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A Novel Subpopulation of B-1 Cells Is Enriched With Autoreactivity in Normal and Lupus-Prone Mice

Xuemei Zhong, PhD[1], **Stanley Lau, BA**[1], **Chunyan Bai, MD**[1], **Nicolas Degauque, PhD**[2], **Nichol E. Holodick, PhD**[3], **Scott J. Steven, BA**[4], **Joseph Tumang, PhD**[3], **Wenda Gao, PhD**[2], and **Thomas L. Rothstein, MD, PhD**[3]

[1]Boston University Medical Center, Boston, Massachusetts

[2]Beth Israel Deaconess Medical Center, and Harvard Medical School, Boston, Massachusetts

[3]The Feinstein Institute for Medical Research, Manhasset, New York

[4]Boston University, Boston, Massachusetts.

Abstract

Objective—B-1 cells have long been suggested to play an important role in lupus. However, reports to date have been controversial regarding their pathogenic or protective roles in different animal models. We undertook this study to investigate a novel subpopulation of B-1 cells and its roles in murine lupus.

Methods—Lymphocyte phenotypes were assessed by flow cytometry. Autoantibody secretion was analyzed by enzyme-linked immunosorbent assay, autoantigen proteome array, and antinuclear antibody assay. Cell proliferation was measured by thymidine incorporation and 5,6-carboxyfluorescein succinimidyl ester dilution. B cell Ig isotype switching was measured by enzyme-linked immunospot assay.

Results—Anti–double-stranded DNA (anti-dsDNA) autoantibodies were preferentially secreted by a subpopulation of CD5+ B-1 cells that expressed programmed death ligand 2 (termed L2pB1 cells). A substantial proportion of hybridoma clones generated from L2pB1 cells reacted to dsDNA. Moreover, these clones were highly cross-reactive with other lupus-related autoantigens. L2pB1 cells were potent antigen-presenting cells and promoted Th17 cell differentiation in vitro. A dramatic increase of circulating L2pB1 cells in lupus-prone BXSB mice was correlated with elevated serum titers of anti-dsDNA antibodies. A significant number of L2pB1 cells preferentially switched to IgG1 and IgG2b when stimulated with interleukin-21.

Conclusion—Our findings identify a novel subpopulation of B-1 cells that is enriched for autoreactive specificities, undergoes isotype switch, manifests enhanced antigen presentation, promotes Th17 cell differentiation, and is preferentially associated with the development of lupus in

Address correspondence and reprint requests to Xuemei Zhong, PhD, Room 437, EBRC, 650 Albany Street, Boston, MA 02118. xuemei.zhong@bmc.org..

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Zhong had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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(current address: EMD Serono Research Center, Billerica, Massachusetts)

a murine model. Together, these findings suggest that L2pB1 cells have the potential to initiate autoimmunity through serologic and T cell–mediated mechanisms.

Systemic lupus erythematosus (SLE) is an extremely complex autoimmune disease with no effective cure. A major clinical manifestation of SLE is the production of a variety of autoantibodies affecting multiple organ systems. Although dysregulation of multiple components of the immune system may be involved at distinct stages of SLE development, antibody-producing B cells have been a key focus of attention. With the recent contradictory and controversial results of pan–B cell depletion therapy in lupus patients, further studies of the pathophysiologic effects of B cells are needed.

In the mouse, B cells can be divided into at least 3 lineages (i.e., conventional B cells [also termed B-2 cells], marginal-zone B cells, and B-1 cells). B-1 cells are a minor population in terms of absolute cell number as compared with B-2 cells. However, B-1 cells constitute a crucial first-line defense against most infections (1,2).

B-1 cells can be divided into B-1a and B-1b cells, depending on the expression of CD5. CD5-expressing B-1a cells are the dominant B-1 cells in the peritoneal cavity, wherein the B-1 cell population is itself enriched relative to the B-2 cell population. B-1a cells are the primary source of natural antibody and T-independent antibody (3,4). Although the full identity and complete characteristics of the human B-1a cell equivalent are yet to be defined, B cells marked by CD5 expression have been reported to be associated with SLE and other autoimmune diseases in humans (5–10). Despite evidence indicating involvement of B-1 cells in lupus, their precise contribution in promoting and/or preventing disease progression still remains controversial (11). This complex state of affairs could result from the putative heterogeneous nature of B-1a cells.

Recently we reported that B-1a cells are phenotypically heterogeneous, in that a substantial portion of B-1a cells, amounting to 50–70%, expresses programmed death ligand 2 (PDL-2), a ligand for the suppressive receptor programmed death 1 (PD-1) (12,13). PDL-2 is much more restricted in expression than the widely distributed PD-1 ligand, PDL-1, and PDL-2 expression has previously been associated solely with activated macrophages and dendritic cells (14,15). Moreover, PDL-2 manifests functional features such as retrograde signaling that are not shared by PDL-1. These features suggest that the role of PDL-2–positive B-1a (L2pB1) cells may differ from that of PDL-2–negative B-1a (L2nB1) cells in both normal immune and autoimmune situations, and this could in turn contribute to confusion regarding the true role of B-1 cells in lupus. Here we report that the recently identified L2pB1 cell population is enriched for autoreactive specificities and for potent antigen presentation capacity and that it is increased in lupus-prone BXSB mice in direct relation to serum anti–double-stranded DNA (anti-dsDNA) titers.

MATERIALS AND METHODS

Mice

BALB/c mice and BXSB mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and maintained in specific pathogen–free animal facilities at Boston University Medical Center. All protocols were approved by the Institutional Animal Care and Use Committee at Boston University School of Medicine.

Cell isolation

Single-cell suspensions were prepared from spleen, peritoneal lavage, and peripheral blood. Erythrocytes were depleted using erythrocyte lysis buffer (Qiagen, Valencia, CA). Subpopulations of B-1 cells were sorted according to B220, CD5, and PDL-2 surface staining

($B220^{low}CD5^{low}$PDL-2–positive and $B220^{low}CD5^{low}$PDL-2–negative) using a MoFlo cytometer (Dako, Carpinteria, CA). Splenic B-2 (B-2s) cells were isolated by cell sorting (B220+CD5–) or were negatively purified using magnetic beads (Miltenyi Biotec, Sunnyvale, CA). All cells were cultured in RPMI 1640 containing 10% fetal calf serum.

T cell proliferation

Proliferation of T cells was measured either by thymidine incorporation during the last 6–10 hours of coculture with B cells or by 5,6-carboxyfluorescein succinimidyl ester (CFSE) dilution, in which T cells were labeled with CFSE prior to coculture with B cells and then assessed for fluorescence by flow cytometry at the end of the culture period.

Flow cytometry

Isolated cells were washed with phosphate buffered saline (PBS) containing 2% fetal bovine serum (Sigma, St. Louis, MO) followed by Fc receptor blockade with anti-CD16/CD32 antibody (2.4G2; BD Biosciences, San Jose, CA). Cell surface markers were then analyzed by staining with fluorescein isothiocyanate (FITC)–, phycoerythrin (PE)–Cy5–, and PE-conjugated monoclonal antibodies specific for B220 (BD Biosciences), CD5 (BD Biosciences), and PDL-2 (eBio-science, San Diego, CA). Data were acquired from an LSRII flow cytometer (BD Biosciences) with FACSDiva software (BD Biosciences) and were analyzed using FlowJo software (Tree Star, San Carlos, CA).

Enzyme-linked immunosorbent assay (ELISA)

ELISA plates were coated with dsDNA (1 μg/well; Sigma), blocked by bovine serum albumin (BSA), and then incubated either with supernatant samples from cultured B cells or hybridomas or with serum samples from BXSB mice at 37°C for 1 hour. DNA-binding antibodies were detected with horseradish peroxidase–conjugated goat anti-mouse total Ig (heavy and light chains) (Southern Biotechnology, Birmingham, AL) followed by 2,2′-azino-bis 3-ethylbenzthiazoline-6-sulfonic acid (Sigma). ELISA plates were analyzed at $\lambda_{405\ nm}$ on an ELISA plate reader.

Antinuclear antibody assay

Serum or supernatant samples were diluted in PBS containing 0.2% BSA as indicated and were added to Antinuclear Antibody (HEp-2 cells) substrate slides (Bion Enterprises, Des Plaines, IL). After incubation at room temperature for 1 hour, slides were washed with PBS and deionized water followed by incubation with FITC-labeled goat anti-mouse antibody (Sigma) at room temperature for 1 hour. Slides were then washed and mounted with Vectashield mounting medium (Vector, Burlingame, CA) and observed using fluorescence microscopy.

Hybridoma generation

L2pB1 cells ($B220^{low}CD5^{low}$ PDL-2–positive) were prepared from peritoneal lavage fluid by fluorescence-activated cell sorting. Sorted cells were then treated with 50 μg/ml lipopolysaccharide (LPS) for 24 hours followed by fusion with the myeloma cell line F0 at a 1:10 ratio (Abgent, Bioggio-Lugano, Switzerland). Hybridomas that secreted antibodies recognizing dsDNA were selected and subcloned in 2 rounds with ELISA screening.

Autoantigen proteome array

Serum and supernatant samples were used for protein array analysis at the Microarray Core Facility of the University of Texas Southwestern Medical Center. Sera from BXSB mice with low and high anti-dsDNA titers were evaluated at 1:100 dilutions, while supernatants of cultured hybridoma clones and from LPS-stimulated L2pB1, L2nB1, and B-2s cells were also

tested without dilution. Each panel on the slides was printed with 62 autoantigens and 7 controls. Slides were scanned with a Genepix 4000B scanner (Molecular Devices, Sunnyvale, CA). Genepix Results files were generated using Genepix Pro 6.0. The background-subtracted median signal intensity of each antigen was normalized to the average intensity of total mouse IgG or IgM, which were included as internal controls. The normalized fluorescence intensity data were used to generate a heat map. Diagrams with row-wise and column-wise clustering were generated using Cluster and Treeview software (http://rana.lbl.gov/EisenSoftware.htm).

B cell isotype switching

L2pB1 cells, L2nB1 cells, and B-2 cells were stimulated with CD40L-CD8 fusion protein as described previously (16) and cultured for 7 days in the presence of 12.5 μg/ml LPS plus 1 or more of the following cytokines: 0.2 ng/ml transforming growth factor β (TGFβ), 5 ng/ml interleukin-21 (IL-21), 50 ng/ml IL-4, and 0.5 μg/ml BAFF (all from R&D Systems, Minneapolis, MN). Ig secretion and class switching were tested by enzyme-linked immunospot (ELISPOT) assay.

ELISPOT assay

MultiScreen-IP plates were coated with goat anti-mouse Ig (heavy and light chains) or rat anti-mouse κ light chain (BD Biosciences). L2pB1 cells, L2nB1 cells, and B-2 cells were stimulated for 7 days as described above. Cells were then harvested and counted. No more than 2×10^5 cells were seeded in each well and cultured for 3 hours for IgM or overnight for IgG1, IgG2a, and IgG2b. Alkaline phosphatase (AP)–conjugated goat anti-mouse IgM (Southern Biotechnology) and biotinylated rat anti-mouse IgM (Southern Biotechnology), rat anti-mouse IgG1 (BD Biosciences), rat anti-mouse IgG2a (BD Biosciences), and rat anti-mouse IgG2b (Southern Biotechnology) were used as secondary antibodies and incubated for 1 hour at room temperature, followed by incubation with AP-conjugated streptavidin for the biotinylated secondary antibodies. Ig-secreting spots were revealed by adding AP substrate. Plates were then dried before imaging. The percentage of Ig-secreting cells was calculated by dividing the number of positive spots by the total number of cells seeded in each well.

RESULTS

L2pB1 cells are enriched for autoreactive specificities

We previously reported that 50–70% of B-1a cells express PDL-2 (here termed L2pB1 cells) (13). One of the hallmarks of B-1a cells is repertoire skewing toward specificity for phosphatidylcholine, encoded by V_H11 and V_H12 (17–19). Strikingly, phosphatidylcholine-binding B-1a cells are found almost exclusively within the L2pB1 population (13). Thus, L2pB1 cells and L2nB1 cells express distinct repertoires. Antiphosphatidylcholine antibody is a vital first-line defense against bacterial infection and also plays a role in senescent erythrocyte clearance as well as in hemolytic autoimmune dyscrasias (1,17,20–22). Like phosphatidylcholine-binding antibody, many B-1 cell–derived natural antibodies recognize both common pathogen structures and autoantigens. We hypothesize that L2pB1 cells may represent an autoreactive-repertoire-skewed population. To test this, we examined L2pB1 cells for autore-activity to antigens beyond phosphatidylcholine, beginning with dsDNA, the hallmark autoantigen in lupus patients.

L2pB1 and L2nB1 cells were sorted from peritoneal lavage of BALB/c mice together with B-2s cells. All B cells were stimulated with LPS for 5–7 days to induce antibody secretion. Culture supernatants were then evaluated by ELISA to detect anti-dsDNA antibody. We found a substantially higher level of anti-dsDNA in L2pB1 cell supernatants than in supernatants from L2nB1 cells and B-2s cells (Figure 1A). To exclude the possibility that L2pB1 cell supernatant contained more antibody overall, we examined total immunoglobulin generated

and found that all cultures contained equivalent levels of immunoglobulin (Figure 1A). Thus, L2pB1 cell–derived antibody is enriched with dsDNA reactivity as compared with either L2nB1 or B-2 cells.

Natural antibodies secreted by B-1a cells have been reported to cross-react with multiple antigens (23). To test if L2pB1 cell–derived anti-DNA autoantibodies also react with other autoantigens, we generated hybridomas from LPS-stimulated L2pB1 cells. After screening by anti-dsDNA ELISA, 10% of the hybridoma clones were positive for anti-dsDNA reactivity. Following 2 rounds of subcloning, hybridoma supernatants were analyzed by autoantigen protein array (Figure 1B). Serum samples from BXSB-Yaa+ lupus-prone mice were used as positive controls. Supernatant samples from LPS-stimulated sort-purified primary L2pB1 cells, L2nB1 cells, and B-2 cells were also included for comparison. We found that serum from BXSB-Yaa+ lupus-prone mice, but not from female BXSB mice, reacted to most of the autoantigens on the array, as expected. Further, like BXSB-Yaa+ mouse sera, supernatant samples from stimulated L2pB1 cells also reacted with multiple autoantigens. In direct contrast, supernatants from L2nB1 cells and B-2 cells failed to recognize most of the autoantigens. These results demonstrate the segregation of B-1a cell autoreactivity to the L2pB1 subpopulation.

More importantly, we found that many of the hybridoma supernatant samples reacted with multiple autoantigens (lanes 1, 4, 7, 8, 9, and 10). Even more striking is that some hybridoma clones reacted to virtually all the signature autoantigens tested, paralleling the serum reactivity of BXSB-Yaa+ lupus-prone mice (lanes 16, 17, 19, and 20).

To verify that the promiscuous autoreactivity of L2pB1-derived hybridomas was not a protein array phenomenon, we retested the supernatant samples by indirect immunofluorescence on HEp-2 cells (Figure 1C). We found that hybridoma immunoglobulin recognized cytosolic, perinuclear, and nuclear antigens in 3 distinct patterns. Thus, clonotypic L2pB1 cell antigen receptors and their soluble Ig products exhibited multi-reactivity toward autoantigens, in contrast to L2nB1 and B-2 cells. The reactivity to HEp-2 cells (Figure 1C) correlated with the level of polyautoantigen binding activity in antigen microarray assay (Figure 1B). This likely resulted from promiscuous self antigen binding of individual immunoglobulin molecules.

L2pB1 cells are potent antigen-presenting cells (APCs)

We previously reported that B-1 cells are strong APCs as compared with B-2 cells (24). To test the antigen-presenting capacity of L2pB1 cells, sort-purified and mitomycin C–treated stimulating populations of L2pB1 cells and L2nB1 cells from BALB/c mice (H-2^d) were cocultured with magnetic-activated cell sorting (MACS)–purified, responding CD4+ T cells from C57BL/6 mice (H-2^b) in a mixed lymphocyte reaction (MLR) for 3 days. We observed that L2pB1 cells produced higher T cell proliferation than did L2nB1 cells, whether measured by thymidine incorporation (Figure 2A) or by CFSE dilution (Figure 2B). Compared with L2nB1 cells, L2pB1 cells stimulated 3 times more responding cells to proliferate (25.6% versus 7.55%) (Figure 2B). To further evaluate T cell activation by B-1 cell subpopulations, supernatant samples from cultures of B cells and T cells were tested for cytokine content by cytokine bead array (Figure 2C). As with T cell proliferation, we found that the levels of IL-2 and tumor necrosis factor α were higher after T cell coculture with L2pB1 cells than after T cell coculture with L2nB1 cells and total B-1 cells. Thus, L2pB1 cells are more efficient than L2nB1 cells in presenting alloantigen to T cells.

Although L2pB1 cells stimulated stronger allo-reactive T cell responses via direct antigen presentation in an MLR, it was not clear if the same would be true for nominal antigen presentation. To test this, CD4+ T cells were MACS-purified from DO11.10 T cell receptor–transgenic mice and cocultured with various B cell subpopulations in the presence of ovalbumin (OVA) peptide. Proliferation of OVA-responding DO11.10 T cells was measured by thymidine

incorporation (Figure 2D). T cells cocultured with peritoneal L2pB1 cells as APCs proliferated to a higher level compared with T cells cocultured with L2nB1 cells and peritoneal B-2 cells. Thus, L2pB1 cells are more efficient than L2nB1 cells in presenting nominal antigens.

L2pB1 cells promote Th17 cell differentiation

We previously reported that in an allogeneic situation, conventional B-2 cells are particularly potent in converting naive T cells into Treg cells, while B-1 cells are inefficient in promoting Treg cell conversion (24). In contrast, B-1 cells are much more potent in promoting Th17 cell differentiation than B-2 cells, even under the same Treg cell–promoting conditions in the presence of IL-2, which has been shown to constrain Th17 cell differentiation (25). To test whether the Th17 cell differentiating activity is enriched in certain subpopulations of B-1 cells, we sorted naive T cells (CD4+CD44–green fluorescent protein [GFP]–negative) from FoxP3–GFP–knockin mice and stimulated them with allogeneic L2pB1 or L2nB1 cells in the presence of TGFβ, IL-6, IL-23, and IL-2. Allogeneic B-2s cells were included as a control. After 4.5 days, intracellular IL-17 and interferon-γ were measured by flow cytometry (Figure 3). We found that both L2pB1 and L2nB1 cells produced substantial and equivalent levels of differentiation to IL-17–producing cells, while B-2 cells were much less effective. Thus, the capacity to induce Th17 cell differentiation is shared by L2pB1 and L2nB1 cells and distinguishes these subpopulations from B-2 cells.

L2pB1 cells in the periphery of lupus-prone mice correlate with anti-dsDNA titer

B-1 cells normally reside in body cavities. Migration of B-1 cells from the peritoneal cavity to the periphery has been associated with lupus development in mice (10). To determine whether L2pB1 cells preferentially express this B-1 cell behavior, we examined B cell populations in the peripheral blood of BXSB mice. Male BXSB-Yaa+ mice manifest accelerated development of a lupus-like autoimmune syndrome as compared with female littermate or male BXSB-Yaa– controls. We compared the levels of L2pB1 cells in the peripheral blood of these mice by flow cytometry (Figure 4A). We found a significant increase of L2pB1 cells in the periphery of male BXSB-Yaa+ mice as compared with both female littermate and BXSB-Yaa– control mice. An increase in peripheral B-1 cells has been associated with aging (26). We evaluated male BXSB-Yaa+ mice and female littermates for L2pB1 cell frequency as a function of age. At age 8 weeks, there was no difference in L2pB1 cell frequency between males and females. However, at age 12 weeks, there was already a significant increase in L2pB1 cell frequency in male BXSB-Yaa+ mice, while in female littermates the frequency remained unchanged (Figure 4B). Since BXSB-Yaa+ mice usually start to show lupus-like syndrome at age 10 weeks, the increase in L2pB1 cells in the periphery is most likely associated with early disease development, and the increase is disease associated but not age related.

One of the hallmarks of lupus is the presence of serum anti-dsDNA autoantibodies. We have shown that L2pB1 cells are especially reactive to multiple lupus-related autoantigens including dsDNA (Figure 1). To test whether the increase of L2pB1 cells in the peripheral blood was related to the level of anti-dsDNA autoantibody in male BXSB-Yaa+ mice, whole blood samples were collected, and serum anti-dsDNA antibodies were measured by ELISA at the same time that L2pB1 cells were evaluated by flow cytometry (Figure 4C). We found a high degree of correlation between L2pB1 cell frequency and anti-dsDNA level.

B-1 cells are known to be the major source of serum IgM (27). To test if the increase of dsDNA-reactive L2pB1 cells in BXSB mice results in elevated serum anti-dsDNA IgM levels, sera of male and female BXSB mice were tested for both anti-dsDNA IgM and IgG levels. ELISA results indicated that both IgM and IgG anti-dsDNA were present and were increased in male BXSB mice as compared with female controls (Figure 4D).

L2pB1 cells preferentially switch Ig isotype to IgG1 and IgG2b in the presence of IL-21

One of the humoral manifestations of disease in BXSB-Yaa mice is the elevated serum level of class-switched Ig isotypes. B-1 cells normally produce IgM and differ from B-2 cells in immunoglobulin isotype switching capacity (28). Since L2pB1 cells are enriched with dsDNA reactivity and the increase of L2pB1 cells correlates with disease onset in BXSB-Yaa mice, we tested if L2pB1 cells could undergo class switch. L2pB1, L2nB1, and B-2 cells were purified as described. Cells were then activated by CD40L as described previously (16) and cultured in the presence of LPS and cytokines as indicated. After 6 days of culture, cells were harvested and subjected to ELISPOT assay for Ig secretion. Our results indicate that in the presence of cytokines, particularly IL-21, >30% of L2pB1 cells could switch to IgG1 or IgG2b (Figure 5). Significantly more L2pB1 cells than L2nB1 and B-2 cells switched to IgG1 and IgG2b. Less than 10% of L2pB1 cells switched to IgG2a (Figure 5).

DISCUSSION

For the past decade, B cells have been a main target of immune therapy for autoimmune diseases such as rheumatoid arthritis (RA) and SLE. However, initial promising results became less impressive in recent large clinical trials. These variable results suggest the need to understand more precisely which type(s) of B cells are involved in disease processes. The recently identified subpopulation of B-1a cells that expresses PDL-2 is here shown to be enriched for autoreactive immunoglobulin, to be especially potent in antigen presentation, and to be fully capable of stimulating Th17 cell differentiation. These newly described features suggest at least 2 mechanisms for the means by which B-1a cells may potentially trigger or perpetuate autoimmunity. One is through the production of self-reactive immunoglobulin with direct pathophysiologic effects. The other is through strong antigen presentation that alters T cell activation and differentiation.

Both CD5+ B-1 cells and conventional B-2 cells can produce autoantibodies, but the disposition and potential pathogenesis of these antibodies are very different. Natural antibodies produced by B-1 cells have been known to recognize common pathogen structures and also to have low affinity for self antigens. This broad specificity is essential for their protective roles during infection and in autoantigen clearance. Conversely, CD5+ B cells have been reported to be responsible for the secretion of rheumatoid factor and anti–single-stranded DNA autoantibody in patients with autoimmune disease (5,6). An increase of CD5+ B cells is correlated with autoantibody production both in RA patients and in (NZB × NZW)F_1 lupus-prone mice, and the lupus susceptibility locus of *Sle2* is responsible for the enlarged B-1 cell pool (7,29). Although an increase of B-1 cell number by *Sle2* expression alone does not guarantee disease onset, combination with *Sle1* and *Sle3* loci did produce a synergistic effect on lupus pathology (30). This again suggests that it may take multiple steps and other factors for a protective-by-design B-1 cell to become pathogenic.

Autoreactive natural antibodies are known to have low affinity for self antigens. However, their pathogenicity can be achieved by higher avidity for damaged tissues with multiple B-1 cell–binding antigens, as well as by isotype switching to IgG. Moreover, it has been reported that CD5+ peripheral blood B cells from patients with childhood SLE showed higher frequencies of recombination-activating gene expression, suggesting that low-affinity autoreactive B-1 cells may serve as a template for the generation of high-affinity, pathogenic autoantibodies by secondary V–D–J recombination (31). Thus, B-1 cell–derived autoantibody may be directly involved in accelerating tissue damage.

We previously reported that characteristic B-1 cell autoantibody that recognizes phosphatidylcholine is produced predominantly by L2pB1 cells. This finding suggested that L2pB1 cells express a unique immunoglobulin repertoire. In the present study, we tested the

hypothesis that L2pB1 cells may be skewed toward an autoreactive repertoire and may therefore be a potential source of pathogenic autoantibodies.

We show here that L2pB1 cells secrete significantly more dsDNA-reactive antibodies than other B cells. Further, L2pB1 cell–derived hybridoma clones positively selected by dsDNA binding showed cross-reactivity to multiple autoantigens. Strikingly, some clones produced immunoglobulin that reacted with almost the entire panel of autoantigens recognized by serum samples from autoimmune mice. This very high degree of polyreactivity for B-1 cell–derived monoclonal autoantibodies has not been previously reported and contrasts with the much more highly restricted specificity of B-2 cell–derived autoantibodies. Considering the possibility of affinity maturation of B-1 cell Ig and the remarkable polyreactivity of L2pB1 cell Ig shown here, the probability of serologic autoreactivity produced by breaking tolerance of a single L2pB1 cell may be greater than the probability of serologic autoreactivity produced by breaking tolerance of a single epitope-specific autoreactive B-2 cell. The risk of these polyreactive L2pB1 cells becoming a potential source of pathogenic autoantibodies is also supported by the finding that anti-dsDNA antibodies with cross-reactivity to glomerular antigens are more likely to be pathogenic than antihistone and antinucleosome antibodies (32–34).

The pathogenicity of autoantibodies also depends on their Ig isotype. According to a study of anti–red blood cell monoclonal antibodies derived from lupus-prone NZB mice, IgG2a and IgG2b are the most pathogenic Ig isotypes (35). We have shown for the first time that B-1 cells, particularly L2pB1 cells, can undergo isotype switch and generate IgG1 and IgG2b but not IgG2a in the presence of IL-21. Interestingly, Bubier and colleagues recently reported that the serum level of IL-21 is elevated in BXSB-Yaa mice and correlates with a marked increase of IgG2b transcripts. Serum levels of IgG1, IgG3, and IgG2b are markedly lower in sera of BXSB-Yaa mice deficient in IL-21 receptor (36). Thus, it is likely that an elevated level of IL-21 will promote class switch of peripheral L2pB1 cells and contribute directly to the IgG autoantibody pool.

However, our findings do not exclude the possibility that L2pB1-derived polyreactive IgM may in some situations also be protective. It has been suggested that among patients with serum anti-DNA antibodies, the copresence of polyreactive IgM antibodies in the serum might confer protection against disease (37). Supporting this possibility, CD5+ B cells were found to be increased in the remission phase of systemic connective tissue diseases, particularly in SLE (38). In summary, L2pB1 cell–derived autoantibodies are quite promiscuous in terms of broad specificity for self antigens. We speculate that the relative pathogenicity of L2pB1 cell–derived autoantibodies is dependent on antibody affinity, polyreactivity, isotype, and the magnitude of inflammatory tissue injury. Further study is required to dissect the pathogenic and protective roles of L2pB1 cell–derived autoantibodies.

Autoantibody secretion is the presumed pathogenic mechanism by which B cells contribute to autoimmune diseases. However, another dimension to the role of B cells in autoimmunity relates to the interaction between B cells and T cells, particularly between B-1 cells and T cells. We previously reported that B-1 cells and conventional B-2 cells differ dramatically in how they influence T cell differentiation. Conventional B-2 cells are excellent in converting naive CD4+ T cells into Treg cells, while B-1 cells preferentially promote Th1 and Th17 cell differentiation. Here we show that L2pB1 cells excel in antigen presentation as compared with L2nB1 and other B cells. Of note, the potent antigen presentation activity of L2pB1 cells is not due to retrograde PDL-2 signaling, because the anti–PDL-2 antibody used during cell sorting did not stimulate autologous NF-κB activation as reported for dendritic cells (data not shown). The strong T cell stimulation by L2pB1 cells seems paradoxical, since PDL-2 has been reported to be a negative regulator for T cells. The function of PDL-2 on L2pB1 cells remains to be determined. It is possible that PDL-2 expression on L2pB1 cells is necessary to restrain

T cell stimulation by autoreactive L2pB1 cells and to promote T cell differentiation. Thus, it can be speculated that down-regulation of PDL-2 on L2pB1 cells might render them even stronger T cell stimulators, increasing the risk of autoimmunity.

In addition to strong antigen presentation, like all B-1 cells, L2pB1 cells promote Th17 cell differentiation in the presence of inflammatory cytokines. Considering the skewed autoreactive repertoire and associated polyreactivity of L2pB1 cell–derived antibodies, the range of autoantigens capable of being presented to T cells by L2pB1 cells is enormous as compared with B-2 cells. Further, L2pB1 cells present those antigens especially efficiently, and they skew the differentiation of T cells to Th17 cells. Thus, it makes a difference whether a particular antigen is presented by an L2pB1 cell or by other B cells. With the new features of L2pB1 cells presented in this report, it is logical to presume that L2pB1 cells can trigger the effector function of autore-active T cells to a much greater extent than other APCs. Further, under normal circumstances, B-1 cells are sequestered in body cavities of mice, but in autoimmune disease, L2pB1 cells are found in increasing numbers in the periphery. It has been reported that with proper stimulation, B-1 cells migrate to the periphery and lymphoid organs (10,26). This provides yet further opportunities for B cell–T cell interaction. Thus, by virtue of autoreactive specificity, efficient antigen presentation, peripheral migration, and induction of Th17 cell differentiation, L2pB1 cells have the full potential to promote autoreactive inflammatory Th17 cell differentiation, which would be expected to contribute to autoimmune dyscrasias.

In summary, we have demonstrated that the novel subpopulation of PDL-2–expressing CD5+ B-1 cells, here termed L2pB1 cells, is especially enriched for autoreactivity and antigen presentation. A peripheral increase of L2pB1 cells is positively correlated with anti-DNA antibody levels in a murine lupus model. These characteristics indicate that L2pB1 may contribute to the pathogenesis of autoimmune disease through both antibody- and T cell–mediated mechanisms. However, the highly polyreactive anti-dsDNA antibodies generated by L2pB1 cells may very well confer a protective role on these cells through clearing autoantigen-containing immune complexes and cellular debris from tissue damage. We speculate that the protective or pathogenic roles of L2pB1 cells may depend upon environmental influences or intrinsic signaling paradigms. Further study is needed to distinguish the roles of L2pB1 cells in health and disease and to dissect the precise contributions of L2pB1 cells to lupus development. It is worth noting that L2pB1 cells are not unique to the mouse. We also found L2pB1 cells in human patients with autoimmune disease (Zhong X: unpublished observations), although their function remains to be tested. Overall, our study has identified a potential new biomarker and cellular target for the design of future diagnostic and therapeutic reagents for lupus.

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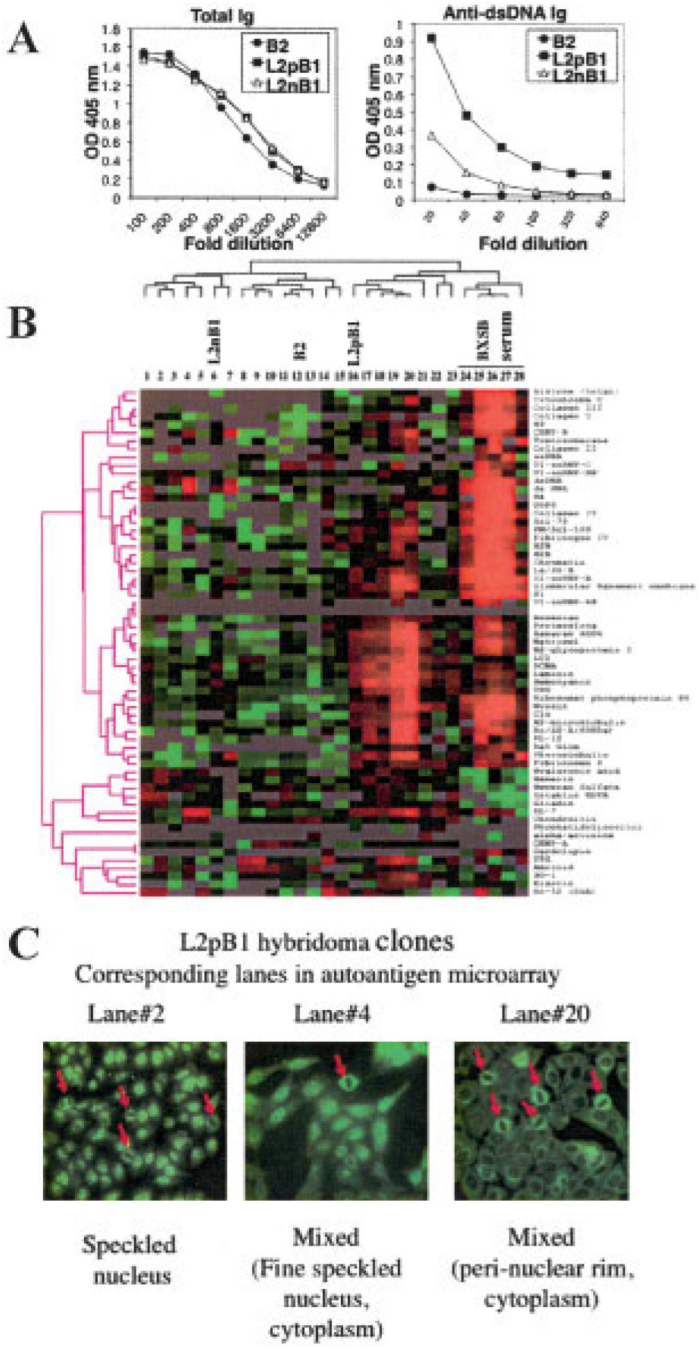

Figure 1.

Programmed death ligand 2 (PDL-2)–positive B-1a cells (L2pB1 cells) are enriched with polyreactivity to autoantigens. **A**, L2pB1 cells, PDL-2–negative B-1a cells (L2nB1 cells), and B-2 cells were stimulated with 25 μg/ml lipopolysaccharide (LPS) for 7 days. Supernatants were harvested and assayed by enzyme-linked immunosorbent assay (ELISA) for total Ig and anti–double-stranded DNA (anti-dsDNA) antibody level. Data are representative of at least 3 independent experiments. **B**, Hybridoma clones were generated by fusion of L2pB1 cells with the myeloma cell line F0. DNA-reactive clones were selected by ELISA. After 2 rounds of subcloning, supernatants of each clone were hybridized to slides printed with 62 autoantigens and 7 controls. Supernatants from LPS-stimulated fluorescence-activated cell–sorted primary

L2nB1 cells (lane 6), L2pB1 cells (lane 16), and B-2 cells (lane 12) were included. Sera from BXSB mice with high anti-dsDNA titer (lanes 25–27) and low anti-dsDNA titer (lanes 24 and 28) by ELISA were also included. **C**, Supernatants from monoclonal L2pB1 hybridomas were collected and incubated on Antinuclear Antibody (HEp-2 cells) substrate slides and developed with fluorescein isothiocyanate–conjugated anti-mouse Ig. Three representative patterns of antibody staining are shown. The corresponding lane numbers of the microarray data are indicated. **Arrows** indicate mitotic figures. Results are representative of more than 3 independent experiments (original magnification × 400.) OD = optical density.

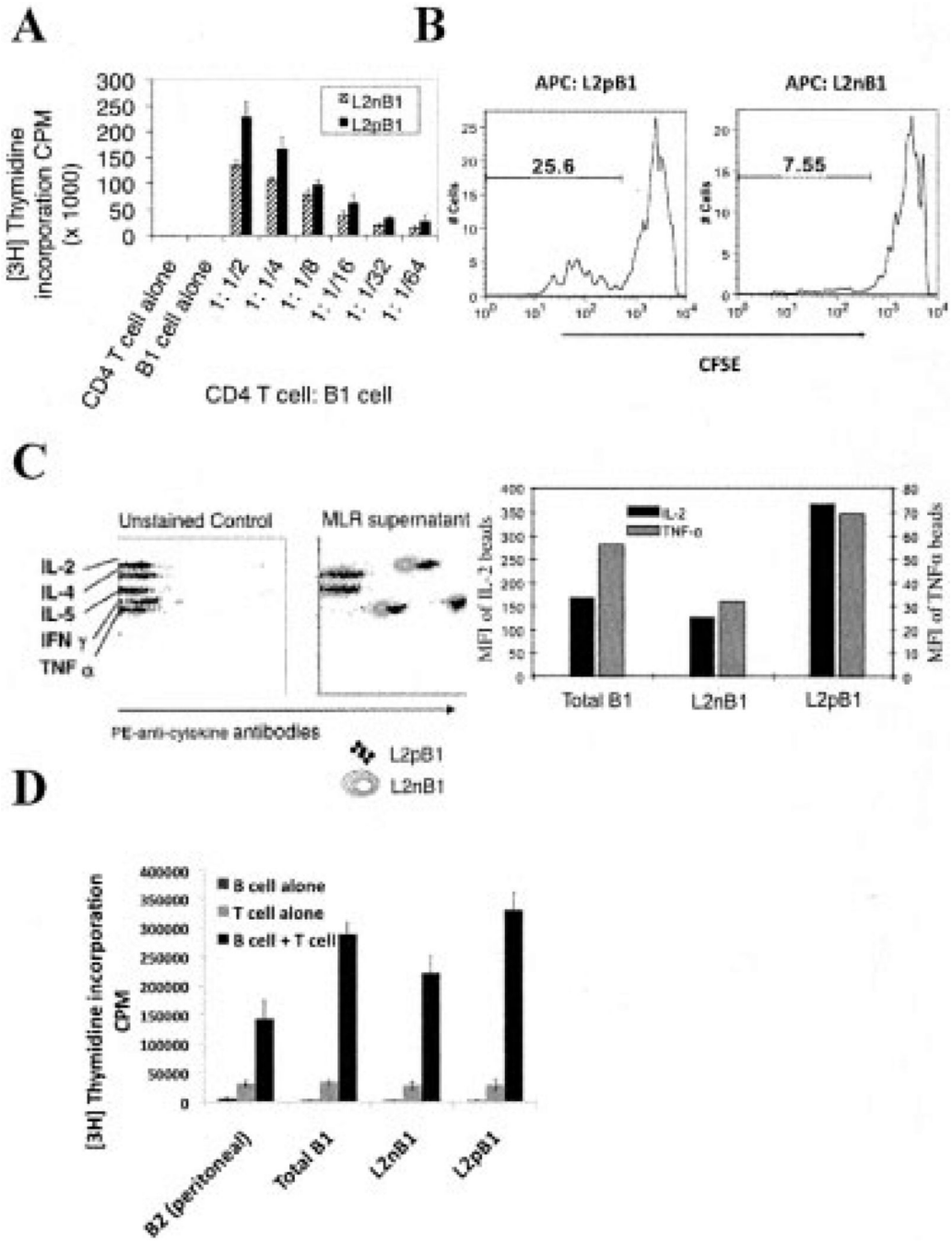

Figure 2.

L2pB1 cells are potent antigen-presenting cells (APCs). **A**, Mixed lymphocyte reaction (MLR) cultures were established by mixing sort-purified B cells from BALB/c mice with splenic CD4 + T cells from C57BL/6 mice. CD4+ T cells (2×10^6/ml) were mixed with L2pB1 or L2nB1 cells at the indicated ratios and cultured for 72 hours. T cell proliferation was measured by ^{3}H-thymidine incorporation. Values are the mean and SD from triplicate wells of each sample. Data shown are representative of 3 independent experiments. **B**, CD4+ T cells labeled with 5,6-carboxyfluorescein succinimidyl ester (CFSE) were mixed with L2pB1 or L2nB1 cells at a 4:1 ratio and cultured for 96 hours. T cell division was analyzed by flow cytometry. Data from 1 of 2 independent experiments are shown. **C**, CD4+ T cells were cocultured with

total B-1a cells, L2pB1 cells, or L2nB1 cells for 72 hours. Supernatants were harvested and cytokines were measured by cytokine bead array. Cytokine levels are indicated by a shift of bead fluorescence along the x-axis. Also shown is a graph of the mean fluorescence intensity (MFI) of interleukin-2 (IL-2) beads and tumor necrosis factor α (TNFα) beads measured by the cytokine bead array assay. Values are the mean. **D**, CD4+ T cells from DO11.10 T cell receptor–transgenic mice were cultured for 5 days with total B-1a cells, L2pB1 cells, L2nB1 cells, or B-2 cells, as indicated, in the presence of 0.5 μM ovalbumin peptide. T cell proliferation was measured by thymidine incorporation. Data are representative of 3 independent experiments.Values are the mean and SD. IFNγ = interferon-γ; PE = phycoerythrin (see Figure 1 for other definitions).

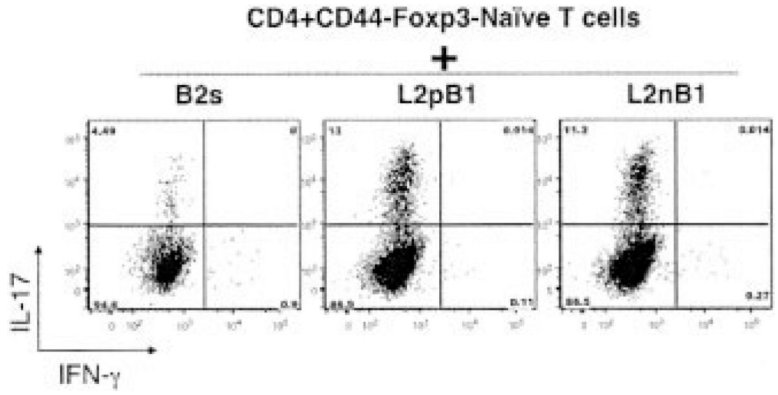

Figure 3.

L2pB1 cells promote Th17 cell differentiation. CD4+ CD44–green fluorescent protein (GFP)–negative naive CD4+ T cells from FoxP3–GFP–knockin mice were sorted, and cultured for 4.5 days with L2pB1 cells, L2nB1 cells, or splenic B-2 cells (B-2s cells), as indicated, in the presence of 2 ng/ml transforming growth factor β, 10 ng/ml interleukin-23 (IL-23), 10 ng/ml IL-6, and 5 ng/ml IL-2. Flow cytometry analysis of intracellular staining for IL-17 and interferon-γ (IFNγ) is shown. See Figure 1 for other definitions.

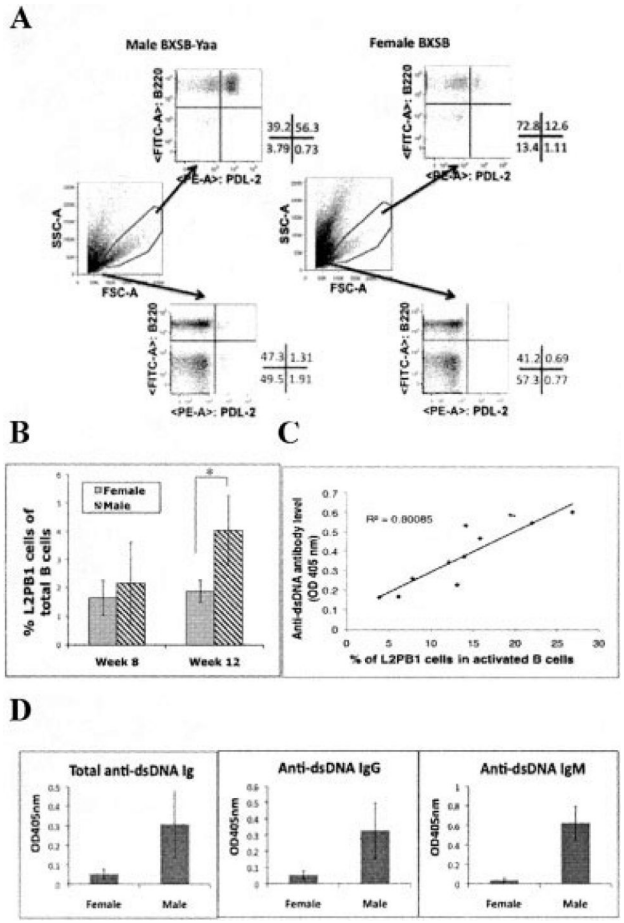

Figure 4.

Peripheral increase of L2pB1 cells in lupus-prone mice correlates with serum anti-dsDNA level. **A**, Peripheral blood samples from male BXSB-Yaa+ mice and littermate female control mice were collected and stained for B220 and PDL-2. Large and small cells, as defined by forward scatter (FSC) and side scatter (SSC), were assessed separately by flow cytometry. **B**, Blood samples were collected from offspring littermate male and female mice at the indicated time points. Frequencies of L2pB1 cells as a percentage of total B cells were assessed by flow cytometry. Values are the mean ± SD. * = $P = 0.03$. **C**, Blood samples from BXSB mice were collected and analyzed by flow cytometry as shown in **A**. The percentages of large B220+ cells that were PDL-2 positive (L2pB1 cells) are shown on the x-axis, and the corresponding anti-dsDNA ELISA OD readings are shown on the y-axis. The coefficient of determination (R^2) is shown. **D**, Sera from male BXSB-Yaa mice and female BXSB control mice were tested for anti-dsDNA autoantibodies by ELISA. Horseradish peroxidase–conjugated anti-mouse Ig heavy and light chain antibodies, anti-mouse IgG antibodies, and anti-mouse IgM antibodies were used as detection antibodies. Mean ± SD OD readings at 405 nm are shown. FITC = fluorescein isothiocyanate; PE = phycoerythrin (see Figure 1 for other definitions).

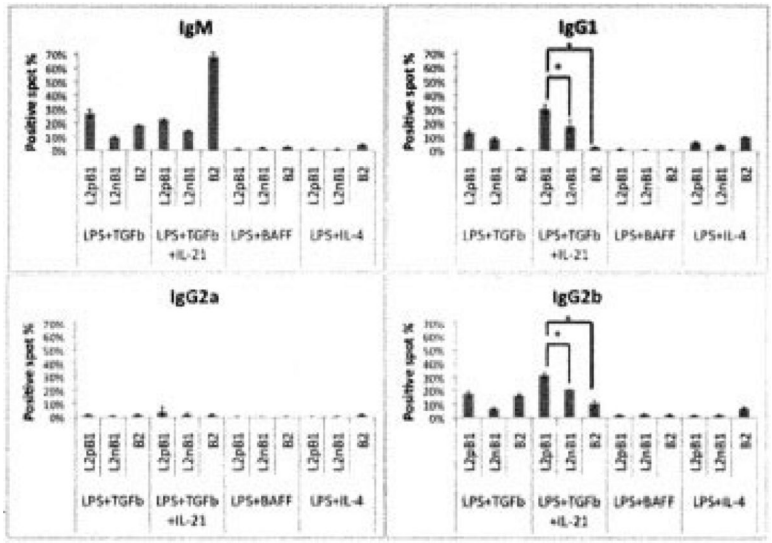

Figure 5.

L2pB1 cells preferentially switch Ig isotype to IgG1 and IgG2b in the presence of interleukin-21 (IL-21). Peritoneal L2pB1 and L2nB1 cells were subjected to fluorescence-activated cell sorting. B-2 cells were purified from spleens using magnetic-activated cell sorting. Cells were then incubated with CD40L-CD8 fusion proteins for 15 minutes followed by anti-CD8 crosslinking. Different combinations of cytokines (12.5 μg/ml LPS, 0.2 ng/ml transforming growth factor β [TGFβ], 5 ng/ml IL-21, 0.5 μg/ml BAFF, 50 ng/ml IL-4) were added to the cell culture, as indicated. Cells were harvested after a 7-day culture and plated on an enzyme-linked immunospot assay plate recoated with anti-mouse antibodies. Anti-IgM, -IgG1, -IgG2a, and -IgG2b antibodies were used to detect Ig-secreting cells. Shown are percentages of cells that secreted different isotypes of Ig. Results are from 1 of 2 experiments. Values are the mean ± SD. * = $P < 0.05$. See Figure 1 for other definitions.