Characterization of anergic anti-DNA B cells: B cell anergy is a T cell-independent and potentially reversible process

Hooman Noorchashm, Anh Bui, Hsiu-Ling Li, Ashlyn Eaton, Laura Mandik-Nayak, Carrie Sokol, Kathryn M. Potts, Ellen Pure and Jan Erikson

The Wistar Institute, 3601 Spruce Street, Philadelphia, PA 19104, USA

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Abstract
Anti-single stranded DNA (ssDNA) and anti-double stranded DNA (dsDNA) B cells are regulated in non-autoimmune mice. In this report we show that while both anti-ssDNA and anti-dsDNA B cells are blocked in their ability to differentiate into antibody-secreting cells, other phenotypic and functional characteristics distinguish them from one another. Splenic anti-ssDNA B cells are found distributed throughout the B cell follicle, and are phenotypically mature and long-lived. On the other hand, splenic anti-dsDNA B cells are short-lived, exhibit an immature and antigen-experienced phenotype, and localize to the T-B interface of the splenic follicle. Functionally, anti-ssDNA B cells proliferate, albeit suboptimally, in response to anti-IgM, lipopolysaccharide (LPS) and CD40L/IL-4 + anti-IgM stimulation, and tyrosine phosphorylate intracellular proteins upon mIgM cross-linking. Anti-dsDNA B cells, on the other hand, are functionally unresponsive to anti-IgM and LPS stimulation, and do not phosphorylate intracellular proteins, including Syk, upon mIg stimulation. Importantly, anti-DNA B cell anergy is maintained in the absence of T cells since both anti-ssDNA and anti-dsDNA B cells are as efficiently regulated in RAG2–/– mice as in their RAG2+/+ counterparts. Interestingly, the severely anergic state of anti-dsDNA B cells is partially reversible upon stimulation with CD40 ligand and IL-4. In response to these signals, anti-dsDNA B cells remain viable, up-regulate cell surface expression of B7-2 and IgM, and restore their ability to proliferate and phosphorylate Syk upon mIg cross-linking. Collectively, these data suggest that anti-DNA B cell anergy encompasses distinct phenotypes which, even in its most severe form, may be reversible upon stimulation with T cell-derived factors.

Introduction
Using Ig transgenic models of B cell tolerance, the regulatory mechanisms by which the B cell compartment achieves a self-tolerant state have been shown to include active negative selection of self-reactive B lymphocytes via antigen-mediated clonal deletion, clonal anergy and receptor editing (1–6). The various reported phenotypes associated with the anergic state of autoreactive B cells in peripheral lymphoid organs suggest that B cell anergy may not be a discrete functional/phenotypic state (3,7–10). Rather, B cell ‘anergy’ is likely a state encompassing a wide spectrum of functional and cell surface phenotypes. The overwhelming majority of studies pertaining to B cell anergy have utilized the hen egg lysozyme (HEL) transgenic model of B cell tolerance. Therefore, it is important to determine how the rules which govern B cell anergy in the anti-HEL system apply to anergic B cells specific for autoantigens targeted in disease.

The presence of self-reactive Ig specificities in the serum of individuals with autoimmune disorders, such as systemic lupus erythematosus (SLE), argues for a dysregulation in the negative selection mechanism(s) to which autoreactive B cells are normally subject. The presence of an array of different autoantibodies collectively termed ‘anti-nuclear antibodies’ (ANA) is one of the diagnostic hallmarks of SLE (11). Paramount among the specificities contained within the spectrum of ANA are anti-DNA antibodies. Operationally, we define anti-single-strand (ss) DNA Ig as ones that bind DNA in an ELISA but are ANA(–), whereas anti-double-strand (ds) DNA Ig are positive in both assays. It is important to note that the
relevant form of the antigen in vivo has not been identified. However, it has been suggested that apoptotic cells may be an important source of the autoantigens targeted in SLE (12).

In order to gain insight into the normal regulation of anti-DNA B cells, Ig H + L chain transgenics encoding anti-DNA specificities have been introduced into non-autoimmune mice. We utilized the VH3H9 H chain transgenics, cloned from an anti-dsDNA antibody-producing hybridoma generated from a lupus-prone MRL-lpr/lpr mouse (13). This heavy chain has been repeatedly identified in anti-ssDNA and anti-dsDNA antibodies from MRL-lpr/lpr mice in association with a spectrum of different light chains (14, 15). In VH3H9 H chain-only transgenic mice, pairing of the transgenic H chain with a variety of different endogenous L chains leads to the generation of both anti-DNA and non-DNA specificities (14,16–18). Thus, the VH3H9 H chain transgenic mice provide the unique opportunity to study the fate of anti-DNA B cells in the context of a diverse B cell repertoire. In these non-autoimmune mice, a population of anti-dsDNA B cells (VH3H9/V4.1) is found in the periphery, and exhibit a phenotype suggestive of antigen-mediated developmental arrest and a reduced in vivo lifespan compared to non-autoreactive B cells in the same mice. Moreover, these anti-dsDNA B cells are found concentrated at the T-B junction of the splenic white pulp (9).

The VH3H9 H chain transgenic mice have been mated to L chain transgenic mice to generate mice harboring an increased frequency of B cells with a given specificity. Mating of VH3H9 H chain transgenic mice with Vλ4 L chain transgenic mice leads to the generation of anti-dsDNA B cells which have been shown to undergo receptor editing or deletion in the bone marrow (6,19). On the other hand, the VH3H9 H chain in conjunction with the Vκ4 L chain encodes an anti-ssDNA Ig, whereas the VH3H9 H chain in combination with a mutated Vκ2 L chain generates an anti-dsDNA Ig. In both cases B cells expressing the transgene encoded specificities persist in the periphery in a regulated fashion (7,8). The distinct fates of the different anti-dsDNA B cells, deletion in the case of VH3H9/Vκ4 and anergy in the case of VH3H9/Vλ2 and VH3H9/Vκ1, most likely reflect heterogeneity among the ‘anti-dsDNA’ B cells in terms of the in vivo antigen being recognized. An alternative explanation for the persistence of anti-dsDNA B cells in VH3H9/Vλ2 and VH3H9/Vκ1 transgenic mice is that such autoreactive B cells are either blocked in receptor editing or have exhausted rearrangements at the κ loci and, as such, are exported from the bone marrow. The observation that VH3H9/Vκ4 B cells still undergo deletion in the bone marrow when on a RAG1−/− background, where receptor editing is blocked, argues against this later possibility (19).

The VH3H9 H chain transgenic mice have been mated with different L chain transgenic mice on the RAG2−/− background to generate mice harboring monoclonal anti-ssDNA or anti-dsDNA B cell repertoires (7,8). The development of these RAG2−/− Ig transgenic mice has allowed us to address the role of T cells in the induction and maintenance of B cell anergy. In addition, the generation of such mice has allowed us to functionally and phenotypically characterize the status of anti-DNA B cells in non-autoimmune mice. Here we detail two distinct cell surface and functional phenotypes associated with anti-ssDNA and anti-dsDNA B cells. Both described phenotypes differ from that previously reported for anergic B cells in the anti-HEL model of B cell tolerance, reinforcing that B cell anergy is not a discrete state but rather encompasses a spectrum of phenotypes. Importantly, examination of the functional capabilities of anergic anti-DNA B cells indicates that some aspects of the anergic phenotype can be reversed upon provision of helper T cell-derived factors. The presence of potentially recruitable anergic anti-DNA B cells in secondary lymphoid organs of non-autoimmune mice may represent a substrate for dysregulation of B cell tolerance leading to autoimmunity.

Methods

Mice

BALB/c mice were obtained from Harlan Sprague Dawley (Indianapolis, IN). The VH3H9/Vκ8 BALB/c, VH3H9/Vκ8 RAG2−/− and VH3H9/Vκ2 RAG2−/− mice were bred and maintained as described previously (7,8). All animals were maintained in the animal facility at The Wistar Institute (Philadelphia, PA). In all described work, age-matched BALB/c mice or Tg(−) littermates were used as controls. All mice were screened by PCR on DNA extracted from tail tissue for the presence of the relevant transgenes as previously described (8,18).

Transfer of carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled B cells

Spleens were removed from the Tg(+) and Tg(−) mice, and single cell suspensions were prepared. Erythrocytes were removed by hypotonic lysis using a hypotonic red blood cell lysis buffer (Sigma, St Louis, MO) and the cells were counted. To enrich for B cells in the Tg(−) BALB/c mice, T cells were depleted by complement-mediated lysis using Low-Tox guinea pig complement (Accurate Chemical, Westbury, NY) following incubation with antibodies from the hybridoma supernatants GK1.5 (anti-CD4) and 3.168.8 (anti-CD8). The cells were labeled with the vital dye CFSE (Molecular Probes, Eugene, OR) and passed through a VarioMACS column. In the other method, T cells were depleted by complement-mediated lysis using Low-Tox guinea pig complement (Accurate Chemical, Westbury, NY) following incubation with antibodies from the hybridoma supernatants GK1.5 (anti-CD4) and 3.168.8 (anti-CD8). The cells were labeled with the vital dye CFSE (Molecular Probes, Eugene, OR) and passed through a VarioMACS column. In the other method, T cells were depleted by complement-mediated lysis using Low-Tox guinea pig complement (Accurate Chemical) after incubation with the following cocktail of mAb from hybridoma supernatants: anti-CD4 (GK1.5), anti-CD8 (3.168.8) and anti-Thy-1 (J1)). The presence of the VH3H9/Vκ2 and VH3H9/Vκ8 H + L chain transgenics on the RAG2−/− background precluded the need for T cell depletion of splenocytes from these mice.

Cell preparations

Single-cell suspensions were prepared from bone marrow, spleen and lymph nodes, and subjected to red blood cell lysis using a hypotonic red blood cell lysis buffer (Sigma). Two different approaches were utilized for T cell depletion to enrich for B cells used in the described experiments. In one method, splenocytes from Tg(−) BALB/c and VH3H9/Vκ8 mice were incubated with anti-Thy-1.2 MACS beads (Miltenyi Biotech, Auburn, CA) and passed through a VarioMACS column. In the other method, T cells were depleted by complement-mediated lysis using Low-Tox guinea pig complement (Accurate Chemical) after incubation with the following cocktail of mAb from hybridoma supernatants: anti-CD4 (GK1.5), anti-CD8 (3.168.8) and anti-Thy-1 (J1)). The presence of the VH3H9/Vκ2 and VH3H9/Vκ8 H + L chain transgenics on the RAG2−/− background precluded the need for T cell depletion of splenocytes from these mice.
Flow cytometry

Cells (1x10^6) were surface stained according to the previously described protocol (23). The following antibodies were used: RA3-6B2–biotin (anti-B220), 1D3–FITC (anti-CD19), 7G6–FITC (anti-CD21/35), Cy3.4.1–FITC (anti-CD22), 3/23–FITC (anti-CD40), Mel-14–FITC (anti-L-selectin), M1/69–FITC (anti-HSA), IM7–FITC (anti-CD44), GL-1–FITC or -phycocerythrin (PE) (anti-B7-2), B3B4–FITC (anti-CD23) (PharMingen, San Diego, CA), polyclonal anti-IgM–PE (Southern Biotechnologies, Birmingham, AL) and streptavidin–Red670 (Gibco/ BRL, Gaithersburg, MD). All samples were analyzed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) using CellQuest software. Between 10,000 and 20,000 events were collected within a live lymphoid gate set based on forward and side scatter.

Immunohistochemistry and immunofluorescence

Spleens were suspended in OCT, frozen in 2-methyl-butane cooled with liquid nitrogen, sectioned and fixed with acetone. The sections were blocked using PBS/5% normal goat serum/0.1% Tween 20 and then stained with alkaline phosphatase (AP)-labeled goat anti-mouse IgM (Southern Biotechnologies) and purified MOMA-1 (Bachem Bioscience, King of Prussia, PA). MOMA-1 stained sections were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rat antibody (Southern Biotechnologies). HRP and AP were developed using the substrate 3-amino-9-ethylcarbazole and Fast-Blue BB base (Sigma), respectively. For immunofluorescence, the spleens were sectioned (6–8 mm sections), fixed in cold acetone and stored at −20°C until use. For staining, slides were allowed to air dry and then sections were rehydrated for 20 min in PBS. Sections were blocked using PBS/5% normal goat serum/0.1% Tween 20 and then stained with RA3-6B2–biotin (anti-B220) (grown as a supernatant) diluted in PBS/5% normal goat serum/0.1% Tween 20. Following three 5 min washes in PBS/0.1% Tween 20, streptavidin–Red670 was used as the secondary reagent to detect the anti-B220–biotin. Following three washes with PBS/0.1% Tween 20, sections were mounted with Fluoromount-G (Southern Biotechnology). The sections were then visualized under a fluorescent microscope.

ELISA

Total Ig concentration in serum and IgM concentration in culture supernatants were determined using an indirect solid-phase ELISA assay, as described (18). To determine total Ig concentration of serum, unlabeled goat anti-mouse total Ig antibody (Southern Biotechnologies) was used as the primary antibody and AP-conjugated goat anti-mouse IgM + anti-total Ig was used for developing. To determine the IgM concentration of culture supernatants, unconjugated goat anti-mouse total Ig was used as the primary antibody, and developing was done with AP-conjugated goat anti-mouse IgM (Southern Biotechnologies). In both cases, Ig concentration was determined by comparing samples to a standard curve generated by a titrated isotype-matched antibody.

Proliferation assays

Splenic B cells were enriched by complement-mediated T cell depletion as described above. B cells were cultured in triplicate at 1x10^6 B cells/ml in 100 µl total volume in microtiter plates for 48 h. B cells were stimulated with various combinations of the following stimuli: phorbol myristate acetate (PMA) (10 ng/ml) (Sigma), ionomycin (1 µg/ml) (Sigma), LPS (50 µg/ml) (Sigma), goat anti-mouse IgM (100 µg/ml) (Pierce, Rockford, IL), CD40 ligand (CD40L)–CD8 fusion protein (1.2 dilution of culture supernatant) (a gift of P. Lane, Basel Institute for Immunology), purified anti-CD8 (3.168.8) (5 µg/ml), rIL-4 (5 ng/ml) (Genzyme Diagnostics, Cambridge, MA) or equivalent Baculovirus IL-4 (1:500). The concentration of each stimulus listed above was determined using dose–response curves generated to determine the conditions yielding optimal proliferation. The culture stimuli were assessed over the following ranges: (i) PMA (10 ng/ml) with ionomycin (0–2 µg/ml), (ii) LPS (0–50 µg/ml), (iii) goat anti-mouse IgM F(ab')2 (0–100 µg/ml), (iv) CD40L–CD8 fusion protein (1:2 dilution of culture supernatant), anti-CD8 (5 µg/ml) and rIL-4 (0–40 ng/ml), and (v) CD40L–CD8 fusion protein (1:2 dilution of culture supernatant), anti-CD8 (5 µg/ml), rIL-4 (0–40 ng/ml) and goat anti-mouse IgM F(ab')2 (0–100 µg/ml). In all cases where the CD40L–CD8 fusion protein was used, anti-CD8 (5 µg/ml) was also added in order to achieve cross-linking of CD40 on B cells. [3H]Thymidine incorporation was measured after an additional 16 h incubation.

ELISPOT

B cells from Tg(–), VH3H9/V8, VH3H9/V2 RAG2+/- and VH3H9/V2 RAG2+/- transgenic mice were cultured in 24 well plates at 1x10^6 B cells/ml for 3 days using the following conditions: (i) medium alone, (ii) LPS (50 µg/ml) or (iii) CD40L–CD8 fusion protein supernatant (1:2), purified anti-CD8 (3.168.8) (5 µg/ml), and Baculovirus IL-4 and IL-5 (1:500). Cell were harvested and following two washes in medium/1% FCS, plated at 5.6x10^5 cells/well and diluted serially 1:3 in Multiscreen HA mixed cellulose ester membrane plates (Millipore, Bedford, MA) coated with unlabeled goat anti-mouse total Ig (Southern Biotechnologies). The Ig secreted by the plated cells was detected by AP-conjugated goat anti-mouse total Ig as secondary antibodies (Southern Biotechnologies) and visualized using NBT–BCIP substrate (Sigma).

In vitro B cell activation for flow cytometric analysis

Splenocytes were T depleted using the VarioMACS system described above. Purified B cell suspensions were resuspended at 1.5x10^6 cells/ml and cultured in 2 ml of RPMI 1640/10% FCS. Three different culture conditions were used: (i) medium alone, (ii) CD40L–CD8 fusion protein supernatant (1:2), anti-CD8 (5 µg/ml) and rIL-4 (5 ng/ml), and (iii) CD40L–CD8 fusion protein supernatant (1:2), anti-CD8 (5 µg/ml), rIL-4 (5 ng/ml) and goat anti-mouse IgM F(ab')2 fragments (12.5 µg/ml). Following 60 h of culture at 37°C, the cells were washed 3 times with RPMI 1640/10% FCS and stained for FACS analysis as described above.

Immunoprecipitation and immunoblotting

For detection of protein tyrosine phosphorylation of Syk in post-nuclear extracts, cells were stimulated with F(ab')2 goat anti-mouse IgM (10 µg/ml) for 3 min at 37°C. Cells were quickly chilled on ice and immediately pelleted by centrifugation. Cell
pellets were lysed in 500 µl ice-cold lysis buffer (1×PBS, 1 mM PMSF, 100 µg/ml soy bean trypsin inhibitor, 20 µg/ml aprotinin, 100 µg/ml leupeptin, 1% NP-40, 0.1% deoxycholate, 10 mM NaF, 10 mM sodium pyrophosphate and 100 mM sodium orthovanadate) for 15 min on ice. Cell lysates were clarified by centrifugation at 15,000 r.p.m. for 10 min. Clarified cell lysates were normalized based on protein concentration as determined using the BCA kit (Pierce, Rockford, IL) and equal amounts of protein were subjected to immunoprecipitation or immunoblotting for each lysate. For Syk immunoprecipitation, the post-nuclear extracts (equivalent to ~2×10⁷ cells) were incubated directly with anti-Syk antibody (a generous gift from Dr. J. Bolen, DNAX, Palo Alto, CA) at 4°C overnight. Protein A–agarose beads (Gibco/BRL) were added for an additional 3 h incubation at 4°C. The beads were washed twice with lysis buffer followed by two washes with PBS. Bound proteins were eluted by boiling in Laemmli buffer containing 0.4% DTT and resolved by 8% SDS–PAGE. The proteins were transferred to PVDF membranes and subjected to immunoblotting. The blots were blocked with TNB buffer (30 mM Tris, pH 7.6, 75 mM NaCl, 3% BSA) overnight at 4°C. Polyclonal rabbit anti-Syk or monoclonal anti-phosphotyrosine antibody (4G10) (UBI, Lake Placid, NY) was added at the concentrations suggested by the manufacturer. For phosphotyrosine immunoblotting, the blots were further incubated with 15 µg/ml polyclonal rabbit anti-mouse antibody for 1 h at 4°C. The blots were then incubated with 1 µCi/ml iodinated Protein A for 45 min at room temperature followed by autoradiography.

Results

Peripheral anti-dsDNA B cells, but not anti-ssDNA B cells, exhibit a cell surface phenotype indicative of antigen-mediated developmental arrest

The use of Ig transgenics has allowed us to create mice with a monoclonal B cell repertoire specific for ssDNA or dsDNA. We generated Ig transgenics by mating VH3H9 H chain transgenics with either V8-8 L chain transgenics (21) to generate mice with an anti-ssDNA repertoire or with V8-2 L chain transgenic mice (22) to generate mice with an anti-dsDNA repertoire. These Ig transgenics vary in their ability to inhibit rearrangement of endogenous Ig. The VH3H9 H chain and the V8-8 L chain transgenics efficiently inhibit endogenous H or L chain rearrangement respectively, whereas the V8-2 L chain transgenic is a poor inhibitor of endogenous L chain rearrangement (18,22). Therefore, in order to study the fates of anti-ssDNA and anti-dsDNA B cells in monoclonal B cell repertoires, as well as to determine the contribution of T cells to B cell anergy, the VH3H9/V8-8 (H + L) and VH3H9/V8-2 (H + L) transgenics were bred onto the RAG2⁻/⁻ background (7,8). A comparison of VH3H9/V8-8 (H + L) on the RAG2⁺/+ backgrounds was included to control for the RAG2⁻/⁻ background. B cells were present in the peripheral lymphoid organs of both the VH3H9/V8-8 (H + L) and VH3H9/V8-2 (H + L) mice regardless of RAG2 status (Fig. 1A).

Using flow cytometry, the cell surface expression of a panel of B cell developmental and activation markers by anti-dsDNA and anti-ssDNA B cells was characterized. These markers included IgM, CD21/35, CD22, CD23, CD40, CD44, HSA, B220 and cell size. Expression of CD21/35, CD22, CD23, CD40 and B220 increases as B cells mature. On the other hand, expression of HSA and CD44 decreases as B cells mature, and is up-regulated again upon activation (23–26). Anti-dsDNA B cells in the monoclonal VH3H9/V8-2 mice exhibit a 10-fold reduction in total surface Ig expression compared to Tg(-) B cells (Fig. 1A) (8). In contrast, splenic anti-ssDNA B cells with and without RAG2 express only a 2-fold decrease in total surface Ig when compared to Tg(-) splenic B cells (Fig. 1A) (7). CD21/35, CD22, CD23, CD40 and B220 on splenic anti-dsDNA B cells are reduced relative to non-transgenic splenic B cells, suggesting that these cells have undergone a developmental arrest (Fig. 1B). Furthermore, we detected elevated expression of HSA and CD44 on splenic anti-dsDNA B cells. The increased CD44 expression by anti-dsDNA B cells, taken together with the increased expression of MHC class II, decreased expression of L-selectin (data not shown), increased cell size and decreased surface Ig (Fig. 1A) provide evidence for antigen-mediated developmental arrest and activation. Interestingly, the cell surface phenotype of anti-dsDNA B cells bears some resemblance to bone marrow ‘transitional’ B cells with respect to their expression of CD23 and HSA (27). Similar to anti-ds DNA B cells, transitional B cells are CD23low, CD44hi and HSAhi. However, whereas the ‘transitional’ B cells described by Caresini et al. are bcl-2⁻, anti-dsDNA B cells are bcl-2⁺ (data not shown).
Fig. 1. Flow cytometric analysis of bone marrow and splenic B cells within the lymphoid gate from Tg(–), VH3H9/V\(\lambda\)2 RAG2\(^{–/–}\), VH3H9/V\(\kappa\)8 RAG2\(^{–/–}\) and VH3H9/V\(\kappa\)8 transgenic mice. Presented data are from a single representative experiment from five different experiments. (A) IgM versus B220 double staining of bone marrow and splenic lymphoid cells from Tg(–), VH3H9/V\(\lambda\)2 RAG2\(^{–/–}\), VH3H9/V\(\kappa\)8 RAG2\(^{–/–}\) and VH3H9/V\(\kappa\)8 transgenic mice. (B) B220\(^{+}\) splenocytes from VH3H9/V\(\lambda\)2 RAG\(^{–/–}\), VH3H9/V\(\kappa\)8 RAG\(^{–/–}\) and VH3H9/V\(\kappa\)8 transgenic mice were analyzed for their expression of CD21/35, CD22, CD23, CD40, CD44, HSA, B220 and cell size. In each histogram the expression of each marker for transgenic B cells (bold line) was compared to that of splenic B cells from a Tg(–) littermate (solid line) in the same experiment.
Transferred cells:

Tg(-) BALB/c  VH3H9/V\kappa8 RAG2-/-  VH3H9/V\lambda2 RAG2-/-

Fig. 2. Localization of transferred B cells. Splenic sections from CB.17 mice receiving BALB/c (1), VH3H9/V\kappa8 RAG 2/- (2) or VH3H9/V\lambda2 RAG2-/- (3) spleen cells were stained with anti-B220–biotin/streptavidin–Red670. Transferred cells were labeled with CFSE prior to transfer and are visible in green/yellow. These are representative sections from a minimum of \(n = 3\) for each genotype. Original magnification: \(\times 200\).

In contrast to anti-dsDNA B cells, the phenotype of anti-ssDNA B cells from both RAG2-/- and RAG2\(^{+/-}\) mice did not indicate that these cells had undergone developmental arrest or activation. We found no consistent differences in the expression of CD22, CD23, CD40, CD44, HSA and B220 on anti-ssDNA B cells as compared to splenic B cells from Tg(-) mice (Fig. 1B).

While, for the most part, the phenotype of anti-ssDNA B cells was comparable on the RAG2-/- and RAG2\(^{+/-}\) backgrounds, there were some minor differences (Fig. 1B). The expression of CD44 and B220 were slightly higher on the RAG2-/- background compared to the RAG2\(^{+/-}\) background. A recent report described an expanded population of IgM\(^{hi}\) B cells with high expression of CD43 in VH3H9/V\kappa8 RAG1-/- mice (19). This phenotype was interpreted as evidence for an ‘activated’ status of a minor population of the VH3H9/V\kappa8 B cells. Examination of CD43 expression on B cells from anti-ssDNA transgenics (VH3H9/V\kappa8 RAG2-/-) from our colony, however, revealed uniform expression of CD43 on all splenic B cells comparable to that seen on non-transgenic B cells with no expansion of a CD43\(^{hi}\) B cell subset (data not shown).

The most striking difference we observe between mice with and without the RAG2 mutation is that the splenic anti-ssDNA B cell compartment in some RAG2-/- mice contains an exaggerated population of CD21/35\(^{hi}\) B cells (Fig. 1B) which are also IgM\(^{hi}\) B cells (data not shown). The phenotype of these cells is reminiscent of that described for B cells located in splenic marginal zones (28).

Localization of anti-DNA B cells

To determine where anti-DNA B cells reside relative to recirculating mature B cells, the Ig transgenic B cells from RAG2-/- mice were transferred into non-transgenic recipients. CFSE-labeled VH3H9/V\lambda2 B cells adoptively transferred into non-transgenic BALB/c mice localize to the T–B interface of the splenic follicle (Fig. 2). This localization is reminiscent of that we have previously documented for another anti-dsDNA B cell population in VH3H9 heavy chain-only transgenic mice (VH3H9/V\lambda1) (9). In contrast, CFSE-labeled anti-ssDNA B cells adoptively transferred into non-transgenic BALB/c mice are widely distributed throughout the B cell follicle (Fig. 2), again suggesting that anti-ssDNA B cells are, by many criteria, similar to the mature recirculating B cells found in non-transgenic mice.

To determine if the developmentally arrested phenotype...
and shortened in vivo life-span of anti-dsDNA B cells (8) correlated with a reduction in B cells in the marginal zones, spleen sections from anti-dsDNA Ig transgenic RAG2^{+/-} mice were compared with those from anti-ssDNA transgenic RAG2^{+/-} mice. Anti-ssDNA Ig transgenic mice, both on the RAG2^{+/-} and RAG2^{1/1} backgrounds, had splenic marginal zones as demarcated by the presence of B cells within and outside of the MOMA-1 staining which defines marginal zone metallophilic macrophages (Fig. 3). In contrast, the B cell follicles from anti-dsDNA Ig transgenic RAG2^{+/-} mice had no identifiable marginal zone B cells.

CD40L and IL-4 reverse the functional unresponsiveness of splenic anti-dsDNA B cells and leads to their activation but not differentiation into antibody-secreting cells (ASC)

The functional status of peripheral anti-dsDNA and anti-ssDNA B cells to a variety of stimuli was characterized. Both anti-dsDNA and anti-ssDNA B cells are capable of proliferating as shown by the response to PMA/ionomycin (Fig. 4). A consistently greater proliferative response to PMA/ionomycin stimulation was noted on the part of anti-ssDNA B cells from RAG2^{+/-} mice as compared to RAG2^{1/1} anti-ssDNA B cells, anti-dsDNA B cells and Tg(-) B cells. The reason for the heightened responsiveness of anti-ssDNA B cells from RAG2^{+/-} versus RAG2^{1/1} mice to PMA/ionomycin is unclear but this pattern was also observed in response to stimulation with LPS. Weigert's group has similarly reported, using a VH3H9 site-directed transgenic(3H9R), in association with the V_{κ}8 transgenic, that B cells from RAG^{+/-} mice appear more responsive than their RAG^{1/1} counterparts (19). We have considered the possibility that this proliferative difference may be due to the fact the RAG2^{+/-} B cells were enriched by complement-mediated lysis of T cells, whereas the RAG2^{+/-} B cells were not exposed to complement. Nonetheless, in most cases (three of five experiments) anti-ssDNA B cells from RAG2^{+/-} and RAG2^{1/1} responded suboptimally to LPS when compared with Tg(-) B cells. In contrast, the 3H9R/V_{κ}8 RAG^{+/-} B cells are reported to be hyper-responsive to LPS. It is unclear what accounts for this difference. In sharp contrast and unlike anti-ssDNA B cells, anti-dsDNA B cells were strikingly refractory to stimulation with anti-IgM and LPS as evidenced by the absence of proliferation.

Importantly, cross-linking CD40 in the presence of rIL-4 reversed the unresponsiveness of peripheral anti-dsDNA B cells to anti-IgM stimulation (Fig. 4). Anti-dsDNA B cells responded maximally to anti-IgM cross-linking when co-cultured with CD40L–CD8 and rIL-4. However, even under these conditions, the response of anti-dsDNA B cells was significantly less than that of Tg(-) B cells. On the other hand, the response of anti-ssDNA B cells was overall comparable (in four separate experiments) to that of Tg(-) B cells when stimulated with CD40L–CD8 and rIL-4 with or without anti-IgM.

We found that the anti-dsDNA B cells increase in size upon culture with CD40L–CD8 and rIL-4 (Fig. 5A). Thus, while co-culture with CD40L and rIL-4 does not induce significant DNA synthesis by anti-dsDNA B cells (Fig. 4), it does lead to
Characterization of anergic anti-DNA B cells

A

Medium Alone

CD40L-CD8 + rIL4

Tg (-) BALB/c

VH3H9/Vλ2 RAG2/-
(Anti-dsDNA)

Size

B

Medium Alone

CD40L-CD8 + rIL4

Tg (-) BALB/c

VH3H9/Vλ2 RAG2/-
(Anti-dsDNA)

MFI=8
MFI=111
MFI=13
MFI=84
MFI=793
MFI=12
MFI=461

B7-2

C

Medium Alone

CD40L-CD8 + rIL4

Tg (-) BALB/c

VH3H9/Vλ2 RAG2/-
(Anti-dsDNA)

mIg(IgM+IgD)
increased size and enhanced viability of these cells in culture as measured by the cell scatter profile after the stimulation period (Fig. 5A). Interestingly, anti-dsDNA B cells stimulated with CD40L and rIL-4 concomitantly up-regulate the co-stimulatory molecule B7-2 when compared to cells cultured in medium alone (Fig. 5B). In addition, stimulation of anergic anti-dsDNA B cells with CD40L and rIL-4 led to the up-regulation of the mlg expression level (Fig. 5C).

Despite the ability of anti-dsDNA B cells to proliferate and up-regulate B7-2 in response to CD40L/IL-4 and mlgM cross-linking, they remain incapable of differentiating into ASC in response to CD40L/IL-4 with or without IL-5 (Fig. 6A and B). This reduced ability to differentiate into ASC is also observed in the case of anti-ssDNA B cells (Fig. 6). Consistent with our in vitro data on antibody secretion, both anti-dsDNA and anti-ssDNA Ig transgenic RAG2−/− mice have a drastically reduced amount of serum Ig (Table 1). The higher levels of serum Ig present in anti-ssDNA B cells (VH3H9/Vλ) is not derived from the transgene encoded Ig (18) but rather originates from endogenously rearranged lymphocytes. It is important to consider that another explanation for the absence of transgene-encoded anti-DNA serum Ig in these mice might be an increased in vivo clearance of such self-reactive Ig specificities. The suboptimal ASC numbers argue against this, however, and suggest an intrinsic B cell block in ASC formation. This interpretation needs to be tempered because it cannot be ruled out that the diminished ASC formation is, in someway, inherent to the VH3H9 H chain + L chain transgenes and not related to antigen encounter. The observation that both VH3H9 H chain alone and Vλ8 L chain alone transgenic B cells are capable of antibody secretion, however, suggests that this is not uniformly the case (16,18,21). Nevertheless, given that the VH3H9/Vλ8 and VH3H9/Vλ2 B cells cannot be studied in the absence of antigen, the possibility remains that the diminished ASC production is a feature of these transgenics, per se. With this formidable caveat in mind, we refer to the VH3H9/Vλ8 B cells as anergic based on their suboptimal ability to generate ASC.

Anti-dsDNA but not anti-ssDNA B cells are deficient in their ability to phosphorylate intracellular proteins, including Syk, upon mlgM cross-linking

Activation of Tg(−) B cells with anti-IgM induced tyrosine phosphorylation of a number of intracellular proteins, including Syk (Fig. 7) (29–35). Anti-ssDNA were indistinguishable from resting Tg(−) B cells in their ability to tyrosine phosphorylate Syk and other intracellular proteins upon mlgM cross-linking (Fig. 7A). In contrast, anti-dsDNA B cells had a greatly diminished capacity to tyrosine phosphorylate total intracellular proteins, including Syk (Fig. 7A and B). The finding that anti-dsDNA B cells are unable to tyrosine phosphorylate intracellular proteins is consistent with the inability of anti-dsDNA B cells to proliferate upon mlgM cross-linking.

Given that stimulation with CD40L and IL-4 partially restores the proliferative capacity of anti-dsDNA B cells to anti-IgM stimulation and also elevates cell surface IgM levels (Figs 4 and 5C), we evaluated the ability of B cells so treated to signal through their slg. Indeed, in vitro treatment of anti-dsDNA B cells with CD40L and rIL-4 restored mlg-mediated phosphorylation of intracellular proteins, including Syk (Fig. 7B). We have not yet determined whether the diminished Ig signaling seen for the untreated anti-dsDNA B cells was due to an intrinsic uncoupling of the mlg signaling pathway in anergic anti-dsDNA B cells or due to antigen-mediated down-modulation of membrane Ig.

Discussion

We have characterized the two distinct phenotypes of anti-ssDNA and anti-dsDNA B cells in non-autoimmune mice. While the phenotypes of anti-dsDNA and anti-ssDNA B cells are similar in their reduced ability to generate ASC and secrete transgene-encoded Ig into serum, they differ notably in other ways. Anti-ssDNA B cells are phenotypically mature, long-lived and widely distributed throughout the splenic B cell follicle. These cells proliferate in response to anti-IgM, LPS, CD40L/IL-4 + anti-IgM and normally tyrosine phosphorylate intracellular proteins upon mlgM cross-linking. On the other hand, splenic anti-dsDNA B cells localize to the T–B interface of the follicle, have an immature but antigen-experienced phenotype with a shortened in vivo life-span, and are functionally incapable of responding to anti-IgM and LPS stimulation. Remarkably, anti-dsDNA (VH3H9/Vλ2) transgenic B cells in a monoclonal repertoire very closely resemble anti-dsDNA B cells (VH3H9/Vλ1) in the diverse repertoire of the VH3H9 H chain alone transgenic mice with respect to both cell surface phenotype and in vivo turnover (8,9). Importantly, anti-dsDNA B cell anergy is maintained in the absence of T cells since both anti-ssDNA and anti-dsDNA B cells are as efficiently regulated in RAG2−/− mice as in their RAG2+/+ counterparts. Similarly, in the anti-H-2K Ig transgenic model it was demonstrated that T cells were not required for deletion of autoreactive B cells (36). Furthermore, a recent study showed that follicular exclusion of autoantigen-reactive anti-HEL B cells is dependent on the presence of competitor B cells but not T cells (37). Therefore, it appears that the regulation of B cell deletion and anergy can proceed normally in the absence of T cells.

Fig. 5. Flow cytometric analysis of cultured splenic B cells from VH3H9/Vλ2 RAG2+/− transgenic or Tg(−) BALB/c mice. B cells were cultured for 60 h with or without CD40L–CD8 and rIL-4 as described in Methods and then analyzed by flow cytometry. Presented data are representative of four experiments. (A) When cultured with CD40L–CD8 and rIL-4, splenic B cells from VH3H9/Vλ2 RAG2+/− transgenic and Tg(−) BALB/c mice increase in size as compared to cultures with medium alone. Gated cells are live and blasts cells. (B) Splenic B cells from VH3H9/Vλ2 RAG2+/− transgenic and Tg(−) BALB/c mice increase their expression of B7-2 when cultured with CD40L–CD8 + rIL-4 but not medium alone. In each histogram, the bold line represents staining with anti-B7-2 (GL-1) and the solid line represents staining with an isotype control rat IgG2a antibody. (C) Splenic B cells from VH3H9/Vλ2 RAG2+/− transgenic and Tg(−) BALB/c mice increase their expression of mlg when cultured with CD40L–CD8 + rIL-4 but not medium alone. In each histogram, the mlg expression post-culture (bold line) is compared to that of freshly isolated splenic B cells (solid line) of the indicated genotype.

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Fig. 6. ASC formation and IgM secretion assays. Presented data are accumulated from two separate experiments and a minimum total of two to five mice per genotype. (A) ASC formation by splenic B cells of VH3H9/V κ8 transgenic (stippled bar), VH3H9/V κ8 RAG2–/– (hatched bar) and VH3H9/V λ2 RAG2–/– transgenic (solid black bar) compared with Tg(–) BALB/c mice (solid gray bar) in response to LPS, CD40L–CD81rIL-4 or CD40L–CD81rIL-41rIL-5. Splenic B cells depleted of T cells in RAG2 intact animals were cultured for 3 days and plated at 5.6 x 104 cells/well post-culture. The number of ASC is expressed as ASC per 360 splenic B cells. (B) IgM secretion by splenic B cells of VH3H9/V κ8 transgenic (stippled bar), VH3H9/V κ8 RAG2–/– (hatched bar) and VH3H9/V λ2 RAG2–/– transgenic (solid black bar) compared with Tg(–) BALB/c mice (solid gray bar) in response to LPS, CD40L1rIL-4 or CD40L1rIL-41rIL-5. IgM secretion was determined by ELISA after 7 day of cultures with the indicated stimuli. Data represent means ± SD of triplicate wells.

Table 1. Total serum Ig levels

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Ig level (µg/ml)</th>
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<tbody>
<tr>
<td>Tg(–) BALB/c</td>
<td>2932.76 ± 1968.57 (10)</td>
</tr>
<tr>
<td>VH3H9/V κ8 BALB/c</td>
<td>1609.66 ± 1849.33 (10)</td>
</tr>
<tr>
<td>VH3H9/V κ8 RAG2–/–</td>
<td>8.18 ± 3.87 (10)</td>
</tr>
<tr>
<td>VH3H9/V λ2 RAG2–/–</td>
<td>11.60 ± 9.35 (10)</td>
</tr>
<tr>
<td>Tg(–) RAG2–/–</td>
<td>2.49 ± 0.08 (4)</td>
</tr>
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</table>

Levels of total serum Ig from mice of the genotypes indicated were determined as described in Methods. The number of mice in each group is indicated in parentheses. mice used in this experiment ranged from 8 to 30 weeks in age.

The different fates of the various anti-DNA B cells in non-autoimmune mice (i.e. the two phenotypes of B cell anergy reported here and deletion in the studies of Weigert et al.) most likely reflect differences in affinity/specificity of the transgenic encoded Ig for the target self antigen. While it has been hypothesized that SLE-associated B cells may recognize apoptotic cells that have rearranged their nuclear constituents, definitive experiments demonstrating SLE-associated Ig with specificity for external surfaces of apoptotic cells have not been reported. Interestingly, however, studies using apoptotic cells as immunogens in non-autoimmune prone mice were capable of partially mimicking the autoantibody production characteristic of lupus mice (38). More recent studies by Rosen et al. showing that different means of inducing apoptosis generate unique proteolytic fragments which by extension may alter the display of self-antigens to the immune system highlight the challenge in identifying the relevant apoptotic cells (39).

The two anergic phenotypes described here for anti-DNA B cells are similar to that described for anergic anti-HEL B cells with respect to their inability to generate ASC. With respect to cell surface phenotype, anergic anti-HEL B cells more closely resemble anti-ssDNA B cells in that they are both mature (3). However, in terms of follicular localization to the T–B interface of the splenic follicle, life-span and an inability to efficiently tyrosine phosphorylate intracellular proteins, anergic anti-dsDNA and anti-HEL B cells are remarkably similar (29,40–43). Contrary to anergic anti-dsDNA B cells, however, anergic anti-HEL B cells are reported to proliferate in response to LPS stimulation. Interestingly, a recent study suggests that anti-Sm B cells (specific for the Smith antigen) also undergo developmental arrest in non-autoimmune mice similar to (44) what we have described for anti-dsDNA B cells. Peripheral anti-Sm B cells are reported to be reminiscent of immature B cells in that they were characterized by an elevated cell surface expression of HSA, a reduced expression of CD23 and an increased in vivo turnover rate. Collectively, B cell ‘anergy’ does not appear to be a single phenotypic state; rather, it entails a range of phenotypes exemplified by anergic anti-HEL and anti-DNA B cells. These different phenotypic fates of anergic B cells found in peripheral lymphoid organs of non-autoimmune mice are likely dictated by the nature of the in vivo antigen for which these B cells are specific.

T cells have been implicated in the immunopathogenesis of autoimmune disease in general and in the MRL-lpr/lpr model for many years (45–48). However, it is unknown at
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Fig. 7. (A) Anti-IgM induced tyrosine phosphorylation is down-regulated in VH3H9/V\textsubscript{λ}2 RAG2–/– transgenic B cells but not VH3H9/V\textsubscript{κ}8 RAG2–/– transgenic B cells. Splenic B cells were isolated from VH3H9/V\textsubscript{λ}2 RAG2–/– and VH3H9/V\textsubscript{κ}8 RAG2–/– transgenic mice and their Tg(–) littermates. Splenic B cells (3 \times 10^7 cells/ml) were then stimulated with 10 \(\mu\)g/ml F(ab\textquoteright9)\textsubscript{2} goat anti-mouse IgM antibody at 37°C for 3 min. Cells were then lysed and equal amounts of cell lysates (50 \(\mu\)g) were resolved by SDS–PAGE and immunoblotted with anti-phosphotyrosine antibody. (B) Pretreatment of VH3H9/V\textsubscript{λ}2 RAG2–/– transgenic B cells with CD40L–CD8 and IL-4 rescues the anti-IgM-induced tyrosine phosphorylation of Syk. Splenic B cells were isolated from VH3H9/V\textsubscript{λ}2 RAG2–/– and VH3H9/V\textsubscript{κ}8 RAG2–/– transgenic mice. Freshly isolated B cells were either stimulated as described above (lanes 1–8) or cultured for 72 h in the presence or absence of IL-4 and CD40L–CD8. Cultured B cells were then harvested and stimulated with 10 \(\mu\)g/ml F(ab\textquoteright9)\textsubscript{2} goat anti-mouse IgM antibody for 3 min (lanes 9–12). Equal volumes of cell lysates were immunoprecipitated with polyclonal anti-Syk antibody. The immune complexes were divided and immunoblotted with anti-phosphotyrosine antibody or polyclonal anti-Syk antibody. The presented data are representative of 22 mice in the case of Tg(–) BALB/c mice, 16 mice in the case of VH3H9/V\textsubscript{λ}2 mice and six mice in the case of VH3H9/V\textsubscript{κ}8 mice.

what stage T cells are exerting their effect. The data presented here suggest that functionally unresponsive B cells can be revitalized to respond to B cell-specific stimulation if simultaneously stimulated with T cell help-derived factors. Importantly, we show that anergic anti-dsDNA B cells remain viable, and can be rescued to proliferate and up-regulate B7-2 upon stimulation with CD40L and IL-4. Furthermore, the demonstration that co-culture with CD40L and IL-4 increases the expression of mIgM and rescues the ability of anti-IgM to induce Syk phosphorylation strongly suggests that T cell help can provide a window of opportunity for the rescue of autoreactive B cells. The capacity of T cell help to rescue anergic autoreactive anti-HEL B cells has been in controversy (41,43). On the one hand, it has been reported that T cell help in the form of soluble CD40L and IL-4 or IL-5 rescues anergic anti-HEL B cells leading to secretion of antibodies at a level comparable to or better than those observed in non-anergic B cells (49). On the other hand, the provision of T cell help in the form of antigen-primed or allogeneic T cells in vivo has been shown not to rescue anergic anti-HEL B cells as assessed by their inability to become activated and secrete anti-HEL (41,43). It appears that anergic anti-DNA B cells may represent a compartment of autoreactive B cells with the potential to be rescued if provided with appropriate T cell help. Therefore, it is of great interest to determine whether provision of physiologic T cell help in vivo can lead to the full rescue of anergic anti-DNA B cells and progression to autoimmune disease.

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Abbreviations

ANA anti-nuclear antibody
AP alkaline phosphatase
ASC antigen-secreting cell
CD40L CD40 ligand
CFSE carboxyfluorescein diacetate succinimidyl ester
ds double stranded
HEL hen egg lysozyme
HRP horseradish peroxidase
LPS lipopolysaccharide
PE phycocerythrin
PMA phorbol myristate acetate
SLE systemic lupus erythematosus
ss single stranded

References

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