The Fate of Self-reactive B Cells Depends Primarily on the Degree of Antigen Receptor Engagement and Availability of T Cell Help

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Summary

Self-reactive B cells from tolerant double-transgenic (Dbl-Tg) mice coexpressing hen egg lysozyme (HEL) and rearranged anti-HEL immunoglobulin genes have a relatively short life span when compared to normal B cells, irrespective of whether they are exposed to antigen in multivalent membrane-bound form (mHEL-Dbl-Tg mice) or soluble form (sHEL-Dbl-Tg mice). The factors responsible for determining the fate of these B cells after encounter with self-antigen were investigated using a cell-tracking technique in which anti-HEL Ig-Tg spleen cells were labeled with the intracellular dye 5-carboxyfluorescein diacetate–succinimidyl ester (CFSE) and injected either into non-Tg recipients or a variety of HEL-Tg hosts. In non-Tg recipients, HEL-binding B cells persisted in the circulation and could be detected in the follicles of the spleen for at least 5 d. On transfer into either mHEL-Tg or sHEL-Tg hosts, they underwent activation and then rapidly disappeared from the blood and spleen over the next 3 d, consistent with the short life span reported previously. Immunohistology of spleens from sHEL-Tg recipients indicated that the transferred B cells had migrated to the outer margins of the periarteriolar lymphoid sheath (PALS), where they were detectable for 24 h before being lost. The positioning of B cells in the outer PALS depended on a critical threshold of Ig receptor binding corresponding to a serum HEL concentration between 0.5 and 15 ng/ml, but was not restricted to endogenously expressed HEL in that the same migratory pattern was observed after transfer into non-Tg recipients given exogenous (foreign) HEL. Moreover, bone marrow–derived immature Ig-Tg B cells homed to the outer PALS of sHEL-Tg mice and then disappeared at the same rate as mature B cells, indicating that the stage of maturation did not influence the fate of self-reactive B cells in a tolerant environment. On the other hand, HEL-binding B cells transferred into sHEL-Dbl-Tg recipients persisted over the 3-d period of study, apparently due to insufficient availability of antigen, as indicated by the fact that the degree of Ig receptor downregulation on the transferred B cells was much less than in sHEL-Tg recipients. If T cell help was provided to Ig-Tg B cells at the time of transfer into sHEL-Tg recipients in the form of preactivated $CD4^+$ T cells specific for major histocompatibility complex-peptide complexes on the B cell surface, HEL-binding B cells migrated through the outer PALS of the spleen to the follicle, where they formed germinal centers, or to adjacent red pulp, where they formed proliferative foci and secreted significant amounts of anti-HEL antibody. Taken together, these results indicated that the outcome of the interaction between self-antigen and B cells is largely determined by a combination of the degree of receptor engagement and availability of T cell help.

Autoimmune disorders are frequently characterized by the presence of autoantibodies. The mechanisms whereby B cells are normally prevented from producing such antibodies have been extensively studied in a number of trans-

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/96/05/2313/16 $2.00
Volume 183 May 1996 2313–2328

genic models (1–3), including the hen egg lysozyme (HEL)[1] system (4). In this model, Ig-transgenic (Ig-Tg) mice were created in which the great majority of B cells express high affinity IgM and IgD receptors for HEL. Double-transgenic (Dbl-Tg) mice were then produced by crossing Ig-Tg mice with a second line of transgenic mice expressing HEL as a neo-self antigen in soluble (sHEL-Tg) (4) or membrane-bound (mHEL-Tg) form (5). The fate of newly generated self-reactive B cells in these mice was shown to be critically dependent on the degree of receptor engagement by self antigen. Thus, after exposure to self-antigen in membrane-bound form, B cells were deleted in the bone marrow with a life span of ~15 h (5, 6). By contrast, B cells exposed to soluble self-antigen in oligovalent form persisted and colonized the splenic follicles in a tolerant (anergic) state characterized by downregulation of surface IgM, but not IgD (4). Nevertheless, these cells failed to enter the long-lived B cell compartment and had a markedly reduced life span of ~3–5 d compared with 4–5 wk for normal B cells (7). This reduction in life span was a constant feature of tolerant (self-reactive) B cells throughout life, whereas the mean half-life of nontolerant normal B cells increased with age, presumably because of the ongoing recruitment of cells into the long-lived compartment (8).

The interpretation of these findings favored by our group was that the longevity of self-reactive B cells is related to the degree of receptor engagement by self-antigen (8). Based on a different series of experiments in the same transgenic model, Cyster et al. (9) have presented an alternative hypothesis to explain why self-reactive B cells may be eliminated on reaching peripheral lymphoid tissue. This states that self-reactive B cells are normally excluded from primary follicles and die as a result of competition with the anti–foreign (polyclonal) B cell repertoire. To distinguish between these possibilities, the fate of self reactive B cells in vivo was studied in a series of cell-tracking experiments in which mature or immature Ig-Tg B cells were labeled with the fluorescent dye 5-carboxyfluorescein diacetate–succinimidyl ester (CFSE) (10) and transferred into transgenic recipients expressing HEL as an endogenous (neo-self) antigen or non-Tg recipients in the presence or absence of HEL given as an exogenous (foreign) antigen. The advantage of using CFSE is that labeled cells can be distinguished from host lymphocytes and that up to five to six cell divisions can be readily detected by progressive twofold reductions in CFSE intensity. In the absence of antigen, the Ig-Tg B cells populated the follicles of non-Tg hosts, but on transfer into Tg recipients expressing HEL above a critical threshold, they became activated and migrated to the outer periarteriolar lymphoid sheath (PALS), after which they disappeared within 3 d without making antibody, irrespective of their stage of maturation. The rapid loss of Ig-Tg B cells did not occur on transfer into sHEL-Dbl-Tg recipients, apparently because of insufficient antigen binding by their Ig receptors. When the B cells were given cognate T cell help at the time of transfer into sHEL-Tg recipients, however, they migrated through the outer PALS into the follicles and red pulp and secreted significant amounts of anti-HEL antibody. Thus, the fate of self-reactive B cells depended both on the degree of Ig receptor engagement by self-antigen and on the availability of T cell help.

[1]*Abbreviations used in this paper:* CSFE, 5-carboxyfluorescein diacetate–succinimidyl ester; Dbl-Tg, double-transgenic mice; GC, germinal center; Ig-Tg, Ig-transgenic mice; HEL, hen egg lysozyme; mHEL, membrane-bound form of HEL; OP, outer PALS; PALS, periarteriolar lymphoid sheath; sHEL, soluble form of HEL; TBS, Tris-buffered saline.

Materials and Methods

Mice. Transgenic mouse lines and conventional inbred C57BL/6 (B6) and (B6 × B10.BR)F_1 strains of mice were housed under standard conditions in the Institute's animal facility. Hemizygous Ig-Tg and HEL-Tg lines were maintained on a B6 or (B6 × B10.BR)F_1 background by back-crosses with nontransgenic mice. The Ig-Tg mice belonged to the MD4 line, and the B cells from this line expressed high affinity IgM and IgD receptors for HEL (11). In HEL-Tg mice, HEL was expressed either in soluble form (sHEL-Tg) under control of the mouse metallothionein-1 promoter (lines ML4, ML5) (12) or as an integral membrane protein (mHEL-Tg) under control of the H-2 class I promoter (lines KLK9 and KLK3) (8). Of the two lines of sHEL-Tg mice used, line ML4 expressed low serum levels of HEL (~0.5 ng/ml), but this concentration could be increased to ~100 ng/ml by inducing the metallothionein promoter with 25 mM zinc sulphate in the drinking water (12), while line ML5 expressed higher constitutive levels of HEL (15–20 ng/ml) (7). Unless otherwise stated in the text, sHEL-Tg mice were of the ML5 line. B6 sHEL-Dbl-Tg mice were generated by crossing sHEL-Tg mice with Ig-Tg mice, and were used as recipients of CFSE-labeled cells when they were between 6 and 10 wk old. TCR-Tg mice specific for the COOH-terminal peptide of moth cytochrome C (MCC_{87-103}) in the context of IE^k (13) were bred on an F_1 (B6 × B10.BR) background.

Antigens. HEL (Sigma Chem. Co., St. Louis, MO) was purified as described in (14). A heptadecapeptide (KANERADLIAYLKQATK) identical to the COOH terminus of moth cytochrome C (MCC_{87-103}) was synthesized at the Queensland Institute of Medical Research. TCR-Tg mice were primed with 10 μg MCC_{87-103} i.v. 1 d before harvesting of cells from peripheral lymph nodes and spleens for adoptive transfer.

Antibodies. The following mAbs were used (specificities bracketed): HyHEL5 (HEL [15]); RA3-6B2 (B220 [16]), B3B4 (CD23 [17]), RS3.1 (IgM^a [18]), AF6 15.1 (IgD^a [19]), RL172.4 (CD4 [20]), 3.155 (CD8 [21]), and HO13.4 (Thy 1.1 [22]). HyHEL5 was biotinylated by standard methods. Polyclonal rabbit anti-HEL antiserum was a kind gift from Dr Helen Pritchard-Briscoe (University of Sydney, Sydney, Australia), which was produced by immunizing a rabbit with 1 mg/ml HEL in CFA (Sigma). Before using any antibody preparation, aggregates were removed by centrifugation in an Airfuge™ (Beckman Instruments, Palo Alto, CA) at 28 psi for 15 min.

CFSE Labeling. CFSE (Molecular Probes, Eugene, OR) was diluted to 5 mM in DMSO, aliquotted and stored under desiccating conditions at −20°C until used. Mice were killed by cervical dislocation, and the spleens removed and then gently pressed through a sieve using a syringe plunger. Cells were suspended in RPMI 1640 supplemented with 20 mM Hepes, 0.01 M sodium bicarbonate, 50 mg/liter penicillin, 100 mg/liter streptomycin, and 10% FCS (Commonwealth Serum Laboratories [CSL], Vic-

toria, Australia); this medium will subsequently be referred to as RPMI/10% FCS. Cells were spun down and resuspended in Tris-ammonium chloride lysis buffer solution (2.06 g/liter Tris and 7.47 g/liter ammonium chloride, pH 7.2) for 3 min at room temperature. After underlaying the suspension with FCS, the cells were pelleted by centrifugation and washed once in RPMI 1640 (without supplemental protein), then resuspended at 5×10^7 cells/ml in RPMI 1640 prewarmed to 37°C. The CFSE stock solution was diluted $1/10$ in RPMI 1640 and added to the warmed cell suspension. Different CFSE doses were used to distinguish between cells of Ig-Tg versus non-Tg origin. To achieve the higher fluorescence intensity, 10 μl/ml of the diluted CFSE solution was added to the cell suspension, whereas for the lower intensity, 2.5 μl/ml was used. The suspension was incubated with CFSE for 10 min at 37°C, with inversion every 3–4 min, after which several volumes of ice-cold RPMI/10% FCS were added to stop the reaction. Cells were then washed twice in the same medium and mixed with cells labeled with the alternate CFSE intensity. The ratio of mixing of non-Tg and Ig-Tg cells, as well as the number of cells injected intravenously into recipient mice, is stated in the figure legends. To assist with flow cytometric analysis in certain experiments, the Ig-Tg cells were labeled at one or other CFSE intensity.

Preparation and Staining of Cell Suspensions from Recipient Mice. At each time point after transfer of CFSE-labeled cells, mice for analysis were anesthetized with diethyl ether, bled from the tail vein (0.5 ml into 30 μl heparin or 0.5 ml of Alsever's solution), and killed by cervical dislocation. The spleen and both inguinal lymph nodes (where needed) were removed and placed in PBS supplemented with 2% FCS and 0.1% sodium azide ("staining medium") at 4°C. Approximately one quarter of each spleen was embedded in Tissue Tek OCT (Bayer, Elkhart, IN), snap-frozen in liquid nitrogen, and stored at −70°C, while cell suspensions were made from the lymph nodes and remainder of the spleen. A suspension of PBLs was prepared by centrifuging blood over Ficoll-Hypaque, density 1.0875. Cell suspensions from the spleen, lymph node, and blood were washed twice in staining medium and then labeled with fluorescent antibodies. HEL-binding cells were detected by a three-stage sandwich stain: (*a*) HEL (200 ng/ml; Sigma); (*b*) biotinylated HyHEL5; and (*c*) streptavidin-PE (Caltag Laboratories, South San Francisco, CA), streptavidin–Quantum red (Sigma), or streptavidin APC (Molecular Probes). B220-expressing cells were identified by staining with unconjugated RA3-6B2 then Texas red– or PE-conjugated goat anti–rat IgG (Caltag). After resuspending in staining medium (for immediate analysis) or 1% paraformaldehyde in PBS (for analysis the next day), cells were analyzed on a FACStar™ or FACScan™ flow cytometer (Becton-Dickinson & Co., Mountain View, CA). Listmode data were collected with or without live gating on $CFSE^+$ cells on 10,000–20,000 cellular events and were analyzed using LYSIS 2.0 software (Becton Dickinson).

Immunohistology. Frozen tissue samples were sectioned at 5 μm and allowed to dry overnight before storage at −20°C in foil. For staining, the slides were thawed, fixed in acetone (10 min), and washed twice in Tris-buffered saline (TBS; 7.88 g/liter Tris-HCl and 8.76 g/liter sodium chloride, pH 7.6). Staining was then carried out in 30-min stages (shielded from the light) with two 5-min washes between each. All washes were done with TBS, except for the wash immediately before application of the first antibody, in which the TBS was supplemented with 30% horse serum (CSL). After staining, slides were stored in antifade (2.66 g/liter 1,4-diazabicyclo-[2.2.2]octane [DABCO] in 90% glycerol and 10% PBS) and photographed on a Leitz DMRBE fluorescent microscope (Leica, Wetzlar, Switzerland). HEL-binding cells were detected by a three-stage sandwich stain: (*a*) HEL; (*b*) rabbit-anti-HEL antiserum; and (*c*) sheep anti–rabbit Ig $F(ab')_2$ directly conjugated to fluorescein (Silenus Laboratories, Hawthorn, Victoria, Australia). B220 cells were stained using RA3-6B2 and Texas red–conjugated goat anti–rat IgG (Caltag).

Depletion of Mature B Cells from the Bone Marrow. Bone marrow was harvested from both the tibiae and femora of donor mice by two-directional medullary flushing with HBSS supplemented with 10% FCS (HBSS/10% FCS). After washing twice in this medium, cells were resuspended at ~30 million cells/ml in a 1/100 dilution of biotinylated anti-CD23. Cells were incubated at 4°C for 20 min, then washed twice and resuspended at 30 million cells/ml. Streptavidin-coated magnetic beads (M280-SA; Dynal, Oslo, Norway) were added at a bead/cell ratio of 1:1, which generated a bead/target cell ratio of ~20–30:1. After 30 min of continuous rotation at 4°C, bound cells were removed by two rounds of magnetic depletion (2 min, then 5 min), and were resuspended in either 1 ml heparinized syngeneic nontransgenic mouse blood (to reduce cell clumping) for immediate injection or in RPMI 1640 for CFSE labeling.

Bone Marrow Chimeras. ML5 sHEL-Tg mice aged at least 3 mo were lethally irradiated (950 Rad) and reconstituted by intravenous injection of a minimum of 2.5×10^6 bone marrow cells from MD4 Ig-Tg donors, prepared as described previously (7). The resultant chimeras were used 7 wk after reconstitution.

Culture of Ig-Tg B Cells in Mouse Sera. Spleen cells from MD4 Ig-Tg mice were harvested in RPMI 1640. 1 million cells (25 μl) were added to 150 μl normal mouse serum, serum from sHEL-Tg mice, serum from MD4 × ML5 sHEL-Dbl-Tg mice, RPMI/10% FCS, or RPMI/10% FCS with 20 ng/ml HEL. After incubation at 37°C in 5% CO_2 for 5 h, the cells were stained for expression of IgM^a (biotinylated RS3.1 and streptavidin–Quantum red [Sigma]), IgD^a (fluoresceinated AMS 15.1), and B220 (RA3-6B2 and PE-conjugated goat anti–rat IgG [Caltag]). Three-color analysis was performed on a FACScan®.

Purification of B Cells from Spleen and Peptide Pulsing. Spleen cells from mice on a B6 or F_1 background were depleted of adherent cells by panning on plastic petri dishes (Becton Dickinson) at 37°C for 1 h. The nonadherent cells were gently resuspended, then incubated on ice with rat IgM antibodies to CD4, CD8, and Thy 1.1 (see above), followed by a 30-min incubation with young rabbit complement (C-six Diagnostics, Mequon, WI) at 37°. The cells were washed with cold medium and assessed for purity by flow cytometry, which showed that $CD4^+$ and $CD8^+$ cells represented less than 1% of purified splenocytes. The cells were then resuspended at 5×10^7/ml and were incubated for 2 h at 37°C in RPMI 1640 containing 1 μM MCC_{87-103}. CFSE was added for the final 10 min of the incubation, as described previously, and the cells were then washed three times in cold RPMI before adoptive transfer.

Serum HEL and Anti-HEL Antibody Assays. Anti-HEL IgM^a antibodies were detected by ELISA. 96-well flat-bottomed plates were coated with 10 μg/ml HEL in NPP buffer (1.54 g/liter sodium carbonate, 2.43 g/liter sodium bicarbonate, and 20 mg/liter magnesium chloride.$6H_2O$, pH 9.6), washed in PBS with 0.05% Tween 20, and blocked with 1% BSA (Pentex; Miles Inc., Kankakee, IL) in PBS. Serum samples were added and bound IgM^a was detected with biotinylated RS3.1 in the presence of 1% FCS and 1% skim milk. After a further incubation with alkaline phosphatase–conjugated avidin (Sigma), the plates were developed with 1 mg/ml *p*-nitrophenyl phosphate disodium (Sigma) in NPP buffer. Optical density readings (OD 405 nM) were stan-

dardized against a purified antibody from an anti-HEL IgM^a hybridoma. The serum concentrations of HEL were measured as described previously (7).

Results

Short Life Span of Splenic Ig-Tg B Cells on Encounter with Soluble or Membrane-bound Self-Antigen. The fate of mature Ig-Tg B cells upon encounter with endogenous self-antigen was determined by injecting CFSE-labeled spleen cells from Ig-Tg mice into sHEL-Tg, mHEL-Tg, or non-Tg recipients. Before injection, labeled cells were mixed with spleen cells from non-Tg mice labeled with CFSE at a lower fluorescence intensity that served as an internal control. 18 h after transfer, CFSE$^+$ B cells made up ~1.4% of host lymphocytes in the spleens of non-Tg recipients, a value that decayed slowly over the subsequent 2 d (data not shown). The number of Ig-Tg B cells relative to non-Tg B cells, however, remained constant throughout the 3-d period of observation in the spleen, blood, and lymph nodes (Figs. 1 and 2). By contrast, in sHEL-Tg recipients, there was an early loss of Ig-Tg B cells from the blood and prominence of the same cells in the spleen at day 1 (Figs. 1 and 2), followed by their disappearance during the next 48 h. The disappearance of Ig-Tg B cells from the blood was examined at earlier time points (6–18 h), and was found to be complete by 12 h (Fig. 3). In mHEL-Tg recipients, progressive loss of transferred Ig-Tg cells from both the blood and spleen occurred from the earliest time point (Figs. 1 and 2). Cell surface Ig on transferred B cells showed down-regulation in both sHEL and mHEL recipients, consistent with antigen encounter (Fig. 1). In neither type of recipient was the disappearance of Ig-Tg B cells from the spleen accompanied by late migration to the lymph nodes (Fig. 2, *lower graph*), nor was there any suggestion of cell division, which would have reduced the CFSE intensity per cell (Fig. 1). Thus, cell loss was most likely to have resulted from death in situ. Interestingly, the HEL-binding B cells increased in size in sHEL-Tg recipients and markedly so in mHEL-Tg recipients by comparison with non–HEL-bind-

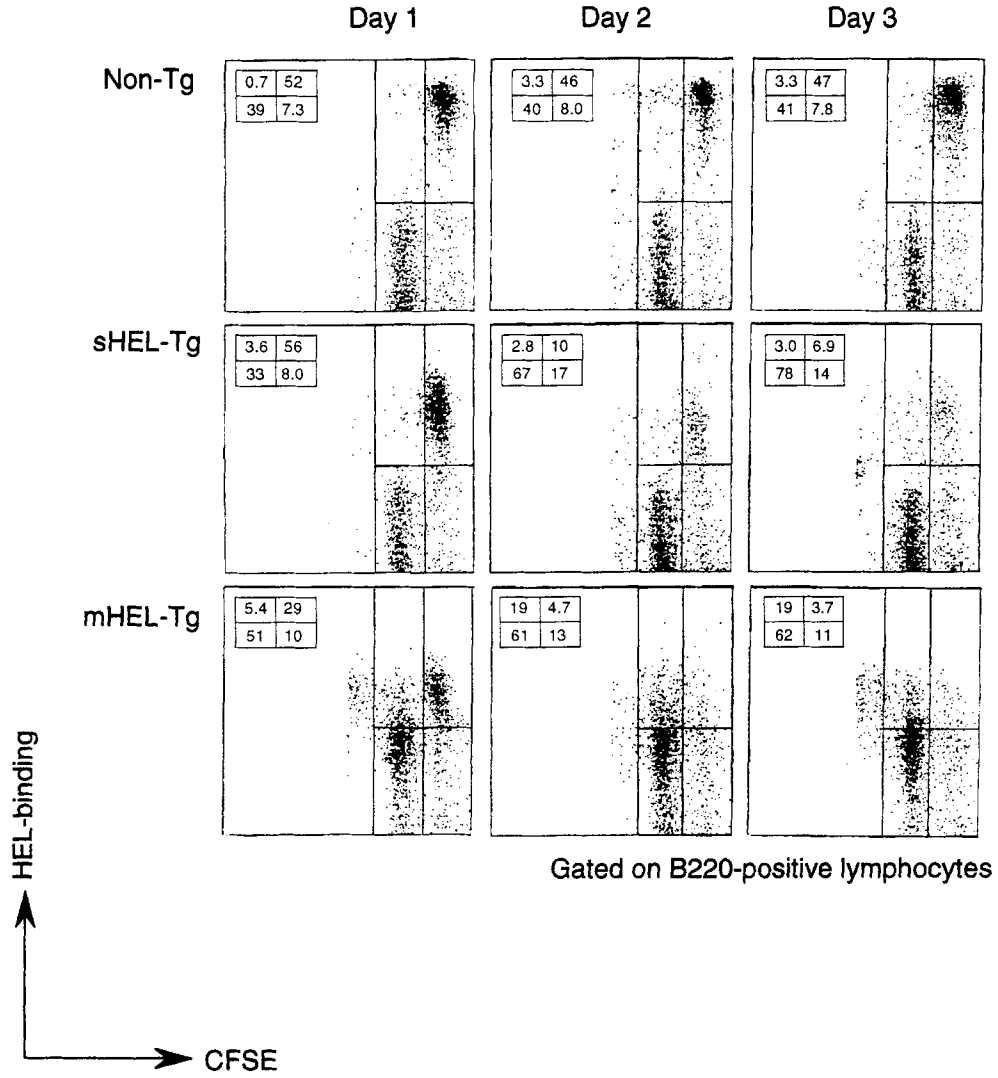


Figure 1. Dot plots of transferred CFSE$^+$ B cells in the spleens of recipient non-Tg (*upper row*), ML5 sHEL-Tg (*middle row*), and KLK9 mHEL-Tg (*lower row*) mice over a 3-d time course. Donor spleen cells from MD4 Ig-Tg mice were labeled CFSE^{hi} and mixed in a ratio of 2.7:1 with spleen cells from non-Tg mice labeled CFSE^{lo}. 30 million cells (based on the prelabeling counts) were injected intravenously; of these, 50–60% were B220$^+$. Data were collected by live gating on CFSE intensity, with subsequent analysis gating on small lymphocytes (by light scatter) and B220 expression. In non-Tg and sHEL-Tg recipients, a minor population of CFSE^{lo} B cells bind HEL at high levels; these presumably represent B cells from Ig-Tg donors that have labeled inefficiently with CFSE. In mHEL-Tg recipients, the non-Tg B cells all show low level HEL-binding, possibly because of the acquisition of small amounts of recipient-derived mHEL after transfer.

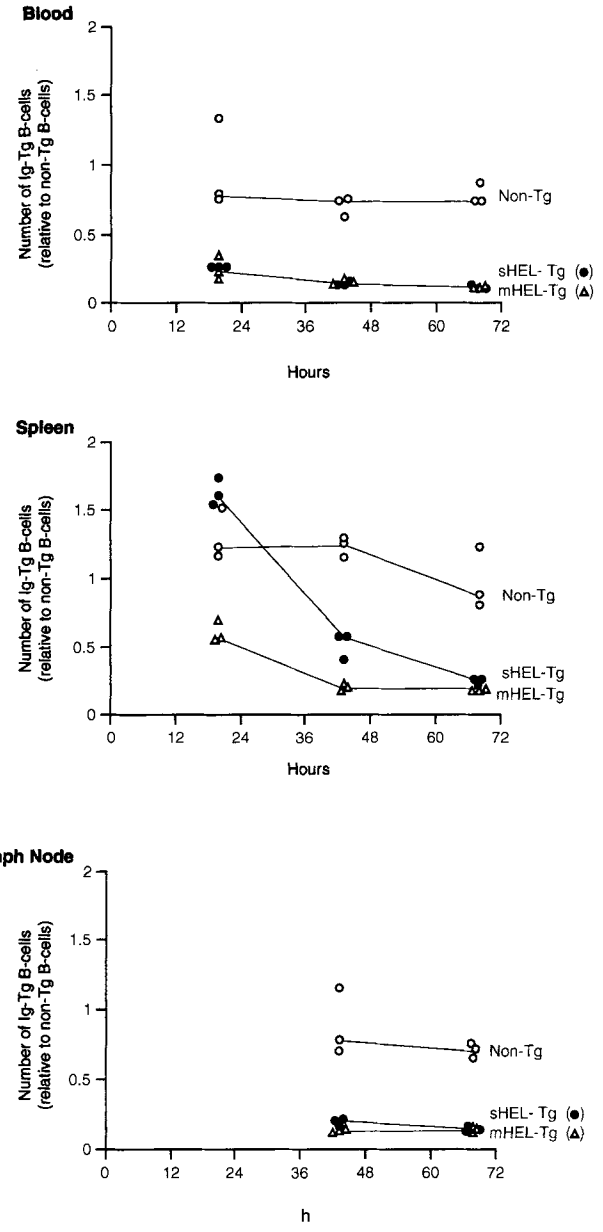


Figure 2. Numbers of Ig-Tg B cells relative to non-Tg B cells after transfer into non-Tg (*open circles*), sHEL-Tg (*closed circles*), or mHEL-Tg (*open triangles*) recipients harvested from the blood (*top*), spleen (*middle*), or lymph node (*bottom*) in the experiment detailed in the legend to Fig. 1. Lines are drawn through median values.

ing control cells from the same mice (Fig. 4), a finding that was suggestive of B cell activation immediately before their disappearance.

Ig-Tg Splenic B Cells Migrate to the Outer PALS (OP) on Encounter with Soluble Antigen. The microanatomical positioning of CFSE$^+$ Ig-Tg B cells during the first 3 d after transfer was determined by staining frozen sections of spleens from non-Tg and sHEL-Tg recipients. In non-Tg recipients, HEL-binding B cells were present in the follicles throughout the study period (Fig. 5, *a* and *b*), whereas in sHEL-Tg recipients, they were largely confined to the outermost margins of the PALS at its interface with the B cell

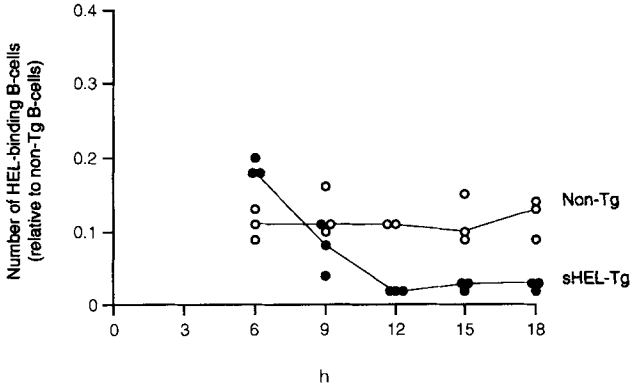


Figure 3. Numbers of HEL-binding Ig-Tg B cells relative to cotransferred non–HEL-binding non-Tg B cells in the blood during the first 18 h after intravenous injection into non-Tg (*open circles*) or sHEL-Tg (*closed circles*) recipients. Spleen cells from Ig-Tg mice were labeled CFSE^{lo} and mixed in a ratio of 1:1 with non-Tg spleen cells labeled CFSE^{hi}. Approximately 40 million cells (based on postlabeling counts) were injected. The number of CFSE^{lo} HEL-binding B cells is expressed relative to the number of CFSE^{hi} non–HEL-binding B cells. Lines are drawn through median values.

zone (Fig. 5, *c* and *d*). By day 3, HEL-binding B cells had decreased markedly in number, consistent with the previous quantitative analysis by flow cytometry (Fig. 2). The localization of Ig-Tg B cells after transfer into mHEL-Tg recipients could not be as readily determined because expression of mHEL by host B cells interfered with the detection of anti–HEL-binding activity on transferred B cells.

Movement into the OP Depends on Ig Receptor Engagement Reaching a Critical Threshold. The increase in the size of Ig-Tg B cells (Fig. 4) and downregulation of their Ig receptors (Fig. 1) after transfer into HEL-expressing recipients suggested that Ig receptor engagement by antigen was a prerequisite for migration into the OP. To confirm this, Ig-Tg B cells were transferred into mice from a different sHEL-Tg line (ML4) that expresses HEL at concentrations below the threshold needed to tolerize the B cell compartment (12). In other words, the B cells in these mice were "ignorant" of self-antigen. As shown in Fig. 5 *e*, the transferred HEL-binding B cells were detected in the follicles, where they persisted during the next 2 d. In a second group of ML4 mice, use was made of the inducibility of the metallothionein promoter to increase the levels of endogenous HEL by zinc feeding (12). This manuever resulted in relocation of HEL-binding B cells to the OP (Fig. 5 *f*) in a manner identical to that observed in ML5 sHEL-Tg recipients expressing higher resting levels of antigen (Fig. 5 *c*).

Thus, the positioning of self-reactive B cells in the OP was shown to depend on engagement of surface Ig by antigen, provided its concentration exceeded a critical threshold, whereas B cells exposed to antigen below that threshold were found in the follicles. These observations raised the question of why a significant number of B cells are found in the follicles of tolerant sHEL-Dbl-Tg mice (11) expressing sufficient levels of self-antigen to engage the Ig receptor. To investigate this paradox, CFSE^{lo} Ig-Tg spleen cells were cotransferred with CFSE^{hi} non-Tg spleen cells

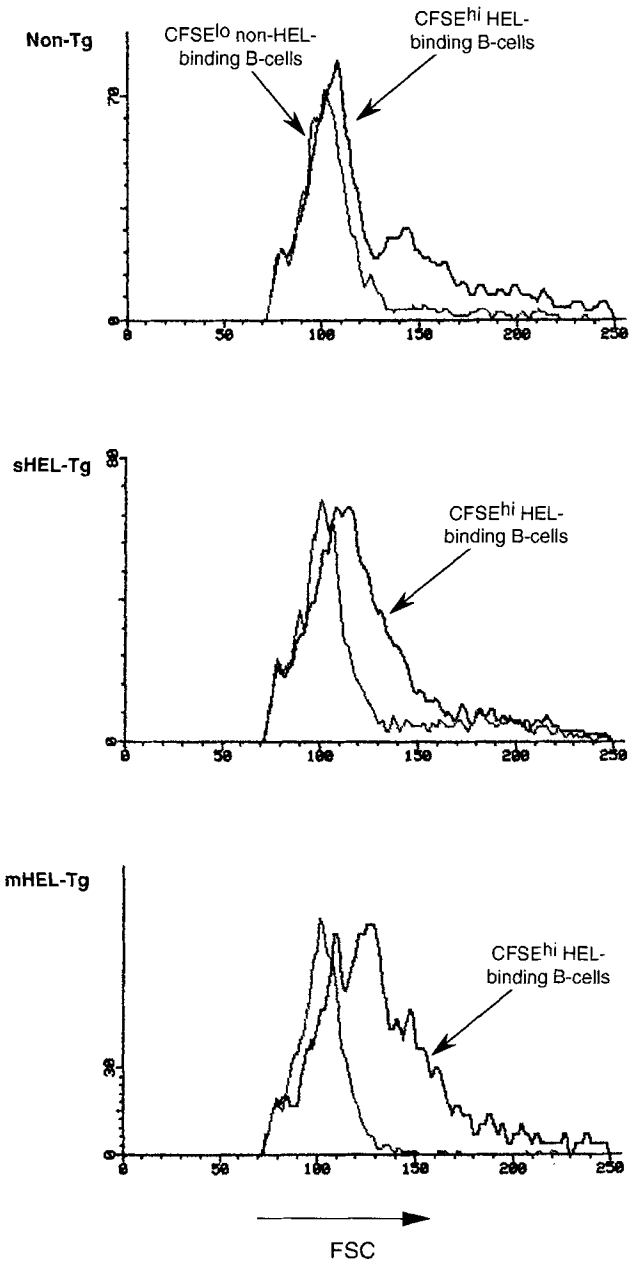


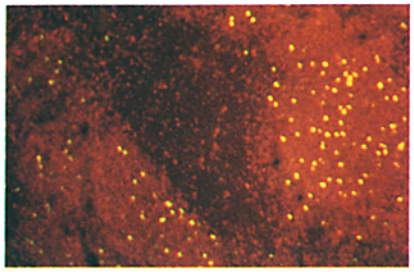
Figure 4. Relative cell size (by forward light scatter) of $CFSE^{lo}$ non-HEL-binding B cells (left histograms as labeled) and $CFSE^{hi}$ HEL-binding B cells (*right histograms*) after transfer into non-Tg (*top*), sHEL-Tg (*middle*), and mHEL-Tg recipients (*bottom*) in the experiment detailed in the legend to Fig. 1.

into three groups of recipients: (*a*) control non-Tg mice; (*b*) sHEL-Tg mice; and (*c*) sHEL-Dbl-Tg mice. 6–10 wk-old recipients were chosen to minimize the number of non-HEL-binding B cells of normal life span that might compete with short-lived HEL-binding (self-reactive) B cells in the follicle (9). In sHEL-Tg recipients, transferred HEL-binding B cells rapidly disappeared from the spleen as before, whereas the cells persisted in the other two groups of recipients (Fig. 6 *A*). Similar results were obtained when tolerant sHEL-Dbl-Tg spleen cells were substituted for Ig-Tg donor spleen cells (Fig. 6 *B*). Interestingly, downregulation of surface Ig on HEL-binding B cells from sHEL-Dbl-Tg recipients was much less (about twofold) compared with sHEL-Tg recipients (10–20-fold, Fig. 6 *C*), suggesting that receptor engagement by antigen was not equivalent in the two types of recipients. Since sHEL-Dbl-Tg mice contain residual amounts of anti-HEL antibody and large numbers of anti-HEL B cells to which HEL can bind (4), the effective antigen concentration in these mice might have been lower than in sHEL-Tg recipients. The finding that the mean concentration of HEL detected by ELISA was 9.5 ng/ml in serum from sHEL-Dbl-Tg mice and 15 ng/ml in serum from sHEL-Tg mice is consistent with this conclusion. To demonstrate formally a reduction in the level of available antigen, Ig-Tg spleen cells were cultured in serum obtained from sHEL-Tg or sHEL-Dbl-Tg mice. After 5 h, downregulation of surface IgM^a and IgD^a was much greater on B cells exposed to serum from sHEL-Tg donors than on B cells exposed to sHEL-Dbl-Tg serum (Fig. 6 *D*). Taken together, these findings suggested that migration of transferred Ig-Tg B cells into the outer PALS required levels of available antigen higher than those present in sHEL-Dbl-Tg mice.

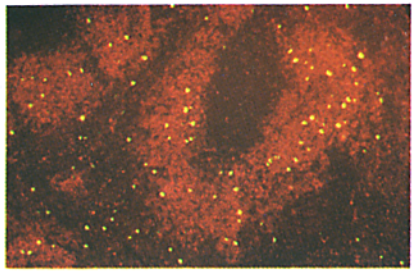
Relocation of Mature Ig-Tg B Cells in Response to Endogenous Antigen Is Identical to Antigen Administered Exogenously. The positional changes of mature HEL-binding B cells upon encounter with endogenous (self) HEL appeared to be identical to the behavior of B cells reported previously after encounter with exogenous (foreign) antigen (23). To confirm this in the current model, unlabeled Ig-Tg B cells were injected into either sHEL-Tg recipients or non-Tg recipients which were then challenged with 50 μg i.p. of exogenous HEL, a dose sufficient to give a serum concentration in excess of 15 ng/ml for ~4 h (data not shown). The repositioning of Ig-Tg B cells in the OP was identical whether the antigen encountered was endogenous (transgenic) or given by injection (Fig. 5, *i–k*). This experiment

Figure 5. Fluorescent micrographs of frozen sections from spleens of recipients after injection of spleen cells from Ig-Tg mice. HEL-binding cells are stained green and $B220^+$ cells are stained red. (*a–d*) CFSE-labeled spleen cells from Ig-Tg mice were injected into non-Tg (*a* and *b*) or sHEL-Tg recipients (*c* and *d*). Two time points are shown, day 1 (*a* and *c*) and day 3 (*b* and *d*). (*e* and *f*) 40 million unlabeled spleen cells from an Ig-Tg mouse were injected into recipients from the low HEL-expressing line of sHEL-Tg mice (ML4), which were then given either ordinary drinking water (*e*) or 25 mM zinc sulphate (*f*) from day 1 to 3, at which time spleens were harvested. (*g* and *h*) Bone marrow cells from Ig-Tg mice were depleted of $CD23^+$ mature B cells and resuspended in syngeneic non-Tg mouse blood. 180 million unlabeled leukocytes were injected into non-Tg (*g*) or sHEL-Tg (*h*) recipients, and spleen sections were prepared 1 d later. A small "island" of $B220^+$ cells is seen to indent the PALS in *H.* (*i–k*) 60 million unlabeled spleen cells from an Ig-Tg mouse were injected into either non-Tg recipients given intraperitoneal saline (*i*), sHEL-Tg recipients given intraperitoneal saline (*j*), or non-Tg recipients given 50 μg intraperitoneal HEL (*k*), and spleen sections were prepared 1 d later. (*l*) The spleen is compartmentalized into red and white pulp, the latter containing distinct T and B cell zones. The periarteriolar lymphoid sheath (PALS) is comprised predominantly of T cells and forms a concentric cuff around the central arteriole. The PALS is surrounded by the follicle (*f*) containing $B220^+$ B cells. The red pulp is separated from the white pulp by the marginal zone composed largely of B cells, which in turn is separated from the inner mantle zone by the marginal sinus. T cell–dependent B cell activation occurs in the outer PALS (OP), which leads to B cell differentiation into two anatomically distinct but clonally related populations, namely proliferative foci (PF) and germinal centers (GC). PF appear first, arising as cellular clusters in the outer PALS which evolve into larger aggregations in the bridging zones and red pulp. GC arise when activated B cells penetrate the mantle zone.

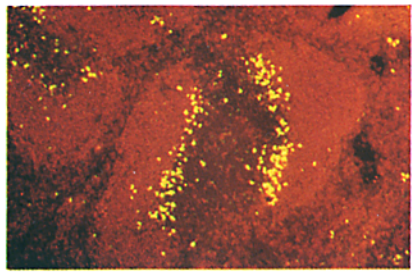
a Ig-Tg spleen → Non-Tg recipient, day 1



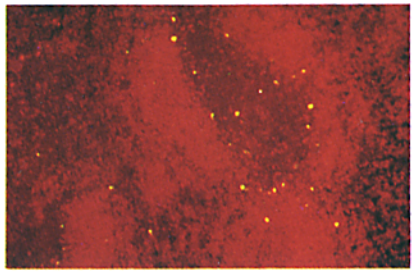
b Ig-Tg spleen → Non-Tg recipient, day 3



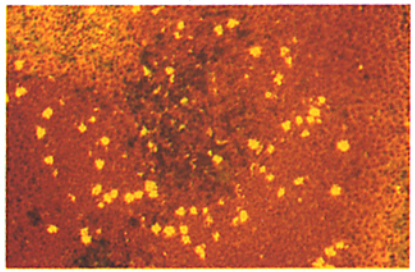
c Ig-Tg spleen → sHEL-Tg recipient, day 1



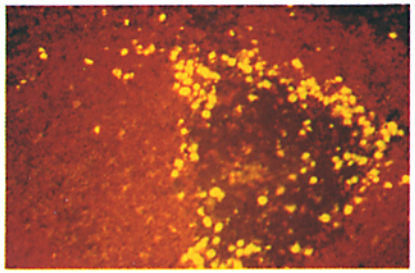
d Ig-Tg spleen → sHEL-Tg recipient, day 3



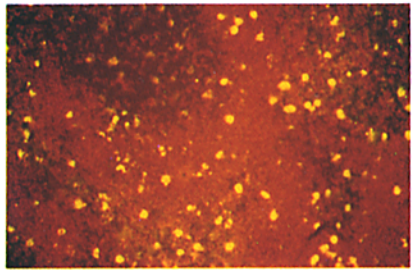
e Ig-Tg spleen → ML4 sHEL-Tg recipient without zinc induction



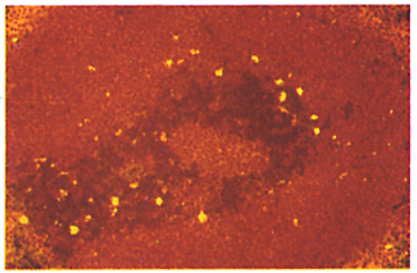
f Ig-Tg spleen → ML4 sHEL-Tg recipient with zinc induction



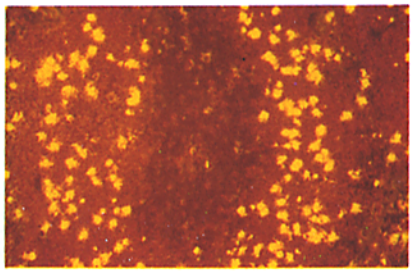
g Bone marrow immature Ig-Tg cells → Non-Tg recipient



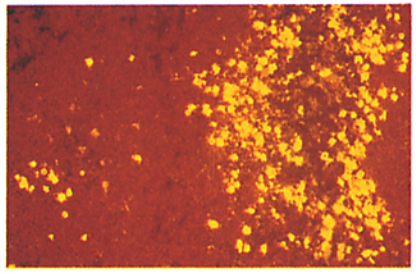
h Bone marrow immature Ig-Tg cells → sHEL-Tg recipient



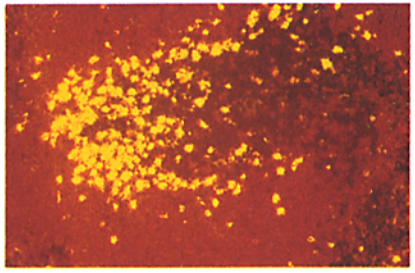
i Ig-Tg spleen → Non-Tg recipient given intraperitoneal PBS



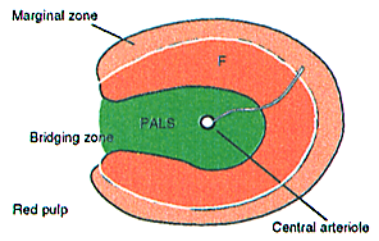
j Ig-Tg spleen → sHEL-Tg recipient given intraperitoneal PBS

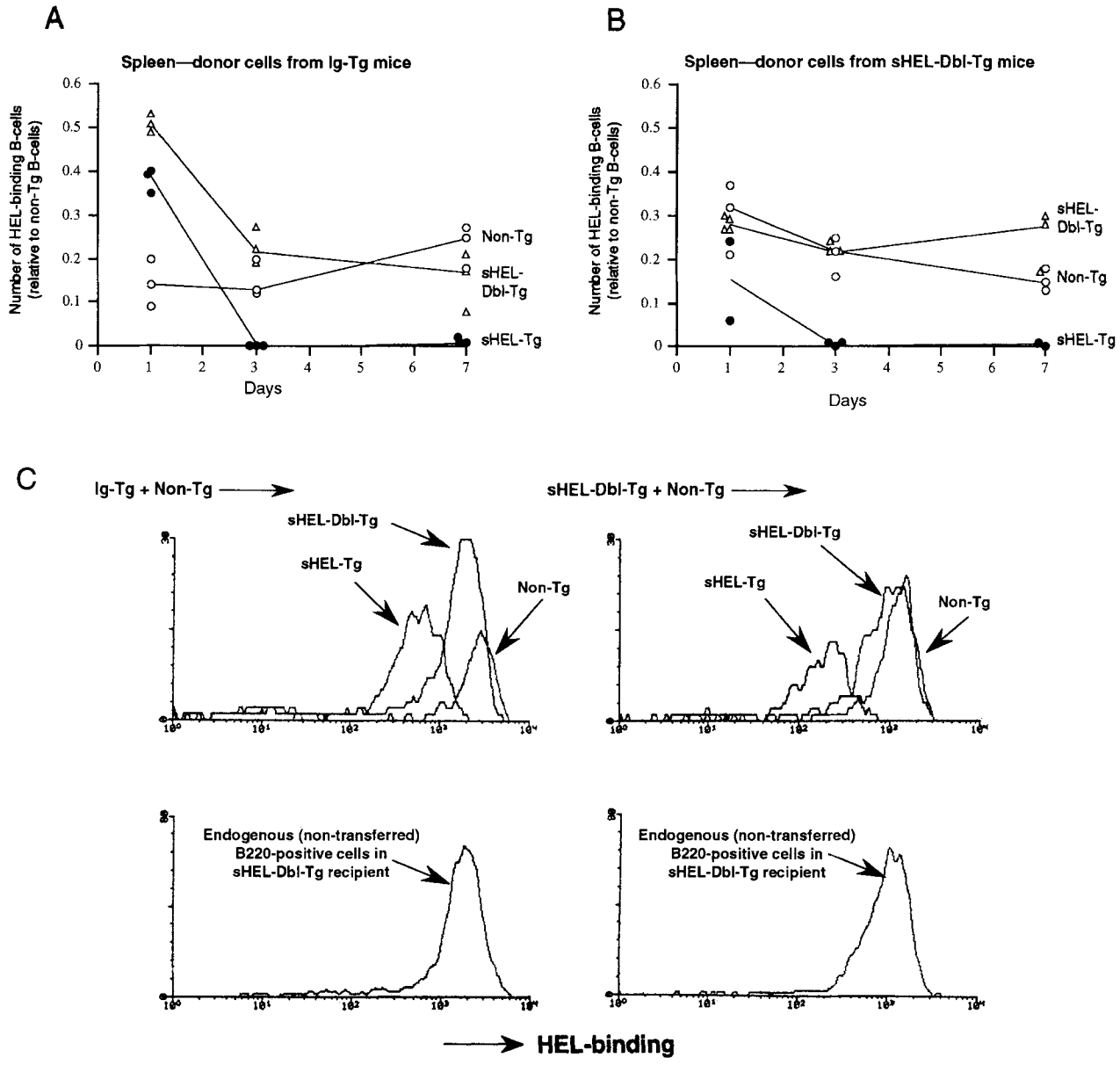


k Ig-Tg spleen → Non-Tg recipient given intraperitoneal HEL



l Schema of splenic follicular structure




A
Spleen—donor cells from Ig-Tg mice
Number of HEL-binding B-cells (relative to non-Tg B-cells)
Days
Non-Tg
sHEL-Dbl-Tg
sHEL-Tg
B
Spleen—donor cells from sHEL-Dbl-Tg mice
Number of HEL-binding B-cells (relative to non-Tg B-cells)
Days
sHEL-Dbl-Tg
Non-Tg
sHEL-Tg
C
Ig-Tg + Non-Tg
sHEL-Dbl-Tg + Non-Tg
sHEL-Dbl-Tg
sHEL-Tg
Non-Tg
Endogenous (non-transferred) B220-positive cells in sHEL-Dbl-Tg recipient
HEL-binding
D
Medium only
Medium + HEL
Normal mouse serum
Serum from sHEL-Tg mice
Serum from sHEL-Dbl-Tg mice
IgDa
IgMa
Gated on B220-positive lymphocytes

also provided further confirmation that CFSE labeling did not influence B cell migratory patterns, since the histological results were similar to those obtained when CFSE-labeled cells were used (Fig. 5, *a–d*).

Repositioning of B Cells in the OP Does Not Depend on the Stage of Maturation. The rapid disappearance of mature Ig-Tg B cells on encounter with antigen after transfer into sHEL-Tg recipients is reminiscent of the short life span of HEL-binding cells when previously measured in tolerant sHEL-Dbl-Tg mice by Brd labeling (7). In the latter mice, however, B cells first encounter self-antigen at an immature stage of their development within the bone marrow (11), whereas in the current experiments, B cells were exposed to self-antigen after reaching maturity. Since immature B cells are thought to be peculiarly sensitive to tolerance induction (24, 25), it was necessary to test whether the stage of maturation might influence B cell positioning and lifespan. This was accomplished by depleting mature $CD23^+$ B cells from suspensions of bone marrow using magnetic beads to leave immature B lineage cells and cells of the other hemopoietic lineages. A mixture of CD23-depleted marrow cells from Ig-Tg and non-Tg mice, labeled with CFSE at different fluorescence intensities, was transferred into non-Tg, sHEL-Tg, or mHEL-Tg recipients.

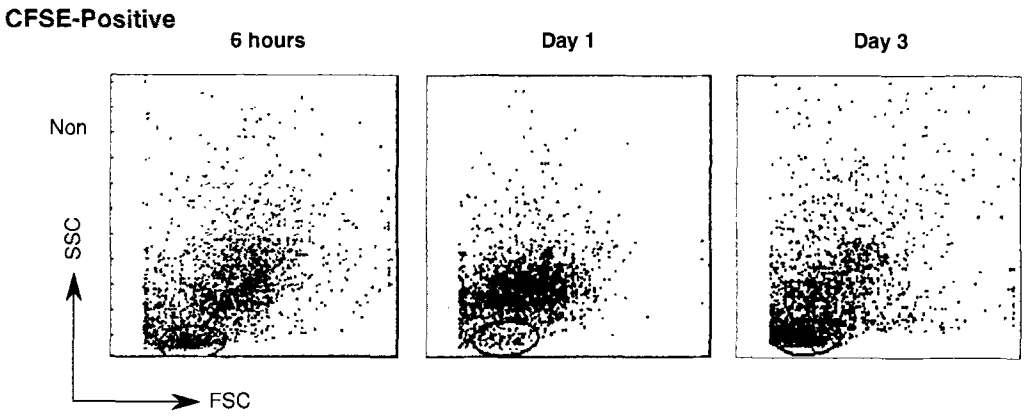
In all recipients, mitotic differentiation of $CFSE^+$ marrow cells continued after transfer, resulting in progressive reduction in cell size (caused by differentiation, Fig. 7 *A*) and CFSE intensity (caused by cell division, Fig. 7 *B*). Nevertheless, the fate of immature B cells that had not divided (i.e., retained their original CFSE intensity) could be followed by restricting analysis to $CFSE^{hi}$ HEL-binding cells. An initial increase in the relative number of $B220^+$ cells from Ig-Tg donors was observed in all recipients between 6 and 18 h (Fig. 7 *C*) because of more rapid differentiation of Ig-Tg B cells by virtue of their prerearranged Ig genes (11). From 18 h onwards, however, immature HEL-binding B cells of the original CFSE intensity disappeared rapidly from the spleens of sHEL-Tg and mHEL-Tg recipients, while persisting in non-Tg mice (Fig. 7, *B* and *C*). A decrease in the number of divided ($CFSE^{lo}$) HEL-binding B cells was also seen in sHEL-Tg compared with normal recipients (Fig. 7 *B*). When the positioning of HEL-binding immature B cells was examined histologically 18 h after transfer, preferential migration into the OP of splenic follicles was again observed in sHEL-Tg recipients (Fig. 5, *g* and *h*) in a manner identical to that of mature B cells (Fig. 5 *c*).

It could be argued that in this experiment, encounter with self-antigen did not take place immediately after the initial expression of Ig receptors, as occurs in sHEL-Dbl-Tg mice. To overcome such a potential problem, radiation chimaeras were created by reconstituting lethally irradiated sHEL-Tg mice with Ig-Tg marrow. 7 wk later, bone marrow cells were harvested and depleted of $CD23^+$ mature B cells, as described previously. After transfer, immature HEL-binding B cells that had been exposed to antigen throughout their development in the marrow were again found predominantly in the OP of sHEL-Tg mice and the follicles of non-Tg recipients (data not shown). Two conclusions can be drawn from these data. First, homing to the PALS was governed by antigen-dependent signaling rather than by the maturational stage of the B cell. Secondly, tolerant B cells that colonize the follicles of sHEL-Dbl-Tg mice can be induced to migrate into the PALS when exposed to the higher levels of available HEL present in sHEL-Tg mice.

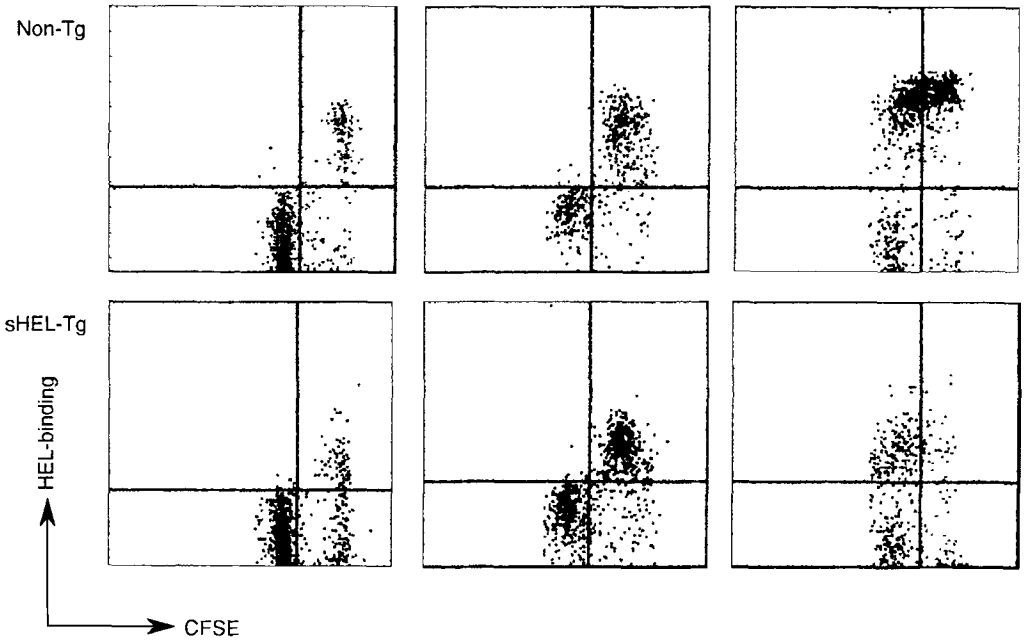
Rescue of Ig-Tg B Cells by Provision of T Cell Help. The transfer experiments described to date have shown that HEL-binding Ig-Tg B cells respond in an identical manner on exposure to self- or foreign antigen by migrating in an activated state (Fig. 4) to the outer PALS (Fig. 5, *c* and *k*). In the case of B cells with specificity for foreign antigen, this is the site where T cell help initiates a productive response (26, 27). On the other hand, self-reactive B cells (i.e., Ig-Tg B cells transferred into sHEL-Tg recipients) receive no helper signals since the T cell compartment of sHEL-Tg mice is known to be tolerant (12). This raised the possibility that a lack of T cell help could have contributed to the rapid loss of self-reactive B cells from the outer PALS. If so, then provision of help might be expected to rescue the transferred self-reactive B cells and allow them to survive and differentiate into antibody-secreting cells. Purified CFSE-labelled HEL-reactive B cells, prepulsed with cytochrome peptide, were cotransferred with activated T cells from TcR-Tg mice specific for cytochrome peptide in the context of IE^k. Both T and B cells were derived from F_1 mice expressing IE^k which is necessary both for positive thymic selection of the transgenic T cells and for presentation of cytochrome peptide by B cells. The T cells were activated by intravenous injection of 10 μg cytochrome peptide 1 d before transfer, a protocol which has been demonstrated to produce high levels of activation in about 80% of $CD4^+$ TcR-Tg T cells (Wikstrom, M., and B. Fazekas de St. Groth, unpublished observations). As a control, activated F_1 T cells were cotransferred with peptide-pulsed CFSE-labelled HEL-reactive B cells from B6 (IE-negative) mice unable to present cytochrome peptide.

Figure 6. Spleen cells from Ig-Tg (*A*) or ML5xMD4 sHEL-Dbl-Tg mice (*B*) were labeled $CFSE^{lo}$ and mixed in a 1:1 ratio with spleen cells from non-Tg mice labeled $CFSE^{hi}$. 30 million cells were injected intravenously into non-Tg, sHEL-Tg, or sHEL-Dbl-Tg mice. The latter were aged between 6 and 10 wk. (*A* and *B*) The number of HEL-binding $CFSE^{lo}$ cells are expressed relative to cotransferred non–HEL-binding $CFSE^{hi}$ cells. Lines are drawn through median values. (*C*) Histograms of transferred $CFSE^+$ B cells obtained from the spleens of non-Tg, sHEL-Tg, and sHEL-Dbl-Tg recipients (as shown) 1 d after transfer. The left panels represent Ig-Tg donors, and the right panels represent sHEL-Dbl-Tg donors. Gating is on small lymphocytes (by light scatter), B220 expression, and low CFSE intensity, the latter representing cells of Ig-Tg (*left*) or sHEL-Dbl-Tg (*right*) origin. The lower histograms represent the degree of HEL binding by endogenous ($CFSE^-$) B cells in sHEL-Dbl-Tg recipients. (*D*) Expression of transgenic IgM^a and IgD^a by Ig-Tg B cells cultured for 5 h in medium (*upper left*), medium with 20 ng/ml HEL (*upper right*), normal mouse serum (*lower left*), serum from sHEL-Tg mice (*lower middle*), or serum from sHEL-Dbl-Tg mice (*lower right*). Gating is on small lymphocytes (by light scatter characteristics) and B220 expression.

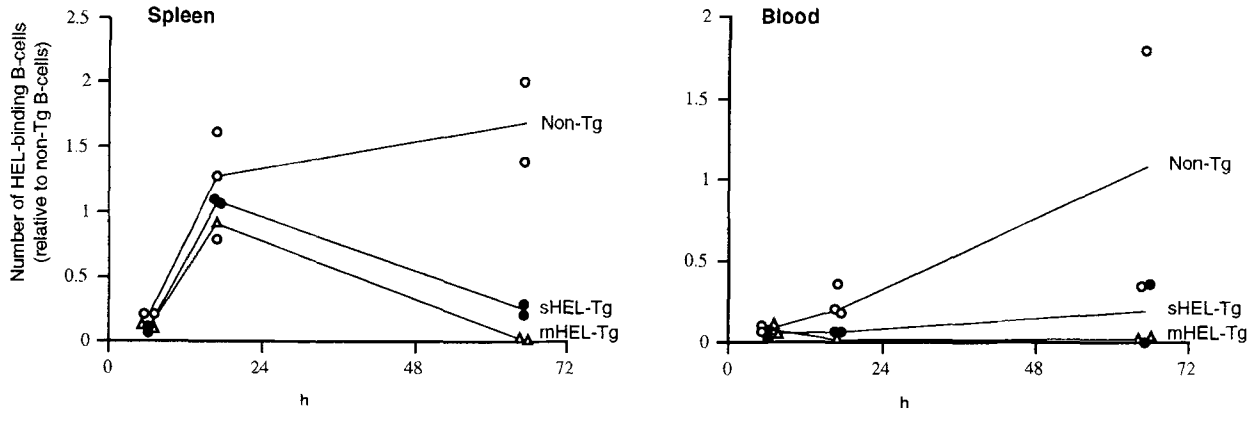
A


CFSE-Positive
6 hours
Day 1
Day 3
Non
SSC
FSC

B


B220-Positive
Non-Tg
sHEL-Tg
HEL-binding
CFSE

C


Spleen
Number of HEL-binding B-cells (relative to non-Tg B-cells)
2.5
2
1.5
1
0.5
0
0
24
48
72
h
Non-Tg
sHEL-Tg
mHEL-Tg
Blood
2
1.5
1
0.5
0
0
24
48
72
h
Non-Tg
sHEL-Tg
mHEL-Tg

24 h after transfer, B6 and F_1 HEL-binding B cells were both located at the interface between the PALS and the follicles (Fig. 8 *A*, *upper panels*), but neither population had divided (Fig. 8 *B*). By day three, F_1 B-cells which were capable of presenting MCC peptide to TcR-Tg T-cells had proliferated throughout the PALS (Fig. 8 *A*, *middle left panel* and Fig. 8 *B*), whereas the majority of B6 HEL-binding B cells had disappeared without undergoing cell division and could not be detected in the lymph nodes (data not shown). 2 d later (day five) a significant proportion of the progeny of the proliferating F_1 HEL-binding B cells were detectable either within the red pulp as proliferative foci of antibody-secreting cells, or in the follicles where they had developed into immature germinal centres (Fig. 8 *A*, *lower left panels*) (28). Although the latter were difficult to discern due to downregulation of surface Ig, they clearly had the appearance of germinal centres based on their location and binding of peanut agglutinin (data not shown). Interestingly, examination of individual follicles taken at the same time point revealed B cells in varying stages of differentiation and movement suggesting that T–B cell interactions in individual follicles may proceed at different rates. When HEL-specific IgM^a antibody was assayed, recipients of F_1 B cells, but not B6 B cells, produced significant amounts of antibody (11.5 μg/ml versus 0.01 μg/ml on day 5). Thus, provided that T cell recognition of B cell MHC peptide complexes takes place, self-reactive B cells can migrate through the T zone into the follicles and red pulp of previously tolerant hosts and mount an antibody response.

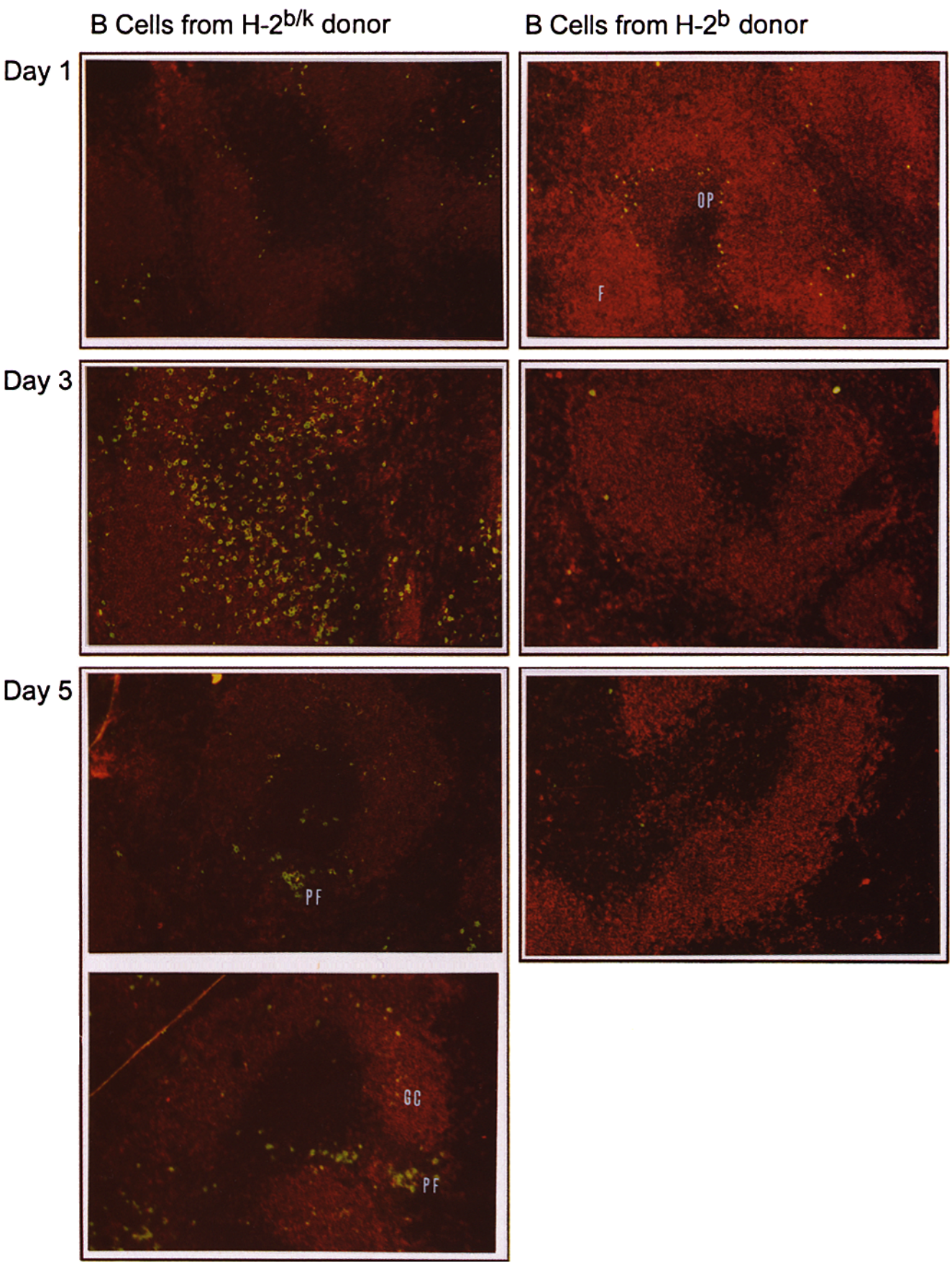
Discussion

The outcome of the interaction between antigen and B cell is thought to depend on several factors. Some factors, such as the stage of maturation and the capacity to interact with other cells in the environment, are intrinsic to the B cell itself, while others are related to the properties of the stimulating antigen and certain extrinsic influences, such as availability of T cell help (24, 29, 30). Collectively, these factors determine whether activation or tolerance occurs and, in the case of tolerance, whether it is mediated by deletion or anergy. According to recent BrdU labeling studies in anti-HEL B cell transgenic mice, a major difference between tolerant and normal or activated (memory) B cells is their life span. Thus, B cells exposed to multivalent mHEL in bone marrow were shown to die rapidly (within 1–2 d) without migrating to peripheral lymphoid tissue (6), and B cells exposed to oligovalent self-antigen in sHEL-Dbl-Tg mice, although they survived long enough to populate peripheral lymphoid tissue, still had a markedly reduced life span (3–5 d). A comparable decrease in the longevity of B cells was also demonstrated by Finkelman et al. (31) after in vivo administration of highly cross-linked anti-IgD to non-Tg mice. By contrast, normal B cells or "ignorant" B cells that had been exposed to subtolerogenic levels of antigen had a life span in the periphery of 4–5 wk (7). In other words, the avidity of antigen exerts some influence on the fate of B cells in terms of their longevity and site of death, but other factors must be important as well. The nature of these factors and the precise positioning of B cells after encounter with antigen were therefore examined in vivo by tracking HEL-binding Ig-Tg-B cells labeled with CFSE after transfer into normal or Tg recipients expressing HEL as a neo–self antigen.

Virgin B cells normally emigrate from the bone marrow to the OP or the marginal zone (27, 32). After exposure to foreign antigen, they are found in the OP and, after receipt of T cell help, undergo activation to form proliferative foci in which isotype switching occurs (27) and GC in which somatic hypermutation and production of memory cells takes place (23, 28, 33–35). When mature CFSE-labeled potentially self-reactive B cells from Ig-Tg mice were transferred into mHEL or (ML5) sHEL-Tg recipients, they increased in size (Fig. 4) and were rapidly recruited to the spleen (Figs. 2 and 3) in a manner identical to foreign antigen–stimulated virgin B cells (Fig. 5 *k*). Not surprisingly, self-reactive immature B cells exposed to sHEL behaved in the same way as mature B cells (Fig. 5, *g* and *h*). On immunohistology, HEL-binding B cells were initially found in the OP in the same location as B cells exposed to HEL as a foreign antigen (Fig. 5 *k*). They disappeared within 2–3 d (Fig. 1) without forming proliferative foci or GC, presumably as a result of death in situ (Fig. 5, *c* and *d*). This outcome was dependent on an encounter with a sufficient concentration of antigen to signal the B cell through its Ig receptors since mature B cells transferred into another sHEL-Tg recipient (ML4), which expressed much lower levels of available antigen (0.5 ng/ml versus 15 ng/ml in ML5), migrated to the follicles, and survived during the 3-d period of study (Fig. 5 *e*). Taken together, these findings indicated that repositioning of B cells in the OP was neither influenced by the stage of maturation nor by the nature of the antigen (self versus foreign), but it did require a critical threshold of antigen. Moreover, the fact that mature B cells were lost as rapidly as immature B cells is con-

Figure 7. Bone marrow suspensions from Ig-Tg and non-Tg mice were depleted of $CD23^+$ mature B cells and then labeled with CFSE, Ig-Tg cells being labeled $CFSE^{hi}$ and non-Tg cells $CFSE^{lo}$. After mixing in a 1:1 ratio (based on prelabeling counts), 33 million leukocytes were injected intravenously into non-Tg, sHEL-Tg, or KLK3 mHEL-Tg recipients. (*A*) Light scatter characteristics of transferred $CFSE^+$ cells recovered from the spleens of non-Tg recipients 6 h (*left*), 1 d (*middle*), or 3 d (*right*) after transfer. Ellipses represent the lymphocyte gate used in *B*. (*B*) Characteristic dot plots of $B220^+$ cells from non-Tg (*upper panels*) or sHEL-Tg (*lower panels*) recipients 6 h (*left*), 1 d (*middle*), or 3 d (*right*) after transfer. Gating is as described in Fig 1. The $CFSE^{hi}$ (Ig-Tg origin) $B220^+$ cells that fail to bind HEL (*lower right quadrants*) presumably represent sIg^- B lineage cells, or in the case of sHEL-Tg recipients, B cells that have downregulated surface Ig. (*C*) The number of HEL-binding $CFSE^{hi}$ B cells (excluding those that had divided to become $CFSE^{lo}$) relative to $CFSE^{lo}$ non–HEL-binding B cells after transfer into non-Tg (*open circles*) or sHEL-Tg (*closed circles*) mice in the spleen (*left*) and blood (*right*). Lines are drawn through median values.

A


B Cells from H-2b/k donor
B Cells from H-2b donor
Day 1
Day 3
Day 5
OP
F
PF
GC
PF

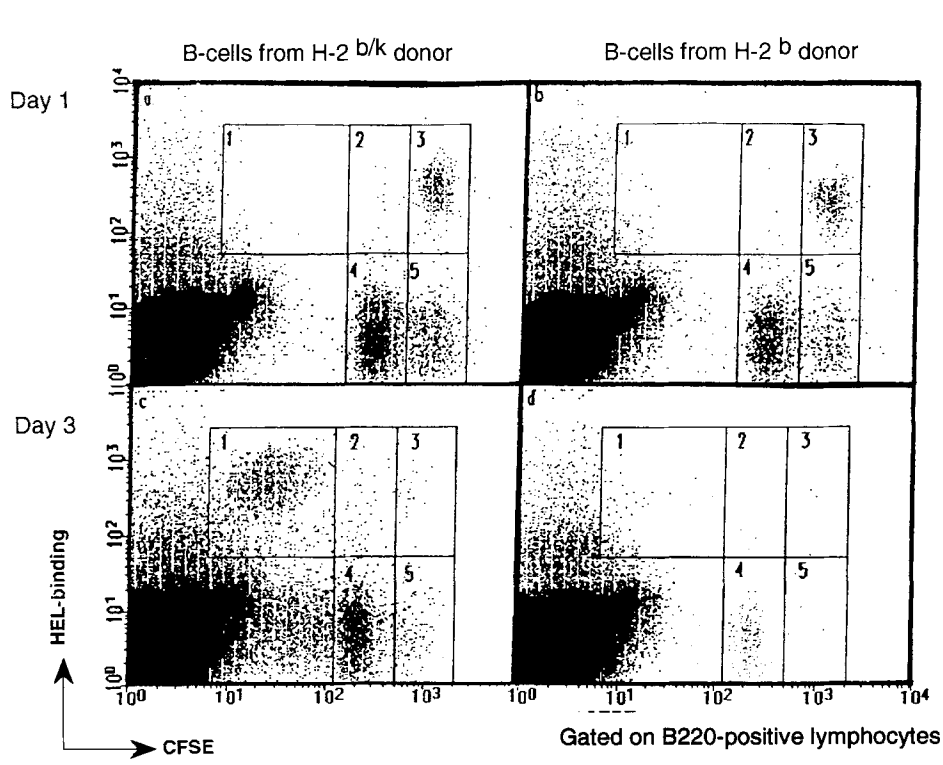


Figure 8. Effect of T cell help on the movement of Ig-Tg B cells transferred into sHEL Tg recipients. (*A*) Fluorescent micrographs of frozen sections from spleens of H-2^{bk} sHEL-Tg mice taken 1, 3, and 5 d after transfer of preactivated anticytochrome TcR-Tg T cells and CFSE-labeled Ig-Tg B cells that had been pulsed in vitro with MCC peptide 87-103. The Ig-Tg B cells were obtained from either H-2^{bk} donors (*left*) or H-2^{b} donors (*right*). HEL-binding B cells are stained green and $B220^+$ cells red. Day 1 sections, H-2^{b} and H-2^{bk} HEL-binding B cells are both positioned in the OP; day 3 sections, H-2^{bk} HEL-binding B cells have proliferated, whereas the great majority of H-2^{b} B-cells have disappeared; day 5 sections from the spleens of recipients given H-2^{bk} B cells reveal the following: (*a*) PF containing antibody-secreting cells (*stained green*) in the T cell zone/red pulp bridge or the red pulp (*top*); and (*b*) immature GC containing HEL^+ B cells with downregulated Ig receptors (*stained orange/yellow*) in the follicle (*bottom*). (*B*) Dot plots of $B220^+$ cells from recipient spleens obtained one (*upper plots*) and three (*lower plots*) days after transfer. Ig-Tg cells were labeled $CFSE^{hi}$ (region 3, day 1) and non-Tg cells were labeled $CFSE^{lo}$ (region 4). Cell division is represented by a decrease in CFSE intensity, as shown in the lower left-hand panel (region 1). The $CFSE^{hi}$ $B220^+$ cells that fail to bind HEL on day 1 (region 5) are probably B cells that have downregulated surface Ig, since by day 3, they behave in an identical fashion to HEL-binding B cells, being deleted in the absence of T cell help (*right-hand panel*) and dividing in response to T cell help (*left-hand panel*, left of region 4).

sistent with previous reports of induction of tolerance in peripheral lymphoid tissue as well as in bone marrow (36, 37).

The requirement for a critical threshold of antigen to direct self-reactive B cells to the OP of tolerant sHEL-Tg recipients raises the question of why they are apparently found in the follicle rather than at this site in the corresponding sHEL-Dbl-Tg mice (11). According to the data presented here (Fig. 6), the concentration of antigen available for engaging Ig receptors on transferred B cells was less than expected in sHEL-Dbl-Tg recipients, possibly because of binding of circulating HEL to anti-HEL Ig-Tg B cells and/or to the small amounts of residual anti-HEL IgM present in the double transgenic mice (4). Consequently, the capacity of HEL to form the oligovalent complexes with serum proteins such as α2-macroglobulin, which are believed to be a prerequisite for cross-linking of Ig receptors by HEL (reference 38 and Fulcher, D.A., and P.W. Peake, unpublished observations), is likely to have been impaired. In the case of intact sHEL-Dbl-Tg mice, sufficient antigen is apparently present in the bone marrow to downregulate surface Ig on newly generated B cells (11) and to impair surface Ig-dependent signaling function (39, 40). On the other hand, this level of antigen appears insufficient to activate these B cells fully (39) or to direct them to the PALS, and instead they migrate into the follicles. Nevertheless, B cells from sHEL-Dbl-Tg mice remain capable of responding in vivo to antigenic signals, as shown by their capacity to migrate into the OP on transfer into sHEL-Tg recipients (Fig. 6). Although a plausible explanation for the failure of B cells from sHEL-Dbl-Tg mice to migrate to the outer PALS, this interpretation relies on the assumption that B cells can undergo three distinct fates on exposure to HEL over a serum concentration range of 0.5–15 ng/ml, which corresponds to an increase in antigen receptor occupancy from <1 to 45% (37); these outcomes are (*a*) ignorance at low concentrations; (*b*) surface Ig downregulation and partial inactivation of antigen-mediated signaling at intermediate concentrations (4, 39); and (*c*) complete downregulation of surface Ig accompanied by an increase in cell size and migration to the OP at the highest antigen concentrations.

An alternative explanation for the positioning of B cells in the follicle rather than the OP of the same line of sHEL-Dbl-Tg mice comes from the work of Cyster et al., who performed similar transfer experiments (9). According to their hypothesis, self-reactive B cells are normally excluded from the follicle by a process of competition with the anti–foreign polyclonal B cell repertoire. On the other hand, in sHEL-Dbl-Tg mice, the follicles are largely populated by B cells with the same single specificity, which means that newly generated anti–HEL B cells can enter unimpeded. Although follicular exclusion is a plausible concept that may in theory play a role in shaping the B cell repertoire as

suggested by Cyster et al. (9), a number of aspects of the phenomenon need to be clarified before it can be regarded as proven. First, no mechanism is currently available to explain how it might operate. Second, it does not take into account the possibility raised here that the persistence of HEL-binding B cells after transfer into sHEL-Dbl-Tg mice, as opposed to their rapid disappearance from sHEL-Tg mice, is a result of the difference in effective antigen concentration between the two types of recipients (Fig. 6). Third, some of the data of Cyster et al. on competition between self-reactive (tolerant) and anti–foreign (normal) B cells in mixed chimeras can be explained by the marked difference in the life span of the two populations of B cells. Finally, one prediction of the follicular exclusion model is that the survival of anergic B cells from sHEL-Dbl-Tg mice transferred into similar sHEL-Dbl-Tg recipients should vary depending on the size of the competing population of nontolerant cells. Survival was identical, however, when anergic cells were transferred into young recipients with almost no competing cells or older recipients in which up to 25% of B cells failed to bind HEL (Fulcher, D.A., and A. Basten, manuscript in preparation).

The decision as to whether a B cell undergoes deletion or anergy is clearly related to the degree of cross-linking of Ig receptors on the B cell surface. On the other hand, none of the antigen-related or cellular factors described to date can fully explain what is responsible for the decision between activation and tolerance. Since B cells, irrespective of their specificity for self- or foreign antigen, can migrate to the OP on encounter with antigen, it is not unreasonable to suggest on a priori grounds that the purpose of doing so is to optimize the chance of interacting with T cells, and that a failure to receive T cell help within a given time frame may result in death. The capacity of activated T cells from anticytochrome TCR-Tg mice to rescue self-reactive B cells after transfer into sHEL-Tg recipients as shown here is consistent with this hypothesis (Fig. 8). Indeed in the presence of cognate T cell help, self-reactive B cells not only survived and proliferated, but migrated out of the T cell zone to form GC in follicles and proliferative foci in the red pulp despite the continued presence of self-antigen. At the same time, some of them secreted antibody while others populated the marginal zone presumably as memory cells (data not shown).

Many previous experiments (41–45) have shown that physical association of T and B cell epitopes is required for optimal T–B cell collaboration to take place in vivo. In the experimental system used here, T–B cell collaboration between HEL-specific B cells and cytochrome-specific T cells was observed after cytochrome peptide–pulsing the B cells in vitro before adoptive transfer. On the other hand, administration of cytochrome peptide in vivo was ineffective (Cook, M.C., M. Wikstrom, and B. Fazekas de St. Groth, unpublished observations), thereby confirming the failure of "bystander" help to substitute for cognate help in vivo. Full T–B cell collaboration was also seen after immunisation in vivo with a recombinant protein expressing linked HEL and cytochrome epitopes (Cook, M.C., A. Basten, and B. Fazekas de St. Groth, manuscript in preparation). Thus, cytochrome peptide pulsing of HEL-specific B-cells in vitro, followed by antigen receptor engagement in vivo, generated a B cell phenotype that could be mimicked by administration of linked, but not unlinked, HEL and cytochrome epitopes in vivo. Presumably, the explanation of this phenomenon lies in the ability of in vitro pulsing to achieve relatively higher surface levels of peptide–MHC complexes. Consequently, the failure of unlinked T and B cell epitopes to induce a significant T–B cell collaboration in vivo is caused by the inability of B cells to achieve sufficient levels of peptide–MHC complexes in the absence of either Ig-mediated endocytosis (46, 47) or peptide pulsing in vitro. Consistent with this interpretation is our previous demonstration that at least 10,000-fold more antigen is required by a polyclonal B cell population than by HEL-specific B cells to induce the same degree of stimulation of HEL-specific T cells (40).

A key role for T cell help in determining the fate of antigen-stimulated B cells is supported by two earlier observations. First, provision of T cell signals to tolerant B cells from sHEL-Dbl-Tg mice in the form of either CD40 ligand in vitro (39, 40) or HEL-primed T cells with adjuvant in vivo (40) was shown to result in complete reversal of tolerance despite the fact that these cells had a partial defect in costimulatory function. Second, B cell tolerance in HEL-Tg mice was associated with a hyporesponsive T cell compartment caused by the lower antigen threshold required for induction of T cell tolerance (12). Thus, the primary determinant for induction of B cell tolerance in vivo may be a lack of T cell help rather than an intrinsic ability of B cells to be rendered tolerant upon exposure to self-antigen. Provision of help via epitopes to which T cells remain responsive could then lead to a breakdown in self tolerance and autoimmunity without any change in the intrinsic state of the B cells. The fact that such an event could well occur, e.g., during coincidental infection, points to the need for an additional T independent mechanism of tolerance induction capable of operating after initiation of an autoimmune response. Antigen-driven B cell death in GC reported recently by several groups (48–50) could well provide such a mechanism by interfering with somatic hypermutation and affinity maturation of B cells that have escaped censoring in the OP.

In summary, our current findings, when taken in conjunction with previous data on B cell lifespan (6–8), indicate that the outcome of the interaction between antigen and B cells is largely determined by a combination of the degree of Ig receptor engagement and availability of T cell help. The stronger the self-antigen signal, the shorter the B cell life span and the less likely they are to be stimulated by T cell help (30), a conclusion also supported by recent in vitro experiments with anti-Ig reagents (51). On the other hand, the fact that self-reactive B cells can be rescued by T cell signals after migration into the OP provides a plausible explanation for the occurrence of autoantibody-dependent autoimmunity when B cells encounter antigen with self–B cell and foreign T cell determinants (30, 52, 53).

We would like to thank Jeffrey Crosbie for his help with animal manipulation, Karen Knight and coworkers (Centenary Institute) and Margaret Ferrara and coworkers (Westmead Hospital) for their expert assistance with animal husbandry.

D.A. Fulcher is the recipient of a Medical Research Scholarship from the National Health and Medical Research Council (NHMRC) of Australia, and M.C. Cook is the recipient of a postgraduate scholarship from the University of Sydney Faculty of Medicine. B. Fazekas de St. Growth was supported by the Wellcome Trust and the NHMRC. The work was supported by an NHMRC program grant.

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Received for publication 14 November 1995 and in revised form 9 February 1996.

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