

## Disruption of the *Bcl6* Gene Results in an Impaired Germinal Center Formation

By Tetsuya Fukuda,<sup>\*‡</sup> Takehiko Yoshida,<sup>\*</sup> Seiji Okada,<sup>\*</sup> Masahiko Hatano,<sup>\*</sup> Tohru Miki,<sup>‡</sup> Kazuki Ishibashi,<sup>\*</sup> Shinichiro Okabe,<sup>\*</sup> Haruhiko Koseki,<sup>§</sup> Shinsaku Hirose,<sup>‡</sup> Masaru Taniguchi,<sup>§</sup> Nobuyuki Miyasaka,<sup>‡</sup> and Takeshi Tokuhisa<sup>\*</sup>

From the <sup>\*</sup>Division of Developmental Genetics, Center for Biomedical Science, Chiba University School of Medicine, Chiba 260, Japan; the <sup>‡</sup>First Department of Internal Medicine, Tokyo Medical and Dental University, Tokyo 113, Japan; and the <sup>§</sup>Core Research for Evolutional Science and Technology, Division of Molecular Immunology, Center for Biomedical Science, Chiba University School of Medicine, Chiba 260, Japan

### Summary

The *Bcl6* gene has been identified from the chromosomal translocation breakpoint in B cell lymphomas, and its products are expressed highly in germinal center (GC) B cells. To investigate the function of Bcl6 in lymphocytes, we have generated RAG1-deficient mice reconstituted with bone marrow cells from Bcl6-deficient mice (Bcl6<sup>-/-</sup>RM). Lymphogenesis in primary lymphoid tissues of Bcl6<sup>-/-</sup>RM is normal, and Bcl6<sup>-/-</sup>RM produced control levels of primary IgG1 antibodies specific to T cell-dependent antigens. However, GCs were not found in these mice. This defect was mainly due to the abnormalities of B cells. Therefore, Bcl6 is essential for the differentiation of GC B cells.

Chromosomal translocations involving band 3q27 are often present in B cell lymphomas. The *BCL6* gene was identified from the breakpoint of these translocations (1–3). Rearrangements of the *BCL6* gene were detected in up to 40% of diffuse large cell lymphomas and 10% of follicular lymphomas (4, 5). These rearrangements cause deregulation of the *BCL6* gene as a result of the juxtaposition of the *BCL6* coding region to heterologous promoters (6), and its deregulation may be responsible for lymphomagenesis. BCL6 is a 92- to 98-kD nuclear phosphoprotein (7, 8) that contains six Krüppel-type zinc finger motifs in COOH-terminal region (1–3, 9), which have been shown to bind to specific DNA sequences in vitro (10, 11), and the POZ/BTB domain (12, 13) in NH<sub>2</sub>-terminal region. Because a NH<sub>2</sub>-terminal half of the protein has been demonstrated to contain transrepressor domain(s) in vitro (14–16), BCL6 can function as a sequence-specific transcriptional repressor.

The expression of BCL6 is ubiquitously detected in human tissues (17). As this gene is well conserved between human and mouse (9), the expression pattern has been extensively studied in mouse tissues. Bcl6 is continuously upregulated in keratinocytes and sperm at their terminal stages (18). *Bcl6* expression is also detected in mature lymphoid tissues and transiently upregulated in thymocytes and splenocytes when these cells were stimulated with chemical mediators (PMA plus ionomycin; 9). Furthermore, the ex-

pression is predominant in the germinal center (GC)<sup>1</sup> B cells of human tonsils, but is undetectable in plasma cells (7, 8). However, the function of BCL6 in lymphocytes, especially in GC B cells, is unknown.

When mice are immunized with T cell-dependent antigens, antigen-reactive B cells in the spleen are activated by interaction with CD4<sup>+</sup> helper T cells in periarteriolar lymphoid sheaths (PALS) (19, 20). These activated B cells generate Ab-producing foci in PALS or migrate into follicles to form GCs that are identified by their binding capacity to peanut agglutinin (PNA). Nascent GC B cells undergo massive clonal expansion to form a dark zone occupied by surface Ig (sIg)-negative centroblasts (21, 22). Somatic hypermutations of the Ig gene occur in centroblasts (23–25). These cells differentiate into sIg-positive centrocytes at a light zone in GCs. Centrocytes with higher affinity to self-antigens or with lower affinity to immunized antigen undergo selective apoptosis (26–30), and those with higher

<sup>1</sup>Abbreviations used in this paper: AFC, antibody-forming cell; AP, alkaline phosphatase; Bcl6<sup>-/-</sup>, Bcl6-deficient mice; Bcl6<sup>-/-</sup>RM, RAG1-deficient mice reconstituted with bone marrow cells from Bcl6-deficient mice; BM, bone marrow; BrdU, 5-bromo-2'-deoxyuridine; CD40L, CD40 ligand; CG, chicken  $\gamma$  globulin; GC, germinal center; GST, glutathione-S-transferase; HRP, horseradish peroxidase; NP, 4-hydroxy-3-nitrophenyl acetyl; PALS, periarteriolar lymphoid sheath; PNA, peanut agglutinin; RAG1<sup>-/-</sup>, RAG1-deficient mice; sIg, surface Ig.

affinity to the antigen further differentiate into antibody-forming cells (AFCs) or memory B cells (31, 32).

To investigate the function of Bcl6 in lymphocytes, we have generated Bcl6-deficient mice (Bcl6<sup>-/-</sup>). However, Bcl6<sup>-/-</sup> displayed growth retardation and mostly died with severe myocardial injury by 9 wk old (Yoshida, T., manuscript in preparation). Therefore, we made RAG1-deficient mice (RAG1<sup>-/-</sup>) reconstituted with bone marrow (BM) cells from Bcl6<sup>-/-</sup> (Bcl6<sup>-/-</sup>-RM). As RAG1-deficient lymphocytes cannot develop into a mature form (33), all mature lymphocytes developed in Bcl6<sup>-/-</sup>-RM are derived from BM cells of Bcl6<sup>-/-</sup>. Here, we analyzed the function of lymphocytes developed in Bcl6<sup>-/-</sup>-RM. Although mature lymphocytes were developed in spleen from Bcl6<sup>-/-</sup>-RM, GC formation was impaired in the spleen. We discuss the possible function of Bcl6 in GC B cells.

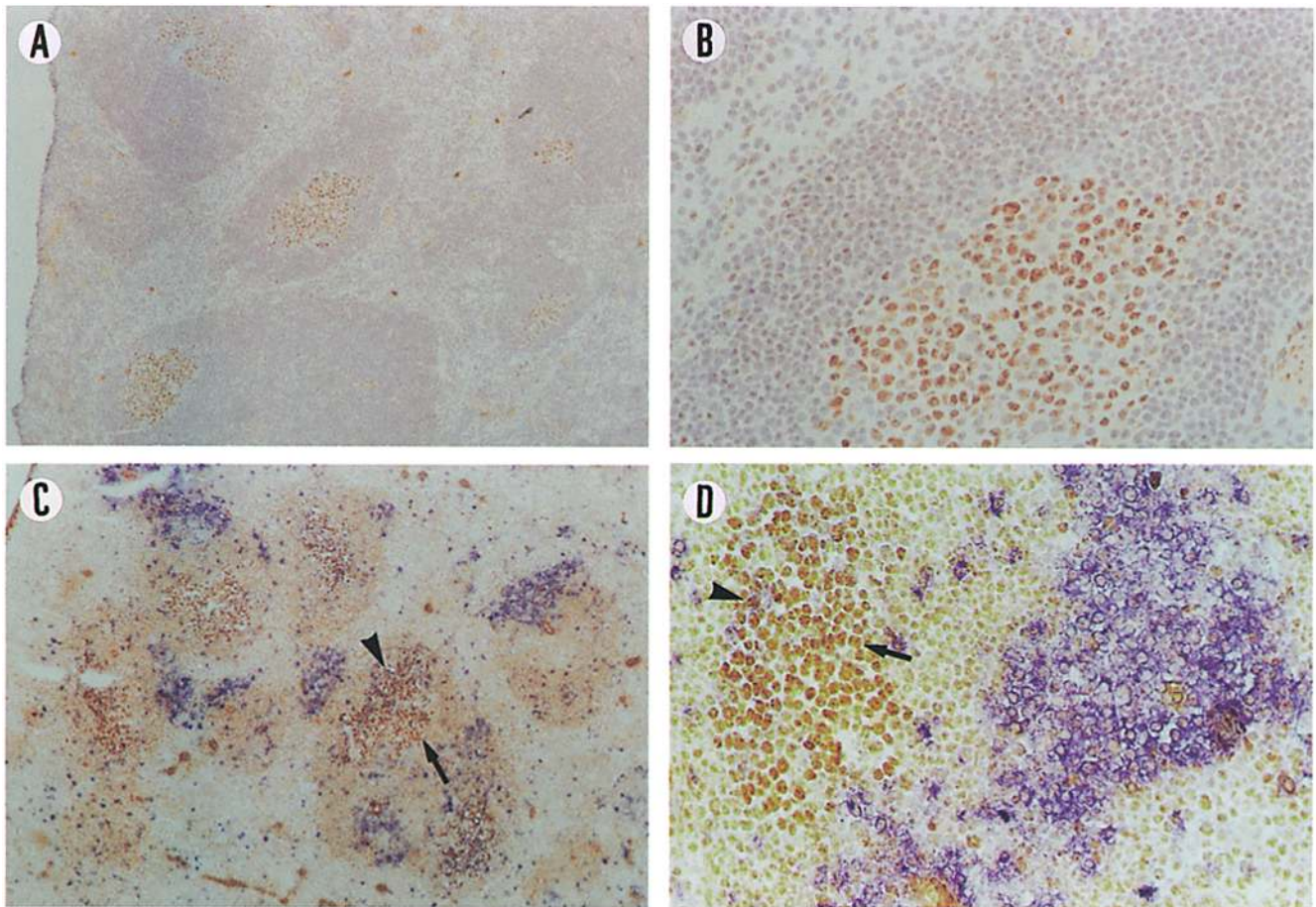
## Materials and Methods

**Animals.** C57BL/6 mice were purchased from Japan SLC Co. Ltd. (Hamamatsu, Japan). Bcl6<sup>-/-</sup> (Yoshida, T., manuscript in preparation) and RAG1<sup>-/-</sup> (33) were described in detail previously.

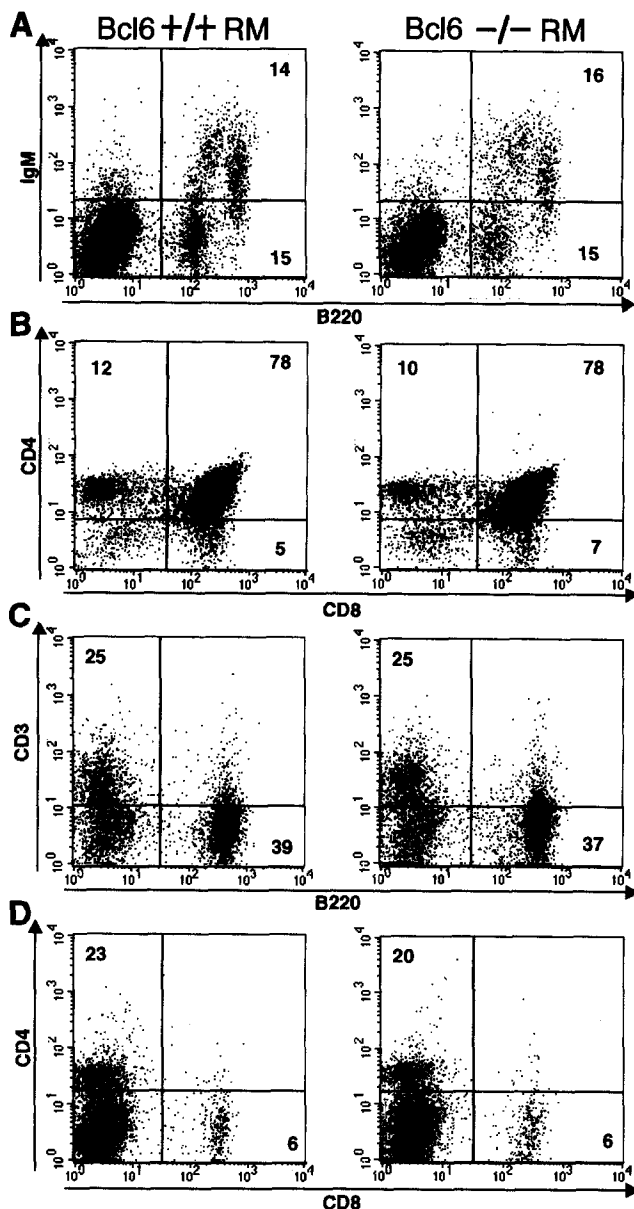
**mAbs and Flow Cytometry.** mAbs used for cell surface staining were FITC-anti-B220 (RA3-6B2; PharMingen, San Diego, CA), -anti-CD8 (Ly-2; PharMingen), biotinylated anti-IgM (II/41; PharMingen), PE-anti-CD4 (L3T4; PharMingen), and -anti-CD3ε (145-2C11; PharMingen). For flow cytometric analysis, cells were stained with antibodies for 30 min on ice. The cells were further incubated with a PE-streptavidin (PharMingen) for 15 min on ice. The stained cells were analyzed by FACSCalibur® (Becton Dickinson, San Jose, CA).

**Generation of Bcl6<sup>-/-</sup>-RM.** RAG1<sup>-/-</sup> (8–16 wk old) were sublethally irradiated (3.5 Gy) and injected with 2–5 × 10<sup>6</sup> BM cells from Bcl6<sup>-/-</sup> or Bcl6<sup>+/+</sup> littermates (3–5 wk old). The reconstitutions were confirmed 8–12 wk after transplantation by identifying CD3<sup>+</sup> and B220<sup>+</sup> mature lymphocytes in peripheral blood from Bcl6<sup>+/+</sup>-RM and Bcl6<sup>-/-</sup>-RM with flow cytometry.

**Immunization.** DNP-OVA was prepared by coupling of OVA (Sigma Chemical Co., St. Louis, MO) with 2,4-dinitrophenylbenzenesulfonic acid under alkaline condition (34). NP-chicken γ globulin (NP-CG) was made by reaction of succinimide ester of 4-hydroxy-3-nitrophenyl acetyl (NP; Genosys Biotechnology, Inc., Woodlands, TX) and CG (Sigma Chemical Co.). Mice were immunized intraperitoneally with 100 μg of alum-precipitated DNP-OVA or NP-CG. Sera were collected from the mice 7 d (for IgM) and 14 d (for IgG1) after immunization.



**Figure 1.** Bcl6 expression in spleen from immunized C57BL/6. Spleen sections from C57BL/6 mice on day 14 (A and B) or day 7 (C and D) after immunization were stained with anti-Bcl6 Ab (A and B) or anti-Bcl6 and anti-λ light chain Abs (C and D). Brown signals in nuclei and membranous blue signals indicate Bcl6 protein and λ light chain, respectively. Sections were counterstained with hematoxylin (A and B). Arrows and arrowheads indicate centroblasts and centrocytes respectively.

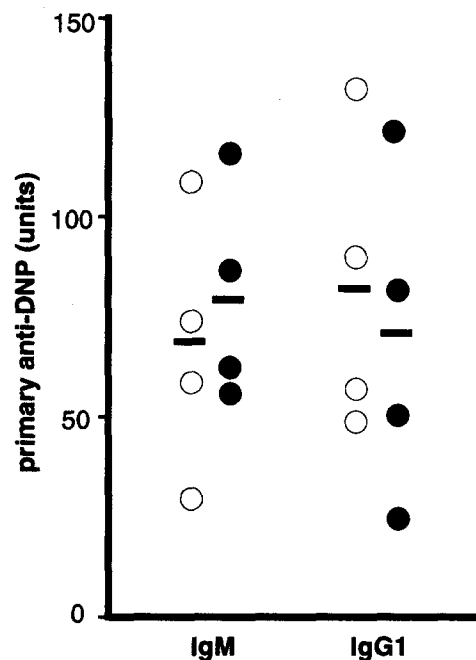


**Figure 2.** Normal development of B and T cells in  $Bcl6^{-/-}$ RM. Representative flow cytometric analysis of BM cells (A), thymocytes (B), and splenocytes (C and D) from  $Bcl6^{+/+}$ RM (left column) and  $Bcl6^{-/-}$ RM (right column). Numbers indicate relative percentages of positive cells within a quadrant.

Amounts of DNP-specific Abs in the sera were measured by ELISA as described previously (34).

**Immunohistochemistry.** A *SmaI*-*XhoI* fragment of murine *Bcl6* cDNA (mBcl6SX) was inserted into pGEX4T-2 vector (Pharmacia Biotech, Piscataway, NJ). The resulting glutathione-S-transferase (GST)-mBcl6SX fusion protein was used to immunize rabbits to raise anti-Bcl6 antibodies. The immune sera were precleared by passage through GST-Sepharose columns and affinity purified with antigen columns on which GST-mBcl6SX protein was coupled to activated CH Sepharose (Pharmacia Biotech).

Spleens were isolated from C57BL/6,  $Bcl6^{-/-}$ RM, or  $Bcl6^{+/+}$ RM after immunization, one third were used for flow cytometric

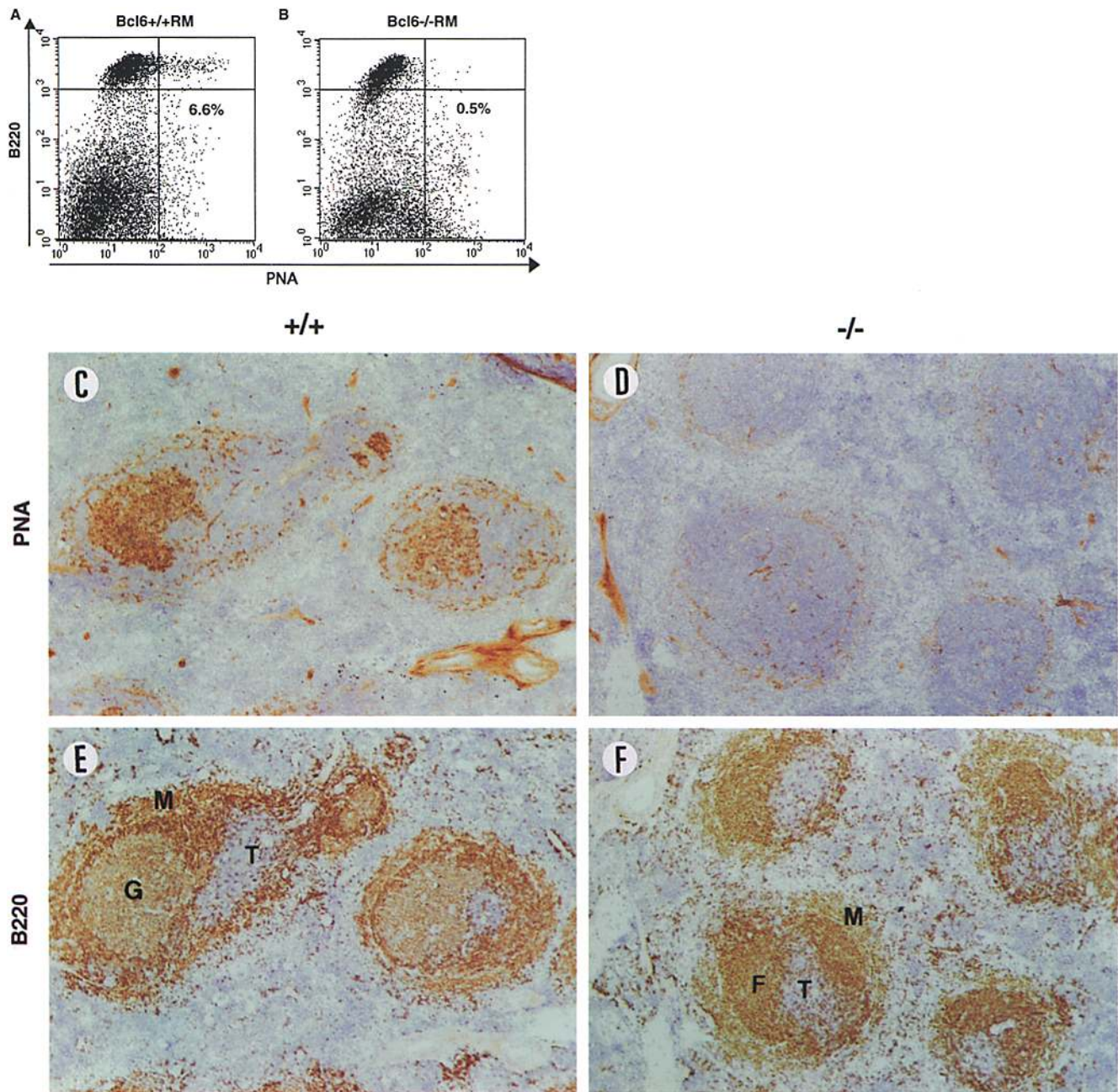


**Figure 3.** Control levels of primary Abs in sera from immunized  $Bcl6^{-/-}$ RM.  $Bcl6^{-/-}$ RM (closed circles) and  $Bcl6^{+/+}$ RM (open circles) were immunized with DNP-OVA. IgM and IgG1 anti-DNP titers in the sera were measured by ELISA.

analysis, and two thirds were embedded in O.C.T. compound (Miles, Incorporated, Elkhart, IN) and frozen in liquid nitrogen. Serial frozen sections (6  $\mu$ m) were fixed in cold acetone and the activity of endogenous peroxidase was quenched with 3%  $H_2O_2$  in methanol. Sections were stained with anti-Bcl6 Ab, PNA coupled to horseradish peroxidase (HRP) (EY Laboratories, San Mateo, CA), biotinylated anti-B220 Ab (PharMingen), or alkaline phosphatase (AP)-labeled goat anti-mouse  $\lambda$  light chain Ab (Southern Biotechnology Associates, Inc., Birmingham, AL) as the first reagents. A biotinylated goat anti-rabbit antibody (Nichirei, Tokyo, Japan) and streptABCComplex-HRP (Dako, Carpinteria, CA) were used as the second- and third-phase reagents, respectively. Bound HRP and AP activities were visualized with the diaminobenzidine detection kit (DAB kit; Nichirei) and the fast blue kit (Nichirei), respectively.

**Preparation of Splenic B and T Cells.** Splenic B cells were enriched by depleting non-B lineage cells from spleen cells. In brief, spleen cells were incubated with mixture of biotinylated mAbs to CD3, CD4, CD8, TER119 (PharMingen), and CD11b (Mac-1) (M1/70; PharMingen). These cells were subsequently reacted with streptavidin-coated immunomagnetic beads (BioMag; PerSeptive Diagnostics, Cambridge, MA). Labeled cells were removed by applying them in a magnetic field. The resulting B cell fraction contained >95% of B220<sup>+</sup> cells and <0.5% of CD3<sup>+</sup> cells. Splenic T cells were also enriched by a similar manner with mAbs to B220, TER119, and Mac-1. Resulting T cells contained <0.5% of B220<sup>+</sup> cells. The mixture of separated B and T cells ( $10^7$  cells each) were injected intravenously into RAG1<sup>-/-</sup>. The mice were immunized with DNP-OVA 1 d after transplantation. Spleens were isolated on day 14 after immunization.

**BrdU Incorporation Assay.** Proliferating cells in spleen were histologically detected by 5-bromo-2'-deoxyuridine (BrdU) incorporation assay (31). BrdU (2 mg; Boehringer Mannheim, Indianapolis, IN) was injected intraperitoneally in  $Bcl6^{-/-}$ RM or



**Figure 4.** GC formation is impaired in spleen from  $Bcl6^{-/-}$ RM. Splenocytes from  $Bcl6^{+/+}$ RM (A) and  $Bcl6^{-/-}$ RM (B) immunized with DNP-OVA on day 14 after immunization were stained with PNA and anti-B220 Ab. The numbers in the figures indicate percentages of  $PNA^{high} B220^{+}$  cells relative to total  $B220^{+}$  cells. Splenic sections from the immunized  $Bcl6^{+/+}$ RM (C and E) and  $Bcl6^{-/-}$ RM (D and F) were stained with PNA (C and D) or anti-B220 Ab (E and F) (brown). Hematoxylin counterstain. G, germinal center; T, T cell-rich PALS; M, marginal zone; F, primary follicle.

$Bcl6^{+/+}$ RM on day 3 after immunization with DNP-OVA. Spleens were isolated 2 h after BrdU injection and their sections were incubated with PNA-HRP for identifying GC B cells. After color development with DAB, the sections were incubated in 2N HCl at 37°C for 1 h, and then reacted with mouse mAb to BrdU (Boehringer Mannheim). The sections were further incubated with biotinylated rabbit anti-mouse Ig Ab (Dako), followed by staining with streptABComplex-AP (Dako). The fast blue kit (Nichirei) was used for chromogen. Therefore, blue color staining indicates both BrdU uptake in nuclei and Ig on B cells.

**Proliferation Assay.** Splenic B cells ( $5 \times 10^4$ /well) from  $Bcl6^{-/-}$ RM were cultured with LPS (10  $\mu$ g/ml; Sigma Chemical Co.), IL-4 (10<sup>2</sup> U/ml; culture supernatant of X63Ag8-653 cells transfected with murine IL-4 gene; reference 35), goat anti-IgM Ab (anti- $\mu$ ; 10  $\mu$ g/ml; Jackson ImmunoResearch Laboratories, West Grove, PA), or CD40 ligand (CD40L)-transfected Chinese hamster ovary cells (a gift from H. Yagita, Juntendo University, Tokyo, Japan;  $2 \times 10^4$  cells fixed with paraformaldehyde per well) in 96-well flat-bottomed microplates for 48 h. The cultures were pulsed with 1  $\mu$ Ci of [<sup>3</sup>H]thymidine (Amersham International, Ay-

lesbury, UK) for 6 h, and [ $^3\text{H}$ ]thymidine uptake was measured in a scintillation counter.

## Results

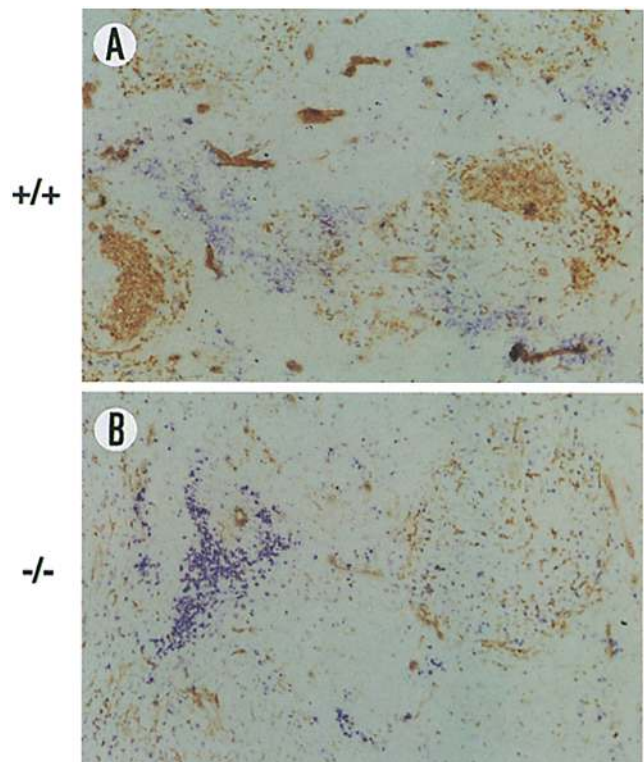
*Bcl6 Is Strongly Detected in GC, but Not in PALS-associated Foci.* We have made polyclonal antibodies specific for mouse Bcl6 and immunohistologically analyzed the expression in GCs from spleens of immunized mice. The GC reaction in spleen reaches its maximum by days 10–12 and keeps the same high levels until day 21 after immunization (31, 36). Therefore, expression of Bcl6 was examined in spleens of normal C57BL/6 mice immunized with T cell-dependent antigen, DNP-OVA, on day 14 after immunization (Fig. 1, A and B). Bcl6 was detected strongly in nuclei of most of GC B cells and weakly in those of other lymphocytes in white pulps.

After activation by helper T cells in PALS, antigen-reactive B cells migrate into follicles to form GCs or generate Ab-producing foci in PALS. The PALS-associated foci can be identified by immunohistological staining of spleens from C57BL/6 mice immunized with an immunogenic conjugate of NP with anti- $\lambda$  light chain antibody (36). The peak of the focus formation occurs on about day 10 after immunization (36). Thus, Bcl6 expression in PALS-associated foci from the mice immunized with NP-CG was analyzed on day 7 after immunization. Although strong Bcl6 expression was detected in GCs, it was not detected in the PALS-associated foci (Fig. 1, C and D). Anti- $\lambda$  light chain antibody used in this staining also distinguished centroblasts (sIg $^-$ ) from centrocytes (sIg $^+$ ) in GCs, in both of which Bcl6 expression was strongly upregulated.

*Germinal Center Formation Is Impaired in Bcl6 $^{-/-}$ RM.* Since Bcl6 $^{-/-}$  mostly died at young age, the function of Bcl6 in lymphocyte development was investigated in RAG1 $^{-/-}$  reconstituted with BM cells from Bcl6 $^{-/-}$  (Bcl6 $^{-/-}$ RM). Flow cytometric analysis of BM cells and thymocytes from Bcl6 $^{-/-}$ RM 3 mo after transplantation revealed no abnormality in early lymphocyte development (Fig. 2, A and B). Percentages of B220 $^+$  B cells, CD4 $^+$ , or CD8 $^+$  T cells in spleens from the Bcl6 $^{-/-}$ RM were also comparable to those from Bcl6 $^{+/+}$ RM (Fig. 2, C and D). Although Bcl6 expression can be detected in murine BM cells, thymocytes, and splenocytes (9), these data indicate that Bcl6 is not essential for lymphogenesis in primary lymphoid tissues.

We then analyzed the function of mature lymphocytes in Bcl6 $^{-/-}$ RM by immunizing them with DNP-OVA. Titers of primary IgM and IgG1 anti-DNP Abs in sera from the mice on day 7 (IgM) and on day 14 (IgG1) after immunization were measured by ELISA. Bcl6 $^{-/-}$ RM produced the primary IgM Abs and IgG1 Abs at levels comparable to those of Bcl6 $^{+/+}$ RM (Fig. 3). These results indicate that Bcl6 is not essential for differentiation of mature B cells into IgG1-producing plasma cells.

GC formation was histologically analyzed in spleens from mice immunized with DNP-OVA on day 14 after immunization. PNA-binding GC B cells were clearly iden-

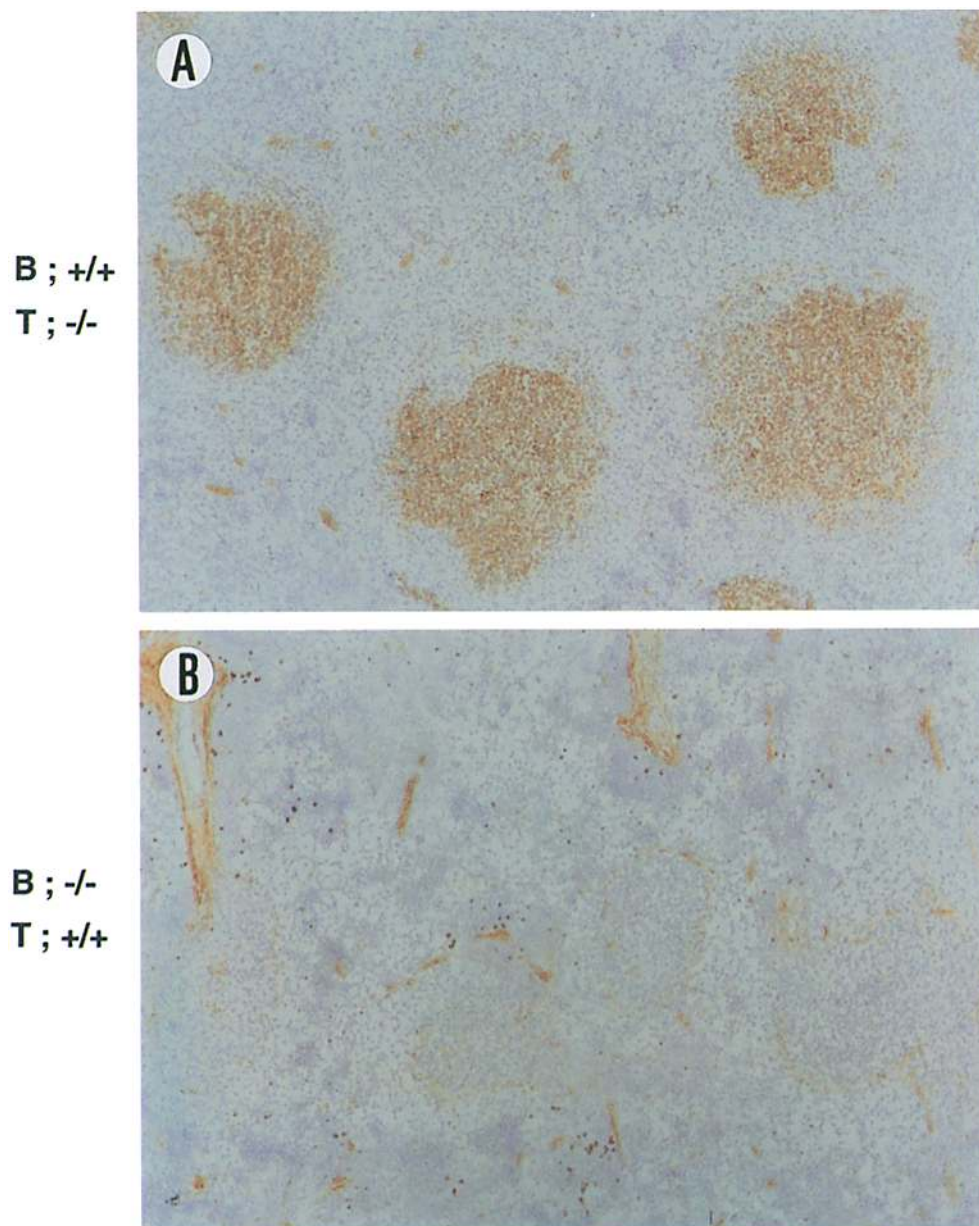


**Figure 5.** PALS-associated focus formation is not impaired in spleen from Bcl6 $^{-/-}$ RM. Splenic sections from Bcl6 $^{+/+}$ RM (A) and Bcl6 $^{-/-}$ RM (B) immunized with NP-CG on day 7 after immunization were stained with anti- $\lambda$  light chain Abs (blue) and PNA (brown).

tified in all of the Bcl6 $^{+/+}$ RM analyzed ( $n = 8$ , Fig. 4, A and C). In contrast, none of the Bcl6 $^{-/-}$ RM spleens ( $n = 8$ ) showed PNA-binding B cells in follicles (Fig. 4, B and D), although primary B cell follicles, marginal zones, and PALS were histologically identified (Fig. 4 F).

Because the great majority of Abs produced in the first 12 d after immunization are derived from PALS-associated foci (37), the focus formation in PALS was histologically analyzed in spleens from Bcl6 $^{-/-}$ RM immunized with NP-CG on day 7 after immunization with anti- $\lambda$  light chain antibody. PALS-associated foci and PNA-binding GCs could be detected in the Bcl6 $^{+/+}$ RM (Fig. 5 A). Although PNA-binding GCs were absent in the Bcl6 $^{-/-}$ RM, PALS-associated foci could be identified (Fig. 5 B). Therefore, Bcl6 is essential for GC formation in follicle but not for focus formation in PALS.

*Bcl6 Is Required in B, but Not in T Cells for GC Formation.* Functions of T cells in GCs are essential for GC formation of B cells (38, 39) and BCL6 is detected in a small fraction of CD4 $^+$  T cells in GCs (7). To determine whether defects in GC formation in Bcl6 $^{-/-}$ RM are due to defects in B or T cells or both, splenic T and B cells were isolated from Bcl6 $^{-/-}$ RM and Bcl6 $^{+/+}$ RM and then co-transferred into RAG1 $^{-/-}$  1 d before immunization with DNP-OVA. GC formation in the spleen was histologically analyzed 2 wk later. GC formation was detected in spleens from mice reconstituted with B cells from Bcl6 $^{+/+}$ RM and T cells from Bcl6 $^{-/-}$ RM (Fig. 6 A). In contrast, co-transfer of B cells



**Figure 6.** B cells are responsible for the defect of GC formation in  $Bcl6^{-/-}$  RM. Spleen sections from the DNP-OVA immunized  $RAG1^{-/-}$  reconstituted with B cells from  $Bcl6^{+/+}$  RM and T cells from  $Bcl6^{-/-}$  RM (A), or with B cells from  $Bcl6^{-/-}$  RM and T cells from  $Bcl6^{+/+}$  RM (B) were stained with PNA (brown). Hematoxylin counterstain.

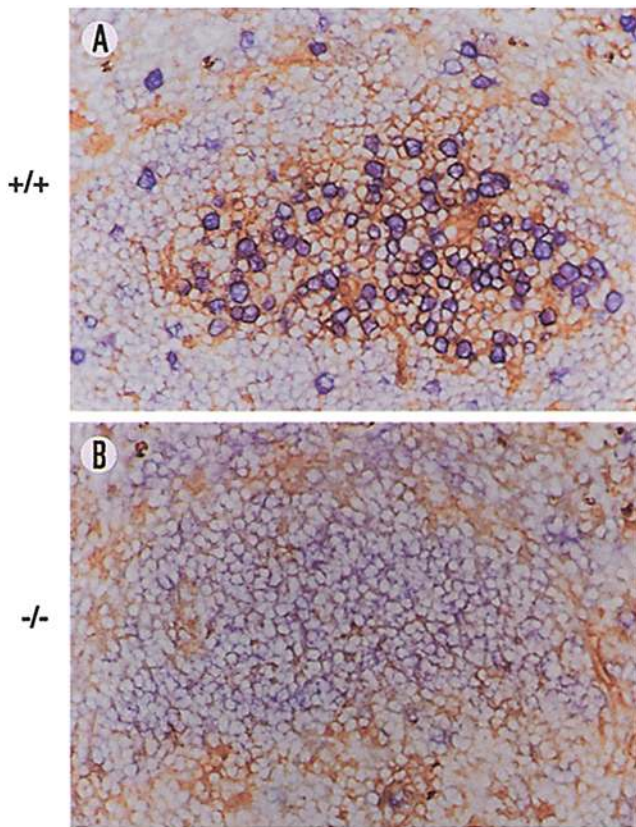
from  $Bcl6^{-/-}$  RM and T cells from  $Bcl6^{+/+}$  RM did not restore GC formation (Fig. 6 B), indicating that  $Bcl6$  expression in B but not in T cells is essential for the GC formation.

*Nascent GC Formation Is Absent in Spleen from  $Bcl6^{-/-}$  RM.* GC first appears in splenic follicles by day 4 and begins to wane by day 21 after immunization (31). It might be possible that GC formation occurs in  $Bcl6^{-/-}$  RM shortly after immunization but cannot be maintained. To examine the possibility, the presence of PNA-binding GCs was examined in spleens from  $Bcl6^{-/-}$  RM immunized with DNP-OVA at an early stage after immunization. Nascent PNA-binding GCs could be identified in the  $Bcl6^{+/+}$  RM spleen on day 3 after immunization (Fig. 7 A) and  $Bcl6$  was detected strongly in nuclei of these GC B cells (data not shown). However, the nascent GCs were not detected at all in the

$Bcl6^{-/-}$  RM on day 3 (Fig. 7 B) and day 4 (data not shown) after immunization.

To exclude the possibility that nascent GC B cells from  $Bcl6^{-/-}$  RM cannot obtain the PNA-binding capacity, the presence of blast cells in S phase in splenic follicles from the  $Bcl6^{-/-}$  RM on day 3 after immunization was histologically analyzed using BrdU incorporation assay, because nascent GC B cells undergo massive clonal expansion in follicles (21, 22). About half the numbers of GC cells in spleen from the  $Bcl6^{+/+}$  RM were positive for BrdU uptake 2 h after pulse (Fig. 7 A). However, no BrdU-positive cells were detected in B cell follicles from the  $Bcl6^{-/-}$  RM spleen (Fig. 7 B), suggesting that antigen-reactive B cells activated by helper T cells in PALS cannot proliferate at all in splenic follicles.

We then examined the capacity for proliferation of ma-

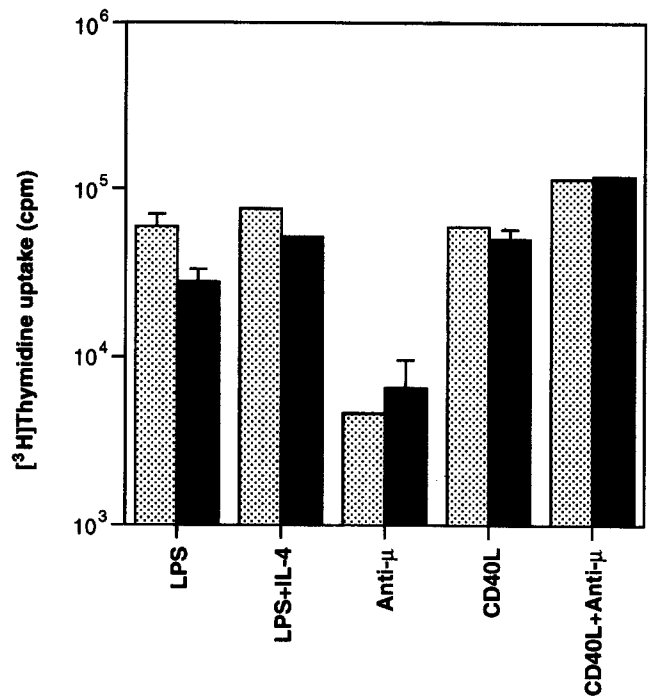


**Figure 7.** Nascent GC is absent in spleen from  $Bcl6^{-/-}$ RM. Spleen sections from  $Bcl6^{+/+}$ RM (A) and  $Bcl6^{-/-}$ RM (B) on day 3 after immunization were stained with anti-BrdU Ab and PNA. Strong blue signals in the nuclei and membranous weak blue signals indicate BrdU uptake and cell surface Igs, respectively. Brown signals in follicle of  $Bcl6^{+/+}$ RM indicate PNA-reactive GC cells.

ture B cells from  $Bcl6^{-/-}$ RM in vitro. Splenic B cells from  $Bcl6^{-/-}$ RM were stimulated with LPS, LPS plus IL-4, anti-IgM Ab, CD40L, or anti-IgM plus CD40L. DNA synthesis of these stimulated cells was analyzed on day 2 after stimulation. Splenic B cells from  $Bcl6^{-/-}$ RM proliferated well against these stimuli similar to those from  $Bcl6^{+/+}$ RM (Fig. 8). Therefore, antigen-reactive B cells in  $Bcl6^{-/-}$ RM may have a capacity to proliferate by antigen stimulations and signals from helper T cells in PALS.

## Discussion

Antigen-reactive B cells activated by helper T cells in PALS further differentiate into AFCs in PALS or GC B cells in follicles. We show here that  $Bcl6$  is essential for differentiation of GC B cells (Fig. 4). However,  $Bcl6$  is not required for the focus formation in PALS by antigen-reactive B cells (Fig. 5). This is also supported by the evidence that  $Bcl6$  expression is not upregulated in PALS-associated foci (Fig. 1). Because primary AFCs and GC B cells are thought to be derived from a common clonal origin (40),  $Bcl6$  may be an essential transcription factor for the differentiation pathway into GC B cells. Although it is still argued



**Figure 8.** Proliferation capacity of splenic B cells from  $Bcl6^{-/-}$ RM. Proliferative responses of splenic B cells from  $Bcl6^{+/+}$ RM (shaded bars) and  $Bcl6^{-/-}$ RM (closed bars) to indicated stimuli. Results represent means and variations (SD) from triplicate cultures. The data presented are representative of three independent experiments.

that these cells originate from distinct cell lineages (41, 42),  $Bcl6$  is a regulator for the differentiation of GC B cells.

$Bcl6$  expression is upregulated in nascent GC B cells (data not shown) and detected in both centroblasts and centrocytes in GCs from normal mice (Fig. 1). Furthermore, GC formation is impaired in  $Bcl6^{-/-}$ RM (Fig. 4). Therefore, signals that upregulate  $Bcl6$  expression in antigen-reactive B cells are essential for the differentiation of GC B cells. Stimulation of mature B cells with CD40L plus anti-IgM Ab induces phenotypic markers on GC B cells (43), and LPS plus IL-4 induces RAG1 and RAG2 expression (44) and Ig isotype class-switching in B cells like GC B cells (45). However, these signals cannot make upregulation of  $Bcl6$  expression in mature B cells (data not shown; reference 46). Interactions of antigen-reactive B cells with helper T cells in PALS through cell surface molecules such as CD40–CD40L and CD86(B7-2)–CD28 are required for the differentiation (47–51). Because the blocking of these interactions inhibits both primary Abs production and GC formation, signals from these interactions may be required for both differentiation pathways. Follicular dendritic cells are unique cells in follicles (19). Helper T cells in GCs have also unique characters that are distinct from those of helper T cells in PALS (52, 53). These cells seem to be required for GC formation by antigen-reactive B cells. Interactions of the B cells with these dendritic cells or GC-helper T cells may be a possible origin of the signals that upregulate  $Bcl6$ . Further study on the signals will be able to elucidate the molecular mechanism of differentiation into GC B cells.

GC formation is also impaired in mice lacking genes other than Bcl6. Lymphotoxin- $\alpha$ - (54) or TNF- $\alpha$ -deficient mice (55) can produce primary IgG1 Abs without GC formation like Bcl6<sup>-/-</sup>RM. The defect in GC formation may be due to the abnormality of microarchitecture of spleens (56). Chemokine receptor, Blr1-deficient mice also produce normal levels of primary IgG1 Abs without functional GCs (57). Although PNA-binding B cells are detected in PALS from the Blr1-deficient mice after immunization, these B cells fail to migrate from PALS into B cell follicles in the spleen. However, mature B cells from Bcl6<sup>-/-</sup>RM can migrate into follicles to form the normal microarchitecture of spleens (Fig. 4 F). Mice lacking CD40 (47, 48), complement receptors (CR1 and CR2) (58, 59), or CD28 (51) display impairments of both GC formation and primary IgG1 production to T cell-dependent antigens, but Bcl6<sup>-/-</sup>RM produced normal levels of the primary IgG1 Abs (Fig. 3). Therefore, the mechanism of the defect in GC formation in Bcl6<sup>-/-</sup>RM is distinct from the mechanisms in these deficient mice.

There are two distinct types of B cells in GCs: centroblasts and centrocytes. Several unique molecular events such as somatic hypermutation of the Ig gene, Ig isotype class-switching, selective cell death, and differentiation into memory B cells occur separately in each type of GC B cells

(60). Bcl6 expression is continuously upregulated in both types of GC B cells (Fig. 1), suggesting that Bcl6 is required not for each specific molecular event in GC B cells but for more fundamental events throughout GC reactions. These events may not be related with proliferation of GC B cells because Bcl6 is strongly detected in both cell cycling centroblasts and resting centrocytes (Fig. 1) and splenic B cells lacking Bcl6 can proliferate to various stimuli (Fig. 8).

BCL6 was identified as an oncogene involved in chromosomal rearrangements in B cell lymphoma. Bcl6 expression is ubiquitous in mature tissues and is upregulated in cardiac myocytes, keratinocytes, and sperm at their terminal stages. Furthermore, Bcl6-deficient mice die with spontaneous death of mature cardiac myocytes, suggesting that mature heart muscle without Bcl6 cannot maintain its differentiation stage. Although the GC stage is not a terminal stage of B cell differentiation, Bcl6 is continuously upregulated in GC B cells (Fig. 1). If the specific function of Bcl6 is shared between B cells at the GC stage and heart muscle at the terminal stage, Bcl6 may be essential for protection of the GC B cells against specific stresses such as somatic hypermutation and selective cell death. Furthermore, this protective function may also contribute to lymphomagenesis of GC B cells by deregulation of BCL6.

---

We thank Dr T. Takemori for discussion, Dr H. Yagita for providing CD40L-transfected CHO cells, Y. Iwata for skillful technical assistance, and E. Furusawa for secretarial services.

This work was supported in part by Grants-in-Aid for Cancer Research from the Ministry of Education, Science, Sports and Culture of Japan and a Research Grant of the Princess Takamatsu Cancer Research Fund (No. 96-22807).

Address correspondence to Takeshi Tokuhisa, Division of Developmental Genetics, Center for Biomedical Science, Chiba University School of Medicine, 1-8-1 Inohana, Chuo-ku, Chiba 260, Japan. Phone: 81-43-226-2181; FAX: 81-43-226-2183; E-mail: tokuhisa@med.m.chiba-u.ac.jp

Received for publication 24 April 1997 and in revised form 23 May 1997.

## References

1. Kerchaert, J.P., C. Deweindt, H. Tilly, S. Quief, G. Lecocq, and C. Bastard. 1993. *LAZ3*, a novel zinc-finger encoding gene, is disrupted by recurring chromosome 3q27 translocations in human lymphomas. *Nature Genet.* 5:66-70.
2. Ye, B.H., F. Lista, D.M. Lo Coco, R. Knoeles, K. Offit, R. S.K. Chaganti, and R. Dalla-Favera. 1993. Alterations of a zinc finger-encoding gene, *BCL-6*, in diffuse large-cell lymphoma. *Science (Wash. DC)*. 262:747-750.
3. Miki, T., N. Kawamata, S. Hirosawa, and N. Aoki. 1994. Gene involved in the 3q27 translocation associated with B-cell lymphoma, *BCL5*, encodes a Krüppel-like zinc-finger protein. *Blood*. 83:26-32.
4. Bastard, C., C. Deweindt, J.P. Kerchaert, B. Leormand, A. Rossi, F. Pezzela, C. Fruchart, C. Duval, M. Monconduit, and H. Tilly. 1994. *LAZ3* rearrangements in non-Hodgkin's lymphoma: correlation with histology, immunophenotype, karyotype, and clinical outcome. *Blood*. 83:2423-2427.
5. Lo Coco, F., B.H. Ye, F. Lista, P. Corradini, K. Offit, R. Knoeles, R.S.K. Chaganti, and R. Dalla-Favera. 1994. Rearrangements of the *BCL6* gene in diffuse large cell non-Hodgkin's lymphoma. *Blood*. 83:1757-1759.
6. Ye, B.H., S. Chaganti, C.C. Chang, H. Niu, P. Corradini, R.S. Chaganti, and R. Dalla-Favera. 1995. Chromosomal translocations cause deregulated BCL6 expression by promoter substitution in B cell lymphoma. *EMBO (Eur. Mol. Biol. Organ.) J.* 14:6209-6217.
7. Cattoretti, G., C.C. Chang, K. Cechova, J. Zhang, B.H. Ye, B. Falini, D.C. Louie, K. Offit, R.S. Chaganti, and R. Dalla-Favera. 1995. BCL-6 protein is expressed in germinal-center B cells. *Blood*. 86:45-53.
8. Onizuka, T., M. Moriyama, T. Yamochi, T. Kuroda, A. Kazama, N. Kanazawa, K. Sato, T. Kato, and S. Mori. 1995. BCL-6 gene product, a 92- to 98-kD nuclear phosphoprotein, is highly expressed in germinal center B cells and their



- neoplastic counterparts. *Blood*. 86:28–37.
9. Fukuda, T., T. Miki, T. Yoshida, M. Hatano, K. Ohashi, S. Hirose, and T. Tokuhisa. 1995. The murine *BCL6* gene is induced in activated lymphocytes as an immediate early gene. *Oncogene*. 11:1657–1663.
  10. Kawamata, N., T. Miki, K. Ohashi, K. Suzuki, T. Fukuda, S. Hirose, and N. Aoki. 1994. Recognition DNA sequence of a novel putative transcription factor, BCL6. *Biochem. Biophys. Res. Commun.* 204:366–374.
  11. Baron, B.W., R.R. Stanger, E. Hume, A. Sadhu, R. Mick, J.P. Kerckaert, C. Deweindt, C. Bastard, G. Nucifora, N. Zeleznik-Le, and W. McKeithan. 1995. BCL6 encodes a sequence-specific DNA-binding protein. *Genes Chromosomes Cancer*. 13:221–224.
  12. Bardwell, V.J., and R. Treisman. 1994. The POZ domain: a conserved protein–protein interaction motif. *Genes Dev.* 8:1664–1677.
  13. Zollman, S., D. Godt, G.G. Prive, J.L. Couderc, and F.A. Laski. 1994. The BTB domain, found primarily in zinc finger proteins, defines an evolutionarily conserved family that includes several developmentally regulated genes in *Drosophila*. *Proc. Natl. Acad. Sci. USA*. 91:10717–10721.
  14. Deweindt, C., O. Albagli, F. Bernardin, P. Dhordain, S. Quief, D. Lantoine, P. Kerckaert, and D. Leprince. 1995. The *LAZ3/BCL6* oncogene encodes a sequence-specific transcriptional inhibitor: a novel function for the BTB/POZ domain as an autonomous repressing domain. *Cell Growth Differ.* 6:1495–1503.
  15. Chang, C.C., B.H. Ye, R.S.K. Chaganti, and R. Dalla-Favera. 1996. BCL-6, a POZ/zinc-finger protein, is a sequence-specific transcriptional repressor. *Proc. Natl. Acad. Sci. USA*. 93:6947–6952.
  16. Seyfert, V.L., D. Allman, Y. He, and L.M. Staudt. 1996. Transcriptional repression by the proto-oncogene BCL-6. *Oncogene*. 12:2331–2342.
  17. Miki, T., N. Kawamata, A. Arai, K. Ohashi, Y. Nakamura, A. Kato, S. Hirose, and N. Aoki. 1994. Molecular cloning of the breakpoint for 3q27 translocation in B-cell lymphomas and leukemias. *Blood*. 83:217–222.
  18. Yoshida, T., T. Fukuda, S. Okabe, M. Hatano, T. Miki, S. Hirose, N. Miyasaka, K. Isono, and T. Tokuhisa. 1996. The *BCL6* gene is predominantly expressed in keratinocytes at their terminal differentiation stage. *Biochem. Biophys. Res. Commun.* 228:216–220.
  19. MacLennan, I.C.M. 1994. Germinal centers. *Annu. Rev. Immunol.* 12:117–139.
  20. Kelsoe, G. 1995. *In situ* studies of the germinal center reaction. *Adv. Immunol.* 60:267–288.
  21. Hanna, M.G. 1964. An autoradiographic study of the germinal center in spleen white pulp during early intervals of the immune response. *Lab. Invest.* 13:95–104.
  22. Kroese, F.G., A.S. Wubbena, H.G. Seijen, and P. Nieuwenhuis. 1987. Germinal centers develop oligoclonally. *Eur. J. Immunol.* 17:1069–1072.
  23. Berek, C., A. Berger, and M. Apel. 1991. Maturation of the immune response in germinal centers. *Cell*. 67:1121–1129.
  24. Jacob, J., G. Kelsoe, K. Rajewsky, and U. Weiss. 1991. Intracloonal generation of antibody mutants in germinal centres. *Nature (Lond.)*. 354:389–392.
  25. Pascual, V., Y.J. Liu, A. Magalski, O. de Bouteiller, J. Banchereau, and J.D. Capra. 1994. Analysis of somatic mutation in five B cell subsets of human tonsil. *J. Exp. Med.* 180:329–339.
  26. Zhang, J., I.C. MacLennan, Y.J. Liu, and P.J. Lane. 1988. Is rapid proliferation in B centroblasts linked to somatic mutation in memory B cell clones? *Immunol. Lett.* 18:297–299.
  27. Weiss, U., and K. Rajewsky. 1990. The repertoire of somatic antibody mutants accumulating in the memory compartment after primary immunization is restricted through affinity maturation and mirrors that expressed in the secondary response. *J. Exp. Med.* 172:1681–1689.
  28. Han, S., B. Zheng, J. Dal Porto, and G. Kelsoe. 1995. *In situ* studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. IV. Affinity-dependent, antigen-driven B cell apoptosis in germinal centers as a mechanism for maintaining self-tolerance. *J. Exp. Med.* 182:1635–1644.
  29. Pulendran, B., G. Kannourakis, S. Nouri, K.G. Smith, and G.J.V. Nossal. 1995. Soluble antigen can cause enhanced apoptosis of germinal-centre B cells. *Nature (Lond.)*. 375:331–334.
  30. Shokat, K.M., and C.C. Goodnow. 1995. Antigen-induced B-cell death and elimination during germinal-centre immune responses. *Nature (Lond.)*. 375:334–338.
  31. Liu, Y.J., J. Zhang, P.J. Lane, E.Y. Chan, and I.C. MacLennan. 1991. Sites of specific B cell activation in primary and secondary responses to T cell-dependent and T cell-independent antigens. *Eur. J. Immunol.* 21:2951–2962.
  32. Arpin, C., J. Dechanet, C. Van Kooten, P. Merville, G. Grouard, F. Briere, J. Banchereau, and Y.J. Liu. 1995. Generation of memory B cells and plasma cells *in vitro*. *Science (Wash. DC)*. 268:720–722.
  33. Mombaerts, P., J. Iacomini, R.S. Johnson, K. Herrup, S. Tonegawa, and E. Papaniannou. 1992. RAG-1-deficient mice have no mature B and T lymphocytes. *Cell*. 68:869–877.
  34. Takao, S., N. Sakai, M. Hatano, T. Koizumi, K. Hanioka, U. Rütther, and T. Tokuhisa. 1991. IgG response is impaired in H2-c-fos transgenic mice. *Int. Immunol.* 3:369–375.
  35. Karasuyama, H., and F. Melchers. 1988. Establishment of mouse cell lines which constitutively secrete large quantities of interleukin 2, 3, 4 or 5, using modified cDNA expression vectors. *Eur. J. Immunol.* 18:97–104.
  36. Jacob, J., R. Kassir, and G. Kelsoe. 1991. *In situ* studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. I. The architecture and dynamics of responding cell populations. *J. Exp. Med.* 173:1165–1175.
  37. Tsiagbe, V.K., P.J. Linton, and G.J. Thorbecke. 1992. The path of memory B-cell development. *Immunol. Rev.* 126:113–141.
  38. Stedra, J., and J. Cerny. 1994. Distinct pathways of B cell differentiation. *J. Immunol.* 152:1718–1726.
  39. van Essen, D., H. Kikutani, and D. Gray. 1995. CD40 ligand-transduced co-stimulation of T cells in the development of helper function. *Nature (Lond.)*. 378:620–623.
  40. Jacob, J., and G. Kelsoe. 1992. *In situ* studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. II. A common clonal origin for periarteriolar lymphoid sheath-associated foci and germinal centers. *J. Exp. Med.* 176:679–687.
  41. Linton, P.J.L., D.J. Decker, and N.R. Klinman. 1989. Primary antibody-forming cells and secondary B cells are generated from separate precursor cell subpopulations. *Cell*. 59:1049–1059.
  42. Linton, P.J., D. Lo, L. Lai, G.J. Thorbecke, and N.R. Klinman. 1992. Among naive precursor cell subpopulations only progenitors of memory B cells originate germinal centers. *Eur. J. Immunol.* 22:1293–1297.
  43. Galibert, L., N. Burdin, B. de Saint-Vis, P. Garrone, C. Van Kooten, J. Banchereau, and F.J. Rousset. 1996. CD40 and B cell antigen receptor dual triggering of resting B lymphocytes

- turns on a partial germinal center phenotype. *J. Exp. Med.* 183: 77–85.
44. Hikida, M., M. Mori, T. Takai, K. Tomochika, K. Hamatani, and H. Ohmori. 1996. Reexpression of RAG-1 and RAG-2 genes in activated mature mouse B cells. *Science (Wash. DC)*. 274:2092–2094.
  45. Coffman, R.L., D.A. Leberman, and P. Rothman. 1993. Mechanism and regulation of immunoglobulin isotype switching. *Adv. Immunol.* 54:229–270.
  46. Allman, D., A. Jain, A. Dent, R.R. Maile, T. Selvaggi, M.R. Kehry, and L.M. Staudt. 1996. BCL-6 expression during B-cell activation. *Blood*. 87:5257–5268.
  47. Castigli, E., F.W. Alt, L. Davidson, A. Bottaro, E. Mizoguchi, A.K. Bhan, and R.S. Geha. 1994. CD40-deficient mice generated by recombination-activating gene-2-deficient blastocyst complementation. *Proc. Natl. Acad. Sci. USA*. 91:12135–12139.
  48. Kawabe, T., T. Naka, K. Yoshida, T. Tanaka, H. Fujiwara, S. Suematsu, N. Yoshida, T. Kishimoto, and H. Kikutani. 1994. The immune responses in CD40-deficient mice: impaired immunoglobulin class switching and germinal center formation. *Immunity*. 1:167–178.
  49. Xu, J., T.M. Foy, J.D. Laman, E.A. Elliott, J.J. Dunn, T.J. Waldschmidt, J. Elsemore, R.J. Noelle, and R.A. Flavell. 1994. Mice deficient for the CD40 ligand. *Immunity*. 1:423–431.
  50. Han, S., K. Hathcock, B. Zheng, T.B. Kepler, R. Hodes, and G. Kelsoe. 1995. Cellular interaction in germinal centers. *J. Immunol.* 155:556–567.
  51. Ferguson, S.E., S. Han, G. Kelsoe, and C.B. Thompson. 1996. CD28 is required for germinal center formation. *J. Immunol.* 156:4576–4581.
  52. Zheng, B., W. Xue, and G. Kelsoe. 1994. Locus-specific somatic hypermutation in germinal centre T cells. *Nature (Lond.)*. 372:556–559.
  53. Zheng, B., S. Han, Q. Zhu, R. Goldsby, and G. Kelsoe. 1996. Alternative pathways for the selection of antigen-specific peripheral T cells. *Nature (Lond.)*. 384:263–266.
  54. Matsumoto, M., S. Mariathasan, M.H. Nahm, F. Baranyay, J.J. Peschon, and D.D. Chaplin. 1996. Role of lymphotoxin and the type I TNF receptor in the formation of germinal centers. *Science (Wash. DC)*. 271:1289–1291.
  55. Pasparakis, M., L. Alexopoulou, V. Episkopou, and G. Kollias. 1996. Immune and inflammatory responses in TNF $\alpha$ -deficient mice: a critical requirement for TNF $\alpha$  in the formation of primary B cell follicles, follicular dendritic cell networks and germinal centers, and in the maturation of the humoral immune response. *J. Exp. Med.* 184:1397–1411.
  56. Liu, Y.J., and J. Banachereau. 1996. Mutant mice without B lymphocyte follicles. *J. Exp. Med.* 184:1207–1211.
  57. Forster, R., A.E. Mattis, E. Kremmer, E. Wolf, G. Brem, and M. Lipp. 1996. A putative chemokine receptor, BLR1, directs B cell migration to defined lymphoid organs and specific anatomic compartments of the spleen. *Cell*. 87:1037–1047.
  58. Ahearn, J.M., M.B. Fischer, D. Croix, S. Goerg, M. Ma, J. Xia, X. Zhou, R.G. Howard, T.L. Rothstein, and M.C. Carroll. 1996. Disruption of the Cr2 locus results in a reduction in B-1a cells and in an impaired B cell response to T-dependent antigen. *Immunity*. 4:251–262.
  59. Croix, D.A., J.M. Ahearn, A.M. Rosengard, S. Han, G. Kelsoe, M. Ma, and C. Carrol. 1996. Antibody response to a T-dependent antigen requires B cell expression of complement receptors. *J. Exp. Med.* 183:1857–1864.
  60. Liu, Y.J., C. Arpin, O. de Bouteiller, C. Guret, J. Banachereau, H. Martinez-Valdez, and S. Lebecque. 1996. Sequential triggering of apoptosis, somatic mutation and isotype switch during germinal center development. *Semin. Immunol.* 8:169–177.