Differences in Their Proliferation and Differentiation between B-1 and B-2 Cell

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ABSTRACT

Background: B cell subset has been divided into B-1 cells and B-2 cells. B-1 cells are found most prominently in the peritoneal cavity, as well as constituting a small proportion of splenic B cells and they are larger and less dense than B-2 cells in morphology. This study was designed to compare the differences in their proliferation and differentiation between B-1 and B-2 cell. **Methods:** We obtained sorted B-1 cells from peritoneal fluid and B-2 cells from spleens of mice. Secreted IgM was measured by enzyme-linked immunosorbent assay. Entering of S phase in response to LPS-stimuli was measured by proliferative assay. Cell cycle analysis by propidium iodide was performed. *p21* expression was assessed by real time PCR. **Results:** Cell proliferation and cell cycle progression in B-1 and B-2 cells, which did not occur in the absence of LPS, required LPS stimulation. After LPS stimulation, B-1 and B-2 cells were shifted to S and G2/M phases. *p21* expression by resting B-1 cells was higher than that of resting B-2 cells. **Conclusion:** B-1 cells differ from conventional B-2 cells in proliferation, differentiation and cell cycle. (Immune Network 2006;6(1):1-5)

Key Words: B-1 cell, B-2 cell, immunology, differentiation, cell cycle, p21

Introduction

B-1 cells constitute a unique set of B cells, with numerous distinguishing phenotypic and functional features. B-1 cells can be distinguished from the more abundant, conventional B (B-2) cells by their expression of the pan-T cell surface glycoprotein, CD5. B-1 cells are responsible for the production of non-immune "natural" immunoglobulin, which provides protection against overwhelming infections prior to adaptive responses, and has been linked to the pathological processes of malignancy and autoimmunity (1). Beyond phenotype, functional differences between B-1 and B-2 cells are apparent in the signals required for cell cycle progression. In response to anti-Ig, B-1 cells fail to enter the cell cycle, whereas B-2 cells are driven into S phase (2). The hyporesponsiveness of B-1 cells to sIg may be due to insufficient activation of PLC and/or modulation of signal transduction by CD5-associated SHP-1 phosphatase (3-5). Conversely, B-1 cells enter S phase in response to treatment with phorbol ester, whereas B-2 cells, which are stimulated by a combination of a phorbol ester and a calcium ionophore, are not stimulated by phorbol ester alone (6). The hyperresponsiveness of B-1 cells to phorbol ester stimulation is reflected in the early induction of cyclin D2 expression and the assembly of active cyclin D2 and cyclin D3 complexes with cdk4/6, which do not occur in PMA-treated B-2 cells (7).

p21 is involved in the regulation of cell growth and in cellular response to DNA damage. This protein has been shown to inhibit cell cycle progression in G1 by binding to G1 cyclin-cyclin dependent kinase complexes and to proliferating cell nuclear antigen (PCNA). In addition, p21 is involved in protecting cells against apoptosis, and, in several cell lineages, p21 expression is essential for maintaining cell survival during differentiation. Expression of p21 is induced by both p53-dependent and -independent mechanisms following stress, and induction of p21 may cause cell cycle arrest and may play a role in maintaining G-2 arrest after DNA damage (8-10).

We therefore evaluated the differences in their immunoglobulin secretion, S phase entering, cell cycle and p21 expression between B-1 and B-2 cells.

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Materials and Methods

Animals. Male BALB/cByJ mice aged 8~14 weeks were housed in our facility for at least 1 week prior to experimentation. Mice were cared for and handled at all times in accordance with institutional guidelines for animal care.

Cell sorting and counting. Unseparated cells were obtained by peritoneal washout and splenic disruption and were maintained at 4°C. Lymphocytes were stained with immunofluorescent antibodies directed against B220 and CD5 and were subjected to fluorescence activated cell sorting (FACS) to obtain highly pure and viable populations of peritoneal B-1 and splenic B-2 cells. Each of these populations was ≥95% pure on re-analysis. B cells were cultured in RPMI 1640 medium (BioWhittaker, Walkersville, MD) supplemented with 5% heat-inactivated fetal bovine serum (Sigma-Aldrich, St. Louis, MO), 10 mM Hepes, pH 7.2 (Calbiochem-Novabiochem, San Diego, CA), 50 μM 2-mercaptoethanol (ME) (Sigma-Aldrich), 2 mM L-glutamine (Invitrogen Co., Japan), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen).

Enzyme-linked immunosorbent assay (ELISA). Total secreted IgM was measured by ELISA. B cells were cultured for 2 days in the presence or absence of 25 μg/ml Salmonella typhimurium lipopolysaccharide (LPS) (Sigma). Supernatants were transferred to 96-well flat-bottom trays coated with goat anti-mouse Ig (H+L) (Southern Biotechnology Associates, Inc., Birmingham, AL) in coating buffer (Na_2CO_3/$NaHCO_3$ plus NaN_3) and incubated overnight at 4°C. Following six washes, horseradish peroxidase-labeled affinity-purified goat anti-mouse IgM in PBS/Tween/BSA was added, and the plates were incubated for 3 hours at room temperature. After further washing, substrate solution (2,2-azio-Bis; Sigma-Aldrich) was added, and, after 5~10 min, the plates were read on a microplate reader (Dynex Technologies, Chantilly, VA) at 414 nm.

Proliferative assay. B-1 and B-2 cells (2.4×10^5/well in 0.2 ml medium) were cultured in flat bottomed microtiter plates (Costar, Cambridge, MA) for 48 hours at 37°C in a CO_2 incubator. During the last 6 hours of culture, the cells were exposed to 0.5 μCi of [^{3}H] thymidine (Dupont Co, NEN Research Products, Boston, MA), and tritium incorporation was measured using a MicroBeta Windows Workstation (1,450 Microbeta Liquid scintillation & Luminescence counter). All experimental and control conditions were carried out in quadruplicate. Results are reported as mean cpm values of quadruplicate samples; SEM values were generally less than 10% of mean values.

Cell cycle Analysis by Propidium Iodide. Cells were trypsinized, collected by centrifugation for 5 min at 1,500 rpm in 15 ml conical tubes, and resuspended in 1 ml PBS. A 30 μl aliquot of each cell suspension was placed in a microfuge tube, along with 30 μl of 0.4% trypan blue, and the number of viable cells was counted using a hemacytometer. An additional 150 μl aliquot of cell suspension was centrifuged for 5 min at 1,500 rpm, and, following removal of the supernatant, the cell pellet was resuspended in 150 μl of PBS, pH 8. To each was added 375 μl of FACS buffer, 250 μl of RNAse, and 100 μl of 0.2% Triton-X 100. Following incubation for 30 min at 37C in 5% CO_2 in air, 100 μl of propidium iodide solution was added to each tube. The tubes were covered with aluminum foil and incubated at room temperature for 30 min, and the cell suspensions were transferred to Falcon 2,063 tubes for FACS analysis. The numbers of cells in the G1, S, and G2/M phases were measured by FACS.

p21 expression. RNA and cDNA were prepared, and p21 message abundance was assessed by PCR amplification using the intron-spanning primers 5'-GTACT TCCTCTGCCCTGCTG-3' (forward) and 5'-CACAG AGTGAGGGCTAAGGC-3' (reverse), with annealing at 58.3°C, for an experimentally optimized number of cycles. p21 expression was normalized relative to β2-microglobulin expression, using the intron-spanning primers 5'- CGGTCGCTTCAGTCGTCAGC-3' (forward) and 5'-CCCAGTAACGGTCTCGGG-3' (reverse), with annealing at 60°C, for an experimentally optimized number of cycles.

Results

B-1, but not B-2, cells secrete IgM spontaneously. B-1 cells cultured in the absence of LPS secreted IgM at day
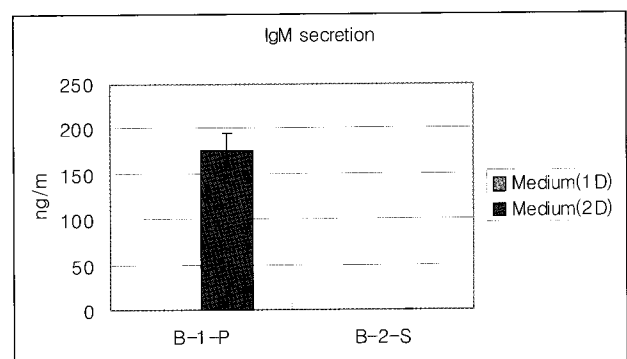

Figure 1. Immunoglobulin production by B cells. 1.7×10^5 cells per each group were cultured in fetal bovine serum-containing culture medium for 2 days. After the culture period, supernatants were collected and tested for secretion of immunglobulin M by using an enzyme-linked immunosorbent assay (ELISA) technique. Data are expressed in nanograms per milliliter of Ig produced and represent the mean±SEM of experiment. B-1 cells secreted IgM without stimulation. B-1-P: peritoneal B-1 cells, B-2-S: splenic B-2 cells.

2, whereas B-2 cells did not secrete IgM without LPS stimulation (Fig. 1).

LPS stimulates entry into S-phase by B-1 and B-2 cells. When we measured [^{3}H]-thymidine incorporation by peritoneal B-1 and splenic B-2 cells exposed to LPS, we found that LPS stimulated entry into S phase in both cell types (Fig. 2).

Cell cycle control in B-1 and B-2 cells. In the absence of LPA stimulation, B-1 and B-2 cells were arrested in G1 phase, whereas, when these cells were stimulated with LPS, they were shifted to S and G2/M phases (Fig. 3).

Peritoneal B-1 cells and splenic B-2 cells differ in expression of p21. Using 3 independently isolated sets of RNA/cDNA, and specific intron-spanning primers, we found that unstimulated peritoneal B-1 cells expressed much more p21 than unstimulated splenic B-2 cells. In contrast, LPS-stimulated peritoneal B-1 cells

expressed less p21 than LPS-stimulated splenic B-2 cells (Fig. 4).

Discussion

B-1 cells spontaneously secrete Ig, which plays a critical role in host immune defenses against infection (1). Although the importance of effector molecules on B-1 cells is understood, the mechanisms regulating natural antibody production are not. Studies of LPS-stimulated B-2 cell plasma cell differentiation have suggested that Ig secretion is regulated by a central cascade controlled by four transcription factors: B cell leukemia/lymphoma-6 (BCL-6), B lymphocyte inducer of maturation program 1 (BLIMP-1), paired box gene 5 (PAX-5), and X-box binding protein 1 (XBP-1) (11-14). Differentiated Ig-secreting B cells express low levels of BCL-6 and PAX-5a, but high levels of BLIMP-1 and XBP-1. In secreting B-1 cells, however, BLIMP-1 and XBP-1 are not up-regulated, suggesting that B-1 cells differentiate according to a
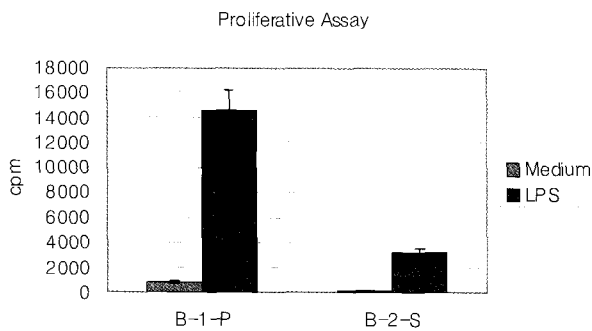

Proliferative Assay

Figure 2. Stimulation of B cell S phase entry. 3×10^4 cells of B-1 and B-2 cells were cultured for 48hours with either medium alone or LPS at 25 μg/ml. Incorporation of label was assessed after exposure to [^{3}H]thymidine during the last 6 hours of culture. Mean values of quadriplicate cultures are shown along with lines indicating the standard errors of the means. LPS: lipopolysaccharide, B-1-P: peritoneal B-1 cells, B-2-S: splenic B-2 cells.
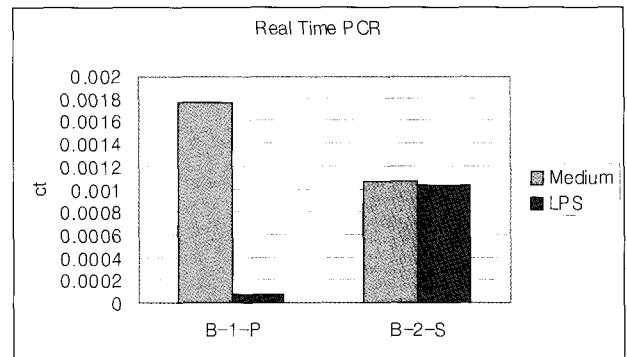

Real Time PCR

Figure 4. *p21* gene of peritoneal B-1 cells and splenic B-2 cells differ. P21 of splenic B-2 cells is not affected by LPS stimulation. LPS: lipopolysaccharide, B-1-P: peritoneal B-1 cells, B-2-S: splenic B-2 cells, ct: relative expression.
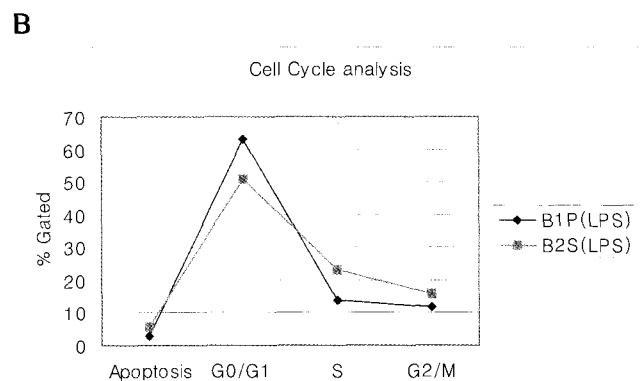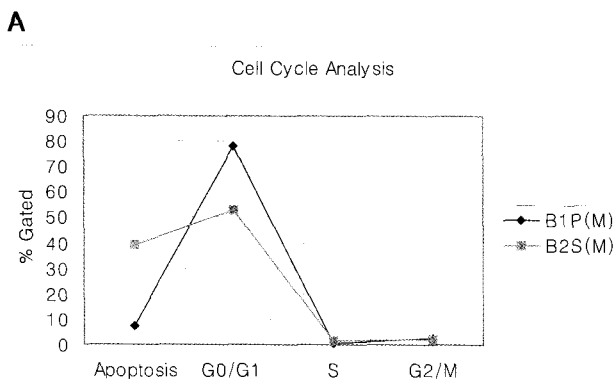
A

Cell Cycle Analysis

B

Cell Cycle analysis

Figure 3. Cell cycle analysis by Propidium Iodide. 1×10^4 cells of B-1 and B-2 cells were cultured in medium (A) and LPS (B) for 2 days. M: medium, LPS: lipopolysaccharide.

mechanism differing from that of Ig-secreting B-2 cells (15). In addition to these 4 transcription factors, it is likely that p21 is associated with natural Ig secretion (16).

When B cells are stimulated by either T cell-dependent or-independent antigens, most undergo apoptosis, with the remainder differentiating into plasma cells or memory cells (17-19). In normal individuals, B-1 cells secrete a preponderance of nonimmune serum IgM, as well as substantial amounts of resting IgA. In adult mice, B-1 cells are the principal lymphocyte population in the peritoneal cavity, as well as constituting a small proportion of splenic B cells. Peritoneal B cells that secrete nonimmune IgM spontaneously do not remain in the peritoneal cavity but migrate into the spleen after activation in vivo, lose Mac-1 expression, and secrete IgM antibodies (20). Differentiation of most cell types, including B cells, requires both the establishment of G1 arrest and the induction of a program related to achieving quiescence (21,22). We found that p21 expression by resting B-1 cells was higher than that of resting B-2 cells, suggesting that spontaneous IgM secretion by B-1 cells may be an effect of p21 expression.

p21 acts as an inhibitor of apoptosis in a number of systems, which may counteract its tumor-suppressive functions as a growth inhibitor (23-25). We found that the survival rate of B-1 cells was about 30% higher than that of B-2 cells, and *p21* is increased significantly. These results have considered the association anti-apoptosis effect of *p21* in B-1 cells and that will be providing furrther evidence that p21 has an anti-apoptotic effect in B-1 cells.

Functional differences between B-1 and B-2 cells are apparent in the signals required for cell cycle progression (26). B-1 cells fail to enter the cell cycle in response to anti-Ig stimulation, whereas B-2 cells are driven into S phase (2). The hyporesponsiveness of B-1 cells to sIg may be due to insufficient activation of phospholipase C and/or modulation of signal transduction by CD5-associated SHP-1 phosphatase (3-5). Conversely, B-1 cells enter S phase after phorbol ester treatment for 24~30 hours, whereas B-2 cells, which are not stimulated by phorbol ester alone, require 54~60 hours of stimulation by both a phorbol ester and a calcium ionophore to enter the cell cycle (6). The hyperresponsiveness of B-1 cells to phorbol ester stimulation is reflected in the early induction of cyclin D2 expression and the assembly of active cyclin D2 and cyclin D3 complexes with cdk4/6, neither of which occurs in phorbol ester-treated B-2 cells (7,27). These differences are associated with the different responses of B-1 and B-2 cells to antigen stimulation. We found that cell proliferation and cell cycle progression by B-1 and B-2

cells, which did not occur in the absence of LPS, required LPS stimulation, and that this was especially evident in B-1 cells. B-1 cell progression into S or G2/M phase was associated with decreased expression of p21, whereas, in B-2 cells, p21 was not altered by cell cycle progression. These findings suggest that p21 is not related to B-2 cell proliferation.

In conclusion, we have shown here that B-1 cells are different with B-2 cells in differentiation, proliferation, cell cycle and p21 expression.

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