen-binding cells is somehow related to the balance of chemokine responsiveness, perhaps reflecting the differences in responsiveness to CXCL13 of naive and anergic B cells. The requirement for competitor B cells to promote rapid elimination of anergic B cells is likely to involve competition for a limiting survival factor, such as BAFF, through a mechanism that does not depend solely on altered distribution of anergic cells. Such a mechanism may help ensure that autoreactive B cells are eliminated in a competitive manner wherever they are located.

Acknowledgments

We thank Karin Reif for comments on the manuscript.

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