Mimicry of CD40 Signals by Epstein-Barr Virus LMP1 in B Lymphocyte Responses

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The effect of the Epstein-Barr virus (EBV) latent membrane protein 1 (LMP1) on the activation and differentiation of normal B cells was investigated. B cells of transgenic mice expressing LMP1 under the control of immunoglobulin promoter/enhancer displayed enhanced expression of activation antigens and spontaneously proliferated and produced antibody. Humoral immune responses of LMP1 transgenic mice in CD40-deficient or normal backgrounds revealed that LMP1 mimics CD40 signals to induce extrafollicular B cell differentiation but, unlike CD40, blocks germinal center formation. Thus, these specific properties of LMP1 may determine the site of primary B cell infection and the state of infection in the natural course of EBV infection, whereas subsequent loss of LMP1 expression may affect the site of persistent latent infection.

Epstein-Barr virus (EBV) is a causative agent of infectious mononucleosis and lymphoproliferative diseases in immunosuppressed patients and is associated with various malignancies such as Burkitt's lymphoma, Hodgkin's lymphoma, and nasopharyngeal carcinoma. Primary EBV infection of B cells results in the expression of EBV-encoded nuclear antigens (EBNAs) and latent membrane proteins (LMPs) and growth transformation (*1*). Characteristics of EBV-infected B cells such as enlarged cell size, expression of activation and adhesion molecules, and antibody secretion are similar to those of B cells stimulated by polyclonal B cell activators, interleukin 4 (IL-4), and CD40 ligation; however, only EBV can immortalize B cells (*2*).

Of the EBV gene-encoded proteins, LMP1 is essential for EBV-mediated transformation and induces most of the changes associated with EBV infection (*3*). LMP1 is an integral membrane protein with six transmembrane domains that facilitate self-aggregation in the plasma membrane (*4*). Its COOH-terminal cytoplasmic region recruits tumor necrosis factor (TNF) receptor–associated factors (TRAFs) that are signal-trans-

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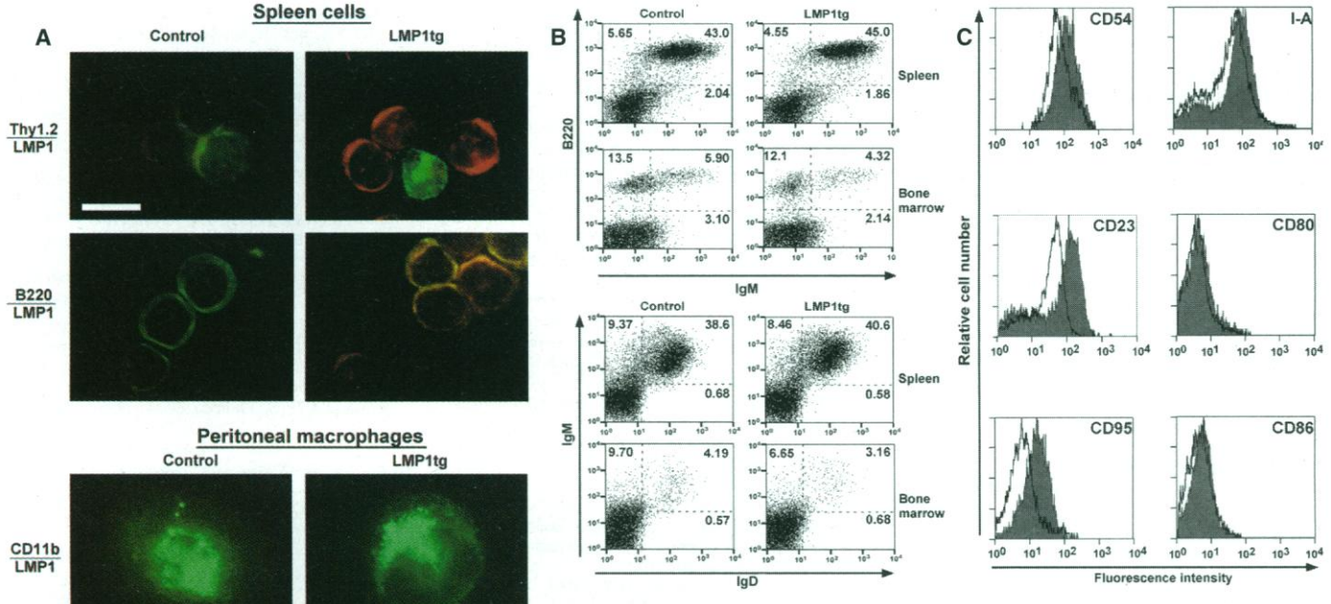

Fig. 1. Immunofluorescence analysis of B lymphocytes in LMP1tg mice. (**A**) B cell–specific expression of transgenic LMP1. Spleen cells were stained by FITC–anti-Thy1.2 or FITC–anti-B220 in combination with biotinylated anti-LMP1, followed by Alexa 594–conjugated streptavidin. Purified macrophages and dendritic cells were also stained by FITC–anti-CD11b or FITC–anti-CD11c, respectively, in combination with biotinylated anti-LMP1, followed by Alexa 594–streptavidin. Only $B220^+$ B cells derived from LMP1tg mice were stained by anti-LMP1 and detected as cells displaying red fluorescence. Scale bar, 10 μm. (**B**) Normal development of LMP1tg B cells. Either splenic or bone marrow–derived mononuclear cells from LMP1tg and the control mice were stained with anti-B220, anti-IgM, and anti-IgD. Population of lymphocytes was defined as forward and side scatters, and their expression patterns of B220 and IgM, or IgM and IgD, are shown. Numbers are percentages of total lymphocytes in each quadrant. (**C**) Enhanced expression of activation markers on LMP1tg B cells. LMP1tg and control splenocytes were stained with monoclonal antibodies specific for B220 and CD54, CD23, CD95, CD80, CD86, or I-A. $B220^+$ cells from LMP1tg mice (shaded area) and control mice (unshaded area) were gated as populations of B lymphocytes, and the magnitudes of their surface marker expressions are shown as histograms. Similar results of these flow cytometric analyses were observed in three separate experiments.

ducing molecules of TNF receptor family proteins, including CD40 on B cells (*5, 6*). LMP1 and CD40 interact with many of the same TRAF molecules; thus, it is likely that LMP1 would engage at least part of the CD40 signal pathway in a ligand-independent manner during EBV infection of B cells. To address the question of whether and how LMP1 mimics CD40 signaling to induce B cell activation and differentiation in vivo, we investigated B cell functions and humoral immune responses of LMP1 transgenic (LMP1tg) mice (*7*) in normal and CD40-deficient ($CD40^{-/-}$) backgrounds (*8, 9*).

We first analyzed the effect of LMP1 expression on the development and activation of B cells in transgenic mice expressing LMP1 (*7*). Because the transgene is under the control of immunoglobulin promoter/enhancer, LMP1 was found to be expressed on $B220^+$ B cells but not on $Thy1.2^+$ T cells in these mice (Fig. 1A) (*10*). Other types of cells prepared from the transgenic mice, such as $CD11b^+$ peritoneal macrophages and $CD11c^+$ splenic dendritic cells, could not be stained with antibody to LMP1 (anti-LMP1). Transgenic LMP1 expression thus appeared to be B cell–specific. Although some fractions of transgenic mice have been shown to develop B cell lymphoma over 12 months (*7*), secondary lymphoid organs such as spleen and lymph nodes of 6- to 8-week-old transgenic mice were macroscopically and histologically normal (*11*). The expression patterns of surface immunoglobulins M and D (IgM and IgD) and ratios of mature and immature B cells in spleen and bone marrow were similar between LMP1tg mice and their control littermates (Fig. 1B) (*10*). However, intercellular adhesion molecule–1 (ICAM-1/CD54), CD23, and Fas/CD95 were more highly expressed in LMP1tg B cells relative to normal B cells. Slight increases in the expression of major histocompatibility complex class II antigen (I-A), CD80, and CD86 were also reproducibly observed on LMP1tg B cells (Fig. 1C). These results indicate that LMP1 expression does not affect antigen-independent B cell development but may activate B cells in vivo.

To further investigate how LMP1 influences B cell activation, we analyzed in vitro proliferation, antibody secretion, and nuclear factor κB (NF-κB) activation of LMP1-expressing B cells (*12*). Thymidine uptake was detected in LMP1tg B cells even without stimulation. Furthermore, IL-4 alone could induce significant enhancement of [^{3}H]thymidine uptake of LMP1tg B cells, whereas proliferation of control B cells also required CD40 ligation (Fig. 2A). LMP1tg B cells also produced substantial amounts of IgG1 as well as IgM in vitro. IL-4 alone could increase amounts of IgM and IgG1 produced by LMP1tg B cells, although antibody secretion of normal B cells was strictly dependent on CD40 ligation (Fig. 2, B and C). LMP1 is known to induce NF-κB activation (*13*). Elevated NF-κB–binding activities were seen in both LMP1tg and $CD40^{-/-}$ LMP1tg B cells even without stimulation (Fig. 2D). However, NF-κB activity was induced in control mice only when stimulated with lipopolysaccharide (LPS) or CD40 ligation. As expected, anti-CD40 failed to induce NF-κB activity in $CD40^{-/-}$ mice.

The flow cytometry and in vitro functional studies of LMP1tg B cells indicate that constitutive LMP1 expression exerts effects on B cells similar to those induced by CD40 ligation, supporting an assumption that LMP1 may mimic CD40 signals when EBV infects and transforms B cells. In addition to in vitro activation, proliferation, and antibody production of B cells, CD40 plays a critical role in the formation of the germinal center (GC), the site of affinity maturation of antibody and generation of memory B cells, both of which are essential for effective humoral responses (*8, 14*). To determine whether LMP1 can mimic CD40 signaling, we investigated the effect of LMP1 on in vivo antibody responses. If LMP1 engages part of the CD40 signal pathway, LMP1 expression may rescue some of the humoral immune responses in $CD40^{-/-}$ mice where Ig class switching and GC formation are defective. LMP1tg and $CD40^{-/-}$ LMP1tg mice were immunized with alum-precipitated (4-hydroxy-3-nitrophenyl)acetyl-conjugated chicken γ-globulin (NP-CGG) (*15*). Anti-NP IgM production was observed in all immunized mice, including $CD40^{-/-}$ mice (Fig. 3A), which is consistent with our previous observation that IgM antibody responses against T-dependent antigen can take place even in the absence of CD40 (*8*). Comparable titers of anti-NP IgG1 were detected in both CD40-expressing control and LMP1tg mice, but not in $CD40^{-/-}$ mice. Interestingly, the anti-NP IgG1 response could be clearly rescued by transgenic LMP1 expression in $CD40^{-/-}$ mice (Fig. 3B). One characteristic of the T-dependent humoral immune response is affinity maturation of antibody. Both CD40-expressing LMP1tg mice and their littermates produced high-affinity anti-NP IgG1 that could bind divalent antigen [bovine serum albumin coupled with two NP haptens (NP_2-BSA)]. On the other hand, anti-NP IgG1 of $CD40^{-/-}$ LMP1tg mice could bind multivalent antigen but not divalent antigen (Fig. 3C), indicating that $CD40^{-/-}$ LMP1tg mice produced only

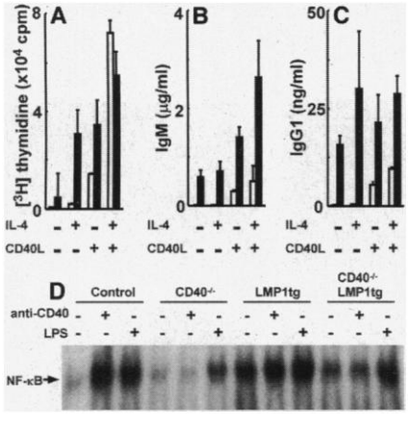

Fig. 2. Effects of LMP1 expression on proliferation, Ig secretion, and NF-κB activation of primary B cells. **(A)** Enhanced in vitro proliferation of LMP1tg B cells. High-density B cells were prepared from LMP1tg mice and their littermates and were cultured with or without IL-4 and CD40L-expressing CHO cells. Data are shown as [^{3}H]thymidine uptake 48 hours after cultivation. **(B** and **C)** Enhanced in vitro IgM and IgG1 production of LMP1tg B cells. Cells were cultured with or without IL-4 and CD40L-expressing CHO cells for 7 days, and amounts of IgM and IgG1 in the culture supernatants were assayed. **(D)** Constitutive activation of NF-κB in LMP1tg B cells. Purified high-density B cells from each LMP1tg mouse were incubated with or without anti-CD40 or LPS. Nuclear extracts were prepared and assayed for NF-κB–binding activity. A sample containing 2.5 μg of nuclear extract per lane was loaded. Similar results of the patterns were seen in three independent experiments. A representative autoradiography is shown.

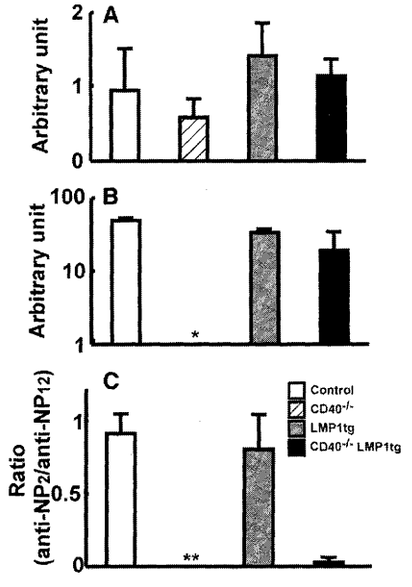

Fig. 3. In vivo T-dependent antibody responses in LMP1tg or $CD40^{-/-}$ LMP1tg mice. Mice were immunized with alum-precipitated NP-CGG; 14 days after immunization, NP-specific immunoglobulins in the sera were measured. **(A)** NP-specific IgM antibody responses. **(B)** NP-specific IgG1 responses (*, not detectable). **(C)** Affinity of NP-specific IgG1 antibodies. Because total NP-specific IgG1 production in $CD40^{-/-}$ mice was not detectable, the ratio is defined as zero (**). More than three mice from each group were used for these experiments.

low-affinity antibodies. Thus, LMP1 could restore class switching but not affinity maturation of antibody.

At the very early stage of antibody response, antigen-specific B cells differentiate into antibody-forming cells in T cell–rich extrafollicular areas of secondary lymphoid organs, particularly along periarteriolar lymphoid sheath (PALS) in spleen (*16*). This B cell differentiation is accompanied by Ig class switching but not affinity maturation. Because most primary anti-NP antibodies bear λ1 chain in the Igh^b mice, PALS-associated B cell foci can be visualized by anti-λ staining (*17*). Spleen sections were prepared 7 days after immunization and stained by anti-λ and anti-γ1 (*18*). B cell foci stained by anti-λ could be observed in immunized control and LMP1tg mice (Fig. 4A). Some λ^+ cells of these mice could be stained by anti-γ1 as well. In $CD40^{-/-}$ and $CD40^{-/-}$ LMP1tg mice, immunoblast-like cells intensely stained by anti-λ were detected dispersedly or as small clusters. Because $CD40^{-/-}$ mice are defective in class switching (*8*), anti-γ1 failed to stain B cell foci of these mice (Fig. 4A), which are probably producing only IgM. On the other hand, a fraction of λ^+ B cells were clearly stained by anti-γ1 in $CD40^{-/-}$ LMP1tg mice (Fig. 4A). This finding is consistent with the results of serum antibody titers and demonstrates that LMP1 expression can rescue PALS-associated B cell differentiation accompanied by Ig class switching in $CD40^{-/-}$ mice.

GC formation was examined 14 days after immunization. Well-developed GCs, which were intensely stained with peanut agglutinin (PNA) (*18*), were observed in the lymphoid follicles of immunized control mice. $CD40^{-/-}$ LMP1tg mice failed to develop GCs, as expected given that they produced only low-affinity anti-NP IgG1 (Fig. 4B). Surprisingly, typical GC formation was not detected in the follicles of LMP1tg even though their sera contained high-affinity anti-NP IgG1 (Fig. 4B). Staining of spleen sections with anti-B220 and anti-Thy1.2 showed that the follicular architectures of both $CD40^{-/-}$ LMP1tg and CD40-positive LMP1tg mice were intact (Fig. 4C), suggesting that LMP1-expressing follicular B cells can differentiate into antibody-producing cells but not form GCs. Thus, LMP1 expression not only fails to rescue GC formation in $CD40^{-/-}$ mice but also actively suppresses GC formation even in the presence of CD40. Although it is not yet known where B cells undergo affinity maturation in LMP1tg mice, similar observations on affinity maturation without GCs have been reported in several mutant mice (*19*). Further studies on hypermutation of the variable region of the Ig chain will be necessary to determine the effect of LMP1 expression on affinity maturation.

Our results show that LMP1 mimics CD40 signals to induce extrafollicular B cell activation and differentiation, accompanied by Ig class switching. However, unlike CD40 signaling, LMP1 blocks B cells from entering the GC pathway. This is consistent with previous observations that in infectious mononucleosis, an exaggerated form of primary EBV infection, EBV^+ B lymphoblasts are detected in extrafollicular areas but not in GCs (*20*), and that $EBNA^+$ cells differentiate toward plasma cells (*21*). It is likely that EBV first infects circulating resting B cells and induces LMP1-dependent extrafollicular B cell activation and differentiation. This contributes to amplification of the viral episome in proliferating cells and will continue until immune responses to EBV, particularly a strong cytotoxic T lymphocyte response, are established. In peripheral blood of EBV carriers, the virus resides latently in long-lived IgD^- memory B cells, which usually develop in GCs (*22*). The EBV-infected memory B cells express only LMP2a but not LMP1.

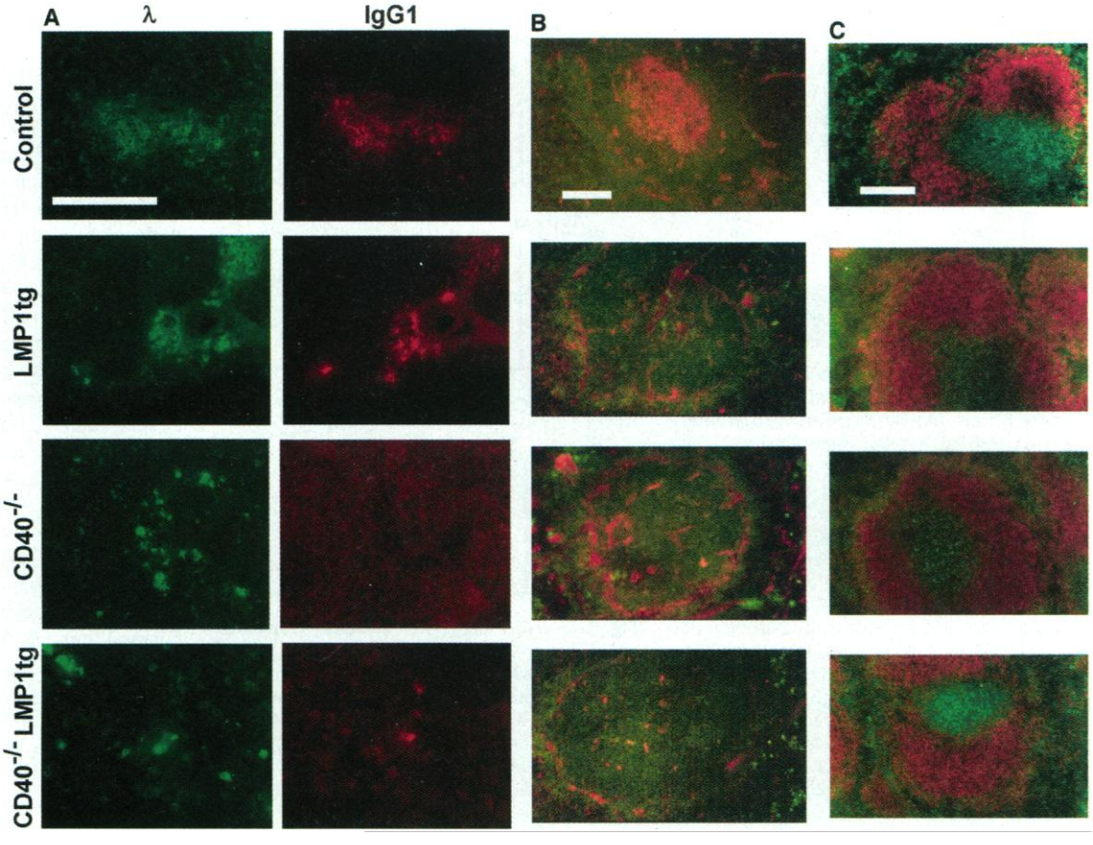

Fig. 4. Immunohistochemical analysis of spleens from LMP1tg or $CD40^{-/-}$ LMP1tg mice during primary immune response. (**A**) Ig class switching in PALS-associated B cell foci of $CD40^{-/-}$ LMP1tg mice. Spleen sections prepared 7 days after immunization were stained with FITC–anti-λ and biotinylated anti-IgG1, followed by Texas Red–conjugated streptavidin. Left and right panels show green (FITC–anti-λ) and red (Texas Red–streptavidin/biotinylated anti-IgG1) fluorescence, respectively. In the $CD40^{-/-}$ spleen, only λ^+ $IgG1^-$ B cells are identified. λ^+ $IgG1^+$ B cells are detected in $CD40^{-/-}$ LMP1tg mice as well as control and LMP1tg mice. Scale bar, 100 μm. (**B**) Defective GC formation in $CD40^{-/-}$ LMP1tg or LMP1tg mice. Spleen sections were prepared 14 days after immunization and stained with FITC–anti-IgM and biotinylated PNA, followed by Texas Red–conjugated streptavidin. Scale bar, 100 μm. (**C**) Spleen sections were stained by FITC–anti-Thy1.2 and biotinylated anti-B220, followed by Texas Red–streptavidin. Scale bar, 100 μm. More than two mice from each group were used.

Similarly, EBV$^+$ Burkitt's lymphomas, transformed counterparts of GC centroblasts, express only EBNA1 but not LMP1 (*23*). Infected B cells express a full spectrum of EBV-encoded products and are vulnerable to the immune system, and it is likely that the ability of LMP1 to prevent these B cells from entering the GC is beneficial for the virus. Only infected B cells that have lost LMP1 expression are eligible to enter the GC pathway; in this way, persistent infection in memory B cells, protected from the host immune system, can be established. This may reflect the loss of expression of the major viral transactivator of LMP1 expression, EBNA2 (*24*). Because antigen reactivity is necessary for B cells to survive under antigen-driven selection in GCs, among B cells that have lost LMP1 expression, only B cells whose corresponding antigens are available can differentiate into memory B cells. This is likely to be a rare event and may account for the low frequency of latently infected B cells in EBV carriers (*25*).

LMP1 and CD40 clearly have differing effects on the induction of GC formation. They share some TRAF molecules such as TRAF1, 2, 3, and 5 as signal transducers (*5*). Both LMP1 and CD40 are associated with Jak3 kinase (*26*). However, there are some differences. CD40, but not LMP1, engages TRAF6 (*6*). LMP1, but not CD40, interacts with TNF receptor 1–associated death domain (TRADD) protein (*27*). The mode of signaling is also different between LMP1 and CD40; that is, LMP1 transduces ligand-independent and constitutive signals (*28*), whereas CD40 signals are ligand-dependent and transient. It is not yet known which interacting molecule(s) or signaling mode is responsible for distinct outcomes of signals. It is also noteworthy that in human B cells LMP1 could down-regulate the expression of BCL-6, a key molecule necessary for GC formation (*29*). Further dissections of LMP1 and CD40 signaling will increase our understanding not only of EBV infection but also of the regulation of the humoral immune response.

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9. LMP1tg mice and CD40-deficient mice were produced as described (*7, 8*). CD40-deficient LMP1tg (CD40$^{-/-}$ LMP1tg) mice were established by crossing CD40$^{-/-}$ mice with LMP1tg mice. The mice we used were all in the background of C57BL/6 and were heterozygous for the transgene. The targeted gene or transgene was identified by polymerase chain reaction of tail DNA. All the mice were maintained in the specific pathogen–free animal facility in accordance with the Osaka University guidelines for animal experimentation.
10. Single-cell suspension was prepared from spleen (by lysing red blood cells with hypotonic buffer) and from bone marrow. Macrophages were prepared from the peritoneum. Splenic dendritic cells were purified as described [J. P. Metlay *et al.*, *J. Exp. Med.* **171**, 1753 (1990)]. For LMP1 staining, cells were fixed with methanol on slide glass and stained by fluorescein isothiocyanate (FITC)–conjugated anti-Thy1.2 (30H12), FITC–anti-B220 (RA3-6B2), FITC–anti-CD11b (Mac-1), or FITC–anti-CD11c (HL3) with biotinylated anti-LMP1 (S-12), followed by Alexa 594–conjugated streptavidin (Molecular Probes). For fluorescence-activated cell sorting analysis, cells were stained with biotinylated anti-CD23 (B3B4), anti-CD95 (Jo2), anti-CD54 (3E2), anti-CD80 (16-10A1), anti-CD86 (GL1), anti–I-A (17/227), or anti-IgD and FITC-conjugated anti-B220 (RA3-6B2) or anti-IgM (R6-60.2), followed by streptavidin-allophycocyanin (Becton-Dickinson). Antibodies were purchased from Pharmingen, except for anti-IgD (Nordic Immunology), anti-LMP1 (provided by E. Kieff), and anti–I-A (provided by M. Kimoto). Flow cytometry was performed on a Facscalibur with Cellquest software (Becton-Dickinson).
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12. Nonadherent splenic B cells were isolated with combination of anti-Thy1.2/CD90 (F7D5, Serotec) and complement. The remaining B cells were further fractionated through percoll gradient to purify high-density B cells, yielding >95% pure B220$^+$ B cells. Purified B cells (10^5 cells per well) were cultured in triplicate in 200 μl of RPMI medium containing 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml), 2 mM L-glutamine, and 50 μM 2-mercaptoethanol in a 96-well plate. Stimulation of B cells was performed with either 5×10^4 cells of irradiated CD40L-expressing CHO cells (provided by H. Yagita) or murine recombinant IL-4 (Genzyme) at 100 U/ml (or both). For proliferation assay, cultures were incubated for 72 hours and [^{3}H]thymidine (1 μCi per well) was added 16 hours before termination of culture. For antibody secretion assay, cells were incubated for 7 days. Amounts of Igs in the culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA) with the sandwich method. For detection of NF-κB activation, 2×10^6 high-density B cells from each mouse spleen were stimulated with medium alone, or with antibody to mouse CD40 (Pharmingen) or LPS (10 μg/ml each), at 37°C for 30 min. Nuclear extracts from stimulated B cells were prepared as described [E. Schreiber, P. Matthias, M. M. Muller, W. Schaffner, *EMBO J.* **7**, 4221 (1988)]. An end-labeled self-complementary oligonucleotide containing the NF-κB site of the mouse H-2K gene was used as a probe for electrophoretic mobility shift assay [T. Fujita, G. P. Nolan, S. Ghosh, D. Baltimore, *Genes Dev.* **6**, 775 (1992)]. Nuclear extract (2.5 μg) was incubated in a buffer containing 25 mM Hepes (pH 8.9), 50 mM KCl, 1 mM EDTA, 1mM dithiothreitol, 10% glycerol, 2.5 μg of poly(deoxyinosine-deoxycytidine), and labeled probe (10^4 cpm) at 30°C for 30 min. Samples were applied to a 5% polyacrylamide gel and fractionated, followed by visualization with autoradiography.
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15. Mice were immunized intraperitoneally with 100 μg of NP_{33}-CGG as an alum-precipitated complex. Total NP-specific isotype antibodies and high-affinity NP-specific IgG1 in the sera at day 14 were measured by ELISA. For detection of NP-specific IgG1, both high-affinity antibodies and total (high- plus low-affinity) antibodies were quantified by means of plates coated with NP_2 and NP_{12}-conjugated BSA, respectively. Bound antibodies were detected with either alkaline phosphatase–conjugated goat antibody to mouse IgM or goat antibody to mouse IgG1 (Southern Biotechnology Associates, Birmingham, AL).
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18. Spleens from mice immunized with NP_{33}-CGG were isolated at the indicated period and were embedded. Frozen sections 6 μm thick were prepared with a cryostat. For the detection of GCs, sections were doubly stained with antibody to mouse IgM (R6-60.2) conjugated with FITC (Pharmingen) and biotinylated PNA (Honen). For PALS-associated B cell foci, sections were stained with biotinylated anti-IgG1 (A85-1, Pharmingen) and FITC–anti-λ (Southern Biotechnology Associates). Biotin-conjugated antibodies were developed with streptavidin-conjugated Texas Red (Gibco BRL).
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References and Notes

[2] **Long-Term Human B Cell Lines Dependent on Interleukin-4 and Antibody to CD40**

Jacques Banchereau; Paolo De Paoli; Alain Vallé; Eric Garcia; Françoise Rousset

Science, New Series, Vol. 251, No. 4989. (Jan. 4, 1991), pp. 70-72.

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[3] **Epstein-Barr Virus Latent Membrane Protein 1 is Essential for B-Lymphocyte Growth Transformation**

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