IL-4/CD40L Co-Stimulation Induces Long-Term Proliferation for CD10-Positive Germinal Center B Cell-Like Diffuse Large B-Cell Lymphoma

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Abstract: Diffuse large B cell lymphoma (DLBCL) is a heterogeneous entity and the explicit mechanism of lymphomagenesis remains to be elucidated. CD40 ligand (CD40L, CD154) and interleukin-4 (IL-4) are important factors regulating B lymphocyte proliferation and differentiation; however, little is known about the effects of these factors on lymphomagenesis in DLBCL. In this study, we investigated the effects of these factors on DLBCL B cell proliferation. Normal (n=2) and DLBCL (n=10) B cells were cultured with a system containing IL-4 and CD40L-expressing NIH3T3 ($CD40L^+$3T3). Normal and tumor cells from six patients stopped growing within three weeks. Tumor cells, obtained from four patients with extranodal lesions, showed continuous proliferation for more than two months. Two cell lines were established from these cells. The cell lines were derived from gastric tissues showing CD10+ germinal center B-cell (GCB) phenotype. Removing IL-4 from the system or adding anti-CD40L blocking antibody inhibited the cell proliferation. An *in vitro* transwell assay showed that direct contact with $CD40L^+$3T3 was required for the proliferation. Our findings suggested that CD40L and IL-4 co-stimulation could induce long-term proliferation in B cells derived from some CD10+ GCB-like DLBCL patients with the same characteristics, and that direct stimulation from tumor microenvironment is important for the cell growth. These cell lines may be useful for investigating the growth mechanism of some type of DLBCL, and should provide new insights concerning pathogenesis of DLBCL.

Keywords: CD40 ligand, CD154, IL-4, cytokine, diffuse large B cell lymphoma, extranodal lymphoma.

INTRODUCTION

Diffuse large B-cell lymphoma (DLBCL) is the most common type of non-Hodgkin's lymphoma (NHL), accounting for 30-40% of newly diagnosed tumors of lymphoid tissues [1]. DLBCL is etiologically, pathologically and cytogenetically a heterogeneous disease. Gene expression profiling has identified three subtypes of DLBCL: germinal-center B cell-like, activated B cell-like, and type 3 diffuse large B-cell lymphoma [2, 3]. These three subtypes show different clinical outcomes which may require different therapeutic approaches. Although many efforts have been made to improve survival [4, 5], some patients with relapse show poor prognosis. To stratify appropriate treatment strategies and develop new potential therapies, an understanding of the pathological and molecular mechanism of the disease is essential.

CD40 is a type-1 transmembrane protein of the tumor necrosis factor receptor superfamily, and is expressed on virtually all mature B lymphocytes and various B lineage neoplasms. Stimulation of CD40 antigen is thought to play an important role in B-cell proliferation, survival, differentiation and function [6], as well as malignant B-cell proliferation [7, 8]. The CD40 ligand (CD40L, or CD154) is expressed primarily on the surface of activated CD4+ T lymphocytes [9, 10]. Additionally, CD40L expression has been shown in several types of lymphoma [11, 12]. It was reported that CD40L expression in B-cell lymphomas may contribute to proliferation of lymphoma cells through activation of intrinsic CD40 [12, 13]. Thus, CD40/CD40L has been suggested as a system contributing to tumor pathogenesis.

IL-4 is thought to increase CD40 antigen expression [14], and CD40L and IL-4 co-stimulation has resulted in long-term B-cell proliferation *in vitro* [15, 16]. CD40L and IL-4 co-stimulation is also reported to promote short-term proliferations of mantle cell lymphoma and a proportion of hairy cell leukemia B cells [7, 17], as well as prolonging proliferations of follicular lymphoma B cells (for up to eight weeks) [18]. However, little is known about the role of CD40L and IL-4 co-stimulation on DLBCL B-cell proliferation. Herein, we investigated the proliferative ability of DLBCL B cells using a CD40L and IL-4 co-stimulation culture system.

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MATERIALS AND METHODOLOGY

Characteristic of Patients and Sample Preparation

This study was approved by the institutional review board of the Aichi Cancer Center. Mononuclear cells from pathologic lymph node or tissue specimens (ten cases), and from peripheral blood (two healthy donors), were obtained (Table **1**) after written informed consent in accordance with the Declaration of Helsinki. Mononuclear cells were isolated by the addition of Ficoll and density centrifugation. All patients were diagnosed on the basis of morphological and immunohistochemical findings in accordance with WHO criteria. Categorization as germinal center B cell-like (GCB) or non-germinal center B cell-like (non-GCB) was performed according to the algorithm using the Hans classification [19].

Culture Conditions and Reagents

The cells were cultured with RPMI 1640 medium containing antibiotics, 10% fatal calf serum (PAA Laboratories GmbH, Austria), 100 nM sodium selenite (Wako Pure Chemical Industries, Osaka, JP), 1 μg/ml cyclosporin A (CsA; Wako Pure Chemical Industries, Osaka, JP) and 2 ng/ml recombinant human IL-4 (PeproTech Inc, Rocky Hill, NJ). NIH3T3 (NIH Swiss mouse embryonic fibroblast cell line) transfected with CD40L ($CD40L^+3T3$), which was kindly supplied by Dr. Lee M. Nadler (Dana-Farber Cancer Institute, Boston, MA, USA) [20], was used for stimulating feeder cells and co-cultured with B cells. $CD40L^+3T3$ was cultured for one to three days at 1×10^5 cells/well using a 12-well plate and 2×10^4 cells/well using a 96-well plate, and then irradiated (100 Gy) on the day B cells were plated. Since one EBV-infected cell is found in about 10^4-10^6 B cells, unseparated mononuclear cells from peripheral blood were plated in different numbers per microculture to established EBV-free CD40-stimulated B cell cultures [16]. The cells were cultured on $CD40L^+3T3$ in the presence of interleukin-4 and CsA in a humidified atmosphere at 37°C with 5% CO_2 in air for three to seven days [16]. The cell number was counted using the trypan blue dye exclusion test in each well, and the cells were transferred to new stimulating feeder cells at the same conditions and with the same concentration of B cells.

Immunophenotyping

Cell surface antigen expression of B-cell marker was studied by flow cytometry using FITC, PE, PerCP or APC labeled CD5 (Becton, Dickinson and Company; BD, Mountain View, CA, USA), CD10 (BD), CD19 (BD), CD20 (BD), and surface immunoglobulin (sIg) (BD). The CD40L expression of feeder cells and established cell lines was evaluated with anti-CD40L antibody (monoclonal antibody CD154, murine IgG_1, TRAP-1 clone; Immunotech, a Beckman Coulter Company, France) using FITC-labeled sIg (goat, DakoCytomation, Glostrup, Denmark) as a secondary antibody. The events acquired by the cytometer varied from sample to sample, because the events depend on the size of the culture system.

Table 1. Patients' Characteristics at Diagnosis

UPN	Age	Sex	Treatment Status	Clinical Features	Sample Analyzed	*Phenotype	Cytogenetic Karyotype	Pathologic Features
1	80	F	newly diagnosed	thyroid, stomach	stomach	CD10+, Igκ+, bcl-2-, p53+	normal	GCB
2	56	M	newly diagnosed	stomach	stomach	CD10+, Igκ+, bcl-2-, bcl-6+ MUM1-, p53+	45, XY, t(11,17)(q13, q21), +12	GCB
3	70	F	newly diagnosed	lymphadenopathy, stomach	stomach	CD10-, Ig-	NA	Non-GCB
4	67	F	newly diagnosed	mediastenal, stomach	stomach	CD10-, Igκ+, bcl-2+, p53±	NA	Non-GCB, with MALT
5	62	M	newly diagnosed	lymphadenopathy, stomach, testis	lymph node	CD10+, Igλ+	NA	GCB, with MALT
6	63	M	newly diagnosed	lymphadenopathy, stomach	lymph node	CD10-, Igκ+, bcl-2+, p53-	normal	Non-GCB
7	69	M	refractory	lymphadenopathy	lymph node	CD10-, Igκ+	NA	Non-GCB
8	48	M	newly diagnosed	lymphadenopathy	lymph node	CD10-, Igλ+, bcl-2+, p53+	NA	Non-GCB
9	46	F	newly diagnosed	breast, bone marrow	breast	CD10-, Igκ+, bcl-2+, bcl-6+, MUM1-, p53±	complex**	GCB
10	40	F	newly diagnosed	breast	breast	CD10+, Igκ+, bcl-2-, p53+	NA	GCB

Abbreviations: Upn, unique patient number; NA, not available; GCB, germinal center B-cell-like.
*CD5 negative, CD19 positive, CD20 positive and CD23 negative in all cases.
**94xx, (add(x)(p11)×2, del(2)(q13q23?)×2, del(2)(q24)×2, add(3)(q21)×2, add(3)(q11), -5, -6, del(6)(p11), add(8)(q22)×2, -9, -10, del(12)(q11q15)×2, -16, -17, (del(18)(q21)?×2, -19, -19, -21, -22, +10mar.

Transwell Culture

CD40L$^+$3T3 cells were plated at 7×10^4/well using a 12-well plate (Transwell, Costar) on day -1. The medium was changed to 1.5 ml of fresh medium on day 0, and the bottom cup with a 0.4-μm diameter microporous membrane was put in place. B cells were inoculated into the inner cup with 0.5 ml of cell suspensions containing 0.5×10^5 cells/ml. The positive control consisted of three conditions: culture with the inner cup without feeder cells, culture without the inner cup with CD40L$^+$3T3 cells, and culture without the inner cup with NIH3T3 untransfected with CD40L (CD40L$^-$3T3) cells. B cells, cultured without the inner cup, were plated with 2 ml of cell suspensions containing 0.5×10^5 cells/ml using a 12-well plate. B-cell number was counted every four days using the trypan blue dye exclusion test in each well, and the cells were transferred to new plates at the same conditions and with the same concentration of B cells. The culture was continued until day 8. Each condition was repeated in duplicate, and the cell number was counted two times in each well.

DNA Extraction and 4×44K Oligo Array Comparative Genomic Hybridization (CGH) Analysis

DNA was extracted from frozen tissue samples using proteinase K treatment and phenol-chloroform extraction. Oligo Array CGH analysis was performed using the Agilent Human Genomic Microarray Kit 4×44K (Agilent Technologies). This high-resolution 60-mer oligonucleotide-based microarray was used in this study and contains about 44,000 probes with an average spatial resolution of 35 kb (Agilent Technologies, Santa Clara, CA). Genomic DNA from the patient and normal male reference DNA were digested with *Alu*I and *Rsa*I (Promega Corporation) and labeled using an Agilent Genomic DNA Labeling kit according to manufacturer's instructions. Patient and reference DNA were labeled with Cy3 and Cy5, respectively. Labeled patient DNA and reference DNA were co-hybridized to arrays for 24 hr at 65°C in a rotating oven (Agilent Technologies) at 20 rpm. Washing was performed according to the Agilent protocol. Arrays were analyzed using an Agilent Microarray Scanner and Agilent Feature Extraction software (9.1). Results were presented by Agilent CGH Analytics software (v3.4.27).

RESULTS

CD40L and IL-4 Co-Stimulation Induce Normal B Lymphocyte Proliferation *In Vitro*

Peripheral blood mononuclear cells from two healthy donors were cultured on the stimulator cells of CD40L$^+$3T3 with IL-4 containing medium. The cells were plated with 2 ml of cell suspensions containing 0.5×10^6/ml using a 12-well plate, and with 200 μl of cell suspensions containing 1.25×10^5/ml, 2.5×10^5/ml, 5.0×10^5/ml or 10.0×10^5/ml using a 96-well plate. Since the best growth curve was obtained using cell suspensions containing an initial concentration of 1.25×10^5/ml with a 96-well plate (data not shown), we chose this condition for further experiments. For the first several days, the cells formed a small cluster on CD40L$^+$3T3 cells, and after a further five to seven days, most cells became large clusters. The cells, adjusted to a concentration of 1.25×10^5/ml, were restimulated every 5 to 7 days with fresh stimulator cells. The cells proliferated to about 110 to 120-fold until day 20. Wiesner *et al*. [16] showed that cultures stimulated with CD40L-expressing murine fibroblasts in the presence of IL-4 can proliferate for more than 900 days. However, the use of our culture system with CD40L$^+$3T3 cells and IL-4 resulted in proliferation for about three weeks, and we observed that cell growth through proliferation rapidly ceased (Fig. **1**).

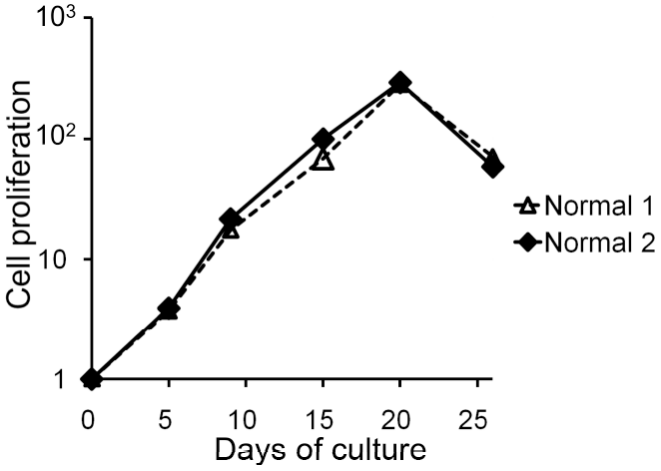


Fig. (1). IL4 and CD40L co-stimulation induced short-term proliferation of B cells *in vitro* from healthy volunteers. CD40L$^+$3T3 stimulating cells and IL-4 induced normal B-cell proliferation for about three weeks. Cells proliferated to about 110 to 120-fold until day 20 and then cell growth ceased on day 26. Abbreviation: IL-4, interleukin-4; CD40L, CD40 ligand; CD40L$^+$3T3, NIH Swiss mouse embryonic fibroblast cell line transfected with CD40L.

Co-Stimulation of CD40L and IL-4 Induces DLBCL Cell Proliferation *In Vitro*

Mononuclear cells from 10 DLBCL samples (five stomach, three lymph node and two breast) were plated with 200 μl of cell suspensions containing 1.25×10^5/ml on a 96-well plate using the stimulator cells of CD40L$^+$3T3 with IL-4 containing medium. Of the ten samples, tumor cells from six patients stopped growing within three weeks. The cells from the remaining four patients have been cultured with continuous proliferation for more than two months, and cell lines were established from two of these cell groups (GDLB-GCB1 and GDLB-GCB2). The cell lines, derived from gastric tissue, showed proliferation for more than three month. The doubling time of the cell lines GDLB-GCB1 [(from UPN (unique patient number)1 patient) and GDLB-GCB2 (from UPN2 patient) was 2.9 and 1.7 days, respectively (Fig. **2a**, **b**). These cell lines showed pathological characteristics of bcl2- and CD10+ germinal center B-cell (GCB)-like DLBCL. Phenotypical analysis of the original samples and established cell lines demonstrated the positive expression of CD10, CD19, CD20 and sIgG, and the negative or weakly positive expression of CD40L (Fig. **3**). UPN 6 and UPN 10 ceased to proliferate within 2.5 to 3 months.

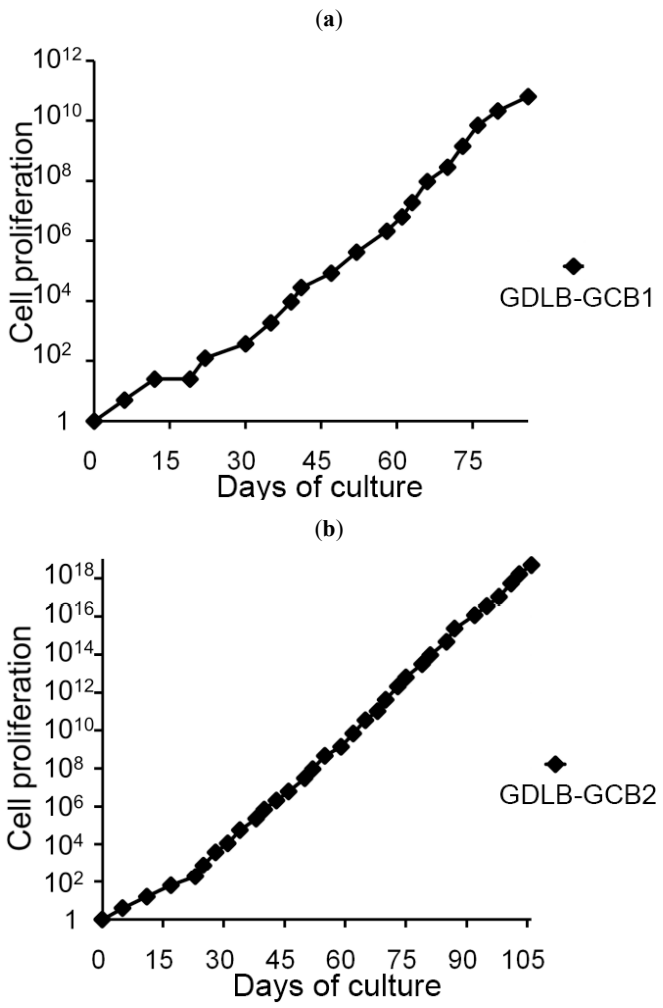


Fig. (2). IL4 and CD40 co-stimulation induced long-term proliferation of DLBCL B cells *in vitro* from DLBCL patients. Of the ten DLBCL cases analyzed, tumor cells from four patients showed continuous proliferation for more than two months, and two cell lines (GDLB-GCB1 and GDLB-GCB2) were established from these cells (**a**, **b**). These cell lines could proliferate for more than three months and could be restored after cryopreservation. The doubling time of the established cell lines of GDLB-GCB1 (from the UPN1 patient) and GDLB-GCB2 (from the UPN2 patient) was 2.9 and 1.7 days, respectively. Abbreviation: IL-4, interleukin-4; CD40L, CD40 ligand; DLBCL, diffuse large B cell lymphoma, UPN, unique patient number.

Proliferation Analysis in the Presence or Absence of Anti-CD40L Antibody and Cytokines

We next examined whether the growth of two established cell lines depended on IL-4 and CD40L. The method of CD40L blocking was conducted as our previous experiments that were used for the other antibodies (Kagami *et al*.) [21]. Removal of IL-4 from the system containing the stimulator cells of $CD40L^{+}3T3$ with IL-4 containing medium resulted in growth inhibition. Proliferation of B cells from the cell lines was also inhibited in medium only or medium containing IL-4 (Fig. **4a**, **b**). When anti-CD40L blocking antibody was added to this culture system, the proliferation of these cell lines was inhibited (Fig. **4a**, **b**). Proliferation of the cells from GDLB-GCB2 was inhibited in a concentration-dependent manner (Fig. **4b**).

Proliferation Analysis with Transwell Culture

The use of an *in vitro* transwell assay, which prevents direct cell-to-cell contact, allows confirmation of whether direct contact with $CD40L^{+}3T3$ cells is required for proliferation of the B-cell lines. The cells inoculated into the inner cup with the transwell assay showed cell growth arrest. Cells of GDLB-GCB2 also stopped growing when the cells were cultured with $CD40L^{-}3T3$ (Fig. **5b**). On the other hand, cells of GDLB-GCB1 stimulated with $CD40L^{-}3T3$ showed cell growth on day 8 (Fig. **5a**). When these cell lines were cultured with $CD40L^{-}3T3$ cells in a 96-well plate, the two cell lines stopped growing as expected.

Array CGH Analysis

Array CGH analysis revealed that the established cell lines showed the identical genomic imbalances as the original samples, indicating that the established cell lines and original samples were derived from the identical clones (Fig. **6**). However, the established cell lines did not have common distinctive genomic imbalances. Array CGH data of the short-term proliferating cells could not be obtained because of the cell death.

DISCUSSION

Established Cell Lines

In this report, we demonstrated that IL-4 and CD40L co-stimulation induced short-term proliferation in DLBCL B cells, and some type of DLBCL B cells showed a long-term proliferation. Interestingly, the three cases including the established two cell lines had common clinicopathological features, presenting with a local extra nodal lesion and a bcl2- CD10+ GCB-like DLBCL phenotype. Moreover, removing IL-4 from the culture system or adding anti-CD40L blocking antibody to the system inhibited proliferation of the cell lines. These results suggested that CD40L and IL-4 co-stimulation played a critical role in long-term proliferation for some type of DLBCL *in vitro*. However, it is possible that more cell lines could have been established under different conditions. It is also conceivable that tumor cell purity influenced the results.

CD40/CD40L Signaling in DLBCL B-Cell Proliferation

The biological behavior, tumor progression and prognosis of patients may be influenced by the tumor microenvironment for some types of malignant lymphomas [22-24]. CD40L is expressed mainly in activated CD4+ T lymphocytes. CD40L binds to CD40 of normal B cells, and induces growth or survival signals of B cells [15, 16]. CD40L expression has also been shown in several types of B-cell lymphomas [11, 12]. Pham *et al*. reported that endogenously expressed CD40L in aggressive B-cell lymphomas binds to the CD40 receptor within cell membrane lipid rafts, and that this CD40 signalosome constitutively activates the canonical NF-κB signaling pathway [12]. In the present study, endogenous CD40L expression was negative in GDLB-GCB1, and weakly positive in GDLB-GCB2. Removing $CD40L^{+}3T3$ feeder cells from the CD40L and IL-4 co-stimulation system or adding anti-CD40L blocking antibody to the system inhibited proliferation of these cell lines. An *in vitro* transwell assay also showed cell growth arrest. These

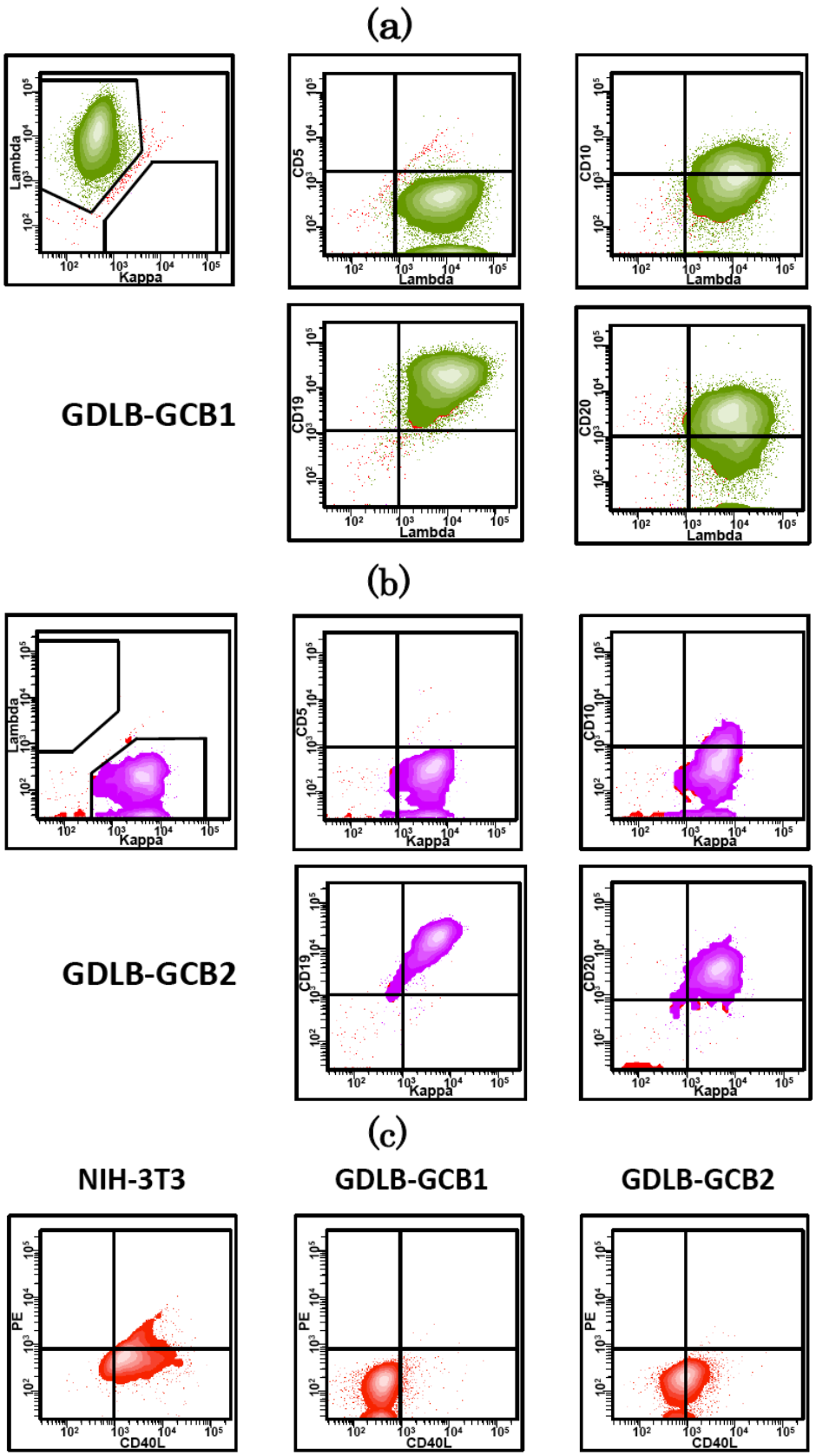


Fig. (3). Flow cytometric analyses of the established cell lines (GDLB-GCB1 and GDLB-GCB2). The original tumor samples and the established cell lines (GDLB-GCB1 and GDLB-GCB2) demonstrated the positive expression of CD10, CD19, CD20 and surface immunoglobulin and the negative expression of CD5 (**a**, **b**). Surface CD40L expression was the negative expression in GDLB-GCB 1 and weakly positive in GDLB-GCB 2 (**c**). $CD40L^{+}3T3$ was used for a positive control of CD40L (**c**). Abbreviation: CD40L, CD40 ligand; $CD40L^{+}3T3$, NIH Swiss mouse embryonic fibroblast cell line transfected with CD40L.

experiments suggested that direct CD40L stimulation from the tumor microenvironment, but not endogenously expressed CD40L, is important for the proliferation of some type of DLBCL.

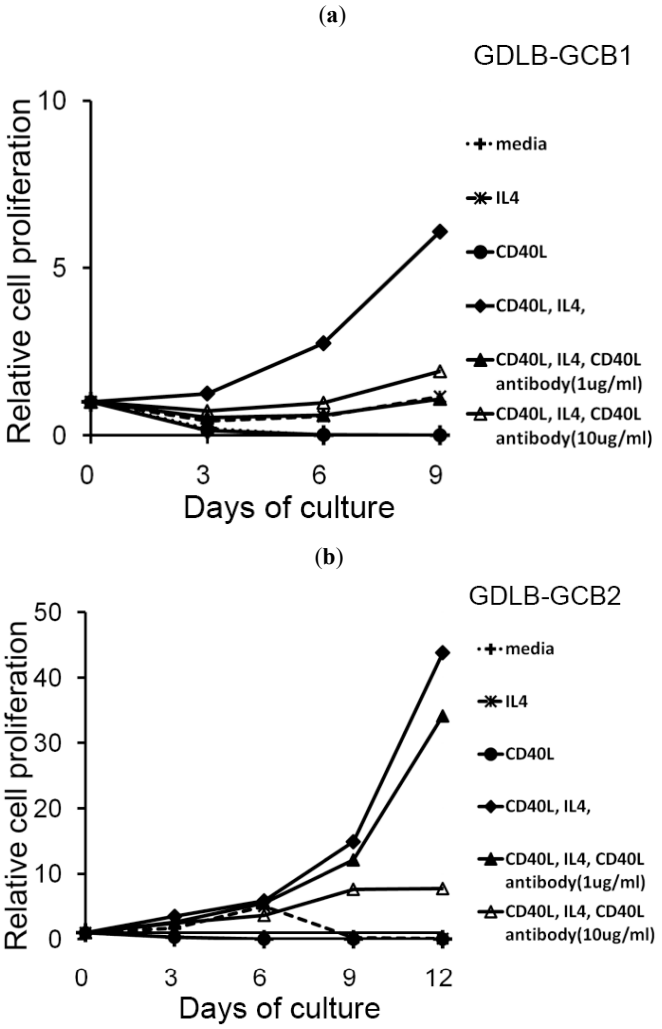


Fig. (4). Growth curves of the established cell lines (GDLB-GCB1 and GDLB-GCB2) in the presence or absence of IL-4 and CD40L stimulation. Removal of IL-4 (●) from the system consisting of $CD40L^{+}3T3$ and IL-4 containing medium (◆) resulted in cell growth arrest. Proliferation of tumor cells also decreased in medium only (┼) or medium containing IL-4 (ж). Addition of anti-CD40 blocking antibody to this culture system inhibited the proliferation of these cell lines (▲)(Δ), and proliferation of the cells from GDLB-GCB2 was inhibited in a concentration-dependent manner. Each condition was repeated in duplicate, and the cell number was counted two times in each well on days 3, 6 and 9. One case (GDLB-GCB2) was counted until day 12. Abbreviation: IL-4, interleukin-4; CD40L, CD40 ligand; $CD40L^{+}3T3$, NIH Swiss mouse embryonic fibroblast cell line transfected with CD40L.

Effect of IL-4 on DLBCL Proliferation

IL-4 is a pleiotropic cytokine regulating differentiation and growth of normal B cells. IL-4 is known to be a potent survival factor for many cell types, and the effect on neoplastic B cells is different for each lymphoma type

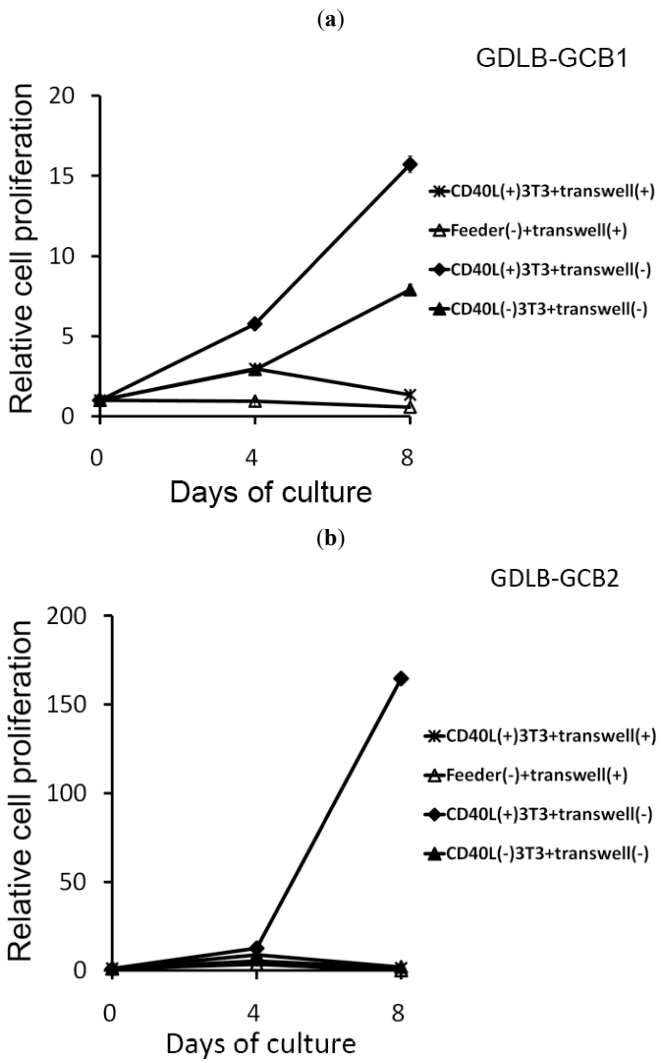
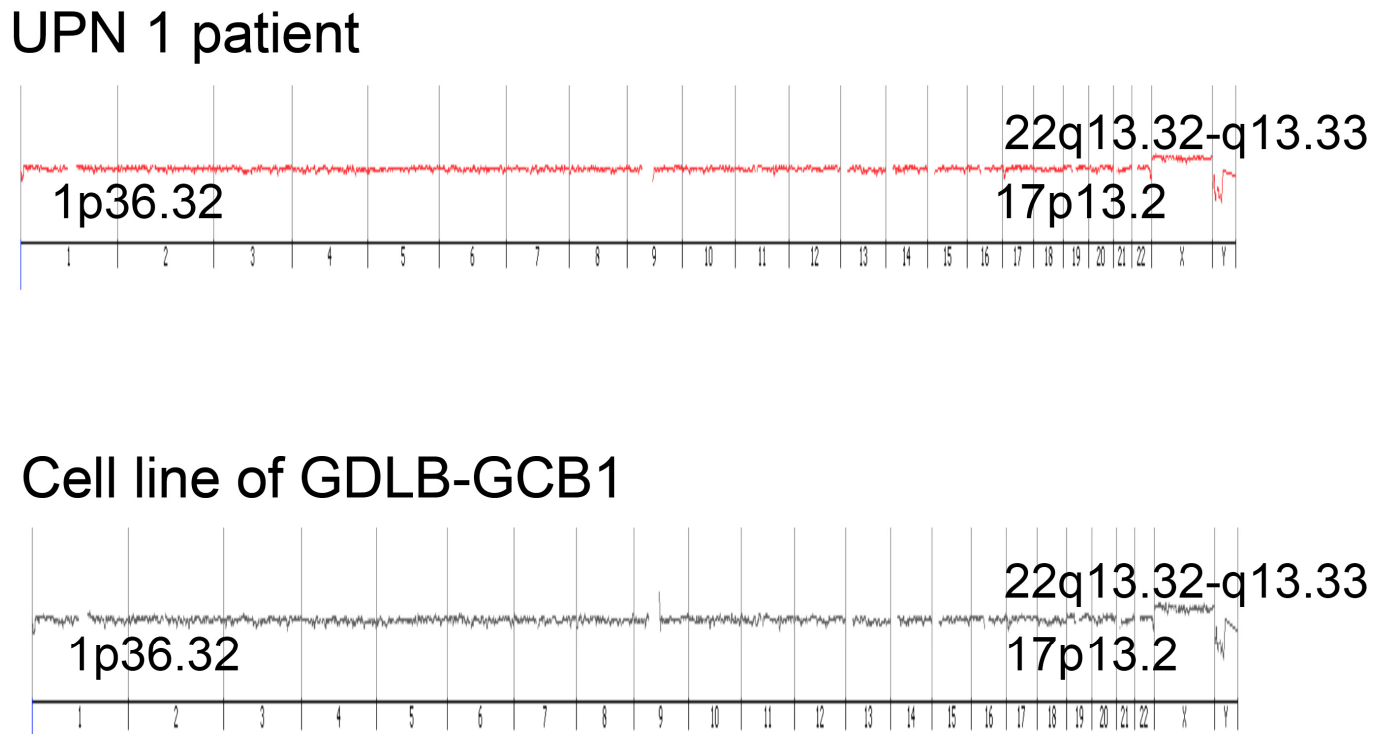


Fig. (5). Growth curves of the established cell lines (GDLB-GCB1 and GDLB-GCB2) without direct contact with feeder cells. When tumor cells and $CD40L^{+}3T3$ cells were separated with a micropore membrane, the proliferation was inhibited (**ж**). The positive control consisted of three conditions: culture with the inner cup without feeder cells (Δ), culture without the inner cup with $CD40L^{+}3T3$ cells (◆), and culture without the inner cup with $CD40L^{-}3T3$ cells (▲). The cells of GDLB-GCB1 stimulated with $CD40L^{-}3T3$ showed cell growth on day 8 (Fig. **5a**). However, when the cell lines were cultured with $CD40L^{-}3T3$ cells in a 96-well plate, the both cell lines stopped growing as expected. Each condition was repeated in duplicate, and the cell number was counted two times in each well on days 4 and 8. Data are presented as the mean ± SE. Abbreviation: CD40L, CD40 ligand; $CD40L^{+}3T3$, NIH Swiss mouse embryonic fibroblast cell line transfected with CD40L; $CD40L^{-}3T3$, NIH Swiss mouse embryonic fibroblast cell line untransfected with CD40L.

[25-28]. Lu *et al.* [29] reported that in GCB-like DLBCL, IL-4 induces expression of its known target genes by activation of STAT6, resulting in cell proliferation. In contrast, in ABC-like DLBCL, IL-4 activates the Akt pathway and results in a decrease of cell proliferation by cell-cycle arrest [29]. In our study, IL-4 was also an important factor for promoting proliferation of the

(**a**)



(**b**)

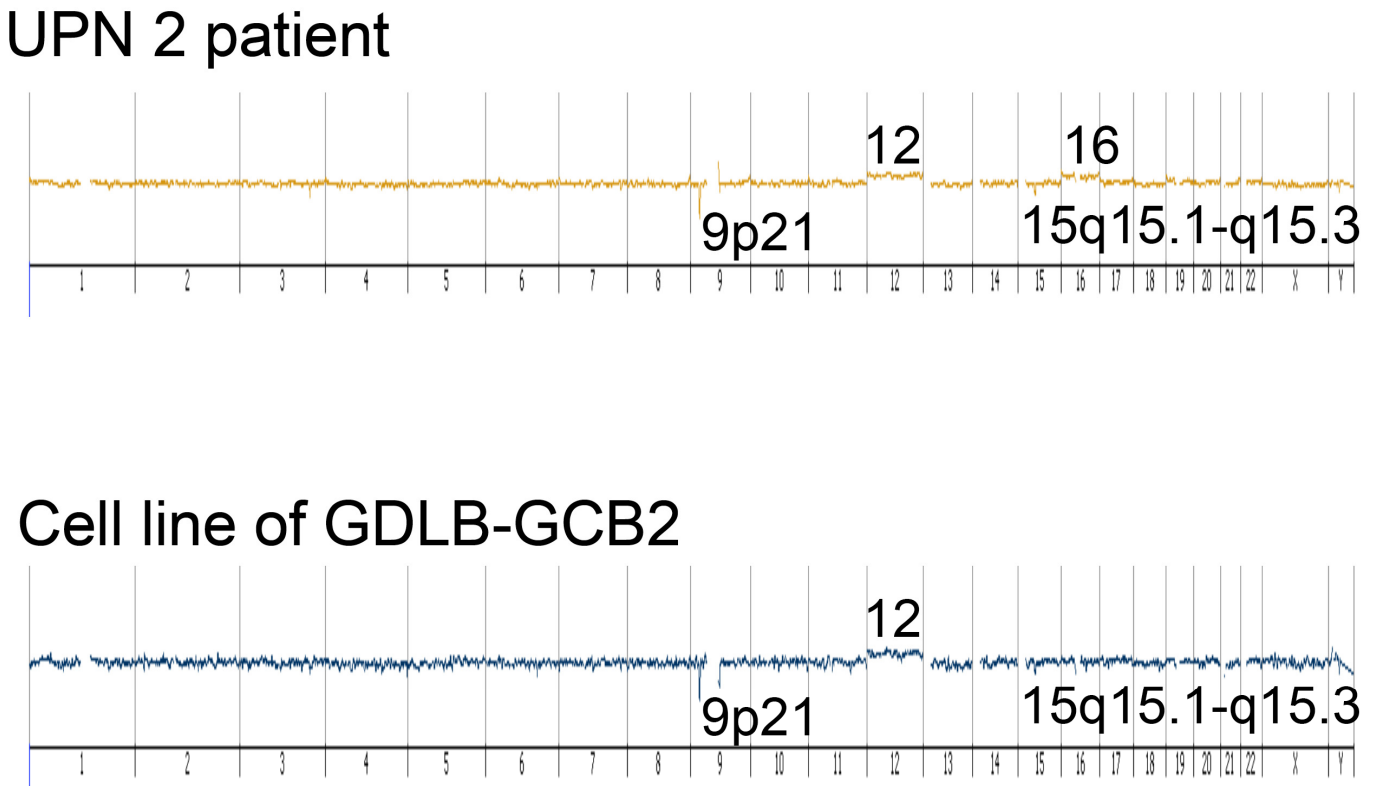


Fig. (6). Genomic imbalances using 4x44K oligo array CGH methods. Array CGH analysis revealed that the established cell lines (GDLB-GCB1 and GDLB-GCB2) showed the identical genomic imbalances as the original samples (UPN1 and 2), indicating that the established cell lines and original samples were derived from the identical clones. Abbreviation: CGH, comparative genomic hybridization; UPN, unique patient number.

established cell lines; however, the stimulation of IL-4 in the absence of CD40L did not show any significant cell growth. For some GCB-like DLBCL patients, CD40L and IL-4 co-stimulation might be associated with lymphoma cell proliferation.

Genetic Characterization and Clinicopathological Features of the Established Cell Lines

Genetic Characterization of the Established Cell Lines

Array CGH analysis showed genetic alteration (deletion of 1p36) in GDLB-GCB1. A recent report revealed a new subtype of FL, which has a characteristic clinicopathological feature of a predominantly diffuse infiltration pattern. It also shows characteristic genetic features with a unifying chromosomal deletion in 1p36 and lack of t(14;18), typical for FL [30]. The report showed that genetic alteration existed in 27 of 29 cases [30]. It is interesting to find that the same genetic aberration were present in our established cell line.

Clinicopathological Features of the Established Cell Lines

In relation to the distinct subtype of FL, the aforementioned report also revealed that many patients had some clinically unique characteristics: low clinical stage, large localized inguinal tumors, and an immunophenotype with frequent expressions of CD10, BCL6 and CD23. The immunophenotype was in accordance with a germinal center B-cell phenotype [30]. In our series, three cases, all representing long-term proliferation, were GCB-like DLBCL and had local extranodal lesions. They also exhibited the same pathological characteristics of bcl2- and CD10+ GCB-like DLBCL, and proliferated in the same system. These results suggested that this group might form a unique entity. Further study is needed to clarify if this type of DLBCL warrants classification as a unique subgroup.

In summary, we demonstrated that CD40L and IL-4 co-stimulation induced the long-term proliferation of some type of DLBCL. Moreover, direct contact with CD40L in the tumor microenvironment is important for cell proliferation. We established DLBCL cell lines stimulated with CD40L and IL-4. These cell lines may be useful for investigating the growth mechanism of GCB-like DLBCL *in vitro* and might provide new insights regarding the pathogenesis of the lymphoma.

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CONFLICT OF INTEREST

The authors indicate no potential conflicts of interest.

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