B-cell anergy is maintained in anti-DNA transgenic NZB/NZW mice

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Abstract

Clonal anergy has been well recognized as an important mechanism of B cell immunologic tolerance. However, the properties of anergic B cells and especially their role in the development of autoimmune disease in susceptible animals have been controversial. Here we show that low-affinity anti-DNA anergic B cells populate the mature B-cell compartment in the mouse spleen in excessive numbers and display paradoxical behavior in response to a combined B-cell receptor/TLR9 activation. Surprisingly, B-cell anergy was maintained in aged NZB/NZW F1 mice that develop a systemic lupus erythematosus (SLE)-like autoimmune disease. In several parameters of anergy, such as calcium mobilization and antibody secretion, the lupus-prone mice appeared more anergic than their non-autoimmune counterparts. We conclude that low-affinity anergic B cells are unlikely to serve as precursors for the high-affinity autoreactive B cells that give rise to pathogenic anti-DNA auto-antibodies in SLE.

Keywords: autoimmunity, lupus, tolerance, transitional B cells

Introduction

Mouse strains with an inherited predisposition to autoimmune disorders provide an approach for studying the breakdown of self-immunological tolerance and the production of auto-antibodies. The NZB/NZW F1 mice are considered to be the murine model most closely resembling human systemic lupus erythematosus (SLE) (1, 2). The lupus-like disease in these mice is more severe in females and is accompanied by high-affinity anti-dsDNA auto-antibodies that are believed to play a role in the development of fatal immune glomerulonephritis (2, 3). Both NZB and NZW parents contribute multiple susceptibility genes to the immune abnormalities of the F1 hybrid mouse (4). These include polyclonal B cell hyperactivity at an early age, the appearance of IgM anti-DNA antibodies at 3–5 months and a spontaneous switch from IgM to IgG anti-DNA antibodies at 5–7 months concomitantly with the onset of severe SLE and renal disease (2, 3).

Several studies on the pathogenesis of human and murine SLE have suggested that intrinsic B-cell defects may be the primary cause for the loss of self-tolerance and development of autoimmune disease (5, 6). Therefore, the mechanisms of B-cell tolerance, particularly those involving clonal deletion of autoreactive B cells (7), clonal anergy (8) and B-cell receptor (BCR) editing (9), have become the focus of intensive investigation.

Anergy is a property of low-affinity B cells that have escaped central tolerance in the bone marrow (i.e. deletion and editing) and migrated to the periphery (reviewed in refs 8, 10). Anergy has been difficult to define due to the great variation in BCR affinity and in the cross-linking capacity of the different autoantigens (8, 10). Functionally, anergic B cells have a reduced capacity to participate in immune responses, particularly a reduced or absent ability to secrete auto-antibodies. Other features of anergic cells may include shortened half-life in vivo, developmental arrest, altered migration and anatomical localization and down-regulation of the BCR. Additionally, anergic B cells are relatively resistant to activation of BCR (e.g. by anti-IgM) and TLR (e.g. by LPS and CpG), or to activation by T cells (e.g. by anti-CD40), as demonstrated by their reduced proliferation, reduced signal transduction, reduced calcium mobilization and reduced activation marker expression (8, 10). Maintenance of B-cell anergy requires the chronic binding of antigen and signal transduction, and many features of anergic cells are rapidly reversed after dissociation of self-antigen (11, 12). Very recently, a population previously thought to identify late-transitional B cells (T3) in the spleen was claimed to represent, instead, anergic B cells in transgenic (Tg) and wild-type mice and was termed ‘An1’ (13).
Anergic B cells have also been identified in anti-DNA transgenic or knock-in mice (14–22). They are usually of low to medium affinity for the autoantigen since high-affinity anti-DNA B cells are deleted in the non-autoimmune animal or edit their autoreactive receptors (17, 23–25). The anti-DNA anergic B cells have been shown to have diminished Ig secretion in response to different stimuli compared with non-Tg controls, reduced proliferation and signal transduction in response to anti-IgM or LPS and reduced surface IgM (15, 16). When the anti-DNA transgene was bred onto the autoimmune MRL-Fas<sup>br/br</sup> mouse, the anergic phenotype was breached, as demonstrated by the presence of DNA-specific antibodies in the serum (18); this was achieved in some B-cell clones, by L-chain rearrangement and by the accumulation of somatic mutations (19).

In order to study the effect of the autoimmune genetic background on the state of B-cell anergy in the lupus-prone NZB/NZW mice, we have utilized our well-defined anti-DNA knock-in mouse lines (17, 21, 25). In these mouse strains, a single anti-DNA H chain (D42H of the NZB/NZW anti-dsDNA D42 hybridoma) or combinations of this H chain with different L chains have been targeted to the appropriate chromosomal loci in the mouse genome. One such combination, D42H/Vk8-Jk5 with an apparent DNA affinity of 1.6 × 10<sup>7</sup> M<sup>−1</sup> bp<sup>−1</sup> (25), has been shown to be strongly resistant to receptor editing on both non-autoimmune and NZB/NZW genetic backgrounds (21, 25). The binding characteristics of D42H/Vk8-Jk5 antibodies were similar in fine specificity for ssDNA and dsDNA and in the speckled pattern of the fluorescent anti-nuclear antibody test to those of some other low-affinity anti-DNA antibodies, such as D42H/Vk1-Jk1 and D42H/Vk4-Jk4 (25). However, transgenic D42H/Vk8-Jk5 mice had much lower serum IgM and IgG anti-DNA-binding activity than D42H or the editing D42H/Vk1-Jk1 NZB/NZW transgenic mice (21). We have, therefore, considered the possibility that the D42H/Vk8 B cells are anergic. A thorough investigation of multiple parameters of B-cell anergy has shown, surprisingly, that the great majority of these cells maintains or even intensifies their anergic state in the diseased animals.

**Methods**

**Mice**

NZB and NZW mice were purchased from Harlan (Oxon, UK). All mice were maintained at the SPF animal facility of the Hebrew University Medical School, Jerusalem. Knock-in mice, transgenic for D42H and Vk8-Jk5, were constructed as described previously (17, 21, 25). Except where specifically indicated otherwise, all mice employed in this study were 7- to 9-month-old females. The Joint Ethics Committee (IACUC) of the Hebrew University and Hadassah Medical Center approved the study protocol for animal welfare. The Hebrew University is an AAALAC International Accredited Center approved the study protocol for animal welfare. The IACUC of the Hebrew University and Hadassah Medical School, Jerusalem. Knock-in mouse lines (17, 21, 25). In these mouse strains, mice, transgenic for D42H and Vk8-Jk5, were constructed as described previously (17, 21, 25). Except where specifically indicated otherwise, all mice employed in this study were 7- to 9-month-old females. The Joint Ethics Committee (IACUC) of the Hebrew University and Hadassah Medical Center approved the study protocol for animal welfare. The Hebrew University is an AAALAC International Accredited Institute.

**Antibody reagents**

FACS reagents: rat anti-mouse CD16/CD32 (Fc Block), CD43-biotin, CD21/35-FITC, IgM-FITC, CXCR4-FITC, streptavidin-Cy (BD Pharmingen, San Diego, CA, USA); goat Fab<sup>‘</sup>- and (Fab‘)<sub>2</sub> anti-mouse μ-chain-FITC (Jackson Laboratories, Baltimore Pike, PA, USA); rat anti-mouse CD45R/B220–Pacific blue (Biolegend, San Diego, CA, USA); rat anti-mouse CD24 (HSA)-biotin, CD23-PE, CD93 AA4.1-APC, IgM PE-Cy7, BAFF-R-FITC (eBioscience, San Diego, CA, USA); rat anti-mouse CD45R/B220-PE, CD19-PE, IgM-FITC and goat (Fab‘)<sub>2</sub> anti-rabbit IgG (H + L)-FITC/PE/APC (SBA, Birmingham, AL, USA).

Reagents for ELISA and ELISPOT: unmodified and HRP-conjugated goat anti-mouse IgM and goat anti-mouse IgG, unmodified and HRP-conjugated rabbit anti-mouse IgM+ IgG (H + L), HRP-conjugated goat anti-mouse IgM+ IgG (H + L), HRP-conjugated streptavidin, AffiniPure (Fab‘)<sub>2</sub> goat anti-mouse IgM, μ-chain-specific, for B-cell activation (Jackson Laboratories) and rabbit anti-idiotypic reagent, D42 V<sub>γ5</sub>-specific RF81 (17).

**Experimental procedures**

Hybridoma production, flow cytometric analysis, reverse transcription (RT)–PCR, sequence determinations and DNA-binding tests were carried out as described previously (17, 21, 25). Splenic CD43<sup>−</sup> B cells were isolated with anti-CD43 magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). ELISPOT assays were carried out as described (26). B-cell apoptosis was measured by FACS with DiOC<sub>4</sub>(3) as described (27); apoptosis by Annexin V was carried out with an apoptosis kit (MBL, Woburn, MA, USA).

In vitro cell proliferation

For thymidine incorporation, MACS-purified B cells were incubated in triplicate microc wells (100 μl, 5 × 10<sup>5</sup> cells per well) with the different activators, endotoxin-free *Escherichia coli* K12 DNA, LPS, ODN 1826 or ODN 1826 control (InvivoGen, San Diego, CA, USA) for 40 h at 37°C. <sup>3</sup>H-thymidine (1 μCi per well) was then added for an additional 8 h incubation and the samples were processed and counted in a Tomtek 96-well harvester. CFSE labeling was carried out with a CFSE cell proliferation kit for flow cytometry (Molecular Probes, Eugene, OR, USA). Incubation with the different activators was done as above. Where indicated, chloroquine, piceatannol (Sigma, St Louis, MO, USA) or PP2 (Calbiochem, San Diego, CA, USA) was included as inhibitor of cell proliferation. Cell generations were resolved using ModFit software (Verity Software House, Topsham, ME, USA).

**Calcium flux**

MACS-purified B cells (5 × 10<sup>7</sup> ml<sup>−1</sup>) were loaded with 1 μM Indo-1AM (Molecular Probes) for 1 h at RT. The cells were washed and stained with the appropriate antibodies (anti-IgM Fab was used to avoid cell activation). Following 3-min incubation at 37°C, the cells were stimulated with 50 μg ml<sup>−1</sup> anti-IgM (Fab‘)<sub>2</sub> for 2 min, then with 10 μg ml<sup>−1</sup> ionomycin for 2 min. Intracellular calcium was evaluated by measuring fluorescence at 405 and 485 nm after excitation at 355 nm with a LSRII flow cytometer (Becton-Dickinson, San Diego, CA, USA). Data were analyzed with FlowJo software (Tree Star Inc., Ashland, OR, USA).
**Western blot analysis**

MACS-purified B cells (1 × 10^7) were stimulated with 10 μg ml^{-1} anti-IgM (Fab'2)i for 1–15 min at 37°C. The cells were lysed in a hypotonic buffer and the lysates resolved on 10% SDS–PAGE and electroblotted onto nitrocellulose membranes. Tyrosine phosphorylation was analyzed with 4G10 anti-phosphotyrosine (Upstate, Lake Placid, NY, USA). Anti-mouse syk and anti-mouse NFκB P65 antibodies were purchased from Santa Cruz Biotechnology. Nuclear extracts were prepared as described (28). Rabbit anti-mouse β-actin (Cell Signaling) was used for protein normalization.

**Results**

**Properties of anti-DNA D42H/Vx8-Jk5 B cells in non-autoimmune genetic background**

We have previously shown that D42H/Vx8-Jk5 Tg B cells are strongly resistant to receptor editing, as compared with D42H/Vx1-Jk1 or D42H/Vx4-Jk4 anti-DNA B cells, which have a similar affinity and fine specificity for the autoantigen (25). Nevertheless, these autoreactive cells were not deleted in the bone marrow. Instead, they had normal numbers of pro-B and immature B cells and reduced numbers of pre-B cells, commensurate with their greatly reduced ability to edit their BCR (25). In the spleen, however, the number of D42H/Vx8 B cells, especially of mature B cells, was dramatically increased (Fig. 1A and Table 1; Table 3 in ref. 25). Since the number of precursor immature B cells was not increased (25), this higher prevalence of splenic B cells suggests a higher resistance to apoptosis and a longer half-life for these cells. Indeed, the percentage of D42H/Vx8 B cells susceptible to induction of apoptosis by mitochondrial inhibitors, such as DiOC6(3), was much lower than that of D42H transgenic B cells (Fig. 1B). Similar results were obtained with AnnexinV/PI staining of D42H and D42H/Vx8 B cells undergoing early apoptosis (data not shown).

We next looked at BCR density as compared with non-transgenic (C57BL/6 × BALB/c)F1 mice and heavy-chain-only D42H Tg mice in the same genetic background. As shown in Fig. 1(C), there was a gradual decrease in BCR (IgM) density in purified B cells from non-Tg mice, through D42H mice to D42H/Vx8 (D8) Tg mice, suggesting that the latter B cells were the most anergic. BCR density could be restored to almost normal levels in D42H mice by TLR agonists, such as LPS, CpG ODN and *E. coli* DNA. BCR densities in D42H/Vx8 B cells were also up-regulated by these agents, although to a lesser extent than their H-chain-transgenic counterparts (Fig. 1C). A non-stimulating (control) CpG ODN and mammalian (calf thymus, CT) DNA were completely inactive in this process. To test whether a chronic exposure to self-antigen (DNA) was necessary for the maintenance of this aspect of the anergic state, the cells were treated with bovine deoxyribonuclease I, previously calibrated (data not shown) to remove all exogenous DNA. The DNase treatment had no effect on BCR density in non-Tg B cells, but led to a marked positive shift in the surface IgM density of D42H/Vx8 cells (Fig. 1D), indicating that a constant self-antigen occupancy of the BCR was indeed required for the anergic state as previously suggested (11, 12).

Since the anergic D42H/Vx8 B cells express a BCR with DNA specificity, we wished to explore the extent of synergy between BCR and TLR9, in response to DNA activation. It has been shown previously that TLR9 signaling directs class-switch recombination toward T_{\text{m}}1-like Ig isotype production (31, 32) and may be important for class-switching to pathogenic auto-antibody isotypes in SLE (33). Consequently, we have measured the effect of *E. coli* and CT DNA on the expression of activation-induced cytidine deaminase (AID) and on the expression of pre- and post-switch IgG2a constant-region transcripts. Surprisingly, the anergic anti-DNA Tg D42H/Vx8 B cells were stimulated by bacterial DNA to a much greater extent than non-transgenic B cells (Fig. 1E). Mammalian CT DNA had only a very small effect. This suggests that the BCR promotes TLR activation by the introduction of bacterial DNA into the cytosol and by recruiting endosomal TLR9. It may also suggest that anergy may be reversed in DNA-specific B cells by the dual action of BCR and TLR. The relative inactivity of mammalian DNA in this mechanism was apparently due to its weak interaction with TLR9, as it was bound with the same or better efficiency to a D42H/Vx8-Jk5 IgM mAb, representing the D42H/Vx8 BCR (Fig. 1F).

The synergy between BCR and TLR in anergic anti-DNA B cells was further tested in cell proliferation by thymidine incorporation assays (Fig. 2A). While LPS activation, which is BCR independent, clearly showed that D42H/Vx8 B cells were anergic, in that they were poorly stimulated to proliferate, as compared with non-transgenic and D42H Tg B cells, the situation was reversed with CpG ODN or *E. coli* DNA (but not CT DNA) activation (Fig. 2A). In these instances, the anergic D42H/Vx8 cells proliferated to the greatest extent, whereas the non-Tg B cells were the least affected. The H-chain-only Tg B cells had an intermediate response to these activators. This clearly suggests a BCR/TLR synergy in a reversal of the anergic state by CpG ODN and bacterial DNA. These results were confirmed by proliferation assays, in which FACS analyses of CFSE dilution were carried out (Fig. 2B). The synergistic BCR/TLR effect was particularly effective when bacterial DNA was used with D42H/Vx8 B cells, as non-transgenic B cells were not induced to proliferate by this high-molecular weight DNA (Fig. 2B and ref. 34); however, the anti-DNA D42H/Vx8 B cells proliferated significantly, presumably due to the BCR cross-linking capacity of bacterial DNA. To further clarify this mechanism, we have used specific BCR and TLR9 inhibitors (Fig. 2C). The TLR9 inhibitor, chloroquine, abolished CpG-induced proliferation of D42H/Vx8 B cells, but was only partly effective in preventing *E. coli* DNA-induced proliferation. In contrast, the syk inhibitor piceatannol (35) was partially inhibitory with both CpG and *E. coli* DNA, suggesting some interference with signal transduction through the BCR (36). When both chloroquine and piceatannol were employed, no proliferation of D42H/Vx8 B cells was observed following *E. coli* DNA activation (Fig. 2C). These experiments suggest that the BCR and TLR9 synergize to produce maximal proliferation of anti-DNA anergic B cells. The Src-family kinase inhibitor PP2, which inhibits BCR internalization (37), completely blocked both CpG-ODN and *E. coli* DNA-induced proliferation (data not shown).
Ig secretion is the most direct functional measure of B-cell activity and anergy. In all our experiments, the spontaneous (non-induced) secretion of IgM or IgG antibodies was lower in D42H/Vj8 than in D42H-only transgenic B cells (Figs 2D and 3D), as measured by the ELISPOT assay. Unlike cell proliferation, however, stimulation of antibody secretion by all BCR and TLR inducers (i.e. anti-IgM, LPS, CpG, E. coli DNA) or by the T-cell help mimicking agent anti-CD40 was uniformly lower in the anergic D42H/Vj8 than in the non-anergic D42H transgenic B cells (Figs 2D and 3D). This indicates that proliferation and class-switch recombination are much more easily induced in anergic B cells than Ig secretion.

It has recently been suggested (13) that in a variety of transgenic mouse models of tolerance induction, as well as in wild-type mice, the late transitional (T3) cell population in the spleen (CD93+ IgMlow CD23+) does not mark a developmental stage as previously thought (38); rather, the population defined by these markers exhibits all the characteristics of anergic cells (8, 13). We have tested this hypothesis with regard to our anti-DNA transgenic D42H/Vj8-Jj5 mice (Fig. 2E and Table 1). Based on the same surface markers used in the previous studies (13, 38), we found that the fraction of transitional cells (CD93+) in the spleen was similar in wild-type (B6 x BALB/c)F1 mice and anergic D42H/Vj8 mice; however, the fraction of T3 within the CD93+ cells was doubled in the anergic strain (Fig. 2E and Table 1). The number of mature B cells in the spleen was markedly increased in D42H/Vx8 mice as compared with wild-type mice and their fraction in the total B220+ population was...
Table 1. Splenic B-cell populations

<table>
<thead>
<tr>
<th>Mouse</th>
<th>No. of total B cells</th>
<th>No. of CD93+ cells</th>
<th>No. of mature B cells</th>
<th>No. of T3 cells</th>
<th>No. of T2 cells</th>
<th>No. of MZ cells</th>
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<tr>
<td>B6</td>
<td>$1.0 \pm 0.2 \times 10^6$</td>
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<td>B/W</td>
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<tr>
<td>D42H</td>
<td>$1.0 \pm 0.2 \times 10^6$</td>
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<td>V8k</td>
<td>$1.0 \pm 0.2 \times 10^6$</td>
<td>$0.1 \pm 0.1 \times 10^6$</td>
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Comparison between non-autoimmune and NZB/NZW anergic D42H/Vxk8 B cells

The D42H and Vxk8-Jk5 targeted genes were bred onto NZB and NZW mice, respectively, for eight generations and then crossed to produce D42H/Vxk8-Jk5 transgenic NZB/NZW F1 mice. Although NZB/NZW mice are lymphopenic (2) and have a reduced number of splenic B cells (Table 1), there was a significant increase in transgenic D42H/Vxk8 B cells on the NZB/NZW background, parallel to that seen in non-autoimmune mice (Table 1). Previous and additional parameters of anergy were employed for comparison of the anergic phenotype on the non-autoimmune and lupus-prone genetic background (Fig. 3). Surprisingly, the BCR (IgM) surface density of B cells from 8-month-old female NZB/NZW D42H/Vxk8 mice had a similar relationship to that of wild-type NZB/NZW mice as did D42H/Vxk8 BCR density to (B6 × BALB)F1 B cells (Fig. 3A and Supplementary Figure 1, available at International Immunology Online). This suggests that most of the B/W cells remained anergic. However, a small fraction of NZB/NZW D42H/Vxk8 B cells repeatedly showed an increased BCR density, suggesting that some B cells escaped anergy and became activated (Fig. 3A). The anergic phenotype was also evident in the nearly identical CFSE proliferation profiles produced by the various activating agents (Supplementary Figure 2, available at International Immunology Online) and a similar up-regulation of AID expression (Supplementary Figure 3, available at International Immunology Online). Additionally, the anti-IgM-induced BCR-dependent phosphorylation of the signal-transducing protein syk as well as the nuclear entry of the NFκB transcription factor p65 (Rel A) were inhibited to a similar extent in D42H/Vxk8 and B/W D42H/Vxk8 B cells as compared with their wild-type counterparts (Fig. 3B).

Impairment of intracellular calcium mobilization in response to BCR aggregation is a well-documented parameter of B-cell anergy (13, 39). A comparison of anti-IgM-induced calcium mobilization in purified B cells from wild-type and transgenic mice showed (Fig. 3C) that NZB/NZW B cells were more stimulated than those of non-autoimmune (B6 × BALB)F1 mice. However, anergic D42H/Vxk8 B cells from NZB/NZW mice were less active in anti-IgM-induced calcium mobilization, not only compared with their wild-type littermates but also with D42H/Vxk8 cells derived from the
non-autoimmune mice. This trend continued and amplified when calcium influx was induced non-specifically by ionomycin, indicating that the anergic state is independent of the mode of activation. Mature B cells exhibited a similar calcium mobilization profile to that of total B cells (Fig. 3C).

Finally, an ELISPOT assay comparing antibody-secreting B cells from anergic D42H/V₉j₈ transgenic (B6 × BALB)F₁ and NZB/NZW mice (Fig. 3D) showed that in response to LPS stimulation, the NZB/NZW B cells were at least as anergic as B cells from non-autoimmune mice. With CpG stimulation, the number of antibody-secreting NZB/NZW D42H/V₉j₈ cells was significantly lower than that of non-autoimmune anergic B cells. We conclude that the anti-DNA transgenic D42H/V₉x8 B cells in female NZB/NZW mice maintain or intensify their anergic phenotype, as compared with the same transgenic cells on a non-autoimmune background.

**Transitional B-cell populations are drastically reduced in diseased NZB/NZW mice**

In order to assess the contribution of transitional B cells, particularly T₃, to the maintenance of the anergic phenotype in NZB/NZW mice, we have measured the splenic transitional B-cell populations in the various wild-type and transgenic mouse strains (Fig. 4A and Table 1) and found, surprisingly, that the CD93⁺ B-cell population was essentially absent from 8-month-old diseased NZB/NZW female mice. This phenomenon was probably associated with disease development, as the decrease in transitional B cells was age dependent (Fig. 4B), but could not be explained by age alone (Table 1 and ref. 40). The disappearance of transitional B cells was not dependent on the CD93 surface marker, as T₁ and T₂ populations alternatively defined by CD21 and CD24 (41) were also drastically reduced in NZB/NZW mice (Supplementary Figure 4, available at International Immunology Online). The decrease in NZB/NZW transitional B cells was probably unrelated to anergy since D42H/V₉x8 NZB/NZW mice had an increased population of T₃ B cells relative to wild-type NZB/NZW mice (Fig. 4A and Table 1). Furthermore, removal of the autoantigen by treatment with DNase I in vitro had no effect on the transitional B-cell populations (Supplementary Figure 4, available at International Immunology Online). In order to better

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**Fig. 2.** Synergy between BCR and TLR9 in anergic anti-DNA B cells. (A) Proliferation of MACS-purified B cells from non-Tg, D42H or D42H/V₉x8-J₅ (D₈) Tg mice as measured by [³H]-thymidine incorporation, following stimulation with LPS, CpG ODN or *Escherichia coli* DNA, as described in Methods. Proliferation following CT DNA stimulation (dashed lines) was negative in all mice. The figure represents an average of three independent experiments, each in triplicate cultures. (B) Proliferation of MACS-purified B cells from non-Tg or D42H/V₉x8-J₅ Tg mice, as measured by FACS analysis of CFSE dilution, following stimulation with 0.3 μg ml⁻¹ CpG ODN or 1 μg ml⁻¹ *E. coli* DNA. Cell divisions were analyzed by ModFit software. (C) Inhibition of D42H/V₉x8-J₅ B-cell proliferation by TLR9 and BCR inhibitors. Chloroquine (1 μg ml⁻¹) or piceatannol (100 μM) was added alone or together to the CFSE-pulsed B cells before incubation with 0.3 μg ml⁻¹ CpG ODN or 1 μg ml⁻¹ *E. coli* DNA (40 h, 37°C). (D) Enumeration of D42H and D42H/V₉x8-J₅ IgG antibody-forming cells by ELISPOT, following stimulation (40 h, 37°C) of MACS-purified B cells with 10 μg ml⁻¹ anti-CD40 mAb or 1 μg ml⁻¹ *E. coli*/CT DNA. Equal numbers of stimulated B cells were incubated with membrane-bound goat anti-mouse IgG Ab overnight at 37°C. The membranes were then incubated with rabbit anti-D42 idiotype antibody and developed as described in Methods. *P < 0.05 by one-tailed Mann–Whitney U-test. (E) FACS analysis of splenic B cells from non-Tg and D42H/V₉x8-J₅ Tg mice. The middle panels are gated on B220⁺ CD93⁺ cells; the right panels are gated on B220⁺ CD93⁻ cells. Transitional (T₁, T₂ and T₃) mature and marginal zone (MZ) B cells are indicated. Each figure represents at least three independent experiments with similar results.
understand this phenomenon, we have looked at several cell surface components that could affect the transition of immature to transitional B cells in NZB/NZW mice. Total bone marrow B220
\(^+\) B cells and immature B cells were reduced 2-fold in NZB/NZW mice as compared with non-autoimmune mice (Supplementary Table 1, available at International Immunology Online). BAFF receptor levels were identical on all tested bone marrow and splenic populations of (B6\(^{3}/2\)BALB)F1 and NZB/NZW mice. However, preliminary experiments have suggested that the levels of the chemokine receptor CXCR4 were higher in BM pre/pro-B and immature B cells in NZB/NZW mice as compared with non-autoimmune mice and increased with age (Supplementary Figure 5, available at International Immunology Online). This could potentially lead to a retention of immature B cells in the bone marrow.

Discussion

Our site-directed transgenic mouse system allows for a single H chain (D42H) with features that strongly support DNA binding (e.g. CDR1 and CDR2 somatic mutations and arginine-rich CDR3, ref. 30) to associate with a variety of L chains to give anti-dsDNA specificity with different affinities (21, 25). Interestingly, a comprehensive picture of all known B-cell tolerance mechanisms emerges from the various H/L combinations on non-autoimmune and lupus-prone genetic backgrounds. Receptor editing probably serves as a first line of defense against autoreactivity to multivalent antigens (42, 43). This mechanism operates efficiently in diseased NZB/NZW mice (21), despite reports on reduced editing in other lupus models (44, 45). However, in lupus-prone mice the editing mechanism plays a negative role as well because diseased NZB/NZW and MRL/lpr mice select very...
strongly for high-affinity anti-DNA B cells, including those arising from the process of editing (21, 46). These high-affinity autoreactive B cells become activated and dominate the IgG anti-DNA immune response of the diseased mouse (21). Low-affinity anti-DNA B cells, such as D42H/Vx8-Jk5 cells (21, 47), play a secondary role in this process but can be converted into high-affinity B cells by L-chain editing and somatic mutation. However, editing is limited by the rearrangement status of the BCR L chain (25, 42), by the efficiency of Cx deletion (49) and by the number of alleles available for secondary rearrangements; consequently, low-affinity autoreactive B cells that are not capable of L-chain editing become anergic and are only rarely recruited to the germinal centers, where they can undergo class-switch recombination and extensive somatic mutation (19, 21). This is evidenced by the relatively low anti-DNA-binding activity in the serum of D42H/Vx8-Jk5 NZB/NZW mice and by the small number of somatically mutated IgG hybridoma antibodies derived from these mice (21). We, therefore, conclude that low-affinity, anergic anti-DNA B cells do not generally serve as precursors for high-affinity anti-DNA antibody-producing cells in these mice.

A second conclusion from our present study and from previous experiments is that like receptor editing, clonal anergy is not abrogated in aged NZB/NZW female mice and consequently clonal deletion remains the dominant protective mechanism against the proliferation of high-affinity B cells, such as D42H/Vx8-Jk5 (47, 48). This is evidenced by their abundance in diseased NZB/NZW mice but complete absence in non-autoimmune D42H Tg mice.

It should be noted that the pathogenic potential of anti-DNA antibodies is a separate issue that is not necessarily linked to DNA affinity (50–52). All H/L combinations involving the D42H transgene were found to be non-pathogenic, and the Tg NZB/NZW female mice appear to be protected by these antibodies from kidney disease and live a normal life span (data not shown).

Anergic D42H/Vx8-Jk5 anti-DNA B cells have unique characteristics that distinguish them from other anergic B cells (8). They are not arrested in their development and do not have a shortened half-life; on the contrary, an excess of presumably long-lived, mature follicular B cells populate the spleen of D42H/Vx8-Jk5 mice. In that respect, these mice behave similarly to other Tg mice that employ the Vx8-Jk5 chain, such as the anti-ssDNA 3H9/Vx8 (15) and the anti-Sm 2-12/Hx8 (53) mice. This supports our previous interpretation (25), that the inability of this L chain to undergo receptor editing, either due to its presence as a conventional transgene (15, 53) or due to the low frequency of Cx deletion in knock-in mice (25) leads these autoreactive B cells to a state of anergy, regardless of their autospecificity.

The anti-dsDNA D42H/Vx8 B cells exhibit many features of anergy, including a profound decrease in BCR density, a diminished stimulation with BCR-independent activators such as LPS and anti-CD40 antibody and reduced BCR and calcium signaling following BCR activation with anti-IgM. However, the response of these anergic cells to TLR9 agonists such as CpG ODN and bacterial DNA was paradoxical, in that their rate of proliferation as well as the up-regulation of AID and class-switch-associated transcripts were substantially higher than those of non-anergic, non-transgenic or partly anergic H-chain-only B cells. These results are best explained by the BCR–TLR9 synergy, previously suggested by several groups of investigators (54–58). Furthermore, the unique mechanism of BCR–TLR9 synergism has now been elucidated (59), where cross-linked, internalized BCR traffics to autophagosome-like compartments and signals to recruit TLR9-containing endosomes to these compartments. The pattern of inhibition by BCR and TLR9 inhibitors in our study is fully compatible with the newly described mechanism. The BCR probably facilitates the internalization of both CpG ODN and E. coli DNA, as the Src-family kinase inhibitor, PP2, that blocks BCR internalization and trafficking (37, 59), completely inhibited anergic B-cell proliferation by both agonists. However, the syk inhibitor 4-aminoantann partially blocked E. coli DNA but not CpG-induced proliferation, suggesting that E. coli DNA activates the BCR and hence the recruitment of TLR9 to the autophagosome-like compartment. CpG-ODN, on the other hand, is unable to cross link the BCR due to its monovalency and is only capable of...
chloroquine-sensitive direct TLR9 activation in the endosome. Notwithstanding the paradoxical enhancing effect of CpG-ODN and *E. coli* DNA on the proliferation of anti-DNA anergic B cells, as compared with naive B cells, the anergic phenotype was maintained with respect to plasma cell differentiation and antibody secretion. This uncoupling between cell proliferation and antibody secretion in anergic B cells has been observed previously (8). In particular, Rui et al. (60) have found that two distinct signaling pathways oppose CpG-induced activation in anti-hen egg lysozyme (HEL) anergic B cells. The BCR–ERK signaling pathway had no impact on CpG-ODN-induced proliferation but it was necessary to mediate a profound inhibitory effect on plasma cell differentiation in anergic cells that were constantly exposed to antigen.

Perhaps the most unexpected finding of this study is the maintenance of B-cell anergy in the lupus-prone NZB/NZW genetic background. This is evident in all tested parameters. Furthermore, in some major aspects of B-cell anergy, such as calcium signaling and antibody-secreting cells, the NZB/NZW anti-DNA B cells appeared more anergic than (B6 × BALB/c)F1 B cells. These results are similar to those of Rathmell and Goodnow (61), who tested their anti-HEL anergic B cells in autoimmune MRL Faspr mice. They are different from the results of Mandik-Nayak et al. (18) and of Santulli-Marotto et al. (62, 63), who observed B-cell activation on the MRL Faspr autoimmune background with anti-DNA $V_j3$H9/V$\alpha$1 and anti-Sm $V_j3$H9/56R (24), that are subject to clonal deletion. However, autoreactive B cells from these transgenic mice were probably not strictly anergic; instead, these cells exhibited maturation arrest and short half-lives (14, 63). We suggest that these transgenic mice are more similar to high-affinity anti-DNA Tg mice, such as D42H/Vx8RF (47) or 3H9/56R (24), that are subject to clonal deletion. On the other hand, the combination of these same H-chain transgenes (i.e. $V_j3$H9 and $V_j2$-12H) with the Vx8-Jk5 L chain gave rise to low-affinity anti-DNA and anti-Sm specificities and exhibited classical anergic properties (16, 53). On the MRL-Faspr background, a small minority of 3H9/V$\alpha$1 transgenes, respectively. However, autoreactive B cells from these transgenic mice were probably not strictly anergic; instead, these cells exhibited maturation arrest and short half-lives (14, 63). We suggest that these transgenic mice are more similar to high-affinity anti-DNA Tg mice, such as D42H/Vx8RF (47) or 3H9/56R (24), that are subject to clonal deletion. On the other hand, the combination of these same H-chain transgenes (i.e. $V_j3$H9 and $V_j2$-12H) with the Vx8-Jk5 L chain gave rise to low-affinity anti-DNA and anti-Sm specificities and exhibited classical anergic properties (16, 53). On the MRL-Faspr background, a small minority of 3H9/Vx8 B cells gave rise to class-switched and somatically mutated anti-dsDNA hybridomas (19). This spontaneous activation of rare anergic B cells that escape immunologic tolerance has also been observed in NZB/NZW mice, especially when tested with non-purified B cells (21), and has led to the erroneous conclusion that anergy is broken in lupus-prone mice. Conversely, it is not easy to explain why anergy should be intensified in diseased mice; one explanation can be provided by the reports on impaired phagocytosis of apoptotic cells in SLE (1, 64). In that situation, excess nuclear material in the circulation could prolong and intensify the anergic state of self-reactive lymphocytes.

We have found no convincing evidence to support the contention (8, 13) that anergic B cells accumulate in the transitional 3 (T3) compartment in the spleen. The D42H/Vx8 anergic B cells accumulated mostly in the mature B-cell compartment. There was a relative increase in T3 B cells; however, this was associated with a decrease in surface IgM that is used as a marker in T3 identification by FACS. In general, transitional B cells were anergic to the same extent as mature B cells in all measured parameters. It is possible that the autoreactive B cells, which have been reported to accumulate in the T3 stage, are those that undergo maturation arrest and do not reach the mature B-cell compartment. This is clearly not the case with anergic D42H/Vx8 B cells. A corollary of this investigation was the striking finding that the whole transitional (T1–T3) B-cell population in the spleen of non-transgenic diseased NZB/NZW mice is drastically reduced. This deficiency increased with age, but was more disease related than age dependent. Our results do not confirm the report (65) that the T3 B-cell population is specifically reduced in murine lupus, including NZB/NZW mice. This report has been interpreted as evidence for a breakdown in B-cell anergy in lupus-prone mice (8, 65). Conversely, the drastic loss of all transitional B cells in the spleen is not proportional to the moderate decrease (~2-fold) in total B220$^+$ and immature B cells in the bone marrow (Supplementary Table 1, available at *International Immunology* Online, and ref. 40). Therefore, in addition to reduced lymphopoiesis, which is well documented for the New Zealand strains (2), a change in the expression of the chemokine receptor CXCR4 could play a role in the migration of transitional B cells to or their maintenance in the spleen of these mice. CXCR4 (CD184) has been shown to have a critical role in hematopoiesis and lymphopoiesis (66). Specifically, CXCR4 binds CXCL12 (SDF-1) and is required for retention of B-cell precursors in the bone marrow. CXCR4-deficient B cells migrate prematurely to splenic follicles (67). Very recently, Wang et al. (68) found that CXCR4 expression is increased on leukocytes of multiple murine lupus strains and is a direct consequence of disease development in these mice. Further work will be needed to study the effect of transitional B-cell depletion on the maintenance and autoreactivity of mature and germinal center B cells in diseased NZB/NZW mice.

**Supplementary data**

Supplementary Figures 1–5 and Table 1 are available at *International Immunology* Online.

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**References**


