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PDCA Expression by B Lymphocytes Reveals Important Functional Attributes

Dass S. Vinay,* Chang H. Kim,† Kyung H. Chang, † and Byoung S. Kwon*†

We have demonstrated in this study the existence of a PDCA-expressing functional B cell population (PDCA⁺ B lymphocytes), which differentiates from activated conventional B (PDCA⁻ Ig⁺⁺) lymphocytes. Stimulation with anti-μ, LPS, CpG oligodeoxynucleotide, HSV-1, or CTLA-4 Ig activates the PDCA⁺ B lymphocytes, leading to cell division and induction of type I IFNs and IDO. Notably, the PDCA⁺ B lymphocytes are capable of Ag-specific Ab production and Ig class switching, which is corroborated by transfer experiments in B- and PDCA⁺ B lymphocyte-deficient μMT mice. Importantly, in lupus-prone MRL-Fas⁺/+ mice, PDCA⁺ B lymphocytes remain the principal source of autoantibodies. The PDCA⁺ B lymphocytes have phenotypes with plasmacytoid dendritic cell markers and are a distinct cell population in that they develop from C-kit⁺B220⁺ pro-B precursors. Thus, our data suggest that not all PDCA⁺ cells are dendritic cell-derived plasmacytoid dendritic cells and that a significant majority is the PDCA⁺ B lymphocyte population having distinct phenotype and function. The Journal of Immunology, 2010, 184: 807–815.

B lymphocytes play critical roles in immune regulation (1). Besides secreting Igs and Abs, B lymphocytes are important for priming T cell responses (2). Depending on the cell surface molecule, cytokine expression, and functionality, B lymphocytes are divided into B1, B2, plasma, memory, marginal zone, and follicular subpopulations, each with unique and sometimes overlapping roles (3). In addition to their neutralizing effect on viruses (4), Abs produced by the autoreactive B lymphocytes contribute to disease severity in autoimmune conditions, including lupus and rheumatoid arthritis (5).

Although plasmacytoid dendritic cells (pDCs), an important class of immune effectors (6, 7), are identified by their expression of PDCA molecules and are considered to be a subset of dendritic cells (DCs) (8), their expression of B lymphocyte-specific molecules raises intriguing questions. Therefore, prompted by their expression of B lymphocyte markers such as B220 and CD19 (9), the fact that primary B lymphocytes, plasma cells, and B lymphocyte lines (A20 and BAF3) express PDCA and that deletion of pDCs results in elimination of CD138⁺ plasma cells (9), we tested whether they are in fact B lymphocytes. As a result, we have identified and characterized a fully functional B lymphocyte subpopulation coexpressing the PDCA marker. The PDCA⁺ B lymphocytes described in this study emerge from a common pro-B precursor, as do conventional B lymphocytes, have phenotypes with pDCs, and are the principal producers of Igs and inducers of type I IFNs.

Materials and Methods

All animal experiments were approved by our Institutional Animal Care and Use Committee.

Mice

C57BL/6 (B6) mice were purchased from the National Cancer Institute (Frederick, MD). B cell-deficient μMT (B6.129S2-Igh-6tm1Cgn) and MRL-Fas⁺/+ (B6.MRL-Fas⁺/+; lpr⁺) mice were obtained from The Jackson Laboratory (Bar Harbor, ME).

Cell isolation

Total PDCA⁺ cells were isolated using anti-PDCA MicroBeads (catalog #130-091-965; Miltenyi Biotec, Auburn, CA). This protocol yields both PDCA⁺ and PDCA⁻ cells. To isolate total PDCA⁺ cells, we used mouse pDC isolation kit II (catalog #130-092-786; Miltenyi Biotec) as observed that this kit yields PDCA⁺ cells. To isolate PDCA⁻ cells, the PDCA⁺ cells were first removed using mouse pDC isolation kit II (catalog #130-092-786; Miltenyi Biotec). The flow-through was collected, passed a second time through a fresh column, stained with Alexa 647 anti-PDCA, and PDCA⁻ cells were sorted (FACSAria, BD Biosciences, San Diego, CA). Purification of PDCA⁺CD43⁻⁺ cells was achieved by first incubating cells in anti-CD43 MicroBeads (Miltenyi Biotec) and passed through a magnetic column. The column-bound cells were used as a source of PDCA⁺CD43⁻⁻ and the flow through as PDCA⁺CD43⁻ cells. The resting B cells were isolated using anti-CD43 MicroBeads (Miltenyi Biotec).

Confocal microscopy

Frozen spleen sections (7 µm) were stained with the indicated Abs (all at 10 µg/ml). After washing, samples were cover slipped with Prolong Gold antifade reagent (Invitrogen, Carlsbad, CA), photographed, and analyzed with a confocal laser-scanning microscope (LSM 510-Meta, Carl Zeiss, Jena, Germany).

Flow cytometry

Unless otherwise stated, all Abs were purchased from eBioscience (San Diego, CA). FcR block (clone 2.4G2; produced in-house) was added to all surface staining mixtures. Flow cytometry was performed using an FACS Calibur (BD Biosciences). Nitrophenyl (NP)-binding B lymphocytes from immunized mice were identified by three-parameter flow cytometry using anti-mouse PDCA MicroBeads (Miltenyi Biotec) and passed through a magnetic column. The column-bound cells were used as a source of PDCA⁺CD43⁻ and the flow through as PDCA⁺CD43⁻ cells. The resting B cells were isolated using anti-CD43 MicroBeads (Miltenyi Biotec).
Precursor analysis
Bone marrow-derived C-kit\(^{+}\)B220\(^{+}\) pro-B precursors were sorted using a FACS Aria (BD Biosciences), layered on subconfluent OP9 cells (American Type Culture Collection, Manassas, VA), and stimulated with murine recombinant Flt3L (200 ng/ml; R&D Systems, Minneapolis, MN) and IL-7 (5 ng/ml; PeproTech, Rocky Hill, NJ). After 7 d, flow cytometry was performed.

In vitro Ig class switching
Purified PDCA\(^{+}\) B or PDCA\(^{-}\) B lymphocytes (5 \(\times\) 10^5) were stimulated with LPS (25 \(\mu\)g/ml; Sigma-Aldrich, St. Louis, MO) alone or in combination with IL-4 (5 ng/ml; Sigma-Aldrich) and IL-7 (5 ng/ml; PeproTech) for 4 d. After 4 d, they were washed and cell surface Igs analyzed by flow cytometry.

RT-PCR
Total RNA was isolated using TRIzol (Invitrogen). The indicated mRNAs were determined by RT-PCR using random hexamer primers specific for the mouse (Table 1).

Cell division
Cells were cultured with CFSE (10 \(\mu\)M; Invitrogen) and stimulated with anti-\(\mu\) (10 \(\mu\)g/ml; F(ab\(^{\prime}\))\(^{2}\) fragment of goat anti-mouse IgM) for 3 d in the presence of murine recombinant Flt3L (200 ng/ml; R&D Systems, Minneapolis, MN) and IL-7 (5 ng/ml; PeproTech) to induce proliferation. Cells were stained and analyzed using a FACS BD Flow Kit (BD Biosciences).

BrdU assay
B6 mice were inoculated i.v. with PBS or CpG ODN 1826 (Invivogen, San Diego, CA) (50 \(\mu\)g/ml) twice a day. After 3 d and 1 h after last BrdU treatment, spleens were collected and stained using a FITC BrdU Flow Kit (BD Biosciences).

Measurement of adenosine deaminase and circular transcripts
Cells were stimulated for 40 h with LPS (25 \(\mu\)g/ml; Sigma-Aldrich) and IL-4 (5 ng/ml; PeproTech) to induce y1 CT and AID. Total RNA extracted with TRIzol (Invitrogen), and RT-PCR was performed using murine AID primers (Table 1). The circular transcripts of I\(\gamma\) were amplified following the protocol of Kinoshita et al. (10).

Anti-NP ELISA
Purified PDCA\(^{+}\) B or PDCA\(^{-}\) B lymphocytes were pulsed by overnight culture with 100 \(\mu\)g/ml NP\(^{39}\)-chicken \(\gamma\) globulin (CGG) (Biosearch Technologies). A total of 1 \(\times\) 10^5 wild-type (wt) Ag-pulsed PDCA\(^{+}\) B or PDCA\(^{-}\) B lymphocytes were injected (i.v.) into syngeneic wt or Rag2^−/− mice. Seven days later, sera were collected and used to measure anti-NP Abs in ELISA plates (Nunc, Roskilde, Denmark) coated with NP\(^{14}\)-BSA (10 \(\mu\)g/ml; Biosearch Technologies).

Induction of IFN-\(\alpha\)
B6 mice were injected (i.v.) with either PBS or CpG oligodeoxynucleotide (ODN) 1826 (Invivogen, San Diego, CA) (50 \(\mu\)g in PBS) (6). Three hours later, spleens were collected, and multicolor flow cytometry was performed. For induction of IFN-\(\alpha\)-dependent cell division, spleen cells (10 \(\times\) 10^6) were stimulated with CpG ODN 1826 (10 \(\mu\)g/ml) or UV-inactivated (1.2 \(\times\) 10^6 \(\mu\)J/cm^2) HSV-1 (KOS strain; 4 \(\times\) 10^4/ml). After 24 h, cells were washed and restimulated with PMA (50 ng/ml), ionomycin (500 ng/ml), and brefeldin A (5 \(\mu\)g/ml) followed by multicolor flow cytometry.

Results
Identification of PDCA\(^{+}\) B lymphocytes
Prompted by B cell-specific molecule expression in pDCs (9), we tested whether these are B lymphocytes or a separate population. Phenotypic analysis of spleen cells revealed the presence of several B lymphocyte-specific markers such as IgM, CD19, AA4.1, and CD79b on PDCA\(^{+}\) cells (Fig. 1A). Calculations revealed \(\sim 28.1 \times 10^6 \pm 4.12\) PDCA\(^{+}\)IgM\(^{+}\) cells and 5.74 \(\times\) 10^5 \(\pm\) 1.22 PDCA\(^{-}\)IgM\(^{+}\) cells per young adult spleen (Fig. 1B, 1C). Lack of staining with FITC rat IgG2b (isotype control for FITC–anti-PDCA Ab) verified our above results (Fig. 1A). Further, we identified two distinct subpopulations of PDCA\(^{+}\) cells where nearly all PDCA\(^{+}\) (closed boxes) and a few PDCA\(^{-}\) (dashed boxes) cell populations coexpressed B cell-specific markers (Fig. 1A). Confocal microscopy of naive spleen sections also identified PDCA\(^{+}\) IgM\(^{+}\) cells (hereafter referred to as PDCA\(^{+}\) B lymphocytes) (Fig. 1D; white arrows). To confirm the existence of PDCA\(^{+}\) B lymphocytes, we purified CD43^− resting B lymphocytes from naive mice treated with EDTA/PBS to dissociate any pDC-B lymphocyte conjugates and found that the purified B lymphocytes exhibited basal PDCA expression (Fig. 1E). Absence of PDCA\(^{+}\) IgM\(^{+}\) cells in B cell-deficient \(\mu\)MT mice confirmed the existence of PDCA\(^{+}\) B lymphocytes (Fig. 1F). It may be mentioned that in \(\mu\)MT mice, frequencies of pDCs (PDCA\(^{+}\)IgM\(^{+}\)) rose significantly over wt controls (Fig. 1F; compare 2.9% in wt mice versus 12.5% in \(\mu\)MT mice). Repeat experiments yielded similar results. We also found similar increase of pDCs (PDCA\(^{+}\)IgM\(^{+}\)) in Rag2^−/− mice (data not shown). The significance of increased pDCs in \(\mu\)MT and Rag2^−/− mice needs additional investigation. To check if the PDCA\(^{+}\) B lymphocytes emerge from the same C-kit\(^{+}\)B220\(^{+}\) pro-B precursors that give rise to conventional B lymphocytes, we gated the bone marrow-derived B220\(^{+}\) cells and found that nearly all C-kit\(^{+}\)PDCA\(^{+}\) and a minority of C-kit\(^{+}\)PDCA\(^{-}\) cells were B220\(^{+}\) (Fig. 1G), indicating that C-kit\(^{+}\)B220\(^{+}\) are the precursors of PDCA\(^{+}\) B lymphocytes. To further confirm this, we cultured bone marrow-derived cells on a monolayer of OP9 cells in the presence of Flt3L and IL-7 for 7 d. Analysis of sorted C-kit\(^{+}\)B220\(^{+}\) cells from the above culture confirmed that they indeed are precursors of PDCA\(^{+}\) B lymphocytes (Fig. 1H). RT-PCR analysis of sorted PDCA\(^{+}\)CD43^− B lymphocytes confirmed the existence of PDCA\(^{+}\) B lymphocytes where expression of a number of B cell-specific molecules (Table 1) was comparable among purified B220\(^{+}\) and resting CD43^− B lymphocytes except for early growth response gene product-1 (EGR1) and ID4, which were found expressed highly in PDCA\(^{+}\)CD43^− B lymphocytes (Fig. 1J). In addition, we have also observed that PDCA\(^{+}\) B (Fig. 1I; PDCA\(^{+}\)CD43^−; right lane) expressed marginally increased PU.1 over conventional B lymphocytes (Fig. 1F; PDCA\(^{+}\)CD43^−; middle lane). Taken together, our data demonstrate the existence of a naturally occurring PDCA\(^{+}\) B lymphocyte subpopulation.

PDCA\(^{+}\) B lymphocytes have phenotypes with pDCs
In Fig. 1E, we demonstrated the existence of PDCA\(^{+}\) B lymphocytes by evaluating purified resting B lymphocytes. We next determined if cells purified based on PDCA expression also express B cell-specific molecules, pDCs, among others, are identified as cells coexpressing PDCA and CD11c (8, 11). We checked if the reported PDCA\(^{+}\) B lymphocytes also express CD11c. Analysis revealed that a fraction of CD19\(^{+}\) (a marker to identify B lymphocytes) cells showed surface CD11c (Fig. 2A; rightmost panel) and that PDCA was found coexpressed on CD19^+CD11c^+ cells (Fig. 2B; second panel from left). Expression of PDCA on B lymphocytes was not coincidental in our findings. Siglec H, a pDC-specific marker (9; Fig. 2C; second panel from left), was also found coexpressed on \(\sim 10\%\) of the CD43^− resting B lymphocytes (Fig. 2C; rightmost panel). CD19 is a B lymphocyte-specific molecule, but proposing PDCA\(^{+}\)CD19^+CD11c^+ as PDCA\(^{+}\) B lymphocytes may lead to confusion, as pDCs are also known to express CD19 (9, 11). To fully resolve the issue of whether pDCs and PDCA\(^{+}\) B lymphocytes are the same or separate cell types, we performed multparameter flow cytometric studies using purified PDCA\(^{+}\) cells. We found two distinct subsets of PDCA\(^{+}\) cells in the spleens of naive mice: PDCA\(^{+}\)CD11c and PDCA\(^{-}\)CD11c (Fig. 2D; leftmost panel). Gates were set around PDCA\(^{+}\)CD11c and PDCA\(^{-}\)CD11c cells (Fig. 2D; second panel...
FIGURE 1. Identification of PDCA+B lymphocytes. A, Spleen cells from naive B6 mice were used for flow cytometry of the indicated markers and frequencies of B lymphocytes expressing PDCA are depicted. One of four independent experiments is shown, n = 3. Where indicated, PDCAlo and PDCAhi cells are shown by dashed and intact boxes, respectively. B and C, Absolute numbers of pDCs (B) and PDCA+B lymphocytes (C) were determined from precounted total viable cell numbers from flow cytometry data and presented as histograms (mean ± SD). n = 3. D, Representative frozen spleen sections were stained with the indicated Abs, and confocal microscopy was performed. Similar results were observed in two other experiments (original magnification ×40). E, CD43− resting B lymphocytes were purified, stained as indicated, and flow cytometry was performed. One of six independent experiments is shown, n = 3. F, Spleen cells from the indicated mouse strains were collected and analyzed. One of three independent experiments is shown, n = 3. G, Bone marrow-derived cells were obtained from naive B6 mice and three-color flow cytometry was performed. H, Total bone marrow-derived cells from G were cultured on a subconfluent OP9 monolayer with Flt3L and IL-7 for 7 d, washed, C-kit+/B220+ sorted, and stained as shown. One of three independent experiments is presented, n = 6. I, Indicated cell populations were purified from naive mice and RT-PCR performed. Shown is the result of samples pooled from five independent experiments and each experiment contained a minimum of six mice.

from left) and identified differential levels of IgD and IgM on these subsets (Fig. 2D; third panel from left). To increase the authenticity of PDCA+B lymphocytes, we next gated PDCA−CD11c+ IgD−IgM+ cells and found several B cell-specific molecules such as CD79b, AA4.1, and Igκ expressed on these gated cells (Fig. 2D; panels 4–6 from left). Although experiments described in Fig. 2D reveal CD11c expression on freshly isolated PDCA+ cells, when activated with LPS or anti-μ/anti-CD40 or CpG ODN 1826, they lose CD11c expression by ∼4-fold within 72 h after stimulation (data not shown). Taken together, these data indicate that although pDCs and PDCA+B lymphocytes have overlapping phenotypes, they are distinct cell types.

PDCA+B lymphocytes undergo BCR-mediated cell division

In order for PDCA+B lymphocytes to be designated as true B lymphocytes, they must possess an important attribute: responsiveness to anti-μ signaling. To test this, PDCAloCD43− and PDCAhiCD43+ cells were purified and stimulated with anti-μ/anti-CD40 and LPS for 96 h (Fig. 3A). Robust cell division in PDCAloCD43− cells confirmed that they possess this attribute (Fig. 3B, upper left quadrant). On the other hand, we found no significant cell division among PDCA+CD43+ cells stimulated with anti-μ and anti-CD40 or with LPS (Fig. 3C, D, upper left quadrant). We found the lack of cells in Fig. 3D (third and fourth panels from left, upper left quadrant) to be very intriguing. We checked whether this was due to cell death or refractiveness to mitogenic stimuli we applied. Careful examination of side and forward scattering profiles of these cells revealed no activation-induced cell blast formation in PDCAhiCD43− cultures (data not shown). These cells, however, remained viable at the end of the 96-h culture period (albeit few; data not shown). Because the data shown in Fig. 3B, D,3G showed no or fewer cells. In addition, when BrdU-treated B6 mice were injected with LPS or goat anti-mouse IgD, which are known to cause B cell activation in vivo (12, 13), we found significant incorporation of BrdU in PDCAloCD43− blasts within these gates, the data in Fig. 3D (third and fourth panels from left) therefore showed no or fewer cells. In addition, when BrdU-treated B6 mice were injected with LPS or goat anti-mouse IgD, which are known to cause B cell activation in vivo (12, 13), we found significant incorporation of BrdU in PDCAloCD43− but not in PDCAhiCD43− cells (Fig. 3E; compare dashed versus closed boxes). These data conclude that PDCAloB but not PDCAhiB lymphocytes show mitogenic activity.

**IFN-α and IDO are expressed by PDCA+B lymphocytes**

pDCs typically produce type I IFNs in response to viral infections and TLR agonists (6, 7). To verify that the same was true of the PDCA+B lymphocytes, we injected wt and age- and sex-matched B- and PDCA+B lymphocyte-deficient μMT mice with CpG ODN (6). Three hours later, spleen cells were collected, and flow cytometry was performed. Gates were set around cells to distinguish multiple cell populations based on the presence or absence of IgM and PDCA. IFN-α expression was observed predominantly in wt PDCA+B IgM+ than in PDCA+B IgM− cells (Fig. 4A). It may be noted that <3% of conventional wt B (PDCA+B IgM+) produced IFN-α (Fig. 4A), suggesting that PDCA+B
(PDCA⁺IgM⁺) lymphocytes are the bulk producers of this cytokine. We checked if this interpretation was true. When B cell-deficient μMT mice, which also lack the PDCA⁺ B lymphocytes (Fig. 1, right panel), were treated in vivo with CpG ODN 1826, we observed a 2-fold reduced expression of total IFN-α by μMT mice (Fig. 4A; compare 25.13 ± 6.61% in wt versus 11.12 ± 4.32% in μMT mice), confirming that PDCA⁺ B lymphocytes indeed are important contributors of IFN-α production. That expression of IFN-α in PDCA⁺IgM⁺ lymphocytes, shown in Fig. 4A, was specific was confirmed by RT-PCR analysis of purified PDCA⁺CD43⁺ B cells from wt and Mt mice impairs their ability to fully express CD79b, AA4.1, and Igk markers. We checked whether these cells express IDO, an immunosuppressive enzyme expressed by activated pDCs (14). When PDCA⁺CD43⁺ B lymphocytes were first activated with anti-μ and rested with CTLA-4-Ig to induce IDO expression (15), both IDO1 and IDO2 levels increased (Fig. 4B). Furthermore, when total spleen cells from naïve mice were cultured in vitro for 24 h with CpG ODN 1826 or UV-inactivated HSV-1 (Fig. 4C), an increase in IFN-α expression was noted in PDCA⁺IgM⁺ (PDCA⁺ B lymphocytes) compared with pDCs (PDCA⁺IgM⁻) (Fig. 4C). Because we had shown that PDCA⁺ B lymphocytes produce type I IFNs, we checked whether these cells express IDO, an immunosuppressive enzyme expressed by activated pDCs (14). When PDCA⁺CD43⁺ B lymphocytes were first activated with anti-μ and rested with CTLA-4-Ig to induce IDO expression (15), both IDO1 and IDO2 levels increased (Fig. 4D). Total spleen cells stimulated with PGE₂, IFN-γ, and TNF-α served as controls for this assay. We also found, for the first time, a several-fold increase in expression of IDO2 in PDCA⁺ B lymphocytes in cultures stimulated with CTLA-4-Ig (Fig. 4D). Because this result hinted that PDCA⁺ B lymphocytes express IDO1 and IDO2, we next verified the authenticity of this result. When total spleen cells from wt and μMT mice were stimulated with LPS or CTLA-4 Ig, we observed that absence of PDCA⁺ IgM⁺ B lymphocytes in μMT mice impairs their ability to fully express inducible IDO1 but not IDO2 (Fig. 4E). Interestingly, LPS but not CTLA-4 Ig failed to induce IDO expression in μMT mice (Fig. 4E). These data are important and suggest that PDCA⁺ B lymphocytes (PDCA⁺IgM⁺), pDCs (PDCA⁺IgM⁻), and conventional B lymphocytes (PDCA⁻IgM⁺) are functionally different and that PDCA⁺ B lymphocytes contribute significantly to IFN-α and IDO expressions.

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**FIGURE 2.** PDCA⁺ B lymphocytes share phenotypes with pDCs. A and B. Total PDCA⁺ cells were purified from naïve B6 mice, stained with indicated markers, and flow cytometry performed. One of three independent experiments is shown. n = 5. Gates (G1–G4) were set around indicated cell populations (A), and PDCA⁺CD43⁺ and B lymphocytes (B) were enumerated. C. PDCA⁺ and CD43⁺ B lymphocytes were purified from naïve mice and used for flow cytometry of the indicated markers. One of three independent experiments is shown. n = 5. D. PDCA⁺ cells were isolated from spleens of naïve mice (leftmost panel), and gates were set around PDCA⁺CD11c⁺ (second panel from left). IgD⁺IgM⁺ cells were selected from gated PDCA⁺CD11c⁺ cells (third panel from left), and gata PDCA⁺CD11c⁺IgM⁺IgD⁺ cells coexpressing CD79b, AA4.1, and Igk markers were identified by multicolor flow cytometry (fourth, fifth, and sixth panels from left). One of three independent experiments is shown. n = 3.
FIGURE 3. PDCA⁺ B lymphocytes undergo BCR-mediated cell division. A–D, Purified PDCA⁺/CD43⁺ (A and B) and PDCA⁺/CD43⁻ (C and D) lymphocytes were labeled with CFSE, stimulated with anti-CD40 IgM, and flow cytometry performed after 96 h. One of three independent experiments is shown. n = 6. E, BrdU-treated B6 mice were administered with indicated agonists, spleen cells were collected after 3 d, and flow cytometry was conducted. One of three independent experiments is presented. n = 3. BrdU incorporating PDCA⁺ and PDCA⁻ cells are indicated by dashed and intact boxes, respectively. Control mice received PBS and BrdU in the same manner.

PDCA⁺ B lymphocytes secrete Igs and produce Ag-specific Abs

We next tested if PDCA⁺ B lymphocytes can secrete Igs and produce Abs. Purified PDCA⁺/CD43⁻ (PDCA⁺ B lymphocytes) and PDCA⁻/CD43⁻ (conventional B lymphocytes) B lymphocytes, when cultured with LPS/IL-4 to induce in vitro Ig class-switching, revealed increased IgG1, AID, and IgG1 circular transscripts expression predominantly in PDCA⁺/CD43⁻ B lymphocytes (Fig. 5A, 5B). Expansion of PDCA⁺ B in PDCA⁻ B lymphocyte cultures and an 8.5% IgG1 expression in these cells (Fig. 5A, upper right panel) points to a possibility that PDCA⁺ B may have differentiated into PDCA⁺ B lymphocytes upon activation. The above finding was verified by experiments utilizing CFSE-labeled PDCA⁺ B lymphocytes where stimulation with anti-μ/anti-CD40 Abs showed significant upregulation of surface PDCA on dividing cells (data not shown). To further understand the role of PDCA⁺ B lymphocytes in humoral responses, we injected wt mice with NP³⁰-CGG/albumin. Seven days after immunization, gated PDCA⁺ but not PDCA⁻ spleen cells displayed increased IgG1 (Fig. 5C, compare upper and lower panels) and syndecan-1 and NP-binding (data not shown). Although PDCA⁺ B lymphocytes showed Ig class switching in the above experiment, it was not clear if these cells participated directly in these responses. To answer this question, we pulsed wt PDCA⁺ B lymphocytes overnight with NP³⁰-CGG and adoptively transferred them into syngeneic wt mice. Sera collected 7 d later from the recipient mice contained increased anti-NP Abs relative to nonimmunized controls (Fig. 5D, 5E). Although in the above wt → wt experiment where transfer of NP³⁰-CGG–pulsed PDCA⁺ B lymphocytes resulted in Ab production, it could be argued that the transferred cells might have cross-presented the Ag to the conventional B lymphocytes of the host mice, and the latter, in turn, might have initiated the anti-NP Ab production. However, the result that transfer of NP³⁰-CGG–pulsed wt PDCA⁺ B lymphocytes into the µMT mice that lack both conventional B and PDCA⁺ B lymphocytes showed anti-NP Ab production (Fig. 5F) indicates that there was no cross-presentation of Ag between the donor PDCA⁺ B and the B cells of the host mice has occurred and that the PDCA⁺ B lymphocytes directly were responsible for the observed Ab production. Although anti-NP Ab production was observed in the µMT mice receiving NP³⁰-CGG–pulsed PDCA⁺ B lymphocytes, it was still not clear if this was mediated by the transferred PDCA⁺ B lymphocytes themselves or was mediated by any newly differentiated PDCA⁺ B lymphocytes that may have been induced. Because we detected two distinct donor-derived cell populations (i.e., PDCA⁺IgM⁺ [60%; Fig. 5G; upper middle panel, upper right quadrant] and PDCA⁺IgM⁻ [39.9%; Fig. 5G; upper middle panel, lower right quadrant]), it was important to investigate which of these two cell populations was responsible for the anti-NP Ab production seen in Fig. 5F. To address this, we have enumerated...
FIGURE 5. PDCA⁺ B lymphocytes produce Ag-specific Abs. A, PDCA⁺ B and PDCA⁺ B lymphocytes were purified, stimulated with LPS or LPS/IL-4 as indicated in Materials and Methods, and in vitro Ig class switching was performed by flow cytometry. One of seven independent experiments is shown. n = 6. B, Purified PDCA⁺CD43⁻ and PDCA⁺CD43⁺ B lymphocytes from spleens of naive mice were stimulated for 40 h with LPS and IL-4. Cells were washed from cultures and stored at −80°C until analysis. Samples pooled from seven independent experiments were subjected to RT-PCR analysis of indicated transcripts. n = 6. C, B6 mice were immunized with NP30-CGG/ alum, and 7 d later spleen cells were collected and labeled as indicated and analyzed by flow cytometry. Shown is IgG1 expression in gated PDCA⁺ and PDCA⁻ cell populations. The experiment was repeated three times. n = 3. D and E, Wt(B6) → wt(B6) mice were adoptively transferred with NP30-CGG–pulsed PDCA⁺ B lymphocytes (●) or were left untreated (○) as mentioned in Materials and Methods. Sera, collected on day 7, were analyzed for presence of anti-NP Abs by ELISA. The values are pooled from three independent experiments (n = 6) and the extent of anti-NP Ab production (absorbance) is shown by the bar graphs (mean ± SD). F, Wt(B6) → μMT (B6) mice were adoptively transferred with NP30-CGG–pulsed PDCA⁺ B (△) or PDCA⁻ (○) B lymphocytes or were left untreated (●). Serum anti-NP Abs were determined by ELISA as in D and E. The values are pooled from three independent experiments (n = 3), and the extent of anti-NP Ab production (absorbance) is shown by the bar graphs (mean ± SD). G, Spleen cells from F were subjected to multiparameter flow cytometry of indicated markers. Gates were set around IgM⁺ cells, and individual PDCA⁺IgM⁺ (upper panels) and PDCA⁻NP⁺ (lower panels) are depicted. One of three independent experiments (n = 3) is shown.

The proportions of NP-binding B cells. Flow cytometry data revealed that NP⁺ cells were found predominantly among the gated IgM⁺PDCA⁺ than in the IgM⁺PDCA⁻ subpopulation (Fig. 5G, lower panels), indicating that the source of anti-NP Abs seen in the recipient μMT mice stemmed from the newly differentiated PDCA⁺ B lymphocyte subpopulation. Taken together, our data that the naturally occurring naive PDCA⁺ B lymphocytes (∼20 × 10⁶/young adult spleen; Fig. 1B), although they produce Abs in vitro when activated (Figure 5A, 5B) or upon adoptive transfer (Fig. 5D, 5E), and the result that activated conventional PDCA⁻ B lymphocytes also expressed surface PDCA (Fig. 5A, upper panels; Fig. 5F, 5G) suggest that PDCA⁺ B lymphocytes are an activated phenotype of conventional B (PDCA⁻) lymphocytes.

PDCA⁺ B lymphocytes are the principal Ab producers in lupus-prone lpr mice

We next assessed the importance of PDCA⁺ B lymphocytes in lupus-prone lpr mice, in which overactive B lymphocyte responses lead to increased autoimmunity production culminating in severe disease. We found increased PDCA⁺CD79b⁺ B lymphocytes in the spleens of 3-, 4-, and 5-mo-old lpr over wt mice (Fig. 6A), which may have expanded from the conventional B (PDCA⁻IgM⁻) B lymphocytes due to the chronic inflammatory vivo milieu known exist in aged lpr mice. In a reciprocal manner, the conventional B lymphocytes (PDCA⁻CD79b⁻) in lpr mice began to constrict in numbers. Thus, by 4 mo of age, a 1.7-fold decrease in the numbers of PDCA⁺CD79b⁺ B lymphocytes was observed (Fig. 6B), which peaked at 5 mo of age (2.78-fold decrease; Fig. 6B) when the experiments were terminated due to deterioration of the general health of lpr mice. Inclusion of CD79b (present study) or Igx (data not shown) over CD19 or B220 as markers to identify PDCA⁺ B lymphocytes in Fig. 6A, 6B was based on the reports that pDCs express CD19 and B220 molecules (7, 9, 16). Inclusion of CD19 or B220 markers to distinguish PDCA⁺ B lymphocytes would therefore lead to confusion whether we are dealing with pDCs or PDCA⁺ B lymphocytes. However, our multiparameter flow cytometric studies confirmed that all of the CD19⁺ cells are positive for CD79b and that the CD19⁺CD79b⁺ cells coexpress PDCA marker (data not shown). In addition, evaluation of freshly isolated spleen cells from groups of mice of various ages revealed increased Ig isotype production (peaking at 4 mo of age) exclusively from gated PDCA⁺ but not PDCA⁻ cell fractions (Fig. 6C–E). Thus, we have identified PDCA⁺ B lymphocytes to be chief producers of autoantibodies in lpr mice.

Discussion

We have shown above that not all PDCA⁺ cells are DC-derived pDCs and that a significant majority is the naturally occurring functional PDCA⁺ B lymphocyte population, which differentiates further from the activated conventional B (PDCA⁻IgM⁻) lymphocytes. In secondary lymphoid organs, the PDCA coexpressing B lymphocytes exist as PDCA⁺ B and PDCA⁻ B lymphocytes where the former outnumbers the latter. Although the PDCA⁺ B lymphocyte shows distinct functional abilities, the PDCA⁻ B lymphocytes have weak proliferative responses, although we attempted activation with anti-μ/anti-CD40 and LPS. Currently, it is not clear why there are functional differences between these two subsets. Due to the limited availability of PDCA⁻ B cells in naive mice, extensive analysis of this subpopulation was not feasible. Also, the PDCA⁻ B cells do not appear to increase significantly in

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number in vivo during inflammation (i.e., in lupus-prone lpr mice). Therefore, in this study, we investigated the role of PDCA\(^{–}\) B lymphocytes (hereafter referred to as PDCA\(^{+}\) B lymphocytes) in health and disease.

Although the PDCA\(^{+}\) B lymphocytes have phenotypes with pDCs including expression of Siglec H, they represent a separate cell population in that they differentiate from C-kit\(^{+}\)B220\(^{+}\) pro-B precursors as conventional B lymphocytes do. This is in contrast to pDC, a subset of DCs (8), which differentiate from a distinct set of precursors (16, 17). Moreover, the PDCA\(^{+}\) B lymphocytes described in this study undergo BCR-mediated cell division, secrete Igs, and show Ig class switch, properties that the pDCs lack. Moreover, others have shown that transcripts of factors, plays important role in a number of cellular processes, including apoptosis in B lymphocytes (29). Also, a functional EGR1 binding site is also shared by the ID4 gene (30). In addition, we have observed a marginally increased expression of PU.1, an Ets family transcription factor, in PDCA\(^{+}\) B lymphocytes (PDCA\(^{+}\) CD43\(^{–}\) ) over conventional B lymphocytes (PDCA\(^{–}\) CD43\(^{–}\) ). There are reports suggesting that PU.1 is required for the differentiation of DCs, B lymphocytes, and pDCs (31). The importance of EGR1, ID4, and PU.1 in the growth and differentiation of PDCA\(^{+}\) B lymphocytes requires additional investigation.

An important finding of the current study is the identification of PDCA\(^{+}\) B lymphocytes as direct contributors to humoral immunity. We found that PDCA\(^{+}\) B lymphocytes possess the capacity to produce Ig isotypes. This claim is based on the result that gated PDCA\(^{+}\) cells expressed anti-NP IgG1 in NP\(^{16\text{O}}\)-CGG–treated mice. Moreover, NP\(^{16\text{O}}\)-CGG–pulsed wt PDCA\(^{+}\) B lymphocytes, when transferred into syngeneic wt mice or into B and PDCA\(^{–}\) B lymphocyte-deficient μMT mice, anti-NP Ig responses were noted in recipient mice, indicating clearly that these cells induce Ig class switch. However, our in vitro data, as well as transfer experiments that showed differentiation of PDCA\(^{+}\) B from PDCA\(^{–}\) B lymphocytes, coupled with the result that the newly differentiated PDCA\(^{+}\) B lymphocytes produced Abs, led us to the conclusion that PDCA is a marker of activated B lymphocytes, although naturally existing PDCA\(^{+}\) B lymphocytes can be found in the naive mice. That the PDCA\(^{+}\) B differentiate from activated PDCA\(^{–}\) B lymphocytes draws support indirectly from our observation that a common C-kit\(^{B220}\) precursor gives rise to both the conventional B and the PDCA\(^{+}\) B lymphocytes. The differentiation of PDCA\(^{+}\) B from PDCA\(^{–}\) B lymphocytes is possible, as PDCA Ag has also been shown to be present in Golgi complexes (9). Thus, cytosolic PDCA in PDCA\(^{\text{surface}}\) CD43\(^{+}\) lymphocytes may have migrated to the cell surface upon activation, resulting in

FIGURE 6. PDCA\(^{+}\) B lymphocytes are the principal Ig producers in lupus-prone lpr mice. A and B, Spleen cells from various age groups of lpr and syngeneic wt mice were subjected to multicolor flow cytometry. Absolute numbers of PDCA\(^{+}\)CD79b\(^{+}\) (A) and PDCA\(^{+}\)CD79b\(^{+}\) (B) cells were calculated from precounted total viable cells. Data from three independent experiments were pooled (\(n = 4\)) and shown as bar graphs (mean ± SD). C–E, Spleen cells collected from various age groups of lpr and syngeneic wt mice were subjected to two-color flow cytometry. Changes in Ig isotype expression among gated PDCA\(^{+}\) and PDCA\(^{–}\) cells are shown as bar graphs (mean ± SD) based on values pooled from three independent experiments. \(n = 4\).
the generation of PDCA$^+$CD43$^-$ lymphocytes. Although depletion studies have demonstrated that elimination of PDCA$^+$ cells eliminates CD138$^+$ plasma B cells (9), studies utilizing PDCA$^{-}$ mice will be required to fully understand the individual roles of the naturally occurring PDCA$^+$ B versus those that differentiate from the activated B lymphocytes in humoral immunity. Our laboratory is currently generating PDCA$^{-}$ mice, and future studies will address the importance of PDCA$^+$ B lymphocytes in the humoral immune responses.

Expression of IDO in PDCA$^+$ B lymphocytes is an important finding implying that PDCA$^+$ B lymphocytes not only evoke positive immune responses (i.e., augmented humoral responses) but also are capable of expressing immunosuppressive IDO when stimulated by various agents. Although the origin of the B lymphocytes was not explored in their studies, Baban et al. (32) showed that CD11c$^+$CD19$^+$ cells express high levels of IDO when stimulated with CTLA-4 Ig. Of note, both CD11c and CD19 are coexpressed on PDCA$^+$CD79b$^+$ B lymphocytes (data not shown).

The significance of IDO$^+$ and IDO$^+$ PDCA$^+$ B lymphocytes in immune regulation and the question of whether they suppress T cell responses by depleting tryptophan are currently under investigation in our laboratories. IFN-α expression by PDCA$^+$IgM$^+$ cells is yet another important finding of the study. Besides naturally occurring IFN-producing pDCs (33), type I IFNs are produced by virtually all cells when activated by bacteria and viruses (34), including B cells (35). The significance of IFN-α production by PDCA$^+$IgM$^+$ lymphocytes needs further investigation, especially in the light of the fact that IFN-α plays important roles in B cell priming and humoral responses (36). Our results with B cell-deficient μMT mice, which also lack PDCA$^+$ B lymphocytes, showed reduced expressions of inducible IFN-α and IDO, authenticating the critical roles the PDCA$^+$ B cells play in immune responses.

The disappearance of conventional B (PDCA$^+$CD79b$^+$) and accumulation of PDCA$^+$ B lymphocytes (PDCA$^+$CD79b$^+$) in lpr mice, particularly as the mice age, indicate that the latter cells orchestrate autoantibody production and account for the disease severity in these mice. In agreement with this, a recent report showed increased blood pDCs in humans suffering from lupus (37). The change from conventional B to PDCA$^+$ B lymphocytes is activation- and age- but not sex-dependent, as both male and female lupus-prone lpr mice shared this feature (data not shown for male lpr mice). We do not understand the basis for this curious finding nor have we checked if PDCA$^+$ B and conventional B lymphocytes numbers increase later, because we terminated the experiments at 5 mo of age due to a deterioration of the general health of lpr mice. However, we noted a correlation between the abundance of PDCA$^+$CD79b$^+$ cells and the extent of Ig isotype production in lpr mice, both of which peaked at 4 mo, substantiating the important role of PDCA$^+$ B lymphocytes in humoral immunity in the lpr mice.

In summary, we have identified that PDCA expression, besides differentiating from the activated conventional (PDCA$^+$IgM$^+$) B lymphocytes, secretes Igs and Abs, shows Ig class switch, secretes type I IFNs, and expresses IDO. Therefore, targeting the PDCA$^+$ B lymphocytes provides a therapeutic opportunity, particularly in those where the production of autoantibodies is primarily responsible for disease severity.

Disclosures

The authors have no financial conflicts of interest.


