

Synergistic activity of *Card11* mutant and *Bcl6* in the development of diffuse large B-cell lymphoma in a mouse model

Taishi Takahara,^{1,3,4} Keitaro Matsuo,¹ Masao Seto,² Shigeo Nakamura³ and Shinobu Tsuzuki^{1,5}

¹Division of Molecular Medicine, Aichi Cancer Center, Research Institute, Nagoya; ²Department of Pathology, Kurume University School of Medicine, Kurume; ³Department of Pathology and Clinical Laboratory, Nagoya University Hospital, Nagoya; ⁴Department of Surgical Pathology, Aichi Medical University Hospital, Nagakute; ⁵Department of Biochemistry, Aichi Medical University School of Medicine, Nagakute, Japan

Key words

Bcl6, *Card11*, diffuse large B cell lymphoma, germinal center, mouse model

Correspondence

Shinobu Tsuzuki, Division of Molecular Medicine, Aichi Cancer Center Research Institute, 1-1 Kanokoden, Chikusa-ku, Nagoya, Aichi 464-8681, Japan.

Tel.: +81-52-762-6111; Fax: +81-52-764-2963;

E-mail: stsuzuki@aichi-cc.jp

or

Department of Biochemistry, Aichi Medical University School of Medicine, 1-1 Karimata, Yasago, Nagakute, Aichi 480-1195, Japan.

Tel.: +81-561-62-3311; Fax: +81-561-61-4056;

E-mail: tsuzukis@aichi-med-u.ac.jp

Funding Information

Japan Society for the Promotion of Science (JP15K09492).

Received April 16, 2016; Revised August 5, 2016; Accepted August 13, 2016

Cancer Sci 107 (2016) 1572–1580

doi: 10.1111/cas.13057

Diffuse large B-cell lymphoma (DLBCL) is the most common subtype of malignant lymphoma; it derives from germinal center B cells. Although DLBCL harbors many genetic alterations, synergistic roles between such alterations in the development of lymphoma are largely undefined. We previously established a mouse model of lymphoma by transplanting gene-transduced germinal center B cells into mice. Here, we chose one of the frequently mutated genes in DLBCL, *Card11* mutant, to explore its possible synergy with other genes, using our lymphoma model. Given that *BCL6* and *BCL2* expression and/or function are often deregulated in human lymphoma, we examined the possible synergy between *Card11*, *Bcl6*, and *Bcl2*. Germinal center B cells were induced *in vitro*, transduced with *Card11* mutant, *Bcl6*, and *Bcl2*, and transplanted. Mice rapidly developed lymphomas, with exogenously transduced *Bcl2* being dispensable. Although some mice developed lymphoma in the absence of transduced *Bcl6*, the absence was compensated by elevated expression of endogenous *Bcl6*. Additionally, the synergy between *Card11* mutant and *Bcl6* in the development of lymphoma was confirmed by the fact that the combination of *Card11* mutant and *Bcl6* caused lymphoma or death significantly earlier and with higher penetrance than *Card11* mutant or *Bcl6* alone. Lymphoma cells expressed interferon regulatory factor 4 and PR domain 1, indicating their differentiation toward plasmablasts, which characterize activated B cell-like DLBCL that represents a clinically aggressive subtype in humans. Thus, our mouse model provides a versatile tool for studying the synergistic roles of altered genes underlying lymphoma development.

Diffuse large B-cell lymphoma is the most common subtype of malignant lymphoma. Genetic abnormalities are heterogeneous between DLBCL clinical samples^(1–3) and a single patient harbors multiple genetic abnormalities, making it difficult to determine the genetic abnormalities specifically required for the development of lymphoma.

A number of transgenic and knockin/knockout mouse models have been generated to clarify the functional roles of the given genetic abnormalities in lymphomagenesis,^(4–6) yet many remain unexplored. To explore the combinatorial effects of multiple genetic abnormalities in lymphomagenesis, such genetically modified mice need to be crossed to give rise to mice harboring the genetic abnormalities of interest in combination. However, this strategy is laborious and time-consuming. In addition, B cells of such genetically modified mice undergo SHM in genes encoding immunoglobulin as well as many other genes involved in lymphomagenesis, which provides an additional layer of difficulty in clarifying the roles of the genetic alterations of interest. In an effort to circumvent these difficulties, we previously established a mouse model of lymphoma using *in vitro*-induced mouse GC B cells. The GC B cells were induced *in vitro*, retrovirally transduced with genes or mutated

genes of interest in combination, and transplanted into immunodeficient mice.^(7,8) In this model, the combinatorial effects of multiple genetic abnormalities can be assessed easily by infecting their corresponding retroviral vectors at once. Somatic hypermutation did not occur in the *in vitro*-induced GC B cells, therefore, the role of the genes or mutated genes of interest in the development of the lymphoma could be assessed.^(7–9)

Diffuse large B cell lymphomas are largely classified into two subtypes based on gene expression profile, the GCB and ABC types.⁽¹⁰⁾ Genetic abnormalities that deregulate functions and/or the expression of *BCL6* and *BCL2* frequently occur in both subtypes of human DLBCL. Chromosomal translocations involving *BCL6* that result in the constitutive expression of *BCL6* in B cells are exclusively found in ABC-DLBCL.⁽⁶⁾ Interestingly however, *BCL6* is transcriptionally upregulated by somatic mutations of *MEF2B*⁽¹¹⁾ or evades EP300/CREBBP-mediated inactivation due to mutations in *EP300/CREBBP* genes,^(12,13) in some GCB-DLBCL cases. Likewise, although chromosomal translocations involving *BCL2* that constitutively elevate *BCL2* expression are found almost exclusively in GCB-DLBCL, gene amplification of *BCL2* is observed in ABC-DLBCL.^(14,15) Moreover, *BCL6* and *BCL2* play critical roles in the development and

maintenance of DLBCL. For example, DLBCL cell lines and BCL6-expressing patient-derived DLBCL cells often depend on BCL6 transcription activity for survival.⁽¹⁶⁾ Elevated BCL2 expression promotes clonogenicity of lymphoblastoid cell lines⁽¹⁷⁾ and elicits lymphoma in some, if not all, mouse lymphoma models.⁽¹⁸⁾ However, enhanced activity of *BCL6* or *BCL2* per se is not sufficient to elicit lymphoma. Transgenic mice carrying a *BCL6* (I μ HABCL6 mice)⁽⁴⁾ or *BCL2* transgene under the control of the IGH genes enhancer take almost 1 year to develop lymphomas.^(18,19) Moreover, lymphomas that developed in these mice presented additional genetic abnormalities^(4,20) such as translocation of *Myc*.⁽¹⁸⁾ Lymphoma development was significantly delayed in I μ HABCL6 mice by genetic inactivation of activation-induced cytidine deaminase⁽²¹⁾ or uracil-DNA glycosylase,⁽²²⁾ both of which induce SHM in cancer-related genes in addition to immunoglobulin genes.⁽²³⁾ Together, these findings suggest that functionally enhanced BCL6 or BCL2 requires additional genetic “hits” to elicit lymphoma.

One such candidate “hit” is a somatic mutation of *CARD11*. *CARD11* is mutated in approximately 10% of DLBCL cases,⁽²⁴⁾ being more prevalent in ABC-DLBCL, but also occurring in GCB-DLBCL.^(1,24) *Card11* mutations occur during the process of lymphoma development in a subset of I μ HABCL6 mice.⁽²⁰⁾ In clinical lymphoma samples, *CARD11* mutations are often accompanied by chromosomal translocations, gene amplifications, or mutations that lead to elevated activity of BCL6 or BCL2 (Fig. S1).⁽¹³⁾ *CARD11* is a critical component of the NF- κ B pathway, transmitting B-cell receptor signal to induce transcriptional activation of NF- κ B target genes. *CARD11* mutations occurring in DLBCL activate the NF- κ B pathway even in the absence of B-cell receptor input, providing survival signals, especially in ABC-DLBCL.⁽²⁴⁾ However, mutations of *CARD11* seem insufficient for the development of lymphoma in humans. Only a limited number of affected persons within a family with a germline *CARD11* mutation develop lymphoma.⁽²⁵⁾

In this study, we investigated the possible synergy between *Card11* mutation, *Bcl6*, and *Bcl2*, using our mouse model.

Materials and Methods

Induction of GC B cells, retroviral infection, and transplantation. Induction of GC B cells was carried out as described previously.^(7,9) Briefly, B220⁺ B cells were isolated from the spleens of C57BL/6N mice using antibodies for B220 (RA3-6B2; eBioscience, San Diego, CA, USA) in combination with MACS columns (Miltenyi Biotec, Bergisch Gladbach, Germany). Isolated B cells were co-cultured with X-irradiated (150 Gy) NIH3T3 cells engineered to express mouse CD40 ligand and mouse Baff (3T3/CD40L/Baff cells) in RPMI-1640 medium (Gibco, Carlsbad, CA, USA) supplemented with 10% FCS, 5×10^{-5} M 2-mercaptoethanol, 1 mM sodium pyruvate, 100 units/mL each of penicillin and streptomycin (Gibco), and recombinant interleukin-4 (1 ng/mL; PeproTech, Rocky Hill, NJ, USA). Four days later, the B cells were transferred onto a new feeder layer and cultured with recombinant interleukin-21 (10 ng/mL; PeproTech) for 4 days to induce GC B cells. The iGCB cells were filtered through 45- μ m nylon mesh to remove feeder cells and spin-infected with the respective virus using RetroNectin (Takara Bio, Shiga, Japan) coated plates twice a day on days 3–5 after initiation of the culture. Cells were seeded back onto the feeder layer after infection. On day 8, gene-transduced iGCB cells (1×10^7 cells) were transplanted i.p. into sublethally irradiated (2 or 2.5 Gy) NOD-SCID mice (NOD.CB17-Prkdc;

Jackson Laboratory, Bar Harbor, ME, USA) (Fig. S2). All animal experiments were carried out according to protocols approved by the Institutional Animal Care and Use Committee at the Aichi Cancer Center (Nagoya, Japan).

Plasmids. cDNA for mouse *Bcl6* was amplified from mouse spleen cDNA by PCR and cloned into MSCV-*Bcl6*-ires-human (h) CD4 retroviral vector. cDNA for mouse *Bcl2* was cloned into MSCV-*Bcl2*-ires-human (h) CD8, as previously described.⁽²⁶⁾ pMX-*Card11*^{L232LI}-ires-GFP was a generous gift from Dr. Keisuke Horikawa (The Australian National University, Canberra, Australia)⁽²⁷⁾ (Fig. S3). These plasmids were co-transfected with MCV-Ecopac plasmid (a generous gift from Dr. Richard Van Etten, Tufts-NEMC Cancer Center, Boston, MA, USA)⁽²⁸⁾ into 293T cells to produce retroviruses.

Flow cytometry. Flow cytometric analysis was undertaken using antibodies for mouse B220 (RA3-6B2), IgG₁ (X-56; Miltenyi Biotec), Fas (15A7), B7-1/CD80 (16-10-A1), IgM (II/41), CD138 (281-2), human CD4 (RPA-T4), and human CD8 (RPA-T8). Anti-mouse IgG₁ antibody (M1-14D12) was used as an isotype-matched control for anti-human CD4 antibody. Antibodies were purchased from eBioscience, except for X-56. Flow cytometric analysis was carried out using a FACSCalibur instrument (BD Biosciences, San Jose, CA, USA) and FlowJo software version 7.6.5 (Tree Star, Ashland, OR, USA).

Western blot analyses. Western blot analyses were carried out using anti-BCL6 (D-8; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-CARD11 (#4440; Cell Signaling Technology, Danvers, MA, USA), anti-IRF4 (M-17; Santa Cruz Biotechnology), anti-PRDM1 (6D3; Santa Cruz Biotechnology), and anti-actin (AC-40; Sigma-Aldrich, St. Louis, MO, USA), anti-BCL2 (3F11; BD Pharmingen, San Diego, CA, USA), anti- α -tubulin (DM1A; Sigma-Aldrich), anti-Lamin B1 (L-5; Thermo Fisher Scientific, Rockford, IL, USA), anti-p65 (C-20; Santa Cruz Biotechnology) antibodies. Cytosolic and nuclear extracts were obtained using the Nuclear Extract Kit (Active Motif, Carlsbad, CA, USA).

Analysis of cell clonality and SHM. To analyze the clonality and SHM of IGH genes in lymphoma cells, isolated genomic DNA was subjected to PCR using Ex Taq (Takara Bio) or KOD-plus2 (Toyobo, Osaka, Japan) along with primer sets that amplify the VDJ regions of IGH genes, as described previously.^(29,30) Combinations of forward (VHA, VHE, or VHJ) and reverse (JH4 intron) primers were used. The resulting PCR products were gel-purified, cloned into the pCR4-TOPO vector (Invitrogen, Carlsbad, CA, USA), and sequenced. Every single plasmid was deemed to represent a distinct molecularly cloned sequence and, here, defined as a molecular clone. VDJ gene usage and somatic mutations were analyzed using IGBLAST (<http://www.ncbi.nlm.nih.gov/igblast/>). To determine the presence of intraclonal heterogeneity in lymphoma cells, confirmed mutations and unconfirmed mutations were defined as follows:⁽³¹⁾ confirmed mutation, substitution mutations observed in more than one molecular clone sharing identical VDJ-rearranged sequence from the same tumor sample; unconfirmed mutation, substitution mutations observed in a single molecular clone from the same tumor sample. Only the confirmed mutations were used for the analysis of intraclonal heterogeneity, or ongoing SHM. The unconfirmed mutations were disregarded because they can be caused by PCR error.

Results

Tumorigenicity of *Card11*^{L232LI}, *Bcl6*, and *Bcl2*-transduced cells. To explore genes or mutated genes that collaborate with

CARD11 mutant in lymphomagenesis, we reviewed published results of next generation sequencing of clinical samples, with special reference to those potentially enhancing the function of *BCL6* or *BCL2*.⁽¹³⁾ Of 12 lymphoma cases harboring *CARD11* mutation, chromosomal translocations involving *BCL6* and *BCL2* existed in three and six cases, respectively, and mutations of *CREBBP* or *MEF2B* existed in two cases each (Fig. S1). The expression of *BCL6* and *BCL2* is under the control of heterotopic enhancer through chromosomal translocations. *CREBBP* mutant lost its ability to inactivate *BCL6* by acetylation,⁽¹²⁾ and *MEF2B* mutant can enhance *BCL6* expression.⁽¹¹⁾ Notably, *BCL6* translocations and mutations of *CREBBP* or *MEF2B* are mutually exclusive, suggesting that they collaborate with *CARD11* mutant in a non-redundant manner in the development of lymphoma. Taken together, *CARD11* mutations often co-exist with functionally enhanced *BCL6* or *BCL2*.

Therefore, we first investigated the possible collaboration between *CARD11* mutant, *BCL6*, and *BCL2* in lymphoma development. To this end, we used iGCB cells as a target for the transduction of *Card11* mutant, *Bcl6*, and *Bcl2* genes, given that DLBCL originates in GC B cells. B220⁺ murine B cells were isolated from the spleen of C57BL/6N mice, induced into GC B cells in culture, and retrovirally transduced with *Card11*^{L232LI}, *Bcl6*, and *Bcl2*. Mouse *Card11*^{L232LI} corresponds to the human *CARD11*^{L225LI}, an activating mutant. *Card11*^{L232LI}, *Bcl6*, and *Bcl2* were programmed to co-express

GFP and the extracellular domains of human CD4 and CD8, respectively, as surrogate markers enabling the identification of the transduced cells by flow cytometry (Fig. S3). Gene-transduced iGCB cells were then transplanted into immunodeficient mice, which were monitored for the development of lymphoma (Fig. S2). Experiments were independently carried out three times, using one (exp. 1), three (exp. 2), and two (exp. 3) mice, respectively. From a total of six mice transplanted with *Card11*^{L232LI}/*Bcl6*/*Bcl2* triply transduced iGCB cells (referred to as “*Card11*^{mut}+*Bcl6* + *Bcl2* #1 to #6”), three mice were dissected while alive when disease signs (either tumor mass coupled with ascites or hepatomegaly) manifested (*Card11*^{mut}+*Bcl6* + *Bcl2* #1 to #3, Table S1). The remaining three mice were found dead, with two mice showing histologically evident lymphomas (*Card11*^{mut}+*Bcl6* + *Bcl2* #5 and #6); histological analysis could not be carried out on one mouse (*Card11*^{mut}+*Bcl6* + *Bcl2* #4). In total, five mice developed lymphomas and one died of unknown cause within 2 months of transplantation (Fig. 1a). Mice that developed lymphomas manifested apparent tumor masses (*Card11*^{mut}+*Bcl6* + *Bcl2* #1 and #2) and/or hepatic involvement (*Card11*^{mut}+*Bcl6* + *Bcl2* #2, #3, #5, and #6), which were accompanied by infiltration of atypical lymphoid cells morphologically consistent with DLBCL (Fig. S4).

To determine which of the three genes (*Card11*^{L232LI}, *Bcl6*, and *Bcl2*) contributed to the development of lymphomas in mice, we examined the expression of surrogate markers, GFP

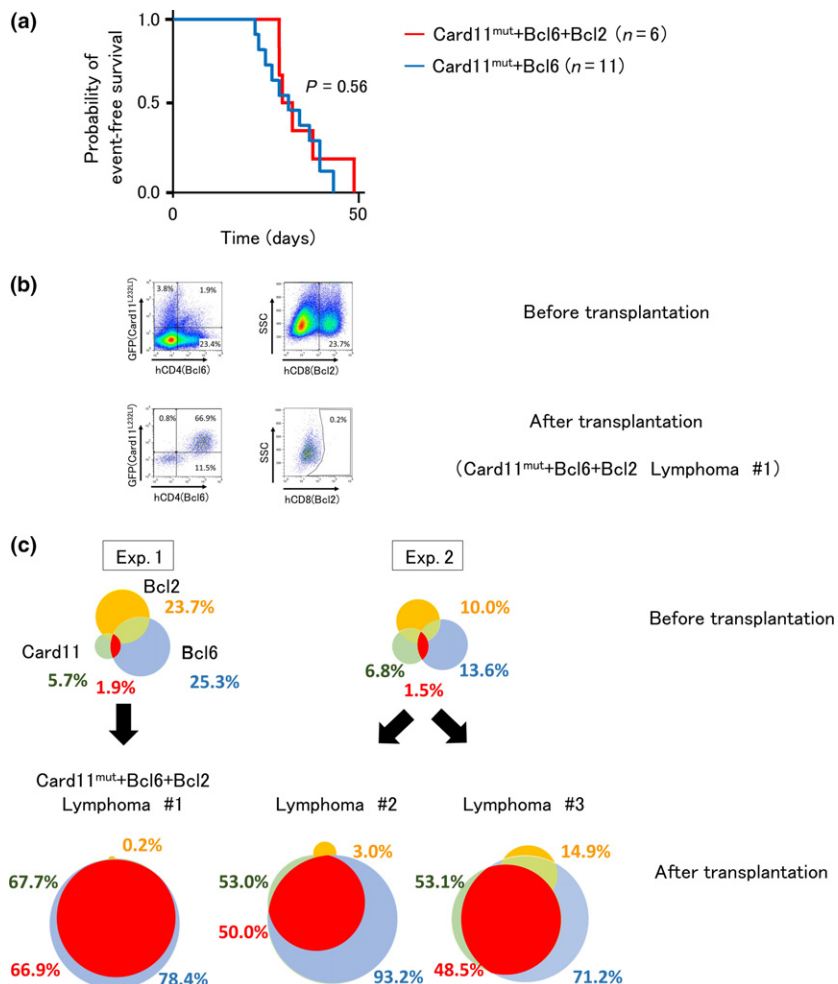


Fig. 1. Tumorigenicity of *Card11*^{L232LI} and *Bcl6* with/without *Bcl2*-transduced germinal center B cells. (a) Kaplan-Meier analysis of the probability of event-free survival of mice transplanted with induced germinal center B (iGCB) cells transduced with *Card11*^{L232LI}, *Bcl6*, and *Bcl2* (red line). An event is defined as the presence of lymphoma on dissection or death. The probability of event-free survival of the mice transplanted with iGCB cells transduced with *Card11*^{L232LI} and *Bcl6* is shown by the blue line. The difference was not statistically significant ($P = 0.56$), as determined by the log-rank test. (b) Flow cytometric analysis of gene-transduced iGCB cells before transplantation (upper panels; experiment [Exp.] 1) and cells in lymphoma that developed after transplantation in a mouse (lower panels). Percentages of cells expressing GFP (representing *Card11*^{L232LI}), hCD4 (*Bcl6*), and hCD8 (*Bcl2*) are depicted and truncated after the decimal point. (c) Venn diagrams showing the percentages of cells expressing exogenously transduced *Card11*^{L232LI} (GFP, green circles), *Bcl6* (hCD4, blue circles), and *Bcl2* (hCD8, yellow circles) before (upper panel) and after transplantation, on lymphoma development (lower panel). One (#1) and two (#2 and #3) mice developed lymphoma in two independent experiments. Percentages of cells co-expressing *Card11* and *Bcl6* are shown in red. The remaining three out of six mice used in the experiment were found dead. Thus, viable cells were unavailable for flow cytometric analysis.

for *CARD11^{L232LI}*, hCD4 for *Bcl6*, and hCD8 for *Bcl2*, on the cells by flow cytometry. As viable tumor cells were only available from the three mice dissected while alive (see description above), our analysis was necessarily confined to them (*Card11^{mut}+Bcl6* + *Bcl2* #1–#3). Before transplantation, the percentages of cells expressing surrogate markers for the three transduced genes were variable and only a small fraction of the cells co-expressed hCD4 or hCD8 along with GFP. In contrast, almost all cells obtained from the developed lymphomas co-expressed both GFP and hCD4, with only a small fraction expressing hCD8, as exemplified in *Card11^{mut}+Bcl6* + *Bcl2* #1 (Fig. 1b). A similar analysis was conducted in *Card11^{mut}+Bcl6* + *Bcl2* #2 and #3 before (Fig. S5, exp. 2) and after lymphoma development (Fig. S6a) and the results are summarized using a Venn diagram (Fig. 1c). These findings suggest that the cells transduced with *Card11^{L232LI}* and *Bcl6* grew selectively among the cells transduced with any possible

combinations of the three genes. *Bcl2* seemingly played only a limited role, if at all, in the development of lymphomas in this model.

Tumorigenicity of *Card11^{L232LI}* and *Bcl6* -transduced cells. We next tested if a combination of *Card11* mutant and *Bcl6* is sufficient for lymphoma development in separate experiments. Induced GCB cells were transduced with the two genes and transplanted. All mice ($n = 11$) developed lymphoma or died within 2 months of transplantation with rapidity and penetrance comparable with those of mice transplanted with cells transduced with *Card11^{L232LI}*, *Bcl2*, and *Bcl6* (referred to as “*Card11^{mut}+Bcl6* #1 to #11”); Fig. 2a, Table S1). Data were combined from four experiments carried out independently, using one (exp. 4), three (exp. 5), four (exp. 6), and three (exp. 7) mice. Seven mice were dissected while alive when disease signs manifested (Table S1, Fig. S7) and tumor cells were isolated and analyzed by flow cytometry

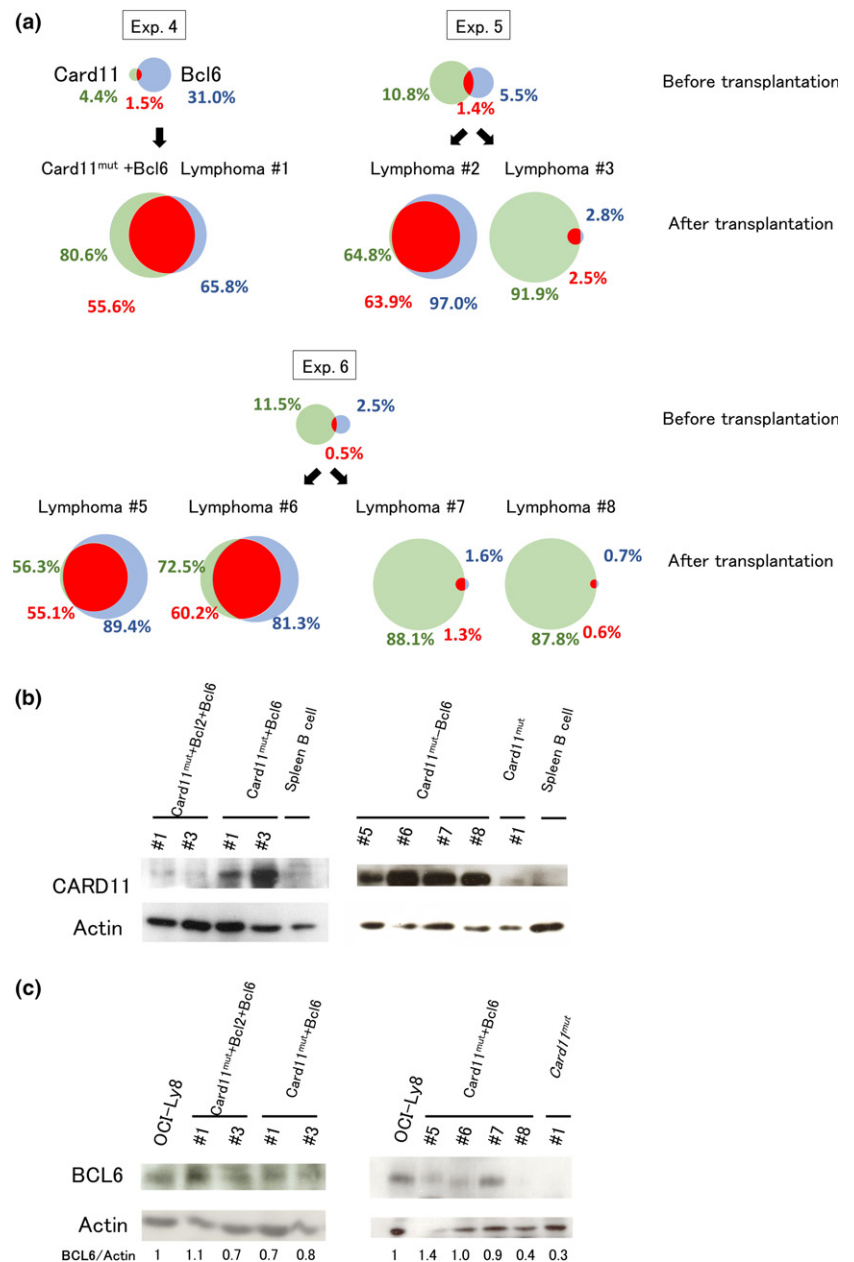


Fig. 2. Synergistic activity of *Card11^{L232LI}* and *Bcl6*. (a) Venn diagrams showing the percentages of cells expressing the surrogate markers for exogenously transduced *Card11^{L232LI}* (GFP, green circles) and *Bcl6* (hCD4, blue circles). Fractions of cells coexpressing GFP and hCD4 are shown in red. The remaining four mice, out of 11 mice used in the experiment (Exp.), were found dead. Thus, viable cells were unavailable for flow cytometric analysis. (b) Expression of CARD11 in the developed lymphoma. (c) BCL6 expression in the developed lymphoma. BCL6 protein levels are normalized to the levels of actin (protein loading control) and BCL6/actin ratios are presented and compared to that of OCI-Ly8, a human lymphoma cell line, being set to 1.0.

(*Card11*^{mut}+*Bcl6* #1–#3, #5–#8; Figs. 2a, S6b). Of the remaining four mice dissected after death, one had histologically evident lymphoma (*Card11*^{mut}+*Bcl6* #11; Fig. S4) and one had no histologically detectable tumor (*Card11*^{mut}+*Bcl6* #10). Detailed analysis could not be undertaken on the remaining two mice (*Card11*^{mut}+*Bcl6* #4 and #9). Flow cytometric analysis revealed that *Card11*^{L232LI}-transduced cells always constituted the major component of lymphomas (Fig. 2a), although the protein expression level of CARD11 was variable (Fig. 2b) between developed lymphoma cells. In contrast, the proportion of *Bcl6*-transduced cells in the developed lymphomas varied between developed lymphomas. Only a small fraction of cells co-expressed both *Card11*^{L232LI} and *Bcl6* before transplantation. Cells co-expressing *Card11*^{L232LI} and *Bcl6* became dominant in four of seven mice (*Card11*^{mut}+*Bcl6* #1, #2, #5, and #6) (Fig. 2a). These findings suggest that exogenously transduced *Card11*^{L232LI} and *Bcl6* synergistically induced lymphomas in these mice, as was the case in mice transplanted with *Card11*^{L232LI}/*Bcl6*/*Bcl2* transduced cells. Nevertheless, in the remaining three mice (*Card11*^{mut}+*Bcl6* #3, #7, and #8), lymphomas mainly consisted of cells expressing only the surrogate marker for *Card11*^{L232LI}, suggesting that *Bcl6* was not required for the development of these lymphomas. However, Western blot analysis revealed that, in two lymphomas (*Card11*^{mut}+*Bcl6* #3 and #7), endogenous BCL6 was expressed at a level comparable to those of lymphoma cells expressing the exogenous *Bcl6* gene with its surrogate marker and in a human GCB-DLBCL cell line, OCI-Ly8, which harbors *BCL6* rearrangement (Fig. 2c).⁽³²⁾ This finding implies that endogenous BCL6 was somehow upregulated, or rare clones with high endogenous BCL6 expression were selected during the course of lymphoma development in these recipient mice. Taken together, these findings suggest that *Card11* mutant collaborates with exogenous or endogenous *Bcl6* for the development of lymphoma.

Interestingly, in one (*Card11*^{mut}+*Bcl6* #8) of seven mice, BCL6 was undetectable in lymphoma cells (Fig. 2c), raising the possibility that transduced *Card11*^{L232LI} might induce lymphoma without the help of BCL6 in a limited number of cases. Indeed, in separate experiments using five mice transplanted with *Card11*^{L232LI} singly transduced cells, although lymphoma developed, the onset was significantly ($P = 1.6 \times 10^{-3}$) delayed and the penetrance was lower than that in mice transplanted with *Card11*^{L232LI}/*Bcl6* co-transduced cells. Only two of five mice receiving *Card11*^{L232LI} singly transduced cells developed lymphoma (referred to as “*Card11*^{mut} #1 and #2”) (Fig. 3; see also Fig. S6c, Table S1). We therefore do not exclude the possibility that *Card11* mutant is able to induce lymphoma by itself, but our results clearly show that *Bcl6* facilitates *Card11*-mediated lymphomagenesis. Three mice receiving *Bcl6* only transduced iGCB cells died of unknown reasons ($n = 1$; 123 days after transplantation) or had no detectable lymphoma when killed ($n = 2$; 132 and 141 days after transplantation), suggesting that BCL6 alone is insufficient to induce lymphoma.

Clonality and properties of lymphomas developed in mice. We next analyzed the clonality of lymphomas. The VDJ regions of IGH genes were PCR amplified, cloned into plasmids, and sequenced. The results revealed that these tumors were oligoclonal (*Card11*^{mut}+*Bcl6* #1) or monoclonal (*Card11*^{mut}+*Bcl6* #2 and #3) (Fig. 4a), suggesting that additional genetic or epigenetic factors, in addition to transduced genes, were needed for the development of lymphoma in our mouse model. *Card11*^{mut}+*Bcl6* #2 and #3 lymphoma had only one VDJ

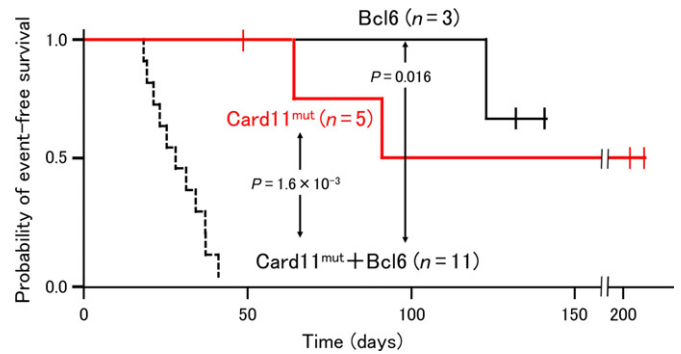


Fig. 3. Tumorigenicity of induced germinal center B cells singly transduced with *Card11*^{L232LI} or *Bcl6*. Kaplan–Meier analysis of the probability of event-free survival of the mice receiving induced germinal center B cells transduced with *Card11*^{L232LI} (red line) or *Bcl6* alone (black line). An event is defined as the presence of lymphoma on dissection or death. The probability of event-free survival was compared with that of mice transplanted with cells transduced with *Card11*^{L232LI} and *Bcl6* (dashed line). The difference in the probability of event-free survival was statistically significant ($P = 1.17 \times 10^{-4}$), as determined by the log-rank test. *Post-hoc* pairwise comparison was undertaken using Bonferroni correction. All *P*-values <0.05 are shown in the figure.

rearranged sequence without confirmed mutations through analysis of multiple plasmid clones, whereas *Card11*^{mut}+*Bcl6* #1 showed three distinct VDJ rearrangements, namely (IGHV1-26*01, IGHD1-1*01, IGHJ2*01), (IGHV1-26*01, IGHD2-3*01, IGHJ3*01), and (IGHV1-80*01, IGHD1-1*01, IGHJ3*02) (Table S2). Among these, only molecular clones using (V: 1-26*01, D: 2-3*01, J: 3*01) presented confirmed mutations, while the others did not. Ten molecular clones using (V: 1-26*01, D: 2-3*01, J: 3*01) presented identical confirmed mutations with no evidence of occurrence of ongoing SHM. The confirmed mutations were unlikely to be induced by PCR error, as they were identified in two independent experiments using different enzymes (Ex Taq and KOD-plus2). These findings indicate that, in our model, lymphomas lacked ongoing SHM.

The tumor cells were positive for B220 (a pan-B-cell marker),⁽³³⁾ IgM, and B7-1 (an activated B-cell marker) (Fig. 4b).⁽³⁴⁾ They also expressed GC markers such as Fas,⁽³⁵⁾ although the expression levels were variable between lymphomas. Intriguingly, these tumor cells also expressed CD138 (a plasma cell marker), albeit weakly, and PRDM1 (a plasma cell differentiating transcription factor), suggesting that they differentiated toward plasmablasts (Fig. 4c).⁽³⁶⁾

Both IRF4 (Fig. 4d) and BCL2 (Fig. S8), known transcriptional targets of the NF- κ B pathway, were highly expressed, consistent with nuclear translocation of p65 (RelA) that shows NF- κ B activation (Fig. S9). Together with high expression of FOXP1 (Fig. 4e), a widely used classifier of ABC-DLBCL in humans,⁽³⁷⁾ these findings show that lymphoma cells developed in mice are consistent with DLBCL. Notably, all four secondary recipient mice transplanted with lymphoma cells derived from the primarily transplanted mice rapidly developed lymphoma within 20 days (Fig. S10), indicating the tumor-propagating activity of the lymphomas; therefore, they are bona fide tumors.

Discussion

In this study, we showed the synergistic activity of *Card11*^{L232LI} and *Bcl6* in lymphomagenesis in a mouse model. Evidence supporting the synergy between the two genes in the

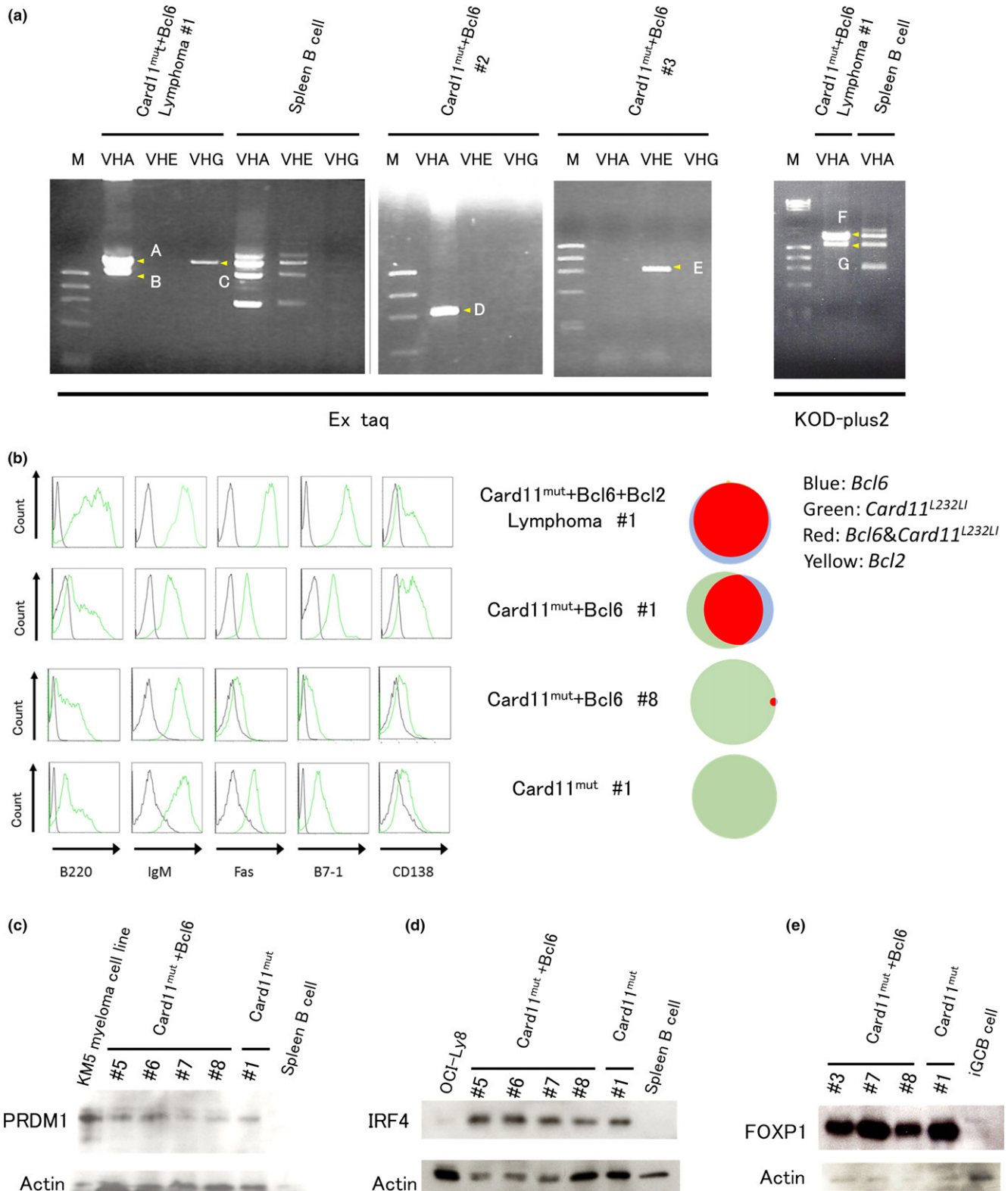


Fig. 4. Clonality and properties of lymphomas. (a) Immunoglobulin heavy chain genes amplified by PCR from genomic DNA of lymphoma cells using combinations of VHA/VHE/VHG (forward) and JH4 intron (reverse) primers. PCR products amplified by Ex Taq or by KOD-plus2 were fractionated by gel electrophoresis and bands were marked by A–E and F–G, respectively (yellow arrowheads). These amplified products were cloned into a plasmid vector and sequenced. Amplified products from genomic DNA of normal, non-transduced spleen B cells were also electrophoresed as a control, to show their polyclonal status. M, markers. (b) Phenotype of the developed lymphoma cells as assessed by flow cytometry. Cells in the developed lymphoma were analyzed for the expression of the indicated molecules by flow cytometry (left). Control samples are represented by black lines. The fractions of cells that expressed each surrogate marker of transduced genes (GFP, *Card11^{L232LI}*; hCD4, *Bcl6*; hCD8, *Bcl2*) of the corresponding tumor samples are represented by the Venn diagram (right). (c–e) Expression of PR domain 1 (PRDM1) (c), interferon regulatory factor 4 (IRF4) (d), and forkhead box P1 (FOXP1) (e) in developed lymphoma, as detected by Western blotting.

development of lymphoma are threefold: (i) iGCB cells doubly transduced with *Card11*^{L232LI} and *Bcl6* grew selectively over singly transduced cells in 7 out of 10 mice receiving cells transduced with *Card11*^{L232LI}/*Bcl6*/*Bcl2* ($n = 3$, Fig. 1c) or *Card11*^{L232LI}/*Bcl6* ($n = 4$, Fig. 2a) and these mice rapidly succumbed to lymphoma; (ii) although some lymphomas (in two of the remaining three mice) did not express the surrogate marker of exogenously transduced *Bcl6*, the expression of intrinsic BCL6 was elevated (Fig. 2c); and (iii) mice transplanted with cells transduced with *Card11*^{L232LI} and *Bcl6* developed lymphoma with significantly higher incidence and shorter latency than those transplanted with cells transduced with either one of the genes (Fig. 3).

Although synergistic, a combination of *Card11* mutant and *Bcl6* seems insufficient for lymphoma development, based on the following observations. First, lymphomas developed in mice that had oligoclonal or monoclonal IGH rearrangements, suggesting that not every single clone but a small number of clones contributed to the development of lymphoma (Fig. 4a). Second, our preliminary use of immunocompetent mice as recipients of *Card11*^{L232LI}/*Bcl6* transduced iGCB cells reduced the incidence of lymphoma compared with immunodeficient mice. Although the reasons for the difference are not clear, evasion from immunosurveillance may facilitate lymphoma development, as suggested by the findings that clinical lymphomas often harbor mutations in immunosurveillance-associated genes.⁽³⁸⁾

Lymphomas developed in our model were consistent with ABC-DLBCL, among others, in that they expressed IRF4^(10,39) and FOXP1,⁽³⁷⁾ molecular markers of ABC-DLBCL, and PRDM1, critically involved in post-GC B cell differentiation.^(40,41) They also showed evidence of NF- κ B activation.⁽⁴²⁾ Lack of ongoing SHM, the hallmark of GCB-DLBCL⁽⁴³⁾ further supports the identity as ABC-DLBCL.

The mechanism by which *Bcl6* and *Card11*^{L232LI} might collaborate in lymphomagenesis remains unclear. Mutant CARD11 forms a complex with BCL10 and MALT1 and activates the NF- κ B pathway, independently of B-cell receptor stimulation.⁽⁴⁴⁾ Although *Card11*^{L232LI} activates NF- κ B signaling as such, the active NF- κ B signaling per se is insufficient for lymphoma development. Expression of constitutive active *IKK2ca* in GC B cells in mice activates NF- κ B, leading to enhanced cell proliferation, however, at the same time, provokes differentiation into plasma cells, failing to induce lymphoma development. The constitutive activation of NF- κ B and the blockade of terminal differentiation into plasma cells therefore appear to be required for the development of ABC-DLBCL.⁽⁴⁵⁾ The activated canonical NF- κ B pathway phosphorylates p65, which translocates into the nucleus, resulting in transcription of its target genes, including *IRF4*,^(41,46) which, in turn, induces expression of *PRDM1*,^(47,48) a transcription factor, which triggers the terminal differentiation into plasma cells. *PRDM1* also functions as a tumor suppressor gene,⁽⁶⁾ as the encoded protein inhibits the survival and proliferation of B cells.⁽⁴⁹⁾ When combined with conditional knockout of *Prdm1*, *IKK2ca* expressed in GC B cells elicits ABC-DLBCL.⁽⁵⁾ The fact that *PRDM1* is biallelically inactivated in human DLBCL⁽⁶⁾ supports the idea that complete, rather than partial repression of *PRDM1* is required for malignant transformation of B cells. Given that BCL6 represses *Prdm1* transcription in cell lines⁽⁵⁰⁾ and that *BCL6* translocation and *PRDM1* inactivation are almost mutually exclusive in human DLBCL cases,⁽⁶⁾ expression of *BCL6* is anticipated to collaborate with the NF-

κ B signaling activated by *CARD11* mutant in lymphomagenesis, but this possibility has not been tested in animal models. Thus, our study provides an experimental evidence for the collaboration of the two genes. Although functions of BCL6 are partially mediated by the repression of *Prdm1*, lymphoma cells in our mouse model expressed PRDM1, albeit at lower levels than a myeloma cell line, suggesting incompleteness of the BCL6-mediated suppression of PRDM1, in stark contrast with PRDM1 knockout mice that recapitulates the biallelic inactivation of PRDM1 in human DLBCL.⁽⁶⁾ Therefore, our findings raise the possibility that *BCL6* may have other functions besides repressing *PRDM1*. Mutant *Card11* activates JNK,⁽²⁷⁾ in addition to the NF- κ B pathway, increasing the amount of phosphorylated c-Jun.⁽⁵¹⁾ BCL6 modulates the transcriptional activity of c-Jun through physical interaction,⁽⁵²⁾ which may represent an additional mechanism underlying the collaboration between *CARD11* and *BCL6*. However, further studies are warranted to investigate the role of this pathway in the development of lymphoma.

Although exogenously expressed BCL2 was dispensable for lymphomagenesis in our model, endogenous BCL2 expression was elevated in lymphoma cells induced by co-transduction of *Card11*^{L232LI} and *Bcl6*. Given that activated NF- κ B directly drives BCL2 expression,^(53–55) elevated BCL2 expression in our lymphoma, even without exogenous *Bcl2*, may reflect NF- κ B activation owing to *Card11*^{L232LI} transduction. This may at least partly explain elevated BCL2 expression in some human ABC-DLBCL cases without *BCL2* translocation.⁽¹⁵⁾ Although some DLBCL cases harbor both *CARD11* mutation and *BCL2* translocation, the sequence of these two events are not always known. In contrast, the two events happened almost simultaneously when induced in our experiments.

A mouse model of lymphoma, in which a mutant *CARD11* is expressed from the embryonic stage in entire B cell fractions, has recently been reported. However, the animals developed lymphoma-like disease very rapidly after birth and with 100% penetrance,⁽⁵¹⁾ in contrast to the low incidence of lymphoma (only two out of five mice) in our mice transplanted with *Card11*^{L232LI} only transduced iGCB cells. The difference in penetrance and rapidity of lymphoma development may reflect the difference in fractions of B cells (pan-B cells vs GC B cells) and the developmental stage (embryonic vs adult) in which *CARD11* is expressed. Human lymphomas usually develop in advanced age and, indeed, even in patients with germline *CARD11* mutation, the development of monoclonal B-cell disease culminates only in their 50s, despite polyclonal B-cell lymphocytosis coupled with splenomegaly and follicular hyperplasia being observed during their childhood.^(25,56) Together, these findings support the notion that *CARD11* mutant needs additional genetic abnormalities to induce lymphoma.

CARD11 mutations are prevalent in ABC-DLBCL,^(1,24) a subtype of DLBCL, with poor prognosis.^(37,57) Although ibrutinib, a Bruton tyrosine kinase inhibitor, is a promising drug for ABC-DLBCL, it is ineffective for ABC-DLBCL cases with *CARD11* mutations,⁽⁵⁸⁾ emphasizing the need for the development of new drugs. Our mouse model may help elucidate the molecular mechanisms underlying the development of DLBCL with *CARD11* mutations to develop effective therapeutic agents.

Acknowledgments

We thank Seiko Sato for help with animal husbandry.

Disclosure Statement

Shinobu Tsuzuki received research grant from the Japan Society for the Promotion of Science (JSPS KAKENHI Grant Number JP15K09492).

Abbreviations

ABC	activated B cell-like
BCL	B-cell lymphoma
CARD11	caspase recruitment domain family member 11
CREBBP	CREB binding protein

DLBCL	diffuse large B-cell lymphoma
exp.	experiment
FOXP1	forkhead box P1
GC	germinal center
GCB	germinal center B cell
iGCB	induced germinal center B cell
IGH	immunoglobulin heavy chain
IRF	interferon regulatory factor
NF- κ B	nuclear factor- κ B
PRDM1	PR domain 1
SHM	somatic hypermutation

References

- Pasqualucci L, Trifonov V, Fabbri G *et al.* Analysis of the coding genome of diffuse large B-cell lymphoma. *Nat Genet* 2011; **43**: 830–7.
- Zhang J, Grubor V, Love CL *et al.* Genetic heterogeneity of diffuse large B-cell lymphoma. *Proc Natl Acad Sci USA* 2013; **110**: 1398–403.
- Morin RD, Mungall K, Pleasance E *et al.* Mutational and structural analysis of diffuse large B-cell lymphoma using whole-genome sequencing. *Blood* 2013; **122**: 1256–65.
- Cattoretti G, Pasqualucci L, Ballon G *et al.* Deregulated BCL6 expression recapitulates the pathogenesis of human diffuse large B cell lymphomas in mice. *Cancer Cell* 2005; **7**: 445–55.
- Calado DP, Zhang B, Srinivasan L *et al.* Constitutive canonical NF- κ B activation cooperates with disruption of BLIMP1 in the pathogenesis of activated B cell-like diffuse large cell lymphoma. *Cancer Cell* 2010; **18**: 580–9.
- Mandelbaum J, Bhagat G, Tang H *et al.* BLIMP1 is a tumor suppressor gene frequently disrupted in activated B cell-like diffuse large B cell lymphoma. *Cancer Cell* 2010; **18**: 568–79.
- Arita K, Maeda-Kasugai Y, Ohshima K *et al.* Generation of mouse models of lymphoid neoplasm using retroviral gene transduction of in vitro-induced germinal center B and T cells. *Exp Hematol* 2013; **41**: 731–41 e9.
- Arita K, Tsuzuki S, Ohshima K, Sugiyama T, Seto M. Synergy of Myc, cell cycle regulators and the Akt pathway in the development of aggressive B-cell lymphoma in a mouse model. *Leukemia* 2014; **28**: 2270–2.
- Nojima T, Haniuda K, Moutai T *et al.* In-vitro derived germinal centre B cells differentially generate memory B or plasma cells in vivo. *Nat Commun* 2011; **2**: 465.
- Alizadeh AA, Eisen MB, Davis RE *et al.* Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. *Nature* 2000; **403**: 503–11.
- Ying CY, Dominguez-Sola D, Fabi M *et al.* MEF2B mutations lead to deregulated expression of the oncogene BCL6 in diffuse large B cell lymphoma. *Nat Immunol* 2013; **14**: 1084–92.
- Pasqualucci L, Dominguez-Sola D, Chiarenza A *et al.* Inactivating mutations of acetyltransferase genes in B-cell lymphoma. *Nature* 2011; **471**: 189–95.
- Morin RD, Mendez-Lago M, Mungall AJ *et al.* Frequent mutation of histone-modifying genes in non-Hodgkin lymphoma. *Nature* 2011; **476**: 298–303.
- Iqbal J, Sanger WG, Horsman DE *et al.* BCL2 translocation defines a unique tumor subset within the germinal center B-cell-like diffuse large B-cell lymphoma. *Am J Pathol* 2004; **165**: 159–66.
- Iqbal J, Neppalli VT, Wright G *et al.* BCL2 expression is a prognostic marker for the activated B-cell-like type of diffuse large B-cell lymphoma. *J Clin Oncol* 2006; **24**: 961–8.
- Cerchetti LC, Ghetu AF, Zhu X *et al.* A small-molecule inhibitor of BCL6 kills DLBCL cells in vitro and in vivo. *Cancer Cell* 2010; **17**: 400–11.
- Nunez G, Seto M, Seremetis S *et al.* Growth- and tumor-promoting effects of deregulated BCL2 in human B-lymphoblastoid cells. *Proc Natl Acad Sci USA* 1989; **86**: 4589–93.
- McDonnell TJ, Korsmeyer SJ. Progression from lymphoid hyperplasia to high-grade malignant lymphoma in mice transgenic for the t(14; 18). *Nature* 1991; **349**: 254–6.
- Strasser A, Harris AW, Bath ML, Cory S. Novel primitive lymphoid tumours induced in transgenic mice by cooperation between myc and bcl-2. *Nature* 1990; **348**: 331–3.
- Zhang B, Calado DP, Wang Z *et al.* An oncogenic role for alternative NF- κ B signaling in DLBCL revealed upon deregulated BCL6 expression. *Cell Rep* 2015; **11**: 715–26.
- Pasqualucci L, Bhagat G, Jankovic M *et al.* AID is required for germinal center-derived lymphomagenesis. *Nat Genet* 2008; **40**: 108–12.
- Gu X, Booth CJ, Liu Z, Strout MP. AID-associated DNA repair pathways regulate malignant transformation in a murine model of BCL6-driven diffuse large B-cell lymphoma. *Blood* 2016; **127**: 102–12.
- Liu M, Duke JL, Richter DJ *et al.* Two levels of protection for the B cell genome during somatic hypermutation. *Nature* 2008; **451**: 841–5.
- Lenz G, Davis RE, Ngo VN *et al.* Oncogenic CARD11 mutations in human diffuse large B cell lymphoma. *Science* 2008; **319**: 1676–9.
- Snow AL, Xiao W, Stinson JR *et al.* Congenital B cell lymphocytosis explained by novel germline CARD11 mutations. *J Exp Med* 2012; **209**: 2247–61.
- Nakagawa M, Tsuzuki S, Honma K, Taguchi O, Seto M. Synergistic effect of Bcl2, Myc and Ccnd1 transforms mouse primary B cells into malignant cells. *Haematologica* 2011; **96**: 1318–26.
- Jeelall YS, Wang JQ, Law HD *et al.* Human lymphoma mutations reveal CARD11 as the switch between self-antigen-induced B cell death or proliferation and autoantibody production. *J Exp Med* 2012; **209**: 1907–17.
- Li S, Ilaria RL Jr, Million RP, Daley GQ, Van Etten RA. The P190, P210, and P230 forms of the BCR/ABL oncogene induce a similar chronic myeloid leukemia-like syndrome in mice but have different lymphoid leukemogenic activity. *J Exp Med* 1999; **189**: 1399–412.
- Ehlich A, Martin V, Muller W, Rajewsky K. Analysis of the B-cell progenitor compartment at the level of single cells. *Curr Biol* 1994; **4**: 573–83.
- Sander S, Calado DP, Srinivasan L *et al.* Synergy between PI3K signaling and MYC in Burkitt lymphomagenesis. *Cancer Cell* 2012; **22**: 167–79.
- Lossos IS, Okada CY, Tibshirani R *et al.* Molecular analysis of immunoglobulin genes in diffuse large B-cell lymphomas. *Blood* 2000; **95**: 1797–803.
- Sanchez-Izquierdo D, Siebert R, Harder L *et al.* Detection of translocations affecting the BCL6 locus in B cell non-Hodgkin's lymphoma by interphase fluorescence in situ hybridization. *Leukemia* 2001; **15**: 1475–84.
- Coffman RL, Weissman IL. A monoclonal antibody that recognizes B cells and B cell precursors in mice. *J Exp Med* 1981; **153**: 269–79.
- Linsley PS, Brady W, Grosmaire L, Aruffo A, Damle NK, Ledbetter JA. Binding of the B cell activation antigen B7 to CD28 costimulates T cell proliferation and interleukin 2 mRNA accumulation. *J Exp Med* 1991; **173**: 721–30.
- Shinall SM, Gonzalez-Fernandez M, Noelle RJ, Waldschmidt TJ. Identification of murine germinal center B cell subsets defined by the expression of surface isotypes and differentiation antigens. *J Immunol* 2000; **164**: 5729–38.
- Shaffer AL, Lin KI, Kuo TC *et al.* Blimp-1 orchestrates plasma cell differentiation by extinguishing the mature B cell gene expression program. *Immunity* 2002; **17**: 51–62.
- Choi WW, Weisenburger DD, Greiner TC *et al.* A new immunostain algorithm classifies diffuse large B-cell lymphoma into molecular subtypes with high accuracy. *Clin Cancer Res* 2009; **15**: 5494–502.
- Challa-Malladi M, Lieu YK, Califano O *et al.* Combined genetic inactivation of beta2-Microglobulin and CD58 reveals frequent escape from immune recognition in diffuse large B cell lymphoma. *Cancer Cell* 2011; **20**: 728–40.
- Hans CP, Weisenburger DD, Greiner TC *et al.* Confirmation of the molecular classification of diffuse large B-cell lymphoma by immunohistochemistry using a tissue microarray. *Blood* 2004; **103**: 275–82.
- Reljic R, Wagner SD, Peakman LJ, Fearon DT. Suppression of signal transducer and activator of transcription 3-dependent B lymphocyte terminal differentiation by BCL-6. *J Exp Med* 2000; **192**: 1841–8.
- Saito M, Gao J, Basso K *et al.* A signaling pathway mediating downregulation of BCL6 in germinal center B cells is blocked by BCL6 gene alterations in B cell lymphoma. *Cancer Cell* 2007; **12**: 280–92.
- Davis RE, Brown KD, Siebenlist U, Staudt LM. Constitutive nuclear factor kappaB activity is required for survival of activated B cell-like diffuse large B cell lymphoma cells. *J Exp Med* 2001; **194**: 1861–74.
- Lossos IS, Alizadeh AA, Eisen MB *et al.* Ongoing immunoglobulin somatic mutation in germinal center B cell-like but not in activated B cell-like diffuse large cell lymphomas. *Proc Natl Acad Sci USA* 2000; **97**: 10209–13.

- 44 Lamason RL, McCully RR, Lew SM, Pomerantz JL. Oncogenic CARD11 mutations induce hyperactive signaling by disrupting autoinhibition by the PKC-responsive inhibitory domain. *Biochemistry* 2010; **49**: 8240–50.
- 45 Basso K, Dalla-Favera R. Germinal centres and B cell lymphomagenesis. *Nat Rev Immunol* 2015; **15**: 172–84.
- 46 Chen LF, Greene WC. Shaping the nuclear action of NF-kappaB. *Nat Rev Mol Cell Biol* 2004; **5**: 392–401.
- 47 Sciammas R, Shaffer AL, Schatz JH, Zhao H, Staudt LM, Singh H. Graded expression of interferon regulatory factor-4 coordinates isotype switching with plasma cell differentiation. *Immunity* 2006; **25**: 225–36.
- 48 Kwon H, Thierry-Mieg D, Thierry-Mieg J *et al.* Analysis of interleukin-21-induced Prdm1 gene regulation reveals functional cooperation of STAT3 and IRF4 transcription factors. *Immunity* 2009; **31**: 941–52.
- 49 Shapiro-Shelef M, Lin KI, McHeyzer-Williams LJ, Liao J, McHeyzer-Williams MG, Calame K. Blimp-1 is required for the formation of immunoglobulin secreting plasma cells and pre-plasma memory B cells. *Immunity* 2003; **19**: 607–20.
- 50 Tunyaplin C, Shaffer AL, Angelin-Duclos CD, Yu X, Staudt LM, Calame KL. Direct repression of prdm1 by Bcl-6 inhibits plasmacytic differentiation. *J Immunol* 2004; **173**: 1158–65.
- 51 Knies N, Alankus B, Weilemann A *et al.* Lymphomagenic CARD11/BCL10/MALT1 signaling drives malignant B-cell proliferation via cooperative NF-kappaB and JNK activation. *Proc Natl Acad Sci USA* 2015; **112**: E7230–8.
- 52 Vasanwala FH, Kusam S, Toney LM, Dent AL. Repression of AP-1 function: a mechanism for the regulation of Blimp-1 expression and B lymphocyte differentiation by the B cell lymphoma-6 protooncogene. *J Immunol* 2002; **169**: 1922–9.
- 53 Catz SD, Johnson JL. Transcriptional regulation of bcl-2 by nuclear factor kappa B and its significance in prostate cancer. *Oncogene* 2001; **20**: 7342–51.
- 54 Zhao B, Barrera LA, Ersing I *et al.* The NF-kappaB genomic landscape in lymphoblastoid B cells. *Cell Rep* 2014; **8**: 1595–606.
- 55 Viatour P, Bentires-Alj M, Chariot A *et al.* NF- kappa B2/p100 induces Bcl-2 expression. *Leukemia* 2003; **17**: 1349–56.
- 56 Brohl AS, Stinson JR, Su HC *et al.* Germline CARD11 mutation in a patient with severe congenital B cell lymphocytosis. *J Clin Immunol* 2015; **35**(1): 32–46.
- 57 Visco C, Li Y, Xu-Monette ZY *et al.* Comprehensive gene expression profiling and immunohistochemical studies support application of immunophenotypic algorithm for molecular subtype classification in diffuse large B-cell lymphoma: a report from the International DLBCL Rituximab-CHOP Consortium Program Study. *Leukemia* 2012; **26**: 2103–13.
- 58 Wilson WH, Young RM, Schmitz R *et al.* Targeting B cell receptor signaling with ibrutinib in diffuse large B cell lymphoma. *Nat Med* 2015; **21**: 922–6.

Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. Distribution of genetic abnormalities coexisting with Card11 mutations.

Fig. S2. Schematic representation of the experimental procedure.

Fig. S3. Schematic representation of the expression vector.

Fig. S4. Histology of developed lymphomas.

Fig. S5. Flow cytometric analysis of germinal center B cells transduced with *Card11*^{L232LI}, *Bcl6*, and *Bcl2* before transplantation.

Fig. S6. Flow cytometric analysis of lymphoma cells.

Fig. S7. Gross anatomy of a mouse with *Card11*^{L232LI}/*Bcl6* doubly transduced lymphoma (Card11^{mut}+Bcl6 #1).

Fig. S8. Expression of BCL2 in developed lymphoma.

Fig. S9. Nuclear translocation of p65.

Fig. S10. Analysis of secondary transplantation of primary lymphoma cells.

Table S1. Summary of the mice analyzed in this study

Table S2. VDJ gene usage and analysis of ongoing somatic hypermutation of lymphoma cells