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CD40 Ligand Stimulation Inhibits the Proliferation of Mantle Cell Lymphoma Lines

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Abstract. *Mantle cell lymphoma (MCL) is a CD5$^+$ non-Hodgkin's B-cell lymphoma characterized by the infiltration of intermediate sized B-cells into the mantle zones. Interaction between CD40L and CD40 is important for B cell proliferation and differentiation. CD40L stimulation can induce both growth arrest and proliferation of B cell lines according to their differentiation state. Previous reports examining the effect of stimulation via the CD40 cascade on ex vivo MCL cells have provided conflicting results. In this study, two MCL lines, SP49 and SP53, were examined for response to CD40L and/or IL-10. Co-cultivation with CD40L-expressing mouse L cells reduced the BrdU incorporation of SP49 and SP53 cells by half to one-third, while BrdU incorporation of control cell lines, including Ramos, BJAB and BALL-1, was not affected or increased. Anti-CD40L antibody blocked the CD40L inhibition of SP49 cell proliferation in a dose-dependent manner in the range from 0 to 20 ng/ml. IL-10 did not affect MCL cell proliferation in the presence or absence of CD40L-expressing cells, while Ramos proliferation was promoted by CD40L and IL-10. These results suggested the possibility that CD40L may also inhibit MCL proliferation in vivo.*

Mantle cell lymphoma (MCL) is a CD5$^+$ non-Hodgkin's B-cell lymphoma characterized by a poor response to therapy and short survival, and is strongly associated with the t (11; 14) chromosomal translocation (1). Overexpression of cyclin D1 due to the translocation is thought to be responsible for the lymphomagenesis (2,3).

Most MCLs comprise intermediate-sized B cells, with histology characterized by lymphoma cell infiltration into the mantle zones. Atrophic germinal centers frequently remain in affected lymph nodes, even in nodes effaced by MCL (1).

CD40 is a well-studied member of the tumor necrosis factor receptor family (4). Cross-linking of CD40 molecules on the cell surface membrane accumulate tumor necrosis factor receptor-associated factor (TRAF) 2 and TRAF3, which then induce NF-Î B activation (5).

The CD40 ligand (CD40L) - CD40 interaction plays an important and pivotal role in B cell proliferation and differentiation (6). CD40 cross-linking induces increased cell size and expression of adhesion molecules. The simultaneous stimulation of CD40 and surface immunoglobulin alters the phenotype of resting B cells to that of germinal center B cells with the expression of CD38, CD86, CD95, carboxypeptidase and CD71 (7). *In vivo* blocking of the CD40-CD40L interaction in mice impedes the development of memory-B cells, but not germinal center formation (8). The reactivity of established B cell lines to CD40L stimulation can vary according to the type of cell studied (9-12). While CD40L stimulation blocked the proliferation of EBV-transformed lymphoblastoid cell lines (12), CD40L stimulation can either promote or arrest the proliferation of Burkitt's lymphoma cell lines (10).

With regard to MCL, both growth arrest and proliferation in response to CD40L have been reported (9-11, 13). Andersen *et al.* reported that human CD40L-trimer alone significantly promoted DNA synthesis in *ex vivo* MCL cells (9). However, Planken *et al.* reported that CD40 stimulation *via* cross-linking by anti-CD40 antibodies failed to promote the proliferation of *ex vivo* MCL cells both with and without IL-4 (10). Castillo *et al.* reported that, whereas the combination of IL-4 and anti-CD40 stimulatory antibody promoted proliferation of *ex vivo* MCL cells, the combination of IL-10 and anti-CD40 stimulatory antibody had no effect (13). Visser *et al.* reported that, in 13 out of 16 cases, *ex vivo* MCL cells proliferated well after exposure to CD40L-transfected cells and that the addition of IL-10 further promoted proliferation (11).

As MCL cell lines are rare, previous studies have tended to use *ex vivo* cells. In this study, we employed two MCL cell lines to assess the effects of CD40L and IL-10 on MCL.

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Key Words: Mantle cell lymphoma, CD40, CD40 ligand, IL-10.

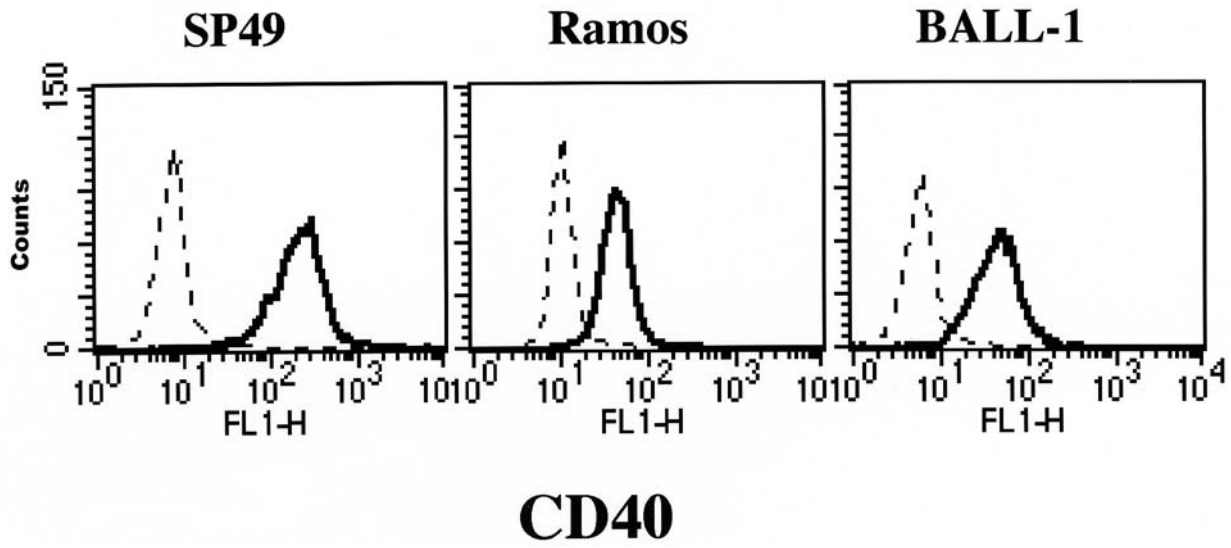

Figure 1. *CD40 expression by SP49, Ramos and BALL-1 cell lines.*

Table I. *Antibodies used in the study.*

Specificity	Clone	Isotype	Source
CD21	1F8	Mouse IgG1	Dako
CD23	MHM6	Mouse IgG1	Dako
CD39	TU66	Mouse IgG2b	BD
CD40	5C3	Mouse IgG1	BD
CD40L	TRAP1	Mouse IgG1	BD
Ki-67		Rabbit	Dako

BD: Becton Dickinson, San Jose, CA, USA; Dako: Dakopatts, Glostrup, Denmark.

Materials and Methods

Cell lines, lymphoma samples and antibodies. SP49 and SP53 cell lines, which were derived from MCL patients (3, 14-16), contain the typical t (11,14) chromosomal translocation and express CD5. The BJAB and Ramos cell lines are EBV-negative Burkitt's lymphoma lines. HL60 is a cell line derived from a promyelocytic leukemia. CD40L-expressing mouse fibrosarcoma L cells (CD40L-L cells) were kindly provided by Pierre Garrone, Schering-Pleough, France (17). Cells were plated into 6-well plates and irradiated at 70 Gy when confluent (18). The MCL and other cell lines were added to the plates at densities of 0.1 ~ 0.2 x10^6 cells/ ml and incubated for 2 days. All cell lines were cultured in Iscove's Modified Dulbecco's Medium (IMDM) with 10% fetal calf serum with and without 10 ng/ml IL-10 (Chemicon International Inc, Temecula, CA, USA). CD40L stimulation was blocked using mouse monoclonal anti-CD40L neutralizing antibody (0 ~ 20 ng/ml, clone TRAP1, BD Pharmingen, San Diego, CA, USA). Other antibodies used are listed in Table I.

Proliferation assay. Proliferation was measured by BrdU incorporation using the Cell Proliferation ELISA, BrdU colormetric kit (Roche Diagnostic Corporation, Indianapolis, IN, USA). Briefly, cells were transferred to 96-well pates and cultured with BrdU for 4 hours. Plates were then centrifuged, washed twice and incorporated BrdU was detected using peroxidase-conjugated anti-BrdU antibody. The amount of incorporated BrdU was estimated by absorbance at 450nm. Each experiment was repeated 3 times.

Immunostaining and FACS analysis. Phenotypes of the hematolymphoid cell lines used were analyzed by flow cytometry using a Scancaliber (Beckton Dickinson) as previously described (19). CD40L expression in lymphoid tissues was detected on the frozen tonsil sections using anti-CD40L (TRAP1, Dakojapan, Kyoto, Japan) and then visualized with rhodamine-conjugated goat IgG-anti-mouse IgG (Leinco Technologies Inc, Manchester, MO, USA) which confirmed the presence of CD40L on the cell surface and cytoplasm of CD40L-L cells.

Results

Immunophenotype of MCL cell lines. Flow cytometric analysis showed that SP49 and SP53 cells were CD21+, CD39+ and CD23-. CD40 was expressed on the cell lines SP49, SP53, Ramos, BALL-1 and BJAB (Figure 1). Exposure to mouse L cells did not affect MCL cell expression of CD21, CD23, or CD39, or the FSC. Typical results obtained for SP49 cells are shown in Figure 2. Exposure to CD40L-L cells induced CD23 expression and increased the number of CD21-positive SP49 and SP53 cells. CD40L exposure increased the cell size, which was monitored by the microscope and Foward Scatter (FSC) of flow cytometry, of both Ramos and SP49 cells (Figure 2).

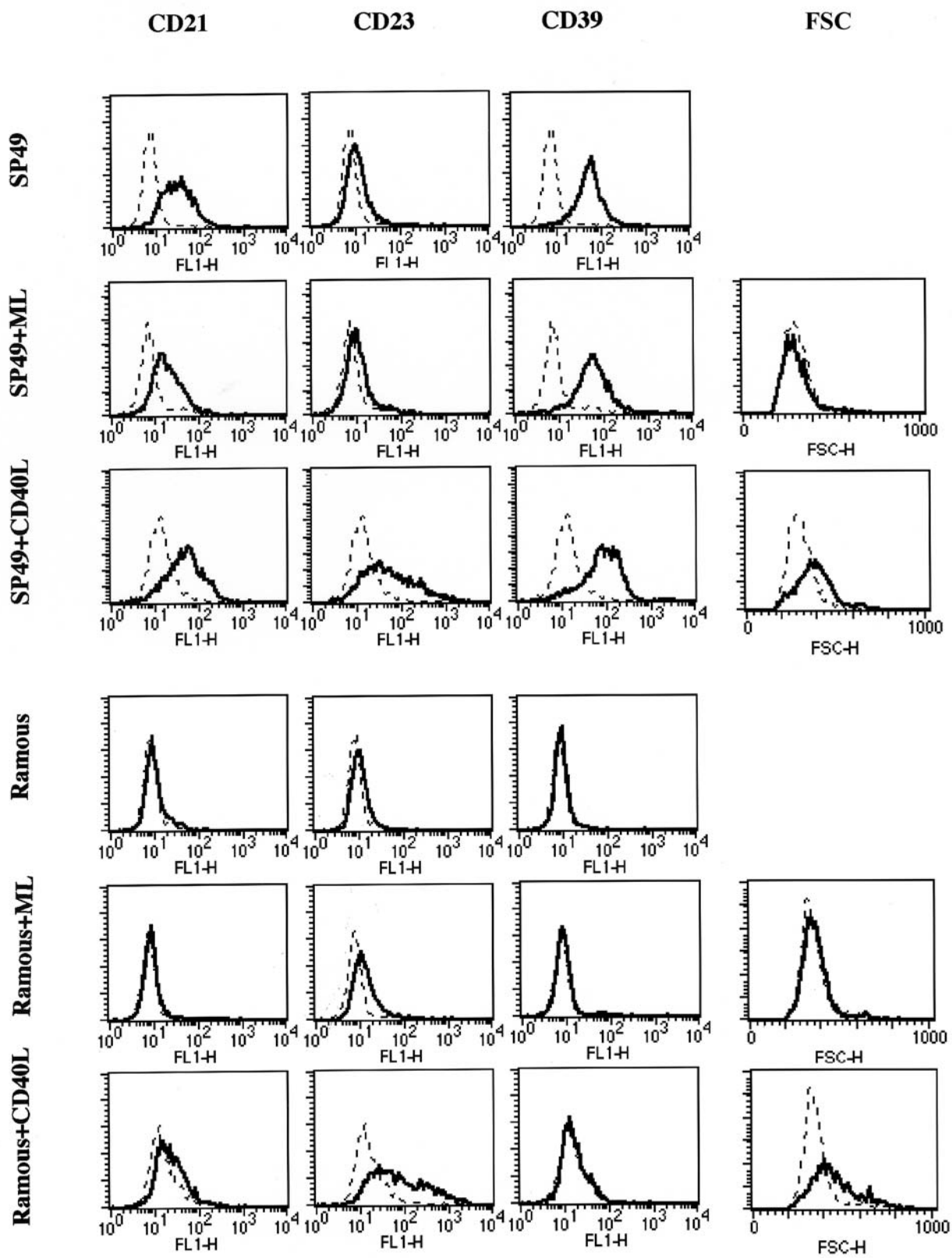

Figure 2. *The phenotypic changes after exposure to CD40L-expressing L-cells. Flow cytometric analyses of CD21, CD23, CD39 and FSC are shown. ML: Non-transfected mouse L cells. CD40L: CD40L-transfected mouse L cells. Broken lines: Control class-matched monoclonal mouse IgG.*

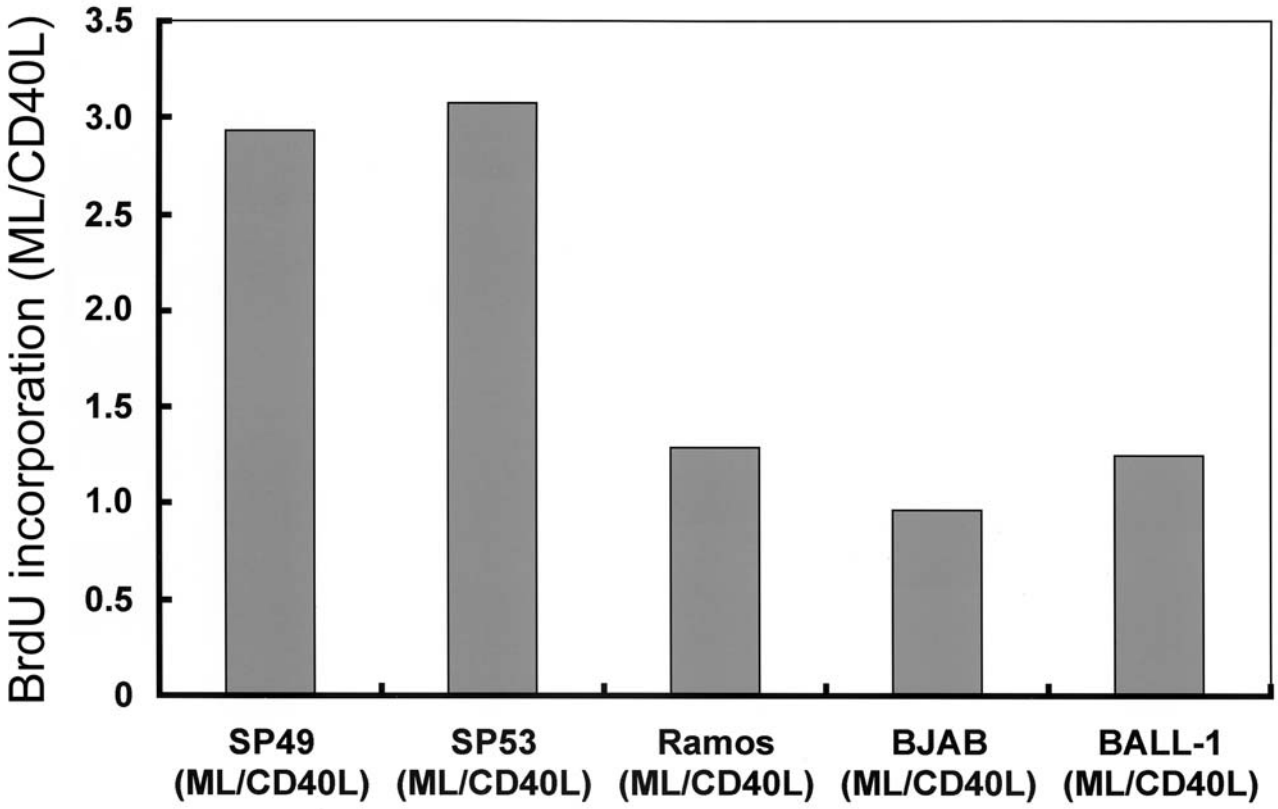

Figure 3. *The inhibitory effect of CD40L on MCL cell lines.*
The Y-axis figures represent BrdU incorporation after exposure to control mouse L cells divided by BrdU incorporation after exposure to CD40L-transfected mouse L cells. Thus, the larger the figure, the stronger the growth inhibition by CD40L stimulation. The results are representative of 3 independent experiments.

CD40L stimulation inhibit the proliferation of SP49 and SP53. After exposure of SP49 and SP53 cells to CD40L-L cells, BrdU incorporation was reduced by 1/2～1/3 compared to cells exposed to mouse L cells only (Figure 3). In contrast, BrdU incorporation by the cell lines BALL-1, BJAB and Ramos were not affected by exposure to CD40L-L cells (Figure 3). The BrdU incorporation rate (mouse L cells/ CD40L-L cells) for each line was 0.584±0.052/0.199±0.055 for SP49, 0.827±0.065/0.269±0.06 for SP53, 1.390±0.11/1.050±0.058 for Ramos, 1.445±0.118/1.506±1.53 for BJAB and 0.982±0.090/ 0.785±0.030 for BALL-1.

Anti-CD40L mouse monoclonal antibody (TRAP1) reduced CD40L-mediated inhibition in a dose-dependent manner, with the addition of 20 ng /ml anti-CD40L overcoming the effect of CD40L on SP49 cells. Anti-CD40L had no effect on SP49 proliferation when co-cultured with mouse L cells (Figure 4). Ki-67 expression was detected by FACS (Figure 5, Table II), which showed that the amount of Ki-67-positive SP49 cells decreased after exposure to CD40L-L cells when compared to mouse L cells. In contrast, Ki-67-positive Ramos cell numbers increased after exposure to CD40L-L cells.

IL-10 did not affect the proliferation of SP49 with or without CD40L stimulation. IL-10 did not affect the proliferation of SP49 and Ramos cells in the absence of CD40L stimulation (Figure 6a). While simultaneous stimulation with both IL-10 and CD40L promoted the proliferation of Ramos cells, IL-10 did not promote the proliferation of CD40L-stimulated SP49 cells, or block CD40L-mediated growth inhibition (Figure 6b).

Discussion

Latent membrane protein-1 (LMP-1) is a potent transforming viral oncogene that plays a central role in B cell transformation by EBV (20). Like CD40, LMP-1 has TRAF2 and TRAF3 binding sites and can accumulate spontaneously in the membrane in the absence of stimulation to function as if it were a constitutively active CD40 (21). However, in some circumstances LMP1 overexpression has been shown to be cytotoxic or inhibit growth (22), and CD40 stimulation has been shown to retard the proliferation of EBV-transformed lymphoblastoid

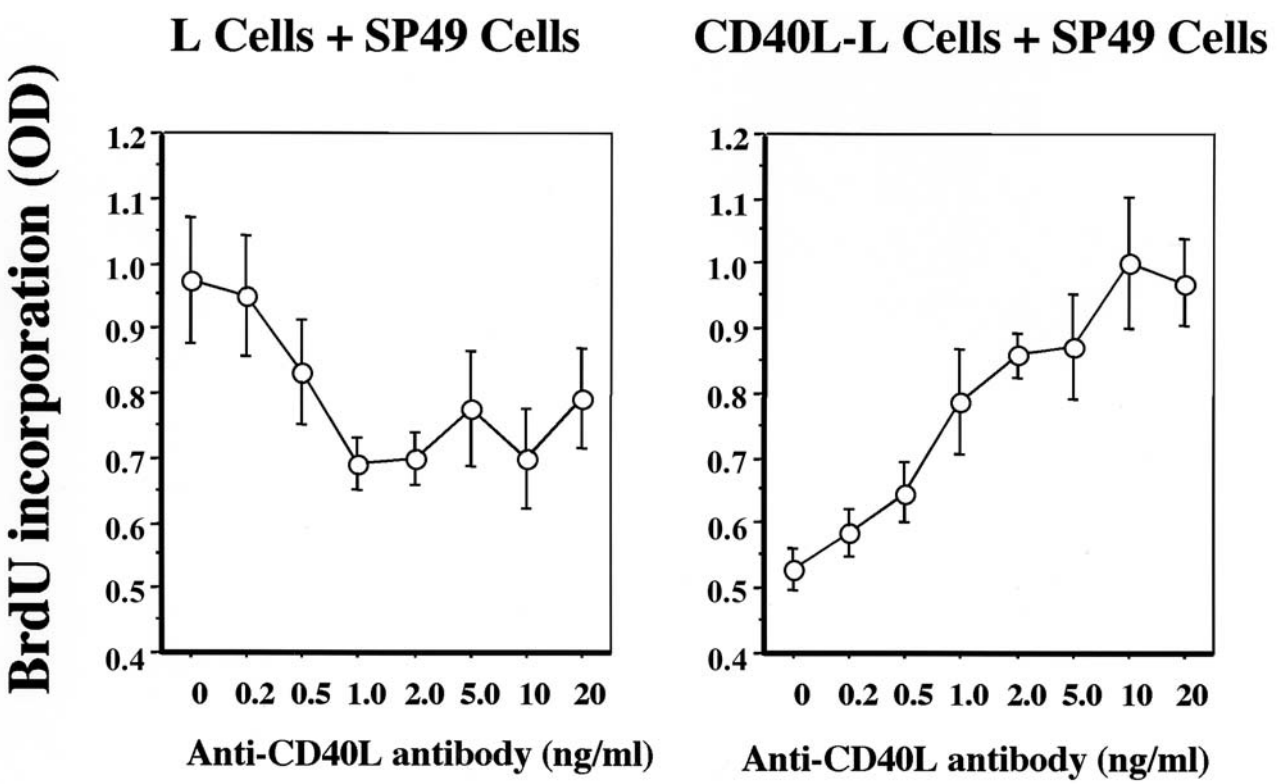

Figure 4. *Anti-CD40L neutralizing antibody blocked the inhibitory effect of CD40L on SP49 cell proliferation in a dose-dependent manner. The horizontal axis indicates the concentration of anti-CD40L antibody (ng/ml), while the vertical axis indicates BrdU incorporation. Note that BrdU incorporation by SP49 cells gradually increases with increasing concentrations of anti-CD40L neutralizing antibody.*

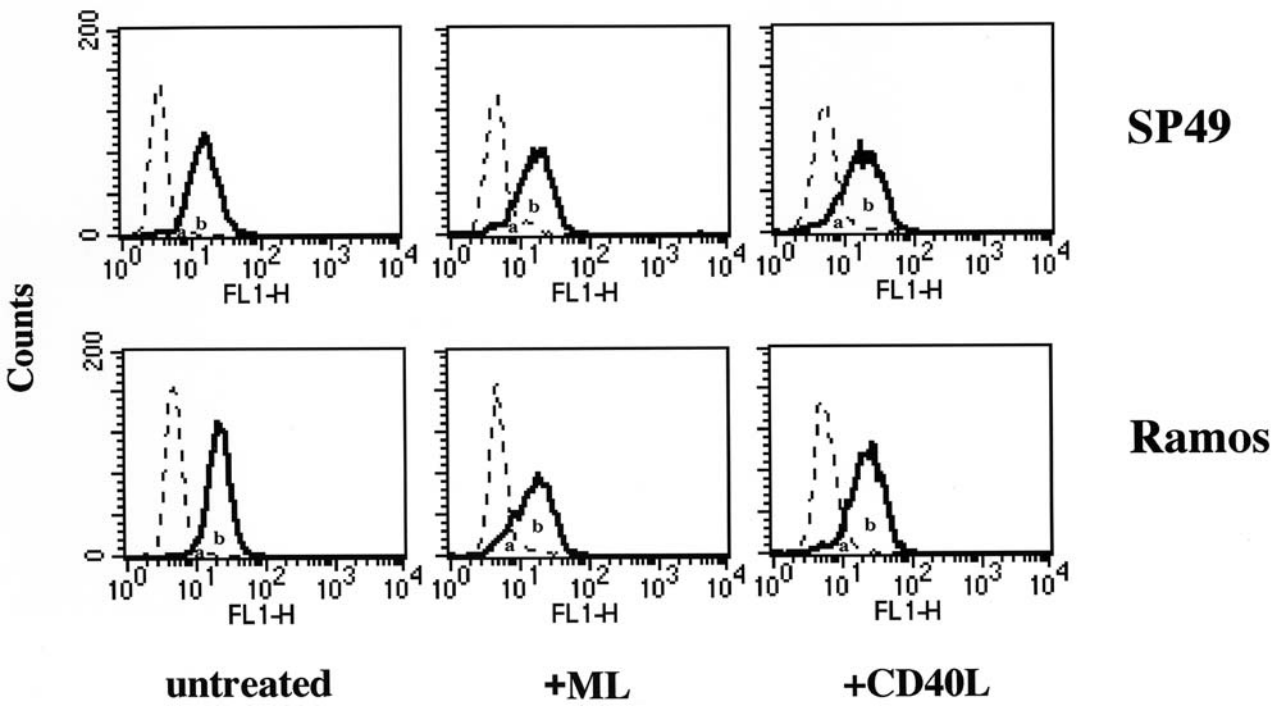

Figure 5. *Flow cytometric analysis of Ki-67 expression on cells exposed to CD40L -expressing mouse L cells. Black lines indicate Ki-67-positive cells and dotted lines unstained cells. The "a" area is the overlap between the black and dotted lines and the "b" area is the black line minus "a". Thus, the "a" and "b" areas represent Ki-67-negative and -positive staining, respectively. Values for areas "a" and "b" are shown in Table II.*

Table II. *The percentage of cells in areas "a" and "b" as indicated in Figure 5.*

	SP49			Ramos		
	untreated	ML	CD40L	untreated	ML	CD40L
"a" (Ki-67- negative)	3.9%	21.8%	25.5%	9.9%	22.3%	15.9%
"b" (Ki-67- positive)	96.1%	78.2%	74.5%	90.1%	77.7%	84.1%

cell lines that express LMP1 (12). Thus, CD40 stimulation can inhibit the proliferation of some cell lines.

It has been reported that EBV-encoded small RNAs can induce IL-10 expression and the autocrine stimulation of Burkitt's lymphoma lines (23), and that IL-10 can promote the proliferation of B cells after CD40L stimulation (24). Therefore, both stimulation through the CD40 cascade and IL-10 secretion are important factors in EBV-mediated B cell transformation.

When peripheral blood B cells become infected by EBV, most B cells are refractory to EBV-mediated transformation such that only a small fraction of B cells are transformed. We have previously reported that EBV infection inhibited the proliferation of the MCL lines SP53 and SP49 (16), and that the EBV-carrying SP53 and SP49 cells express LMP1. As EBV inhibited SP49 and SP53 proliferation, we postulated this inhibition could be due to stimulation and/or IL-10-mediated inhibition.

In the present study, MCL cell line proliferation reduced to about 1/2 ~ 1/3 after exposure to CD40L-L cells. This CD40L effect was specific as anti-CD40L antibody blocked the effect in a dose-dependent manner.

However, the effects of CD40 signaling are not straightforward. Different groups have reported conflicting results concerning the effect of CD40 stimulation on B-cell lymphomas. These differences may be at least partly due to the different methods of stimulation used, for instance, antibody cross-linking of CD40, soluble forms of CD40L, or CD40L-transfected cell lines. The conflicting results may also be due to the instability of *ex vivo* cells, as it is difficult to handle *ex vivo* cells in a reproducible manner. Since MCL cells are not actively proliferating cells *in vivo*, the inhibition of proliferation of *ex vivo* cells is difficult to assess and established MCL cell lines are rare.

Although this study was an *in vitro* experiment, the results suggested that MCL might be influenced by CD40 stimulation *in vivo* and could open a new therapeutic strategy for the treatment of MCL.

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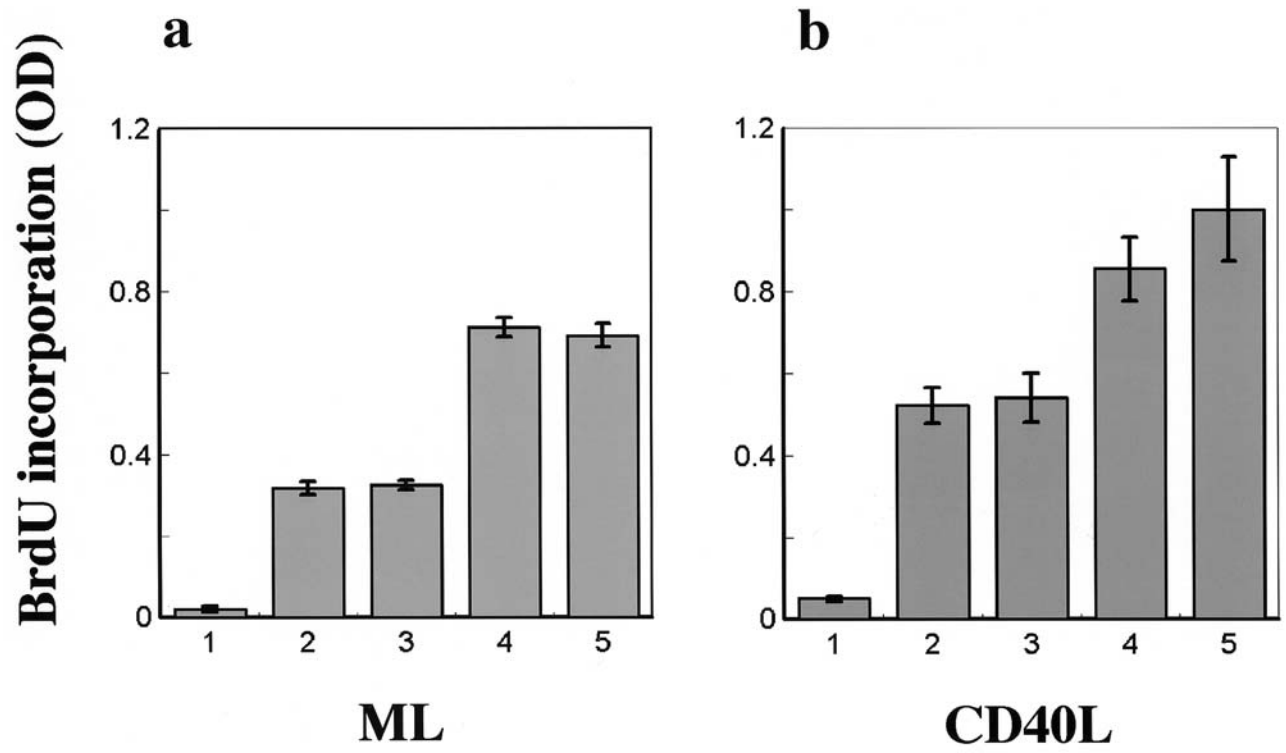

Figure 6. *The effect of IL-10 and CD40L stimulation on SP49 and Ramos cells. (a): IL-10 (10 ng/ml) has no effect on SP49 and Ramos cell growth in the absence of CD40L stimulation. 1: Mouse L-cells (ML), 2:SP49 + ML without IL-10, 3: SP49 + ML with IL-10, 4: Ramos + ML without IL-10, 5: Ramos + ML with IL-10. Vertical axis shows BrdU incorporation. (b): IL-10 with CD40 stimulation promotes BrdU incorporation by Ramos cells but not by SP49 cells. 1: CD40L-transfected L-cells (CD40L), 2: SP49 + CD40L without IL-10, 3: SP49 + CD40L with IL-10, 4: Ramos + CD40L without IL-10, 5: Ramos + CD40L with IL-10. Vertical axis shows BrdU incorporation.*

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