The skewed heavy-chain repertoire in peritoneal B-1 cells is predetermined by the selection via pre-B cell receptor during B cell ontogeny in the fetal liver

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Keywords: B cell development, JH1, surrogate light chain, V\text{H}11, V\text{k}9

Abstract
As many as 5–15% of B-1 cells in the peritoneal cavity of adult mice produce antibodies reactive to phosphatidylcholine (PtC) and the vast majority of them express B cell receptors (BCRs) composed of V\text{H}11-\mu\text{H} chains utilizing the J\text{H}1 segment and V\text{k}9-L chains. This extremely skewed repertoire of PtC-reactive B-1 cells is traditionally attributed to the expansion of particular clones in response to self or exogenous antigens. Here, we show that the strong bias toward the J\text{H}1 usage among V\text{H}11-\mu\text{H} chains is already established prior to the BCR assembly, namely at the transition from the large to the small pre-B cell stage during B cell ontogeny in the fetal liver. Among V\text{H}11-\mu\text{H} clones isolated from large pre-B cells where the J\text{H}1 skewing was not established yet, the J\text{H}1 users showed the highest ability to form pre-B cell receptor (pre-BCR) and to induce cellular proliferation and differentiation when expressed in fetal liver pro-B cells. Thus, the J\text{H}1 users were positively selected and amplified at the pre-BCR checkpoint. When co-expressed with V\text{k}9-L chains to form BCR, the J\text{H}1 users almost exclusively conferred the PtC reactivity on BCR even though other J\text{H} users could also form BCR on the cell surface. Therefore, the pre-BCR-mediated positive selection of the J\text{H}1 users among V\text{H}11-\mu\text{H} chains appears to be beneficial to the efficient generation of ‘innate-type’ PtC-reactive B cells during the fetal B cell development, even before the self-renewal or the antigen-driven clonal expansion of B-1 cells takes place in the peritoneal cavity.

Introduction
A distinct B cell subset, termed B-1, shows enriched expression of self-reactive B cell receptor (BCR) and is responsible for a significant portion of IgM natural antibodies in serum (1, 2). The majority of B cells in the spleen and lymph nodes are referred to as B-2 and express high levels of IgD and lack detectable expression of the pan-T cell surface glycoprotein CD5. In contrast, B-1 cells comprise ~5% of B cells in the spleen and display lower levels of IgD. Many of B-1 cells are CD5\textsuperscript{+} and designated as B-1a cells while their CD5\textsuperscript{−} counterparts are called B-1b cells (3–5). In the adult animal, B-1 cells are enriched in certain anatomical locations, such as the peritoneal and pleural cavities (6). In neonatal spleen, B-1 cells are relatively more abundant. The majority of B-1 cells, particularly B-1a cells, are normally generated during fetal/neonatal development and thereafter maintained by self-renewal throughout the life (3, 4).

B-1a cells are the major producers of natural antibodies to phosphatidylcholine (PtC), a ubiquitous membrane phospholipid found in both mammalian cells and bacteria (7, 8). These natural antibodies have recently been shown to protect against acute bacterial peritonitis induced by cecal ligation and puncture in mice, supporting the idea that natural antibodies produced by B-1 cells play the major role in the innate defense against invading pathogens (9). Cells producing PtC-reactive antibodies comprise 5–15% of B-1 cells in the peritoneal cavity of most mouse strains and can be detected by binding to fluorescent liposomes carrying PtC or bromelain-treated mouse red blood cells (Br-MRBCs) (7, 8, 10). No other antigen specificity has been reported to be shared by such a large number of B cells in unimmunized, normal, nude as well as germ-free mice.

Anti-PtC antibodies are largely encoded by IgH chains utilizing the V\text{H}11 and V\text{H}12 segments in combination with IgL chains utilizing the V\text{k}9 and V\text{k}4 segments, respectively (11–13). Particularly in C57BL/6 mice, >80% of PtC-reactive B-1 cells express V\text{H}11-\mu\text{H} chains, almost exclusively in...
association with V\textsubscript{\kappa}9-L chains (14). The V\textsubscript{\gamma}11 segment is predominantly used for V\textsubscript{\gamma}\textsubscript{1}-D-J\textsubscript{\gamma}1 rearrangements during B cell development in the fetal liver but not in the adult bone marrow (13, 15, 16). N-nucleotide addition is rarely found in the V\textsubscript{\gamma}1-D and D-J\textsubscript{\gamma}1 junctions of V\textsubscript{\gamma}11-\muH chains expressed by PtC-reactive B-1 cells (17, 18). These characteristics of the H chains are consistent with the fetal origin of B-1 cells. On top of these, the J\textsubscript{\gamma}1 among the four J\textsubscript{\gamma} segments are predominantly utilized by those V\textsubscript{\gamma}11-\muH chains (14, 17, 19). It is generally thought that the skewed repertoire of BCR on PtC-reactive B-1 cells, including the overrepresented J\textsubscript{\gamma}1 segment, is the consequence of the antigen-driven clonal selection and/or expansion. The positive selection of B-1 cells with auto-antigens has been clearly demonstrated for Thy-1 glycoprotein-reactive B-1 cells (20).

We previously reported that the repertoire selection of H chains carrying the V\textsubscript{\delta}81X occurs during early B cell development in the fetal liver. The V\textsubscript{\delta}81X is another example of the V\textsubscript{\delta} segments that are predominantly used for V\textsubscript{\delta}1-D-J\textsubscript{\delta} rearrangements in fetal B cell ontology (21). Two types of V\textsubscript{\delta}81X-\muH chains are produced following productive H chain gene rearrangements in the fetal liver; one is capable of pairing with invariant \lambda5/V\textsubscript{pre-B} surrogate light (SL) chains and the other not. SL chains form pre-B cell receptor (pre-BCR) on pre-B cells, in association with \muH chains and signal-transducing module Ig\textsubscript{\kappa}/Ig\textsubscript{\lambda} heterodimers (22). The pre-BCR plays a critical role in early B cell development, and a deficiency in pre-BCR formation or signaling results in severe impairment of B cell development at the transition from pro-B to pre-B cell stages (23). Comparison of SL-pairing and SL-non-pairing V\textsubscript{\delta}81X-\muH chains in the fetal liver revealed that the pre-BCR plays an important role in the positive selection of V\textsubscript{\delta}81X-\muH chains carrying the characteristic sequences of the complementarity-determining region 3 (CDR3) encoded by the V\textsubscript{\delta}1-D-J\textsubscript{\delta} junctions. V\textsubscript{\delta}81X-\muH chains utilizing the J\textsubscript{\delta}2 segment were excluded at the pre-BCR checkpoint while those carrying a histidine residue at the first position of the CDR3 and little or no N-nucleotide addition were preferentially selected. Interestingly, these characteristic, fetal-type V\textsubscript{\delta}81X-\muH chains were also detected in adult spleen, but almost exclusively in marginal zone B cells. This strongly suggested that neonatally generated and selected B cells expressing the stereotyped V\textsubscript{\delta}81X-\muH chains are maintained in the adult marginal zone (21).

These findings prompted us to examine the possibility that the skewed repertoire observed in peritoneal B-1 cells, particularly in V\textsubscript{\kappa}11-\muH chains of PtC-reactive cells, might also be established during fetal ontogeny even before the antigen-driven selection takes place, in contrast to the previous thought. In the present study, we first examined where the bias toward the J\textsubscript{\gamma}1 usage among V\textsubscript{\gamma}11-\muH chains is established during B cell development and then how such a bias can be produced. We further explored the functional significance of the positive selection of the J\textsubscript{\gamma}1 users among others. Our results clearly demonstrate that the skewed V\textsubscript{\gamma}11-\muH repertoire observed in peritoneal B-1 cells is predetermined by the pre-BCR-mediated selection during fetal B cell development and suggest that such a pre-selection in the fetal liver is beneficial to the efficient generation of ‘innate-type’ PtC-reactive B cells.

Methods

Mice

C57BL/6 mice were purchased from CLEA Japan. \muM mice (24) and \lambda5-deficient mice (25) on the C57BL/6 background were bred and maintained under specific pathogen-free conditions in our animal facility. All the experiments in this study were performed according to the Guidelines for Animal Use and Experimentation as set forth by Tokyo Medical and Dental University.

Cell lines and culture

The pro-B cell line 38B9 (26) was cultured in iscove’s modified dulbecco’s medium (IMDM) supplemented with 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 100 U ml\textsuperscript{−1} penicillin-streptomycin and 5 \times 10\textsuperscript{−5} M 2-mercaptoethanol (complete IMDM) at 37°C in 5% CO\textsubscript{2}. The retroviral packaging cell line Plat-E (27) was culture in dulbecco’s modified eagle’s medium supplemented with 10% FCS, 100 U ml\textsuperscript{−1} penicillin-streptomycin, 1 µg ml\textsuperscript{−1} puromycin (Sigma-Aldrich, St Louis, MO, USA) and 10 µg ml\textsuperscript{−1} blasticidin (Invitrogen, Carlsbad, CA, USA).

Antibodies

Anti-\lambda5 mAb (LM34) was conjugated with PE (PROzyme, San Leandro, CA, USA) according to the manufacturer’s instructions. Biotin-labeled mAbs to \kappaL chain (187.1), \lambdaL chain (R26-46), c-kit (2B8), CD2 (RM2-5), IgD (11–26) and human CD8 staining were purchased from BD Pharmingen (San Diego, CA, USA).

Cell sorting

For separating large pre-B and small pre-B populations, B220\textsuperscript{+} cells were enriched (＞95% purity) with anti-B220-conjugated magnetic beads (Miltenyi Biotech, Bergisch Gladbach, Germany) from day 16 fetal livers of C57BL/6 mice and intracellularly stained with labeled mAbs to \kappaL chain, \lambdaL chain, \lambda5 and \muH chain after fixation and permeabilization with Cytofix/Cytoperm (BD Pharmingen). Stained cells were subjected to fractionation and sorting with FACS Vantage (BD Bioscience). Purifications of immature B cell populations are as follows: B220\textsuperscript{+} cells were enriched with anti-B220-conjugated magnetic beads from liver or spleen of neonatal C57BL/6 mice and surface stained with labeled mAbs to \kappaH chain (II/41) and CD19 (1D3) were purchased from BD Pharmingen (San Diego, CA, USA).

Genomic DNA isolation, amplification and sequencing

The sorted cells were treated with PCR lysis buffer (10 mM Tris, 50 mM KCl, 1.8 mM MgCl\textsubscript{2}, 0.5% Tween 20) containing 100 µg ml\textsuperscript{−1} proteinase K (Roche, Basel, Switzerland) at 56°C for 1 h and then heated to 95°C for 10 min. The lysates were used directly for nested PCR to amplify V\textsubscript{\gamma}11-DJ rearranged fragments by using following primers: for the first
V_{H}11-target sequence sense 5'GGAATTCGCGCATGGAGTTGGAACTGAC-3' (or V_{H}11 sense 5'GGAATTCGCGCATGGAGTTGGAACTGAC-3') and J_{H}4 3'-intron antisense 5'CAATCTAGTGCCGAACTTCTTCAC-3'. PCR was performed at 95°C for 120 s, followed by 30 cycles of 95°C for 30 s, 55°C for 30 s and 68°C for 120 s. In the second PCR, 1 μl of the first PCR products was re-amplified with V_{H}11 framework region 1-specific sense primer carrying an Xba I restriction site (5'GTTCACTTGAGGCTACCCTGAGAG-3') and each J_{H}4-specific or a universal J_{H}4-specific anti-sense primers carrying Hind III restriction sites as follows: J_{H}1, 5'TTGGGAAAGCCTTTGACCTCTTCTGAGAGAAGCCGAGGTGAAGGCTCAGGG-3'; J_{H}2, 5'TTGGGAAAGCCTTTGACCTCTTCTGAGAGAAGCCGAGGTGAAGGCTCAGGG-3'; J_{H}3, 5'TTGGGAAAGCCTTTGACCTCTTCTGAGAGAAGCCGAGGTGAAGGCTCAGGG-3'; J_{H}4, 5'TTGGGAAAGCCTTTGACCTCTTCTGAGAGAAGCCGAGGTGAAGGCTCAGGG-3' and universal-JHs, 5'TTGGGAAAGCCTTTGACCTCTTCTGAGAGAAGCCGAGGTGAAGGCTCAGGG-3' (underline shows restriction enzyme site). Amplifications shown in were done separately using each J_{H}4-specific primers as the anti-sense primer, while those shown in Fig. 1 and 2(D) were performed using the universal J_{H}4 primers as the anti-sense primer. PCR was performed at 95°C for 120 s, followed by 30 cycles of 95°C for 20 s, 58°C for 30 s and 68°C for 60 s. The second PCR products were digested with Xba I and Hind III and sub-cloned into the Xba I/Hind III site of the pBS-V_{H}11-μH, a modified pBS-μH vector (21), in which the V_{H}11 sequence (underline shows restriction enzyme site) was created in 17th and 18th codons, and the CDR3 region of each clone was sequenced. In the experiment shown in Fig. 2(B), 1 μl of each sample after the second PCR specific for each J_{H}4 gene was subjected to the PCR amplification of the J_{H}4 3'-intron region with a pair of primers (sense 5'GGAATTCGCGCATGGAGTTGGAACTGAC-3' and anti-sense 5'CAATCTAGTGCCGAACTTCTTCAC-3') for standardization of input DNA templates. The PCR products were subjected to electrophoresis on 1% of agarose gel followed by staining with ethidium bromide. Fluorescence intensity of each J_{H}4 user group among independent V_{H}11 clones judged from the CDR3 sequence (n = 23 from neonatal B cells, n = 18 from adult peritoneal B-1a cells) is shown as a pie chart.

Fig. 1. Strong bias toward J_{H}1 usage in V_{H}11-μH chains is established at transition from the large to small pre-B cell stages in fetal liver B cell development. (A) B220+ cells were enriched from fetal livers at day 16 of gestation and intracellularly stained with combination of biotin-anti-κ and κL chains plus FITC-streptavidin, PE-anti-λ5 and APC-anti-μH chain. L-μH+J_{H}5+ (large pre-B) and L-μH+J_{H}5- (small pre-B) fractions were separately sorted. Post-sort profiles of J_{H}5 and μH staining as wells as forward scatter of cells in each fraction are shown (right panels). (B) Genomic DNAs were isolated from each fraction and subjected to PCR analysis for the J_{H}5 usage in VDJ-rearranged H genes as described in Methods. Relative incidence of individual J_{H}5 segments in V_{H}11- and V_{H}81X-DHJ_{H}-rearrangements isolated from large pre-B cells (gray bars) and small pre-B cells (black bars) is shown. (C) VDJ-rearranged V_{H}11 clones with different J_{H}5 segments were randomly isolated from the PCR products and subjected to sequence analysis. Frequency (%) of productively VDJ-rearranged clones among the J_{H}1, J_{H}2, J_{H}3 or J_{H}4 users isolated from large pre-B cells (white bars) and small pre-B cells (black bars) is shown. The numbers of J_{H}1, J_{H}2, J_{H}3 or J_{H}4 users analyzed were 40, 49, 41 and 51 in large pre-B cells and 31, 32, 34 and 28 in small pre-B cells, respectively. (D) IgM+ B cells were sorted from neonatal spleen of wild-type or IκBα-deficient mice and genomic DNA isolated from the B cells were subjected to PCR and DNA sequencing for analysis of the relative J_{H}5 usage among productively VDJ-rearranged V_{H}11 clones. The frequency (%) of each J_{H}5 user group among independent V_{H}11 clones judged from the CDR3 sequence (n = 16 from wild-type mice, n = 22 from IκBα-deficient mice) is shown as a pie chart.
region. The intensity of the J4μ4 3’-intron PCR products was comparable in the four different Jk users (data not shown). KOD-Plus-polymerase (TOYOBO, Osaka, Japan) was used for all the PCR analyses.

Construction of retroviral vectors and the infection

The BamHIXho fragments of pBS-VH11-μH clones were inserted into the retroviral vector pMX-ires-GFP (28) to express a membrane form of μH chains, as previously described (21). Plat-E cells were cultured in Plat-E medium for 24 h and then transfected with 1 μg of the vector plasmid DNA using Fu-GENE (Roche). The culture supernatants were collected 48 h later, and 38B9 pro-B cell line or pro-B cells from day 16 fetal livers of μMT cells were infected with retroviral vectors as previously described (29).

Flow cytometric analysis

Cells were pre-incubated with anti-CD16/32 mAb (2.4G2, BD PharMingen) on ice for 15 min to prevent the non-specific binding of irrelevant antibodies and surface stained with the indicated combination of antibodies followed by streptavidin-conjugated PE, FITC or APC (BD PharMingen). For intra-cellular staining, cells were fixed and permeabilized with Cytofix/Cytoperm (BD PharMingen) before staining. Stained cells were analyzed with FACSCalibur (BD Bioscience).

Cell survival and proliferation assay

Fetal liver pro-B cells were cultured at 2 × 10^5 cells per ml in the presence of 10 μg ml^{-1} recombinant mouse IL-7 after the infection. The viable cells were identified using trypan blue staining and counted at the indicated time points.

κL chain cloning

Total RNA was isolated from peritoneal or spleen cells using RNAiso (TaKaRa, Shiga, Japan) according to the manufacturer’s protocol, and the cDNA was synthesized with ReverTra Ace (TOYOBO). Reverse transcription–PCR for cloning of Vκ9 (AJ231238), Vκ4 (AJ231225) and Vκ8 (AJ235948) κL chain clones were carried out with 30 cycles of denaturation at 94°C for 10 s, annealing at 58°C for 20 s and extension at 68°C for 2 min, using KOD-Plus (TOYOBO). Primers used for amplification of these cloning were as follows (each Vκ9-, Vκ4- and Vκ8-L chain-specific sense primers contained an EcoRI restriction site while Ck-specific anti-sense primer contained BamH restriction site for sub-cloning): Vκ9, 5’-CCGAATTCGGTCAATTGAGCACTGACGACCACC-3’; Vκ4, 5’-CCGAATTCGCTGATTTTCAAGTGCGATTTATCGTC-3’; Vκ8, 5’-CCGAATTCGGTACAGCCCGAGGCT-3’; and Ck, 5’-CCGATCCGCCTACTATCCGTCCTGATTAT-3’. The restriction enzyme sites are indicated by underline. The PCR products were digested with EcoRI and BamH and sub-cloned into the EcoRI/BamH site of the pMX-ires-hCD8 (30). hCD8+ transfectants were enriched by the i-Mag cell separation system (BD PharMingen, San Diego, CA, USA). The purity of the hCD8+-enriched cells was >90%.

Rosette-forming assay

Br-MRBCs were prepared according to the previous report (31). Briefly, MRBC from C57BL/6 mice were purified by retro-orbital puncture on heparin and centrifugation on Lymphoprep layer for 20 min at 1700 r.p.m. RBCs were incubated as a 50% suspension with bromelain (Sigma–Aldrich) at a final concentration of 20 mg ml^{-1} in PBS for 45 min at 37°C. Peritoneal mac-1^+CD45^+CD43^- B-1a cells, B220^+ spleen cells or transfectants were adjusted to 10^7 cells ml^{-1} with cold Hanks’ medium supplemented with 3% FCS and 0.01 M azide and 1/10 volume of 10% Br-MRBC suspension was then added. Cells were centrifuged for 5 min (500 × g) and re-suspended on a roller at 4°C for 15 min. Images were acquired using an Axioplan 2 imaging (Carl Zeiss Microlmaging, Jena, Germany).

Results

Strong bias toward J4μ1 usage in VH11-μH chains is established at transition from the large to small pre-B cell stages during B cell development in fetal liver

We first compared the usage of the Jk segments among VH11-μH chains expressed in peritoneal B-1a cells from adult mice and immature B cells from neonatal livers, since peritoneal B-1a cells have been demonstrated to originate from fetal/neonatal B cells. Sequence analysis of productively-rearranged H chain genes revealed that neonatal liver B cells displayed the predominant J4μ1 usage (57%) among their VH11-μH chains, just as observed in adult peritoneal B-1a cells in which 72% of VH11-μH chains utilized the J4μ1 segment (Fig. 1). None of the VH11-μH chains examined utilized the J4μ3 segment in both B cell populations although we cannot formally exclude the possibility of underrepresentation of the J4μ3 usage owing to the less compatibility of the primer used to the J4μ3 segment. These observations might be explained by the preferential clonal expansion of the VH11-J4μ1-μH-expressing B cells in neonatal livers, as suggested in adult peritoneal B-1a cells. Alternatively, the strong bias toward the J4μ1 usage might be established during earlier B cell ontogeny, either at the stage of VDJ rearrangements or at the pre-B cell stage where μH chains associate with SL chains to form pre-BCR.

In order to address this issue, we next examined the Jk usage of VH11-μH chains at the pre-B cell stage of the B cell development in fetal livers. Approximately 10% of B220^+ fetal liver B cells in fetuses at 16 days of gestation produce μH chains but little or no L chains yet (Fig. 2A), and therefore they are at the pre-B cell stage in B cell development. Approximately 85% of them are at the early stage of pre-B cells (large pre-B cells), as judged by their expression of Ck5 and higher forward scatter (Fig. 2A), where the pre-BCR-mediated selection is in progress (20). The rest of pre-B cells are at the late stage of pre-B cells (small pre-B cells). Large pre-B cells are c-kit^+CD2^- on their surface while small pre-B cells are c-kit^-CD2^+ (data not shown). Both pre-B cell subsets were separately sorted, and their genomic DNAs were subjected to PCR analyses to compare the Jk usage in VDJ-rearranged VH11 genes in each subset (Fig. 2B, left). The incidence of Jk1 usage was comparable in large pre-B cells and small pre-B cells. In contrast, the incidence of other Jk users, particularly Jk2 and Jk3, was extremely low in small pre-B cells compared with that in large pre-B cells. This bias
toward JH1 usage did not seem to be the common feature of VDJ-rearranged H chain genes in small pre-B cells in the fetal liver or an experimental artifact, because VDJ-rearranged VH81X genes amplified from the same DNA sources under comparable PCR conditions displayed distinct preference in the JH usage (Fig. 2B, right). Drastic reduction in the incidence of JH2 but not other JHs was observed in the VH81X genes isolated from small pre-B cells compared with those from large pre-B cells, in accord with our previous report using a different method (21).

The PCR analysis shown in Fig. 2(B) did not distinguish productively and non-productively VDJ-rearranged VH11 genes. Therefore, we next sequenced an array of VDJ-rearranged VH11 clones utilizing different JHs isolated from large and small pre-B cells. In large pre-B cells, the frequency of productively rearranged VH11 genes was comparable (~80%) between the JH1, JH2, JH3 and JH4 users (Fig. 2C). In small pre-B cells, the frequency was as high as 90% (28/31) in the JH1 users while it was 28% (9/32), 21% (7/34) and 46% (13/28) in the JH2, JH3 and JH4 users, respectively. These results together with the data shown in Fig. 2(B) indicated that the strong bias toward JH1 usage is established in VH11-μH chains at transition from the large to small pre-B cell stages in fetal liver B cell development.

The skewing at this stage strongly suggested the involvement of pre-BCR in the selection of the JH1 users among VH11-μH chains during fetal B cell development. The majority of B cells in the neonatal spleen originate in the fetal liver. Therefore, we compared the JH usage of VH11-μH chains in B cells isolated from neonatal spleens of wild-type and JH3-deficient mice. The frequency of the JH1 users was 32% in λ5-deficient mice, in sharp contrast to 56% in wild-type mice. Moreover, the JH3 users accounted for 14% of the VH11-μH clones in λ5-deficient mice while they were hardly detected in wild-type mice. These results clearly indicated that pre-BCR plays a critical role in the establishment of the biased repertoire of VH11-μH chains prior to the BCR assembly during the fetal/neonatal B cell development.

JH1 users among VH11-μH chains show the highest ability to form pre-BCR

In order to understand the mechanism underlying the pre-BCR-mediated repertoire skewing of VH11-μH chains, an array of VH11-μH clones isolated from the large pre-B cells was individually expressed in the μH SL+ pro-B cell line 38B9 (Fig. 3A and B). Although the amount of total μH chains per cell was comparable among the clones examined as assessed by intracellular staining (Fig. 3A, right panels), the levels of surface μH expression varied greatly among the clones (Fig. 3A, left panel and summarized in Fig. 3B). The average of the mean fluorescence intensity of surface μH expression was 3.3, 12.4, 6.7, 4.1 and 8.1 in the mock, JH1, JH2, JH3 and JH4 transfectants, respectively.

We wondered if the JH1-dependent variation in levels of μH expression on the 38B9 transfectants might not reflect what happens in the pre-BCR expression on fetal liver pre-B cells, since 38B9 is an Abelson murine leukemia virus-transformed cell line although it was established from fetal liver cells. Therefore, we next expressed individual μH clones in pro-B cells freshly isolated from day 16 fetal livers of mice deficient in the membrane exon of the μH chain gene (μMT mice) (Fig. 3C and D). Three representative clones displaying the surface pre-BCR expression nearly at the average level in each JH1 user group (indicated by black dots in Fig. 3B) were analyzed. The average of the mean fluorescence intensity of surface pre-BCR expression was, 2.9, 62.6, 17.2, 3.3 and 26.1 in the mock, JH1, JH2, JH3 and JH4 transfectants of fetal liver pro-B cells, respectively, in good correlation with that observed in the 38B9 transfectants. Thus, the JH1 users on
average showed the highest ability to form pre-BCR. In contrast, the J\(_{\text{H}1}\)3 users displayed little or no surface expression.

**J\(_{\text{H}1}\)1 users among V\(_{\text{H}1}\)11-mH chains show the highest ability to induce proliferation and differentiation of fetal liver pro-B cells**

We next examined the functional consequences of the difference in the pre-BCR-forming ability observed among the V\(_{\text{H}1}\)11-mH clones isolated from the large pre-B cells. Mock and J\(_{\text{H}1}\)3 transfectants of mMT fetal liver pro-B cells showed no significant proliferation during the culture period from day 1 to day 4 post-infection (Fig. 4A and B). In contrast, J\(_{\text{H}1}\)1 transfectants showed prominent proliferation, and the cell number increased ~8 times. J\(_{\text{H}2}\)2 and J\(_{\text{H}4}\)4 transfectants also showed significant proliferation albeit to lesser extent, and the cell number increased only ~2 and ~3 times, respectively. Thus, the extent of proliferation correlated well with the level of pre-BCR expression, following the rule that we previously reported (29).

We further examined the differentiation potential of the individual transfectants (Fig. 4C and D). On day 2 post-infection, the down-regulation of surface c-kit expression was prominent in the J\(_{\text{H}1}\)1 transfectants while it was much less evident in the J\(_{\text{H}2}\)2, J\(_{\text{H}3}\)3 and J\(_{\text{H}4}\)4 transfectants. On day 4 post-infection, as many as 21% of the J\(_{\text{H}1}\)1 transfectants expressed IgM on the cell surface whereas the frequency of surface IgM+ B cells was ~8%, ~2% and ~12% on average in the J\(_{\text{H}2}\)2, J\(_{\text{H}3}\)3 and J\(_{\text{H}4}\)4 transfectants, in good correlation with the level of their pre-BCR expression.

As a consequence of the difference in extents of proliferation and differentiation among the V\(_{\text{H}1}\)11-mH transfectants (Fig. 4A–D), the number of surface IgM+ B cells that differentiated from the pro-B cells differed greatly among them (Fig. 4E). During 3-day culture period, 143, 19, <1 and 42 IgM+ B cells were produced from 100 pro-B cells infected with V\(_{\text{H}1}\)11-mH chains utilizing J\(_{\text{H}1}\)1, J\(_{\text{H}2}\)2, J\(_{\text{H}3}\)3 and J\(_{\text{H}4}\)4, respectively. Thus, cells expressing the J\(_{\text{H}1}\)1 users among V\(_{\text{H}1}\)11-mH chains showed the highest level of pre-BCR expression and hence have great advantage over the other J\(_{\text{H}}\) users in differentiation to B cells.

**J\(_{\text{H}1}\)1 users among V\(_{\text{H}1}\)11-mH chains almost exclusively display the PIC reactivity when assembled with Vk9-L chains**

The vast majority of PtC-reactive B cells in the peritoneal cavity of C57BL/6 mice display BCR composed of V\(_{\text{H}1}\)11-J\(_{\text{H}1}\)1-mH chains and Vk9-L chains (9, 11). Therefore, we reasoned that the positive selection of the J\(_{\text{H}1}\)1 users through pre-BCR could help to exclude V\(_{\text{H}1}\)11-mH chains incapable of pairing with Vk9-L chains in advance, leading to the efficient assembly of the H chains and L chains at the next developmental stage (the small pre-B cell stage) to ensure the reactivity to PtC. To address this issue, 38B9 pro-B cells were co-infected with a Vk9-L clone and a panel of V\(_{\text{H}1}\)11-mH clones utilizing different J\(_{\text{H}1}\)1 segments (Fig. 5). The surface expression of Vk9-L chains was detected on most of transfectants irrespective of their J\(_{\text{H}1}\)1 usage of V\(_{\text{H}1}\)11-mH chains, and the levels of L chain expression were not well correlated with those of pre-BCR expression. This was also the case when other two representative kL chain clones utilizing the Vk4 or Vk8 segment in place of Vk9 were co-expressed with V\(_{\text{H}1}\)11-mH chains. Of note, the J\(_{\text{H}1}\)3 users conferred on 38B9 cells high levels of BCR expression even though they failed to express pre-BCR on the same cells. This was unexpected, since the previous studies demonstrated that mH chains (utilizing V\(_{\text{H}8}\)1X and V\(_{\text{H}12}\)12) incapable of forming pre-BCR fail to assemble with any of L chains examined (32, 33). Indeed, we also found that among mH chains utilizing V\(_{\text{H}7}\)183 or V\(_{\text{H}5}\)558, most of pre-BCR-competent ones were able to assemble with Vk4-, Vk8- and Vk9-L chains to form...
 whereas none of pre-BCR-impotent ones formed BCR with any of the L chains (Fig. 5A and B). Thus, VH11-L chains produced during the fetal B cell development do not follow the rule of pre-BCR–BCR assembly observed in those utilizing other VH segments, and the pre-BCR-mediated JH1 selection does not seem to function as a quality control of VH11-L chains for their ability to assemble with their future partners, L chains.

The surface expression of BCR on all the Vx9-L chain/ Vπ11-μH chain transfectants of 38B9 irrespective of their Jπ1 selection does not seem to function as a quality control of Vπ11-μH chains for their ability to assemble with their future partners, L chains.

The surface expression of BCR on all the Vx9-L chain/ Vπ11-μH chain transfectants of 38B9 irrespective of their Jπ1 usage enabled us to examine the reactivity of each type of BCR to PtC. The PtC reactivity of each transfectant was assayed by its ability to form rosettes with Br-MRBCs. Under our experimental conditions, ~15% of peritoneal B-1a cells (mac-1+ B220lowCD5+) bound Br-MRBCs to form rosettes whereas none of splenic B cells examined showed such rosette formation (Fig. 6A). Two-thirds (6/9) of the Jπ1 clones analyzed showed the rosette formation when co-expressed with Vx9-L chains while none of the Jπ2 and Jπ3 clones did (Fig. 6B and summarized in Table 1). Only one of nine Jπ4 clones formed the rosette. Thus, the Jπ1 users among the Vπ11-μH chains almost exclusively showed the PtC reactivity when assembled with Vx9-L chains. As far as we analyzed, none of Vπ11-μH chains regardless of their JH usage showed the rosette formation when co-expressed with either Vx4- or Vx8-L chains in 38B9 cells (data not shown). Taken together, the positive selection of the Jπ1 users through pre-BCR appears to be beneficial to the efficient generation of PtC-reactive B cells during the fetal B cell development.

Discussion

The innate-type B cells including peritoneal B-1 cells and marginal zone B cells display the restricted repertoire of BCR in general. Natural antibodies produced by such B cells contribute to the early phase of the protective responses to

**Fig. 5.** Most of Vπ11-μH chains can associate with conventional κL chains regardless of the difference in their Jπ1 usage. Three different clones encoding representative κL chains (Vx4, Vx8 and Vx9) were inserted into a retroviral vector pMX-ires-hCD8 to infect 38B9 cells. hCD8+ infected cells were sorted and superinfected with another retroviral vector pMX-ires-GFP encoding different Jπ1 users of Vπ11-μH chains as in Fig. 3 or μH chains utilizing the VJ segments other than Vπ11 (Vπ7183 and J558 family members) including pre-BCR-competent (pre-BCR+) and pre-BCR-impotent (pre-BCR−) clones. Two days after the superinfection, the cells were stained for the surface expression of pre-BCR and κL chains. (A) The data show staining profiles of clones representative of each group. The staining profiles of the GFP+ cells infected with μH vectors (open histograms) and those infected with empty vectors (shaded histograms) are overlaid. The mean fluorescence intensity (MFI) of pre-BCR and κL chain expression is indicated in each panel. (B) All the data of surface κL chain expression in Vπ11-μH transfectants examined (n = 6–9 in each Jπ1 group) are summarized. Each μH clone is indicated as a dot, and the mean value of κL chain expression in each Jπ1 group of Vπ11-μH transfectants is indicated as a horizontal bar.
common pathogens (34). PtC-reactive B-1 cells are one of the best-studied innate-type B cells, and the vast majority of them express BCR composed of VH11-JH1-μH chains and Vx9-L chains (1, 8, 10). This skewed repertoire has long been believed to be the consequence of the antigen-driven clonal selection. The present study clearly demonstrated that the strong bias toward the JH1 usage among VH11-μH clones expressed by peritoneal B-1 cells has already been established in the fetal liver through the pre-BCR-mediated selection during early B cell ontogeny, in an antigen-independent manner, to ensure the efficient generation of PtC-reactive B cells.

Fig. 6. JH1 users among VH11-μH chains almost exclusively display the ability of binding to Br-MRBCs when assembled with Vx9-L chains. (A) Peritoneal B-1a cells (mac-1+ B220+CD5+) and splenic B220+ B cells were incubated with Br-MRBCs as described in Methods. Differential interference contrast photographs were taken to show the binding of Br-MRBCs to B cells (rosette formation). Arrows indicate B cells. A small inset in the left panel shows an enlarged picture of rosette-forming cells. (B) 38B9 cells infected with the Vx9-L chain clone or mock were superinfected with a panel of VH11-μH clones with different JH usage as in Fig. 4 (n = 5–9 in each JH group as shown in Table 1). These cells were incubated with Br-MRBCs or intact MRBCs to see their ability of rosette formation. The cells expressing μH chains were identified as GFP+ cells. The data show photographs of 38B9 transfecteds expressing a μH clone representative of each group together with Vx9-L chain. All the data are summarized in Table 1 that shows the frequency of Br-MRBC-binding clones in each JH group of VH11-μH chains.

Table 1. The frequency of Br-MRBC-binding clones in each JH group of VH11-μH chains

<table>
<thead>
<tr>
<th>L chain</th>
<th>VH11-μH chain</th>
<th>JH1 clones (%)</th>
<th>JH2 clones (%)</th>
<th>JH3 clones (%)</th>
<th>JH4 clones (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock</td>
<td>0/9 (0)</td>
<td>0/7 (0)</td>
<td>0/5 (0)</td>
<td>0/9 (0)</td>
<td></td>
</tr>
<tr>
<td>Vx9</td>
<td>6/9 (67%)</td>
<td>0/7 (0)</td>
<td>0/5 (0)</td>
<td>1/9 (11)</td>
<td></td>
</tr>
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</table>

The critical role for the pre-BCR in the JH1-skewing was highlighted by the observation that 5-deficient mice incapable of forming pre-BCR did not show such a strong bias toward the JH1 usage in their neonatal spleens. Most of VH11-μH chains utilizing JH1 segments other than the JH1 are excluded at the pre-BCR checkpoint during B cell development in the fetal liver of normal mice. The in vitro experiment demonstrated that the JH1 users showed the highest levels of pre-BCR expression and the highest ability to induce cellular proliferation and differentiation when expressed in fetal liver pro-B cells. Thus, the JH1 users are positively selected and amplified at the pre-BCR checkpoint, prior to the assembly into BCR. Besides the JH1-skewing, we often found the identical sequences of the CDR3 in VH11-JH1-μH chains, shared by adult peritoneal B-1 cells and small pre-B cells in the fetal liver (data not shown), supporting the scheme that VH11-μH chains selected at the pre-BCR checkpoint are maintained in peritoneal B-1 cells.

The molecular basis of the JH1 selection at the pre-BCR checkpoint remains to be determined. We previously demonstrated that the histidine residue at the first position of the CDR3 in VH11-μH chains determines the ability of the μH chains to pair with SL chains and hence the fate of pre-B cells via the pre-BCR-mediated selection (21). Another group reported that a glycine in the fourth position of the 10 amino acid-long CDR3 in VH12-μH chains is critically involved in the pre-BCR formation (33, 35, 36). We found that most of pre-BCR-competent VH11-μH chains possess a tyrosine residue at the first position of the CDR3. However, the mutational replacement of the tyrosine to alanine did not show any significant effect on the levels of pre-BCR expression (data not shown), even though the functional consequence of this replacement has not been analyzed.

We previously demonstrated that stereotyped VH81X-μH chains were positively selected via pre-BCR formation during the fetal B cell ontogeny and thereafter maintained in marginal zone B cells in the adult. In the present study, we further extended these findings and explored the functional relevance of the pre-selection of the H chain repertoire at the pre-BCR checkpoint to the generation of innate-type B cells with a particular specificity. Two-thirds of the VH11-μH clones utilizing the JH1 segment showed the reactivity to Br-MRBC when assembled with Vx9-L chains. In contrast, none of the JH2 and JH3 clones analyzed showed the reactivity and only one of the nine JH4 clones did. Thus, the pre-BCR-mediated selection of the JH1 users prior to the BCR assembly gives great advantage in terms of the efficient generation of innate-type B-1 cells with the specificity to PtC, even before the encounter of real antigens. We cannot formally exclude the possibility that endogenous antigens such as PtC are involved in the pre-BCR-mediated selection. However, we believe that this possibility is unlikely because 38B9 cells expressing BCR composed of VH11-JH1-μH chains and Vx9-L chains, but not those expressing pre-BCR composed of VH11-JH1-μH chains and SL chains, displayed the ability to form rosettes with Br-MRBC (data not shown).

An intriguing model has been proposed for the selection of VH11-μH chains at the pre-BCR checkpoint, based on the analysis of two different types of VH11 transgenic mice (37). This model challenged the traditional view of the
pre-BCR-mediated selection and contrasts the possible difference in the selection during the B-2 development in the adult bone marrow versus the B-1 development in the fetal liver. According to this model, in the fetal B cell development unlike in the adult B cell development, μH chains that only weakly associate with SL chains show better proliferation than those capable of efficiently forming pre-BCR (4, 37, 38). We found that V<sub>H</sub>11-μH chains on average, even the J<sub>H</sub>1 users, displayed lesser capacity of pre-BCR formation as compared with μH chains found in adult bone marrow, such as those utilizing the V<sub>H</sub>J<sub>558</sub> segment. However, among the V<sub>H</sub>11-μH chains analyzed, the J<sub>H</sub>1 users showed the highest levels of pre-BCR expression and the highest ability to induce cellular proliferation and differentiation when expressed in fetal liver pro-B cells. This was also the case when they were expressed in pro-B cells derived from the adult bone marrow (data not shown). Therefore, the selection of V<sub>H</sub>11-μH chains in the fetal liver is not exceptional and does follow the traditional rule of the pre-BCR-mediated selection, as observed in that of V<sub>H</sub>81X- and V<sub>H</sub>12-μH chains in the fetal liver as well as other chains in the adult bone marrow (21, 29, 39).

V<sub>k</sub>9-L chains are almost exclusively utilized by PtC-reactive B-1 cells (11–13). We found that the J<sub>H</sub>1 users of V<sub>H</sub>11-μH chains can form BCR in association with not only V<sub>k</sub>9-L chains but also those utilizing other V<sub>k</sub> segments such as V<sub>k</sub>4 and V<sub>k</sub>8, even though the PtC reactivity was detected only in the combination with V<sub>k</sub>9-L chains. A previous study also reported that V<sub>H</sub>11-μH could assemble with V<sub>k</sub>8-, V<sub>k</sub>9-, V<sub>k</sub>19- and V<sub>k</sub>21c-L chains (40). Moreover, we did not detect any significant bias toward the V<sub>k</sub>9-L usage in the surface μL chain-positive B cells that were differentiated in vitro from fetal liver pro-B cells infected with V<sub>H</sub>11-J<sub>H</sub>1-μH clones (data not shown). Therefore, the predominant combination of V<sub>H</sub>11-μH and V<sub>k</sub>9-L chains observed in PtC-reactive B-1 cells does not seem to be the direct consequence of the pre-BCR-mediated selection of the J<sub>H</sub>1 users among V<sub>H</sub>11-μH chains and is most likely due to the anti-gen-driven selection of clones expressing this particular combination of the H and L chains. This is a sharp contrast to the observation in V<sub>H</sub>12-μH chains that are frequently utilized in PtC-reactive B-1 cells of C.B17 mice, where V<sub>H</sub>12-μH chains are incapable of associating with most κ and λ chains except V<sub>k</sub>4/5H-L chains that confer PtC reactivity on BCR in association with V<sub>H</sub>12-μH chains (33, 39, 41). It is intriguing to assume that V<sub>H</sub>11-J<sub>H</sub>1-μH chains display other innate-type specificities to unidentified antigens when assembled with L chains other than V<sub>k</sub>9.

In conclusion, we demonstrated that the skewed repertoire of the μH chains observed in peritoneal B-1 cells of adult mice was predetermined prior to their assembly into BCR through the pre-BCR-mediated selection during fetal B cell development. This antigen-independent pre-selection of the H chain repertoire early in B cell ontogeny appears to give great advantage to the generation of the innate-type specificity in B-1 cells.

Funding

Acknowledgements
We thank T. Kitamura for providing pMX-IRES-GFP and Plat-E and A. Yoshida for their excellent assistance with cell sorting.

Abbreviations
APC allophtocyanin
BCR B cell receptor
Br-MRBC bromelain-treated mouse red blood cell
CDR3 complementarity-determining region 3
IMDM iscover’s modified dulbecco’s medium
pre-BCR pre-B cell receptor
PtC phosphatidylcholine
SL surrogate light

References
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