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Autoreactive B Cells in Lupus-Prone New Zealand Black Mice Exhibit Aberrant Survival and Proliferation in the Presence of Self-Antigen In Vivo[1]

Nan-Hua Chang,* Ralph MacLeod,* and Joan E. Wither[2]*[†‡]

To identify defects in B cell tolerance that may contribute to the production of autoantibodies in New Zealand Black (NZB) mice, we crossed soluble hen egg white lysozyme (sHEL) and anti-HEL Ig transgenes (Ig Tg) onto the NZB background. In this study, we have examined one of the first checkpoints involved in maintenance of peripheral B cell tolerance, follicular exclusion and elimination of self-reactive B cells in the absence of T cell help. Freshly isolated anti-HEL Ig Tg B cells were labeled with CFSE, adoptively transferred into sHEL recipients, and the fate of self-reactive anti-HEL Ig Tg B cells was followed using flow cytometry and immunofluorescence microscopy. Although anti-HEL Ig Tg B cells from NZB mice are appropriately excluded from B cell follicles in NZB sHEL recipient mice, they demonstrate aberrant survival, proliferation, and generation of anti-HEL Ab-producing cells. This abnormal response results from an intrinsic defect in NZB B cells, requires the presence of CD4^{+} T cells, and is facilitated by the splenic environment in NZB mice. Thus, NZB mice have immune defects that interact synergistically to allow autoreactive B cells to become activated despite the presence of tolerizing autoantigens.   *The Journal of Immunology,* 2004, 172: 1553–1560.

Systemic lupus erythematosus (SLE)[3] is a generalized autoimmune disease characterized by production of pathogenic autoantibodies directed predominantly toward nuclear Ags. The New Zealand Black (NZB), NZB.H-2[bm12], and (NZB × New Zealand White (NZW))F$_1$ (NZB/W) mouse strains spontaneously develop an autoimmune condition that is considered to be an excellent model of this disease. In NZB mice, lupus-like autoimmune disease is characterized by production of anti-RBC, -lymphocyte, and -ssDNA Abs leading to hemolytic anemia and mild glomerulonephritis late in life (reviewed in Ref. 1). Although these mice do not develop the rapidly progressive immune-complex-mediated glomerulonephritis found in NZB.H-2[bm12] and NZB/W mice, this appears to result solely from the lack of a MHC locus that facilitates nephritogenic autoantibody production (2).

The capacity of NZB mice to produce diverse autoantibodies suggests that these mice possess a generalized defect in self-tolerance. Although both B and T cell defects could contribute to the loss of tolerance in these mice, studies of T cell tolerance have been negative with normal clonal deletion and clonal anergy induction being demonstrated (3–7). In contrast, NZB mice have a number of immunological abnormalities that suggest that B cell tolerance mechanisms may be defective. These include polyclonal B cell activation in vivo (8–12), IgM hypergammaglobulinemia (13, 14), and altered B cell function in vitro (15–17). In this study, we examine one of the earliest B cell tolerance checkpoints involved in the maintenance of peripheral tolerance by crossing the well-characterized anti-hen egg white lysozyme (HEL) Ig and soluble HEL (sHEL) transgenes onto the NZB background (18).

Negative regulation of self-reactive B cells can occur at multiple checkpoints in the bone marrow or periphery (18, 19). In the periphery, B cells that have not successfully edited their self-reactive receptor and escaped clonal deletion within the bone marrow are excluded from the B cell follicle arresting in the outer T cell zone or periarteriolar lymphoid sheath (PALS) (20, 21). In the absence of T cell help, for example when there is T cell tolerance to the self-Ag, these B cells are eliminated from the peripheral repertoire (20–22). Although naive self-reactive B cells that have contacted Ag can proliferate and differentiate to plasma cells with appropriate T cell help, provision of T cell help to some types of anergic B cells, depending upon the Ag that has induced tolerance, can result in Fas-mediated apoptosis and a block in terminal differentiation of the B cell (21–24).

In this study, we demonstrate that, in contrast to anti-HEL Ig transgenic (Tg) B cells from nonautoimmune C57BL/6 (B6) mice, anti-HEL Ig Tg B cells from NZB mice survive, proliferate, and differentiate into anti-HEL Ab-producing cells when transferred into NZB sHEL recipient mice. This abnormal response results from an intrinsic defect in NZB B cells, requires the presence of CD4^{+} T cells, and is facilitated by the NZB splenic environment. Thus, the immune mechanisms that lead to exclusion and elimination of autoreactive B cells from the peripheral repertoire are defective in NZB mice.

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[3] Abbreviations used in this paper: SLE, systemic lupus erythematosus; HEL, hen egg white lysozyme; sHEL, soluble HEL; Tg, transgenic; PALS, periarteriolar lymphoid sheath; PNA, peanut agglutinin.

Materials and Methods

Mice

B6, B6.H-2[d], and B6 mice expressing transgenes encoding sHEL (ML5) or IgM/IgD H and L chains specific for HEL (anti-HEL Ig Tg; MD4) were

purchased from The Jackson Laboratory (Bar Harbor, ME). NZB mice were purchased from Harlan Sprague Dawley (Indianapolis, IN). Backcrossing of transgenes onto the NZB and B6.H-2^d background was performed in the Toronto Western Hospital animal facility. Offspring were genotyped by PCR using primers specific for the variable region of the Ig H chain and sHEL transgenes (25). NZB anti-HEL Ig Tg and sHEL transgenic mice were produced using the speed congenic technique. Fully backcrossed mice were obtained in six generations for the sHEL transgene and in seven generations for the Ig transgene. All of the mice were housed in microisolators.

Flow cytometry staining and analysis

Briefly, 1×10^6 RBC-depleted spleen cells were incubated with 10 μg/ml mouse IgG (Sigma-Aldrich, St. Louis, MO) for 15 min to block FcRs and then stained with various combinations of directly conjugated mAbs. Following washing, allophycocyanin-conjugated streptavidin (BD PharMingen, San Diego, CA) was used to reveal biotin-conjugated Ab staining. Dead cells were excluded by staining with propidium iodide (0.6 μg/ml; Sigma-Aldrich). Flow cytometry of the stained cells was performed using a dual laser FACSCalibur (BD Biosciences, Mountain View, CA) and analyzed using CellQuest (BD Biosciences) software. The following directly conjugated mAbs were purchased from BD PharMingen: biotin anti-IgM^a (DS-1), PE anti-IgM^a, PE anti-CD23 (B3B4), PE anti-CD69 (H1.2F3), and FITC anti-CD21 (7G6). Biotin-, PE-, and FITC-conjugated anti-B220 mAbs were purchased from Cedarlane Laboratories (Hornby, Ontario, Canada). Biotinylated peanut agglutinin (PNA) was obtained from Sigma-Aldrich. Biotinylated HEL was prepared using an EZ-Link Sulfo-NHS-LC Biotinylation kit (Pierce, Rockford, IL). All isotype controls were purchased from Cedarlane Laboratories except for hamster IgG controls, which were obtained from BD PharMingen.

Adoptive transfers

Donor cells were isolated from the spleens of NZB or B6 anti-HEL Ig Tg or non-Tg mice. Following lysis of erythrocytes, spleen cells were depleted of T cells by treatment with a mixture of supernatants from hybridomas producing anti-Thy1.2 (HO-13-4), anti-CD4 (GK1.5), and anti-CD8 (3.155) mAbs for 30 min at 4°C followed by the addition of guinea pig complement (Cedarlane Laboratories) for an additional 1 h at 37°C. For some experiments, resting or activated B cells were isolated by fractionation over a discontinuous Percoll (Pharmacia, Peapack, NJ) gradient (17). Marginal zone B cells (CD23$^-$CD21^{high}) were purified by cell sorting following staining with anti-CD23 and CD21 mAb using a MoFlow instrument (DakoCytomation, Carpinteria, CA). Donor B cells were labeled with 2.5 or 5 μM CFSE (Molecular Probes, Eugene, OR) (22). In brief, the cells were washed twice in serum-free RPMI 1640, resuspended at 5×10^7/ml in RPMI 1640, and incubated for 10 min at 37°C with CFSE. Labeling was quenched with two washes of ice-cold RPMI 1640-10% FCS. Recipient NZB, NZB.H-2$^{b/d}$, B6, B6.H-2^d, (NZB $\times$ B6)F$_1$, or (NZB $\times$ B6.H-2^d)F$_1$ sHEL or non-Tg mice were injected with 0.2-ml aliquots of $1-3 \times 10^7$ CFSE-labeled (days 1 and 3) or unstained (days 7 and 21) B cells via the lateral tail vein. Spleens were isolated from mice sacrificed at 1, 3, 7, or 21 days following transfer. One half of the spleen was used for flow cytometry and ELISPOT assays, and the other half was frozen in OCT compound (Sakura Finetek, Torrance, CA) for sectioning.

CD4$^+$ T cell depletion

CD4$^+$ T cells were depleted from recipient NZB and (NZB $\times$ B6)F$_1$ sHEL mice as previously described (26). Mice received 0.5 mg of purified anti-CD4 GK1.5 mAb for 2 consecutive days (this protocol was sufficient to deplete >95% of CD4$^+$ T cells). On day 5, CFSE-labeled B cells were transferred into the mice.

ELISPOT assays

Ab-producing cells were detected in the spleens of recipient mice as previously described (27). Briefly, 96-well MultiScreen-HA plates (Millipore, Bedford, MA) were coated with PBS or HEL (50 μg/ml; Sigma-Aldrich). Following blocking with 5% FCS in PBS, freshly isolated splenocytes were plated at 10^6 cells and 10^5 cells/well and incubated for 48 h at 37°C. The cells were washed away with PBS/Tween 20 (0.05%) and biotinylated anti-mouse IgM^a, diluted in 0.1% BSA/PBS/Tween 20, was added to the wells for 2 h at room temperature. Following washing, plates were incubated with alkaline phosphatase-conjugated streptavidin (BD PharMingen), washed again, and substrate added (Sigma Fast 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium; Sigma-Aldrich). Individual spots, reflecting Ab-producing cells, were visualized using a stereomicroscope.

Staining of splenic tissue sections

Cryostat sections (6 μm) were fixed in acetone, washed with PBS, and blocked with PBS/5% normal goat serum. Day 1 and 3 sections were stained with biotin-conjugated anti-B220 (Cedarlane Laboratories), and day 7 sections were stained with FITC-conjugated anti-B220 and biotinylated anti-IgM^a or with FITC-conjugated anti-IgM^a and biotinylated PNA. Biotinylated Ab staining was revealed with rhodamine (tetramethylrhodamine)-conjugated streptavidin (Molecular Probes). Stained sections were mounted with Mowiol (a generous gift from Dr. M. Fehlings, Toronto Western Research Institute, Toronto, Canada) and tissue fluorescence was visualized using a Zeiss Axioplan 2 imaging microscope (Zeiss, Oberkochen, Germany). Digital images were obtained using the manufacturer's imaging system.

Results

Normal early activation and follicular exclusion of NZB anti-HEL Ig Tg B cells following transfer into sHEL recipient mice

In normal B6 mice, transfer of naive anti-HEL Ig Tg B cells into sHEL recipients results in transient activation of transferred B cells, arrest of the cells at the T-B interface, and elimination in the absence of T cell help (20–22). To assess this mechanism of tolerance in NZB mice, anti-HEL Ig and sHEL transgenes were crossed onto the NZB background, and the fate of naive anti-HEL Ig Tg B cells was examined following transfer into NZB sHEL recipient mice. To this end, $1-3 \times 10^7$ CFSE-labeled (days 1 and 3) or unlabeled (days 7 and 21) T cell-depleted naive anti-HEL Ig Tg splenocytes from B6 or NZB mice were transferred into syngeneic non-Tg or sHEL recipients. Mice were sacrificed at 1, 3, 7, or 21 days following transfer, and the fate of the transferred anti-HEL Ig Tg B cells was examined by flow cytometry, tissue immunofluorescence, and ELISPOT assays. Activation of transferred splenic anti-HEL Ig Tg B cells was assessed 1 day following transfer, by flow cytometry, following staining with anti-B220 and -IgM^a or -CD69. As shown in Fig. 1, contact with sHEL resulted in decreased IgM^a expression, increased cell size, and an increased proportion of cells expressing the early activation marker CD69, for both B6 and NZB mice. Although IgM^a expression was significantly higher on transferred NZB B cells (IgM^a mean fluorescence intensity: B6, 113.5 ± 75.5; NZB, 233.9 ± 14.4; $p = 0.026$), increases in cell size and the proportion of CD69$^+$ cells were comparable. Thus, early activation events appear to be similar in transferred B6 and NZB B cells.

We next sought to determine whether transferred NZB anti-HEL Ig Tg B cells localize appropriately in sHEL recipient mice. For the most part, B and T cells are located in discrete areas of the spleen called the B cell follicle and PALS, respectively. In the absence of Ag, naive B cells migrate into the B cell follicle and enter the recirculating pool, whereas contact with Ag leads to exclusion from the B cell follicle and arrest in the outer PALS (20–22). As shown in Fig. 2, 1 day following transfer, NZB anti-HEL Ig Tg B cells were appropriately scattered throughout the splenic B cell follicle in non-Tg recipients and restricted to the outer PALS in sHEL recipients.

Enhanced survival and proliferation of transferred NZB anti-HEL Ig Tg B cells in sHEL recipient mice

Previous studies indicate that by 3 days following transfer of anti-HEL Ig Tg B cells into sHEL recipients, the majority of transferred cells have been eliminated (20–22). Elimination of transferred B cells is the consequence of a lack of T cell help in sHEL recipient mice and can be overcome by provision of exogenous T cell help in the form of nontolerant HEL-specific T cells or by transfer into allogeneic recipients (22, 23). To determine whether anti-HEL Ig Tg B cells are appropriately eliminated following transfer into
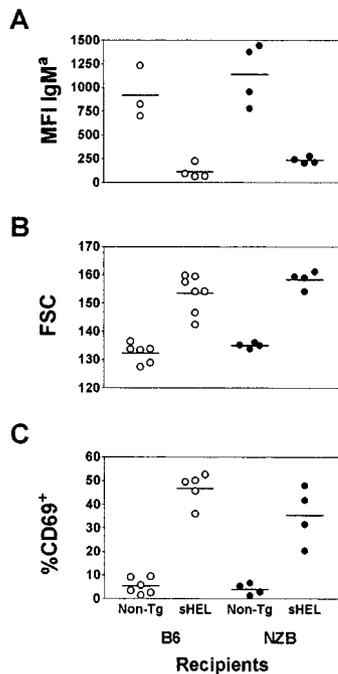
FIGURE 1. Phenotype of anti-HEL Ig Tg B cells 1 day following transfer into sHEL or non-Tg recipient mice. Scatter plots showing the results of a flow cytometric analysis of transferred B cells in the spleens of recipient mice injected 1 day earlier with $1–3 \times 10^7$ T cell-depleted CFSE-labeled splenocytes. Results shown are for gated CFSE$^+$B220$^+$ propidium iodide-excluding cells stained with anti-IgM^a or -CD69. Each circle represents the determination from an individual mouse with B6 mice shown as open circles and NZB mice as filled circles. Horizontal lines indicate the mean for each population examined. *A,* Mean fluorescence intensity (MFI) for anti-IgM^a staining of gated cells. *B,* Forward scatter (FSC) of gated cells. *C,* Percentage of gated cells with CD69 levels above background staining with an isotype-matched control.
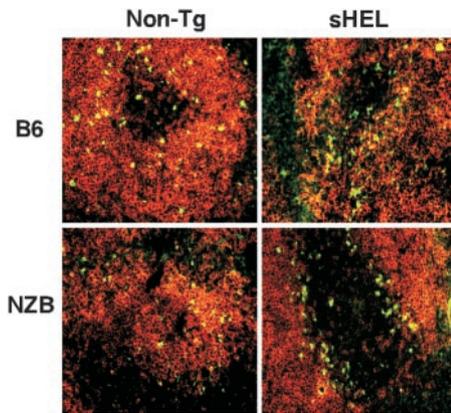

FIGURE 2. NZB anti-HEL Ig Tg B cells transferred into sHEL Tg recipient mice localize appropriately at the T-B interface. Immunofluorescence microscopy was performed on splenic sections from non-Tg and sHEL recipient mice injected 1 day earlier with $1–3 \times 10^7$ T cell-depleted CFSE-labeled splenocytes from anti-HEL Ig Tg mice. CFSE-labeled B cells are green and B220$^+$ cells, delineating the B cell follicle, are red (rhodamine).

NZB sHEL recipient mice, spleens were obtained from mice sacrificed 3 days following B cell transfer and the number of surviving CFSE$^+$ transferred cells was quantitated by flow cytometry. In agreement with earlier studies, the majority of B6 anti-HEL Ig Tg B cells had been eliminated in the B6 sHEL recipient mice (Fig. 3, *A–C*). In striking contrast to the rapid elimination of anti-HEL Ig Tg B cells in B6 sHEL mice, a significant proportion of NZB anti-HEL Ig Tg B cells had survived and proliferated (Fig. 3, *A–C*). This difference was highly significant ($p < 0.0001$). Close to 90% of B6 anti-HEL Ig Tg B cells were eliminated in B6 sHEL mice, whereas 58% of NZB anti-HEL Ig Tg B cells survived in NZB sHEL mice. Notably, increased survival and proliferation of NZB anti-HEL Ig Tg B cells was only seen in sHEL recipient mice and not in non-Tg NZB recipients, indicating that receptor engagement and/or follicular arrest is necessary for this aberrant response.

To determine whether transferred anti-HEL Ig Tg B cells can differentiate into Ab-forming cells in NZB mice, we quantitated the number of IgM^{a+} anti-HEL-secreting cells in the spleen 3 days following transfer, by ELISPOT (Fig. 3*D*). As expected, B6 anti-HEL Ig Tg B cells did not differentiate into Ab-forming cells in sHEL recipient mice. In contrast, significant numbers of IgM^{a+} anti-HEL-secreting cells were found in NZB sHEL recipients. Although a few IgM^{a+} anti-HEL-secreting cells were seen in NZB non-Tg recipients, the number of IgM^{a+} anti-HEL-secreting cells was clearly increased in NZB sHEL recipient mice. This finding indicates that the IgM^{a+} anti-HEL-secreting cells in sHEL mice were not simply transferred plasma cells, but were induced in response to sHEL in the recipient.

Survival and proliferation of Ig Tg NZB B cells results, in part, from an intrinsic B cell defect and is CD4$^+$ T cell dependent

The aberrant survival and proliferation of NZB anti-HEL Ig Tg B cells could result from an intrinsic B cell defect or an altered splenic environment in these mice. To discriminate between these two possibilities, we transferred B6 and NZB anti-HEL Ig Tg B cells into the same (NZB × B6)F$_1$ recipient mouse strain. Three days after transfer, NZB anti-HEL Ig Tg B cells showed the same abnormal survival (Fig. 4), proliferation, and production of HEL-specific Ab-forming cells (data not shown) in sHEL (NZB × B6)F$_1$ recipients as NZB recipients. In contrast, B6 anti-HEL Ig Tg B cells were eliminated just as rapidly as they had been in B6 sHEL recipient mice, indicating that the abnormal response of NZB anti-HEL Ig Tg B cells is due, at least in part, to an intrinsic B cell defect.

Although mice expressing sHEL as an autoantigen are generally considered to lack HEL-specific Th cells, the proliferation of NZB anti-HEL Ig Tg B cells in sHEL recipient mice resembled that reported for transferred B6 anti-HEL Ig Tg B cells provided with exogenous T cell help (22, 28). We therefore investigated the role of T cells in the enhanced survival and proliferation of transferred NZB anti-HEL Ig Tg B cells in sHEL recipient mice. Recipient mice were depleted of CD4$^+$ T cells by administration of two doses of anti-CD4 mAb (500 μg i.p.), and anti-HEL Ig Tg B cells were transferred 5 days later. Flow cytometry confirmed that >95% of CD4$^+$ T cells were depleted by this treatment. As shown in Fig. 4, transferred NZB anti-HEL Ig Tg B cells were eliminated similar to their B6 counterparts in CD4$^+$ T cell-depleted (NZB × B6)F$_1$ sHEL recipient mice.

Because B6 and NZB mice have different MHC haplotypes, H-2^b and H-2^d, respectively, we speculated whether the enhanced proliferation and survival of NZB anti-HEL Ig Tg B cells could result solely from the MHC differences in these two strains of mice. To address this issue, anti-HEL Ig and sHEL transgenes were crossed onto the B6.H-2^d genetic background and B6.H-2^d anti-HEL Ig Tg B cells were transferred into B6.H-2^d or (NZB × B6)F$_1$ sHEL recipients. This revealed that, similar to B6 cells, the majority of B6.H-2^d anti-HEL Ig Tg B cells were eliminated in B6.H-2^d and (NZB × B6)F$_1$ recipients (Fig. 5).
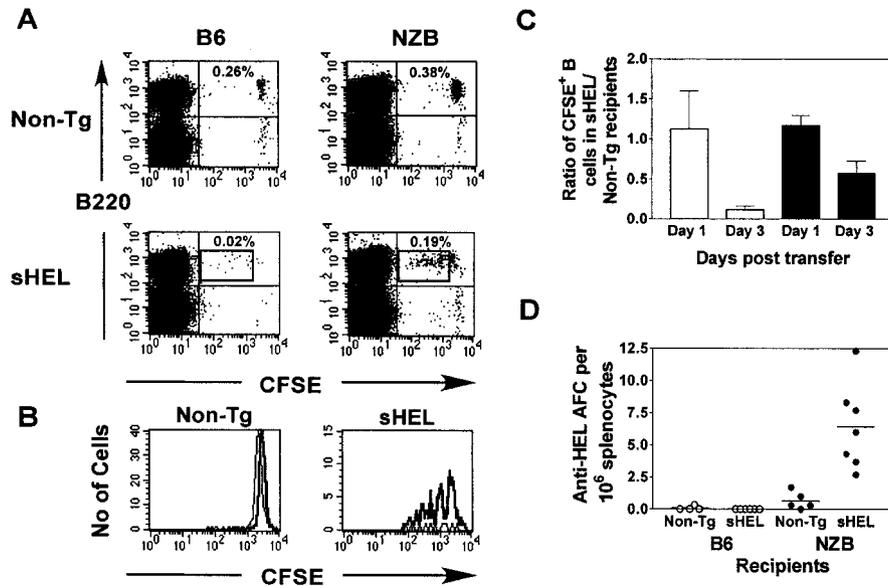
FIGURE 3. Abnormal proliferation and differentiation of NZB anti-HEL Ig Tg B cells on day 3 following transfer into sHEL recipients. *A*, Two-color flow cytometric analysis of splenocytes 3 days following transfer of 2×10^7 T cell-depleted CFSE-labeled splenocytes from anti-HEL Ig Tg mice into non-Tg or sHEL recipient mice. Divided B cells are indicated by the rectangles. Numbers on each plot indicate the percent $B220^+CFSE^+$ splenocytes. *B*, Histograms, gated on $B220^+$ cells showing CFSE staining. Histograms show results following transfer of anti-HEL Ig Tg B cells into non-Tg or sHEL recipient mice, as indicated, with thin-lined histograms representing B6 mice and thick-lined histograms representing NZB mice. Note that NZB anti-HEL Ig Tg B cells transferred into sHEL recipients have undergone up to four cycles of proliferation. *C*, Bar graph comparing survival of $CFSE^+$ anti-HEL Ig Tg B cells in sHEL recipient mice with that in non-Tg mice on days 1 and 3 following transfer. The percent $B220^+CFSE^+$ B cells was quantitated by flow cytometry as shown in *A*. Results are expressed as a ratio of the percent $B220^+CFSE^+$ B cells in sHEL mice divided by the mean percent $B220^+CFSE^+$ B cells in non-Tg mice in the same experiment. Open and filled bars show the results for B6 and NZB mice, respectively. Error bars indicate the SD. *D*, The number of IgM^{a+} anti-HEL Ab-producing cells was quantitated by ELISPOT. Each circle represents the determination from a single recipient mouse. Open and filled circles indicate B6 and NZB mice, respectively, with horizontal lines indicating the mean for each group.

Taken together, these findings indicate that NZB mice have an intrinsic B cell defect that results from background non-MHC differences between these mice and B6 mice and leads to $CD4^+$ T cell-dependent aberrant survival and proliferation of anti-HEL Ig Tg B cells in sHEL recipient mice, despite apparent tolerance to sHEL.

Persistence of NZB anti-HEL Ig Tg B cells in NZB but not (NZB × B6)F₁ sHEL recipient mice

We next assessed whether the aberrant response of transferred NZB anti-HEL Ig Tg B cells in sHEL recipient mice results in sustained survival of transferred cells. To this end, non-Tg and sHEL recipient mice were sacrificed 7 days after transfer of unlabeled NZB anti-HEL Ig Tg B cells. Immunofluorescence microscopy of splenic sections from (NZB × B6)F₁ and NZB sHEL

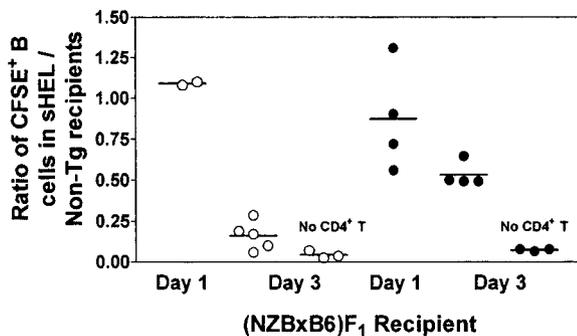**FIGURE 4.** T cell-dependent abnormal survival of NZB anti-HEL Ig Tg B cells following transfer into (NZB × B6)F₁ sHEL recipients. Scatter plot comparing survival of $CFSE^+$ anti-HEL Ig Tg B cells in sHEL recipient mice with that in non-Tg mice, in the presence or absence of $CD4^+$ T cells, 3 days following transfer. $CD4^+$ T cells were depleted by administration of anti-CD4 mAb i.p. The percent $B220^+CFSE^+$ B cells was quantitated by flow cytometry. Results are expressed as a ratio of the percent $B220^+CFSE^+$ B cells in sHEL mice divided by the mean percent $B220^+CFSE^+$ B cells in non-Tg mice in the same experiment. Open and filled circles show the results for B6 and NZB mice, respectively, with horizontal lines indicating the mean for each group.
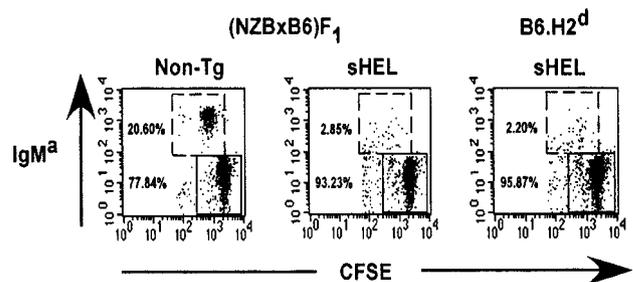
FIGURE 5. MHC differences between B6 and NZB mice do not account for the increased survival and proliferation of NZB anti-HEL Ig Tg B cells. Three-color flow cytometric analysis of splenocytes 3 days following transfer of 2×10^7 T cell-depleted $CFSE^{low}$ (2.5 μM) anti-HEL Ig Tg and $CFSE^{high}$ (5.0 μM) non-Tg B cells from B6.H-2^d mice into (NZB × B6)F₁ or B6.H-2^d, non-Tg, or sHEL recipient mice. Cells were stained with anti-B220 and -IgMᵃ. Dot plots shown are gated on $B220^+$ cells, with $CFSE^-$ cells gated out. Recipient mice are indicated above each dot plot. Boxes indicate $B220^+$ cells that are anti-HEL Ig Tg ($CFSE^{low}$, IgM^{a+}) (dashed) or non-Tg ($CFSE^{high}$, IgM^{a-}) (solid), with the percentage of each population shown next to the box.
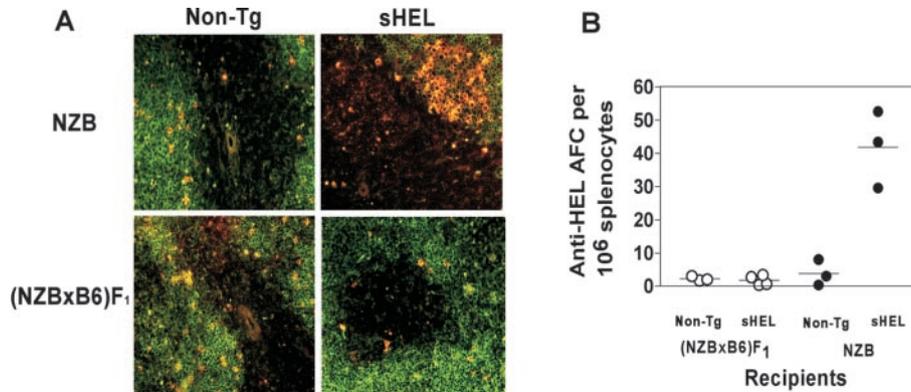
FIGURE 6. Sustained proliferation of NZB anti-HEL Ig Tg B cells and differentiation to HEL-Ab-forming cells in NZB sHEL recipient mice. *A*, NZB anti-HEL Ig Tg B cells were transferred into (NZB × B6)F$_1$ and NZB recipient mice. Immunofluorescence microscopy was performed on splenic sections from non-Tg and sHEL recipient mice injected 7 days earlier with 2–3 × 10^7 T cell-depleted splenocytes from anti-HEL Ig Tg mice. IgM^{a+} B cells have been stained red (rhodamine) and B220$^+$ cells green (FITC). Note proliferating focus of IgM^{a+} B cells at the T-B interface in NZB sHEL recipient mice. *B*, The number of IgM^{a+} anti-HEL Ab-producing cells was quantitated by ELISPOT. Each circle represents the determination from a single recipient mouse. Open and filled circles indicate (NZB × B6)F$_1$ and NZB mice, respectively, with horizontal lines indicating the mean for each group.

recipients, revealed that there were few remaining anti-HEL Ig Tg B cells in (NZB × B6)F$_1$ sHEL mice (Fig. 6*A*). In marked contrast, NZB sHEL recipients had numerous proliferative foci of anti-HEL Ig Tg B cells. These foci were located at the T-B interface, as were the few remaining cells in (NZB × B6)F$_1$ sHEL recipients. The location of the proliferative foci suggested that they were within germinal centers. This possibility was confirmed by staining with PNA and anti-IgM^a which revealed that all of the proliferative foci were PNA$^+$ (data not shown).

To determine whether there was similar persistence of Ab-forming cells in NZB sHEL recipients, we quantitated the number of IgM^{a+} anti-HEL-secreting cells at 7 days following transfer (Fig. 6*B*). Although an increased number of splenic IgM^{a+} anti-HEL-secreting B cells was no longer observed in (NZB × B6)F$_1$ sHEL recipients, the number of IgM^{a+} anti-HEL-secreting B cells in NZB sHEL recipients was increased 5- to 10-fold over that seen at 3 days following transfer. It should be noted, that the capacity of NZB sHEL mice to support this abnormality develops early in life and is already present in 2-mo-old NZB sHEL mice. Furthermore, this support can last for prolonged periods of time. In a subset of mice (two of four) examined at 21 days after transfer, the number of IgM^{a+} anti-HEL-secreting cells remained increased and elevated serum levels of anti-HEL Abs could be detected.

The differential ability of NZB and (NZB × B6)F$_1$ sHEL mice to support the abnormal persistence of NZB anti-HEL Ig Tg B cells reflects both MHC and background gene differences

To further explore the origin of the differential ability of NZB and (NZB × B6)F$_1$ sHEL recipient mice to support sustained proliferation and differentiation of NZB anti-HEL Ig Tg B cells, we transferred unlabeled NZB anti-HEL Ig Tg B cells into NZB.H-2$^{b/d}$ and (NZB × B6.H-2^d)F$_1$ sHEL recipients and quantitated the number of splenic anti-HEL Ab-producing cells 7 days later (Fig. 7). In contrast to (NZB × B6)F$_1$ sHEL recipients, NZB.H-2$^{b/d}$ sHEL recipients had significantly elevated numbers of anti-HEL Ab-producing cells, indicating that NZB non-MHC background gene homozygosity promotes the development of this immune abnormality (NZB.H-2$^{b/d}$, 15.61 ± 2.234; (NZB × B6)F$_1$, 1.67 ± 0.609; $p = 0.0093$). Nevertheless, the number of anti-HEL Ab-producing cells was significantly reduced in NZB.H-2$^{b/d}$ when compared with NZB sHEL recipients (NZB.H-2$^{b/d}$, 15.61 ± 2.243; NZB, 41.98 ± 3.788; $p = 0.0024$), suggesting that the decreased support for activation and differentiation of the H-2^d-
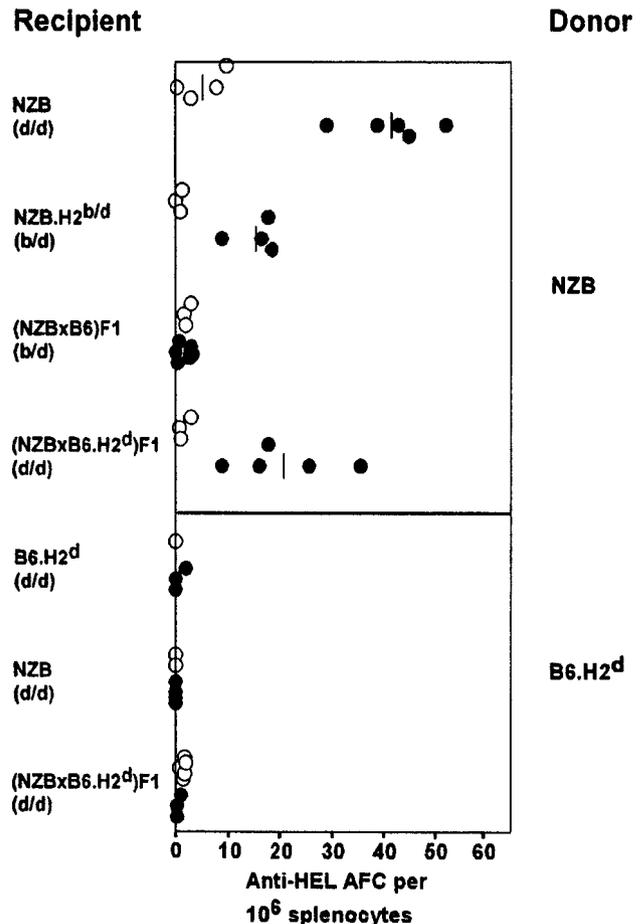

FIGURE 7. Both MHC and background gene NZB homozygosity in sHEL recipient mice promote the persistence of transferred NZB anti-HEL Ig Tg B cells. Briefly, 2–3 × 10^7 T cell-depleted splenocytes from NZB or B6.H-2^d anti-HEL Ig Tg donor mice (shown on the *right*) were transferred into various recipient mouse strains (shown on the *left* with MHC haplotype given in parentheses beneath). The number of IgM^{a+} anti-HEL Ab-producing cells was quantitated 7 days later by ELISPOT. Each circle represents the determination from a single recipient mouse. Open and filled circles indicate non-Tg and sHEL recipient mice, respectively, with lines indicating the mean for each group.
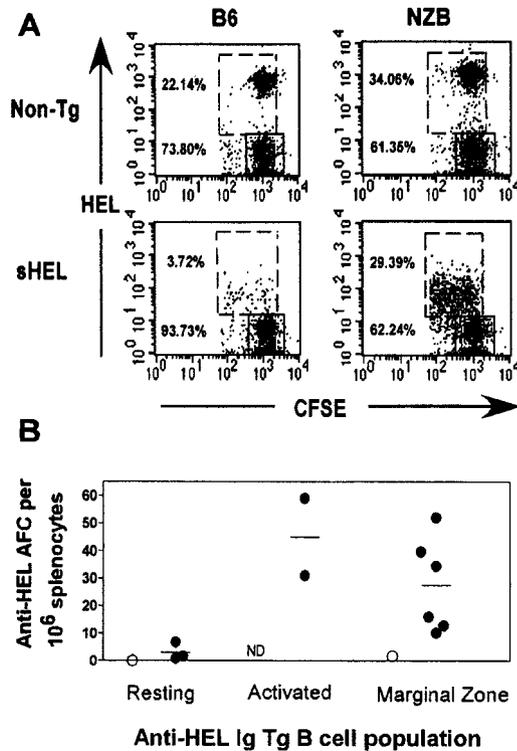
FIGURE 8. Identification of NZB anti-HEL Ig Tg B cell populations exhibiting aberrant proliferation and differentiation. *A,* Resting anti-HEL Ig Tg B cells from NZB mice exhibit aberrant proliferation 3 days following transfer into (NZB × B6)F_1 recipient mice. Three-color flow cytometric analysis of splenocytes 3 days following transfer of ~1 × 10^7 resting CFSE^{low} (2.5 μM) anti-HEL Ig Tg and CFSE^{high} (5.0 mM) non-Tg B cells from B6 or NZB mice into non-Tg or sHEL (NZB × B6)F_1 recipients. Cells were stained with anti-B220 and HEL. Dot plots shown are gated on B220$^+$ cells, with CFSE$^-$ cells gated out. Boxes indicate B220$^+$ cells that are anti-HEL Ig Tg (CFSE^{low}, HEL$^+$) (dashed) or non-Tg (CFSE^{high}, HEL$^-$) (solid), with the percentage of each population shown next to the box. *B,* NZB anti-HEL Ig Tg B cell populations enriched for marginal zone B cells lead to persistence of anti-HEL Ab-producing cells in NZB sHEL recipient mice. Approximately 1 × 10^7 resting (high density, predominantly follicular) and activated (low density, predominantly marginal zone) B cells or 1–5 × 10^6 sorted purified marginal zone B cells from anti-HEL Ig Tg mice were transferred into NZB non-Tg or sHEL recipient mice. The number of IgM^{a+} anti-HEL Ab-producing cells was quantitated 7 days later by ELISPOT. Each circle represents the determination from an individual mouse. Open and filled circles indicate non-Tg and sHEL recipient mice, respectively, with horizontal lines indicating the mean for each group.

bearing NZB anti-HEL Ig Tg B cells in (NZB × B6)F_1 sHEL recipient mice may also result from an altered T cell repertoire. This concept is further supported by results of transfers into (NZB × B6.H-2^d)F_1 sHEL recipients where significant numbers of anti-HEL Ab-producing cells ((NZB × B6.H-2^d)F_1, 21.05 ± 4.613; (NZB × B6)F_1,1.67 ± 0.609; $p = 0.0141$) were detected, although not to the levels seen in NZB sHEL recipients ((NZB × B6.H-2^d)F_1, 21.05 ± 4.613; NZB, 41.98 ± 3.788; $p = 0.0099$). Thus, in NZB mice non-MHC genes act in concert with the H-2^d haplotype to promote the altered activation of transferred anti-HEL Ig Tg B cells.

Consistent with results in previous sections, the ability of the NZB splenic environment to support altered B cell activation is critically dependent on the NZB intrinsic B cell defect, since transfer of B6.H-2^d anti-HEL Ig Tg B cells into B6.H-2^d, (NZB ×

B6.H-2^d)F_1, or NZB sHEL recipients did not lead to development of anti-HEL Ab-producing cells (Fig. 7).

Transfer of resting anti-HEL Ig Tg B cells into sHEL recipient mice results in early aberrant survival and proliferation, but does not lead to a persistent proliferative response or anti-HEL Ab production

The transferred population of anti-HEL Ig Tg B cells is comprised of several different B cell subsets including: T1, T2, marginal zone, and mature follicular B cells. The rapid generation of anti-HEL-secreting B cells in sHEL recipient mice strongly suggested that marginal zone B cells, which are known to be rapidly recruited into Ab responses (29), were leading to this manifestation of aberrant activation. However, it was not clear whether the aberrant proliferation of NZB anti-HEL Ig Tg B cells was also restricted to this subset. The resting B cell population contains T1 and mature follicular B cells, but excludes marginal zone and T2 B cells (17, 27). Therefore, to address this question, resting B cells were isolated from anti-HEL Ig Tg B6 and NZB mice, labeled with CFSE (2.5 μM), and transferred into sHEL or non-Tg (NZB × B6)F_1 recipient mice. As an internal control, transferred cells were mixed with non-Tg resting B cells labeled with a higher concentration of CFSE (5 μM). This revealed that NZB anti-HEL Ig Tg resting B cells demonstrated the same aberrant survival and proliferation as the total population 3 days following transfer (Fig. 8A).

We next sought to determine whether transfer of resting NZB anti-HEL Ig Tg B cells could lead to the development of proliferative foci and anti-HEL Ab-producing cells in NZB sHEL recipient mice. At 3 days following transfer of resting anti-HEL Ig Tg NZB B cells, anti-HEL Ab-producing cells could not be detected and by 7 days there were few surviving IgM^{a+} B cells in sHEL recipient mice (data not shown). In marked contrast, as early as 3 days following transfer of low-density B cell fractions containing marginal zone and T2 B cells, anti-HEL Ab-producing cells could be readily detected (Fig. 8B). Transfer of sorted purified marginal zone B cells confirmed the role of this cell population in the sustained development of anti-HEL Ab-producing cells (Fig. 8B) and proliferative foci (data not shown).

Discussion

The presence of activated IgM- and IgG-secreting self-reactive B cells in NZB mice strongly suggests that B cell tolerance processes are defective; however, the nature of the tolerance defects has been elusive. In this study, we demonstrate that NZB mice have a defect in the earliest checkpoint involved in the maintenance of peripheral tolerance, follicular exclusion and elimination of self-reactive B cells in the absence of T cell help. We show that although naive self-reactive NZB B cells migrate appropriately to the T-B interface following contact with Ag, they survive, proliferate, and differentiate to Ab-forming cells, despite the presence of putatively tolerizing self-Ag. This aberrant response appears to result from an intrinsic NZB B cell functional defect and is facilitated by the NZB splenic environment.

Enhanced survival and proliferation of NZB anti-HEL Ig Tg B cells at 3 days following transfer was seen in all sHEL recipients tested and was present in the resting B cell subset. This phenotype was not shared by B6.H-2^d B cells, indicating that NZB mice have an intrinsic B cell defect that results from genetic polymorphisms that are localized outside the MHC locus. The CD4$^+$ T cell dependence of this defect suggests that it affects the ability of NZB B cells to activate T cells and/or to respond to T cell-derived stimuli. Previous work by ourselves and others, examining NZB resting B cell function in vitro, has defined a number of abnormalities that may be relevant to this point. We have shown that resting B

cells from NZB mice express higher levels of the costimulatory molecules B7.1 and B7.2 following CD40 engagement (17). This abnormality could lead to increased costimulation of self-reactive (sHEL or autoantigen specific) T cells during T-B collaboration resulting in enhanced survival and/or cytokine production by these cells in sHEL recipient mice. Alternatively, resting B cells from B6 and NZB mice may activate self-reactive T cells equivalently, but may differ in their capacity to proliferate in response to limited T cell signals. This is consistent with the observation that NZB resting B cells are hyperproliferative following stimulation with T cell-derived cytokines, such as IL-4, IL-5, and IFN-γ (17), or cross-linking of cell surface IgM and MHC class II (15).

Transfer of resting NZB anti-HEL Ig Tg B cells into sHEL recipient mice did not lead to development of Ab-secreting cells on day 3. Since the resting B cell fraction contains predominantly mature follicular and T1 B cells, it is likely that the aberrant generation of anti-HEL Ab-secreting cells results from a defect in the marginal zone or T2 B cell populations in these mice. Of these two populations, the kinetics of the anti-HEL Ab response are most compatible with a marginal zone B cell defect. Marginal zone B cells are capable of rapidly differentiating into plasma cells following contact with blood-borne Ags, with a peak at 3–4 days (30). Although these kinetics were originally described for T-independent Ags, it is likely that similar rapid kinetics are seen for T-dependent responses because marginal zone B cells acquire the ability to costimulate T cells in <4 h (31).

Our experiments clearly implicate the marginal zone B cell population in the $CD4^+$ T cell-dependent generation of proliferative foci and anti-HEL Ab-secreting cells 7 days following transfer of NZB anti-HEL Ig Tg B cells into sHEL recipient mice. Several findings argue that marginal zone B cell function is abnormal in NZB mice. NZB marginal zone B cells demonstrate enhanced proliferation following stimulation with anti-Ig and -MHC class II Abs (15). Additionally, NZB mice have an increased proportion of marginal zone B cells and these cells have a more "activated" phenotype, with increased levels of costimulatory molecules, than those from nonautoimmune mouse strains (27). Similar phenotypic abnormalities are observed in NZB anti-HEL Ig Tg mice (V. Roy and J. E. Wither, unpublished observations). Mounting evidence suggests that recruitment of B cells into the marginal zone compartment involves positive selection, which is critically dependent upon signaling thresholds in the B cells (reviewed in Ref. 31). Thus, the expansion and increased activation of the marginal zone compartment in NZB mice suggest that signaling is enhanced in these cells.

What is the nature of the T cell help that provides support for the abnormal NZB B cell response in sHEL recipient mice? There are several possibilities. sHEL-reactive T cells may become activated in sHEL recipient mice following interaction with activated NZB anti-HEL Ig Tg B cells. Previous experiments indicate that anergic HEL-specific T cells in sHEL mice can become activated with a strong immunogenic stimulus (32, 33). Activated NZB, but not B6 or B6.H-2^d, B cells may be able to provide this stimulus and thus NZB B cells may solicit their own T cell help. This possibility is compatible with studies in MRL-*lpr/lpr* and NZB/W lupus-prone mouse models where B cells have been shown to play an important role in the expansion of $CD4^+$ memory T cells, presumably through activation of self-reactive T cells (Refs. 34 and 35 and V. Roy and J. E. Wither, unpublished observations). Alternatively, autoreactive T cells could provide support for NZB anti-HEL Ig Tg B cell proliferation and differentiation in sHEL recipient mice. In this case, activated NZB anti-HEL Ig Tg B cells in sHEL recipient mice could interact with activated autoreactive T cells already present in recipient mice or, alternatively, solicit their own

help activating naive autoreactive T cells. Finally, the presence of activated T cells in the environment may provide support to NZB anti-HEL Ig Tg B cells in the absence of a specific recognition event through bystander mechanisms such as secretion of cytokines.

The differential ability of (NZB × B6)F_1 recipients to support NZB anti-HEL Ig Tg B cell proliferation and anti-HEL Ab production on days 3 and 7 following cell transfer suggests that the T cell signals involved in the early and sustained responses differ qualitatively or quantitatively. The observation that NZB MHC and non-MHC background gene homozygosity has little impact on the early proliferative abnormality suggests that direct interactions between self-reactive T cells and NZB anti-HEL Ig Tg B cells may not be required for this response. Instead, the early proliferative abnormality may reflect an abnormal response to the cytokine milieu in the outer PALS. Preliminary results examining NZB anti-HEL Ig Tg B cell proliferation following transfer into sHEL DO11.10 TCR Tg mice are consistent with this possibility. Although >90% of $CD4^+$ T cells express an OVA-specific TCR in these mice, anti-HEL Ig Tg B cell proliferation was equivalent to that seen in sHEL recipient mice.

The requirements for T cell support of a more prolonged response (≥7 days) following transfer of NZB anti-HEL Ig Tg B cells are more stringent. In this case, both NZB MHC and non-MHC background gene homozygosity appear to act in an additive fashion to facilitate a sustained response. The observation that H-2^d sHEL recipient mice have more anti-HEL Ab-forming cells than corresponding background gene-matched H-$2^{b/d}$ sHEL mice suggests that H-2^d class II-restricted $CD4^+$ T cells are providing support for sustained anti-HEL Ab production.

Regardless of MHC haplotype, the homozygous NZB splenic environment better supports sustained anti-HEL Ab production than the F_1 environment. NZB mice have an increased proportion of splenic $CD4^+$ T cells with a memory phenotype (J. E. Wither, unpublished observations) consistent with enhanced activation of autoreactive T cells. It is possible that this increased T cell activation leads to increased local levels of cytokines that facilitate anti-HEL Ab production through bystander activation. Alternatively, defective T cell tolerance mechanisms in NZB mice may lead to increased activation of the sHEL-specific or autoreactive T cells that provide support for sustained anti-HEL Ab production. Consistent with this possibility, NZB mice possess a number of T cell and thymic abnormalities that could impact on central tolerance (36–38).

Although our studies have examined the response to a neo-self-Ag, our findings are generally relevant to the activation of autoreactive B cells. Low-affinity autoreactive B cells can evade central tolerance mechanisms, resulting in the exit of naive autoreactive B cells to the periphery (39). Indeed, experiments suggest that the marginal zone population may be enriched for B cells with low affinity for self-Ags (reviewed in Ref. 31). Following contact with the relevant autoantigen, naive autoreactive B cells would be expected to rapidly migrate to the T-B interface, where they await T cells signals. For many nuclear Ag-reactive B cells, including those specific for La, small nuclear ribonucleoprotein, ssDNA, and to some extent dsDNA, T cell tolerance constitutes a major checkpoint preventing production of autoantibodies (40–43). Thus, in normal mice, autoreactive B cells that have been induced to migrate to the outer PALS fail to receive T cell help and are eliminated. In this study, we have shown that in marked contrast to normal mice, autoreactive B cells from NZB mice are able to effectively interact with autoreactive T cells, resulting in proliferation, recruitment into germinal centers, and differentiation of

the B cells to autoantibody-secreting cells. This abnormal T-B collaboration between self-reactive cells provides a potential explanation for the spontaneous development of germinal centers in young germfree NZB mice (44). In addition, our findings suggest that the aberrant B cell activation and generation of IgM autoantibody-secreting cells in NZB mice is likely to be T cell dependent. Although traditionally polyclonal B cell activation in NZB mice has been viewed as T cell independent, recent findings in NZB.CD4$^{-/-}$ mice provide support for the role of T cells in this process (45).

Acknowledgments

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