

Effects of Epstein-Barr virus infection on the development of multiple myeloma after liver transplantation

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Reduced cellular immune function in patients after liver transplantation easily results in many types of viral infections, such as Epstein-Barr virus. Epstein-Barr virus is a γ -herpesvirus and is related to many malignant diseases, especially epithelial and lymph tumors. The abnormal interaction of cluster of differentiation 40 with cluster of differentiation 40 ligand and expression of cluster of differentiation 40 ligand are considered closely related to the development of myeloma cells. This study explored the influence and mechanism of Epstein-Barr virus infection on the phenotype and biological behavior of myeloma cells after liver transplantation. Flow cytometry was used to detect coexpression of cluster of differentiation 40 and cluster of differentiation 40 ligand in 10 samples of freshly isolated multiple myeloma cells. Cluster of differentiation 40 and cluster of differentiation 40 ligand were coexpressed in sample Nos. 5, 8, 9, and 10, particularly in sample No. 5. Western blot analysis was used to detect the expression of the Epstein-Barr virus antigens latent membrane protein 1 and Epstein-Barr virus nuclear antigen 2 in the multiple myeloma cell line RPMI 8226 infected with Epstein-Barr virus. The antigen expression indicated that Epstein-Barr virus can infect multiple myeloma virus cells *in vitro*. Reverse transcription-polymerase chain reaction revealed up-regulated expression of cluster of differentiation 40 ligand on the infected RPMI 8226 cells, which may be involved in the anti-apoptosis activity of the infected cells. Confocal microscopy showed that pairs of molecules of cluster of differentiation 40, cluster of differentiation 40 ligand, and latent membrane protein 1 were colocalized on the surface of the infected cells. CXC chemokine receptor 4 was upregulated on the RPMI 8226 cells after Epstein-Barr virus infection. The migratory ability of the infected cells improved in the presence of the chemokine stromal cell-derived factor-1 α . Anti-apoptosis and migration are known important biological characteristics of malignant cells. Our results indicate the involvement of Epstein-Barr virus in the origin and development of multiple myeloma. The risk of multiple myeloma increases when Epstein-Barr virus infects B cells in the germinal center, which may result in an anti-apoptosis effect of B cells and an improved ability to migrate from the germinal center to peripheral blood.

liver transplantation, CD40, CD40L, multiple myeloma, EBV

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After liver transplantation, patients are immunosuppressed [1] and become susceptible to all types of infection. A common viral infection is caused by Epstein-Barr virus (EBV). The clinical presentation of this infection can vary from no symptoms to serious fulminant infection [2]. Patients usually recover spontaneously from the infection, but complications such as hepatitis, infectious mononucleosis, and post-transplant lymphoproliferative disorder can develop. Under immunosuppression after liver transplantation, T-cell function is suppressed, allowing monoclonality hyperplasia of B cells and tumors to occur [2,3].

EBV is a γ -herpesvirus that is related to many malignant diseases, particularly to tumors that are epithelial and lymphatic in origin. EBV can infect B cells *in vitro* and transform them into immortalized lymphoblastoid cell lines. The viral antigens expressed by lymphoblastoid cell lines include the EBV-determined nuclear antigens (EBNA-1 to -6), EBV-encoded latent membrane proteins (LMP1 and LMP2), and EBV-encoded RNAs [4]. LMP1 plays an important role in B-cell transformation. However, B-cell multiplication *in vitro* cannot be maintained by the expression of LMP1 alone, and a second signal is required [5]. Cluster of differentiation 40 (CD40) is the main accessory of LMP1 signal transduction.

CD40 is a type-I transmembrane glycoprotein that belongs to the tumor necrosis factor (TNF) receptor (TNFR) superfamily. CD40 is expressed in the different stages of B-cell differentiation [6] and in malignant tumor induction by B cells, such as in multiple myeloma (MM). CD40 ligand (CD40L) is mainly expressed in active CD4⁺ T cells, but was recently also found to be expressed in normal or tumor B cells [7,8]. The interaction between abnormal CD40 and CD40L proteins enables myeloma cells to survive [9].

MM is a malignant tumor that originates from plasma cells in the final stage of B-cell differentiation [10]. Tumor cell multiplication mostly occurs in the marrow, and tumor cells can account for 15%–90% of the total cells. The exact etiology of MM is unclear, but it may be related to herpesvirus infection [11]. Consequently, EBV has received much attention in recent years.

This study was designed to detect the frequency of CD40 and CD40L coexpression by flow cytometry (FCM) in freshly isolated MM cells from 10 post-liver-transplant patients who developed EBV infection complicated by MM. The *ex vivo* development of EBV infection complicated by MM was studied. The *ex vivo* changes in the phenotypic and biological behavior of EBV-infected RPMI 8226 cells were also examined, with focus on CD40 and CD40L coexpression. The aims were to explore the mechanism of the malignant transformation of plasma cells into malignant MM cells and the effects of EBV infection on MM development.

1 Materials and methods

1.1 Reagents, cell strains, and bone marrow samples

CD40 monoclonal antibody (mAb) (5C11, 3G3-FITC), CD40L mAb (4F1), LMP1 mAb, EBNA-2 polyclonal antibody, and CXC chemokine receptor 4 (CXCR4) mAb were purchased from Santa Cruz Biotechnology Co., Ltd., USA. Fluorescein isothiocyanate (FITC)-labeled anti-mouse antibody was purchased from Immunotech Corporation, France. RNA extraction kits and Western blot coloration kits were purchased from Shanghai Huashun Corporation, China. Reverse transcription-polymerase chain reaction (RT-PCR) kits were purchased from Takara Corporation, Japan. Annexin-FITC was purchased from Boehringer Mannheim Corporation, Germany. Transwell trays were purchased from Corning Costar Corporation, Germany. Chemokine stromal cell-derived factor-1 α (SDF-1 α) was purchased from BD Corporation, USA. Fresh bone marrow samples from 10 post-liver-transplant patients with a history of EBV infection and MM were obtained from Living Body Liver Research Institute, China. The MM cell strain RPMI 8226, the cell strain B95-8 from EBV, and the B-cell lymphoma cell lines Raji and Daudi were purchased from ATCC Corporation, USA. RPMI 1640 culture medium was purchased from Gibco Corporation, USA. All cell strains were tested against mycoplasma contamination using enzyme-linked immuno sorbent assay (ELISA) kits from Boehringer Mannheim Corporation, Germany.

Fresh bone marrow samples were obtained from 10 post-liver-transplant patients who developed EBV infection complicated by MM. Diagnosis of MM was performed according to internationally recognized criteria (International Myeloma Working Group, 2003). There were seven male and three female participants. Their ages ranged from 14 years to 45 years (mean, 27 years). The hepatitis markers were 7/3 for B/C, 4/6 for HBV DNA +/-, 8/2/0 for Child-Pugh grade A/B/C, and 8/2 for alpha fetoprotein +/- . Preoperative transarterial chemoembolization was performed in one patient. All patients had a single tumor <3 cm in size in a single lobe of the liver. The tumor invaded either the left or right branch of the portal vein in four patients. Serum EBV-IgM was negative in all 10 patients before surgery.

For immunotherapy, seven patients received cyclosporin A and prednisone, whereas three patients received tacrolimus and prednisone. All patients were given Stoss therapy (100–200 mg methylprednisolone sodium succinate) for liver rejection. The patients who were positive for hepatitis B surface antigen were treated with lamivudine. Seven patients developed nonproductive cough, fever, dyspnea, and infiltrative lesions upon chest radiography on postoperative days 9, 11, 12, 14, 19, and 28. Three patients developed

refractory diarrhea, myalgia, arthralgia, nausea, and vomiting on postoperative days 23, 25, and 27, respectively. All 10 patients had fever, and 4 had increased pharyngeal secretion. Seven patients had enlarged lymph nodes: three in the neck and four around the mandible. Two patients had liver enlargement, three had spleen enlargement, and four had both liver and spleen enlargement. Skin rashes were observed in three patients; bronchitis and bronchopneumonia occurred in two. All 10 patients had EBV infection with positive serum for EBV-IgM by ELISA. All patients were diagnosed with EBV infection and treated accordingly, and all eventually recovered. Clinical manifestations of MM developed, and MM was diagnosed 9–29 months after liver transplantation. Specimens of freshly isolated MM cells were obtained from the bone marrow of these patients. Informed consent from each patient was obtained according to the Declaration of Helsinki as agreed upon by the ethical committee of Affiliated Jiangsu Cancer Hospital of Nanjing Medical University.

1.2 FCM analysis of CD40 and CD40L expression in MM cells

Freshly isolated MM cells were collected from the 10 liver transplant patients with EBV infection using a standard Ficoll-Histopaque procedure. Approximately 3 μL each of FITC-conjugated CD40 mAb and R-phycoerythrin-conjugated CD40L or CD138 mAb were added to 100 μL cell suspension (1×10^6 cells) according to the manufacturer's instructions. After 30 min of incubation at room temperature in darkness, the stained samples were treated for 10 min in darkness with 1 mL of Fluorescence Activated Cell Sorter (FACS) lysing solution, which was diluted with MilliQ water at a ratio of 1:9. The samples were then washed with phosphate buffered saline (PBS) and centrifuged at $450 \times g$ for 5 min at room temperature. The cells were fixed in 300 μL of polyformaldehyde solution in darkness for 15 min at room temperature or overnight at 4°C , then washed with FACSFlow solution. The cell pellets were resuspended in FACSFlow solution and analyzed with a FACScan flow cytometer (BD Biosciences, San Jose, CA).

1.3 Preparation of EBV

Based on the method of Oh *et al.* [12], B95-8 cells ($5 \times 10^5 \text{ mL}^{-1}$) in the exponential growth phase were stimulated using 100 nmol L^{-1} of 12-O-tetradecanoylphorbol-13-acetate (TPA) for 2 h. The cells were washed three times with HEPES-buffered saline to remove any remaining TPA in the solution. After cell culture for 48 h, the supernatant was collected by centrifugation at $850 \times g$ for 3 min. The supernatant was passed through a 0.22- μm filter and stored in 1-mL aliquots at 4°C in centrifuge tubes.

1.4 Infection of EBV

The rate of infection of RPMI 8226 cells increased with enriched EBV and prolonged infection time. RPMI 8226 cells (3×10^6) in the exponential growth phase were centrifuged for 5 min, and the supernatant was discarded after each run. Approximately 3 mL of cell suspension containing the prepared virus was cultured for 16 h. The cells were then washed twice with the culture medium to remove the virus. A solution of RPMI 1640 containing 10% fetal calf serum (FCS) was added to continue the cell culture. A control group was established using EBV inactivated by ultraviolet (UV) light (15 W, 60-cm distance, and 60-min irradiation) to infect RPMI 8226 cells.

1.5 Cell phenotype analysis of antibody immunofluorescence

At 48 h after the RPMI 8226 cells were infected with EBV, immunofluorescence assays were performed. The treatment group comprised the EBV-infected cells. The control groups included the RPMI 8226 cells cultured in RPMI 1640 with 10% FCS and without EBV infection or with UV-inactivated EBV infection. The cell density of both groups was adjusted to $5 \times 10^6 \text{ mL}^{-1}$. Next, 100 μL aliquots of the cell cultures were incubated with either 1 μg of CD40 mAb (5C11), 1 μg of CD40L mAb (4F1), or 1 μg of LMP1 mAb at 4°C for 30 min. The cells were washed twice with PBS and incubated with FITC-labeled anti-mouse antibody at 4°C for 30 min. After washing again twice with PBS, the cells were suspended in 0.5 mL of PBS and analyzed with FCM. The experiment was repeated three times for each group, and the average value was obtained.

1.6 RT-PCR

At 24 h after EBV infection, the total number of cells in the cultures was adjusted to $5 \times 10^6 \text{ mL}^{-1}$. RPMI 8226 cells with UV-inactivated EBV infection were used as a control. Total RNA was extracted according to the instructions in the RNA extraction kits. After reverse transcription of complementary DNA, PCR amplification of CD40L was performed using a total of 34 cycles (94°C for 30 s, 56°C for 30 s, and 72°C for 45 s in each cycle). The upstream primer for CD40L was 5'-GCCGAATTCGAAAACAGCTTT-3', whereas the downstream primer was 5'-GCCTCTAGAAATCAGAGT-TTGAGT-3' (Immunotech, France). The upstream primer for β -actin was 5'-TCATGAAGTGTGACGTGGACATC-3', and the downstream primer was 5'-TCTAGTTCTAGTAA-CGAGGAGGAC-3'. For the internal standard, 30 cycles of β -actin amplification were performed as follows: 94°C for 30 s, 53°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 3 min. Electrophoresis was performed with 1% sepharose gels to obtain the PCR products.

1.7 Western blot analysis

Approximately 1×10^6 RPMI 8226 cells from each of the two groups were obtained and washed twice with PBS. RPMI 8226 cells with UV-inactivated EBV infection were used as controls. The cells were resuspended in cold RIPA buffer ($1 \times$ PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate), and then protease inhibitor (10 mg mL^{-1} phenylmethanesulfonyl fluoride, $30 \text{ }\mu\text{g mL}^{-1}$ Aprotinin, and 100 mmol L^{-1} Na_3VO_4) was added to the suspension. The cells were placed on ice for 30 min, aspirated several times with a 1-mL syringe, and centrifuged at $1000 \times g$ at 4°C for 20 min. The supernatant to be used for sodium dodecyl sulfate polyacrylamide gel electrophoresis was transferred to a nitric acid fibrous membrane and sealed for 1 h with 1% confining liquid. LMP1 mAb or EBNA-2 polyclonal antibody was added, and the membrane was incubated for 1 h. The unconjugated antibodies were washed with Tris-buffered saline with Tween (TBST). After adding alkaline phosphatase-labeled goat anti-mouse antibody, the membrane was again incubated for 1 h and washed three times with TBST. The corresponding substrate was added to produce the desired color (details can be found in the instructions from BD Corporation, USA). The equivalent protein loading and transfer efficiency were verified by staining the membranes for glyceraldehyde-3-phosphate dehydrogenase.

1.8 Cell cycle detection and apoptosis analysis

Cell cycle detection with propidium iodide (PI) staining: The cell concentration of the cultures was adjusted to $5 \times 10^6 \text{ mL}^{-1}$. From each culture, $100 \text{ }\mu\text{L}$ cell suspension was collected and mixed with $500 \text{ }\mu\text{L}$ PI buffer ($450 \text{ }\mu\text{L}$ H_2O , 0.05 mol L^{-1} sodium citrate, $50 \text{ }\mu\text{g}$ of PI, 0.05% Triton 100, and $100 \text{ }\mu\text{g mL}^{-1}$ Rnase) placed at 4°C for 30 min in darkness and analyzed with FCM.

Apoptosis analysis: Apoptotic cells were detected using PI and Annexin-V by double labeling. Approximately 5×10^5 cells were washed twice with PBS and resuspended in $500 \text{ }\mu\text{L}$ of cooled PI buffer. To each suspension, $5 \text{ }\mu\text{L}$ FITC-Annexin-V and $5 \text{ }\mu\text{L}$ PI were added. The suspensions were incubated at 4°C for 10 min in darkness and then analyzed with FCM (details can be found in the instructions from Immunotech, France).

1.9 Detection of cell migration ability toward SDF-1 α

Transwell plates with $8\text{-}\mu\text{m}$ pore sizes were divided into four groups to detect the ability of the cultured cells to migrate toward the chemokine SDF-1 α . Group A had cells with EBV(-) RPMI 8226+SDF-1 α (-) in RPMI 1640 containing 10% FCS, Group B had EBV(+) RPMI 8226+SDF-1 α (-) in RPMI 1640 containing 10% FCS, Group C

had EBV(-) RPMI 8226+SDF-1 α (+) in RPMI 1640 containing 10% FCS, and Group D had EBV(+) RPMI 8226+SDF-1 α (+) in RPMI 1640 containing 10% FCS. In the upper chamber, approximately 10^5 cells were added to each group. In the lower chamber, $600 \text{ }\mu\text{L}$ 10% FCS in RPMI 1640 containing 200 ng mL^{-1} SDF-1 α was added. After incubation at 37°C for 4 h, the cells were collected and counted with FCM to observe the cell migration ability in the different groups.

1.10 Laser confocal microscopy

Cells at a concentration of $1 \times 10^5 \text{ mL}^{-1}$ were washed with PBS, and $100 \text{ }\mu\text{L}$ of fixation liquid was added to resuspend the cells. The suspensions were kept at room temperature for 15–30 min. After adding 1 mL PBS/Bull Serum Albumin (BSA) (PBS containing 1 mg mL^{-1} BSA), the cells were resuspended, centrifuged, and washed twice. The cells were then divided into two groups. To one group, $100 \text{ }\mu\text{L}$ of reaction liquid I (containing $10 \text{ }\mu\text{g mL}^{-1}$ CD40 polyclonal antibody and $10 \text{ }\mu\text{g mL}^{-1}$ LMP1 mAb in PBS/BSA) was added to resuspend the cells, which were kept at room temperature for 30–60 min. Next, 1 mL of PBS/BSA was added and the cells were resuspended, centrifuged, and washed twice. Thereafter, $100 \text{ }\mu\text{L}$ reaction liquid II (containing $20 \text{ }\mu\text{g mL}^{-1}$ Cy5-goat anti-rabbit IgG and Cy3-goat anti-mouse IgG in PBS/BSA) were added, and the cells were resuspended, kept at room temperature for 30–45 min, and washed. To the other group, $100 \text{ }\mu\text{L}$ of reaction liquid I (containing $10 \text{ }\mu\text{g mL}^{-1}$ CD40L mAb 4F1 in PBS/BSA) was added, and the cells were resuspended and kept at room temperature for 30–60 min. Next, 1 mL PBS/BSA was added and the cells were resuspended, centrifuged, and washed twice. After adding $100 \text{ }\mu\text{L}$ of reaction liquid II (containing $20 \text{ }\mu\text{g mL}^{-1}$ Cy3-goat anti-mouse IgG PBS/BSA), the cells were resuspended, kept at room temperature for 30–45 min, and washed. Next, $100 \text{ }\mu\text{L}$ of reaction liquid III (containing $10 \text{ }\mu\text{g mL}^{-1}$ 3G3-FITC in PBS/BSA) was added and the cells were resuspended, kept at room temperature for 30–45 min, and washed. Both groups of cells were washed once, $20 \text{ }\mu\text{L}$ of covering liquid was added, and the cells were resuspended. Approximately $10 \text{ }\mu\text{L}$ of covering liquid containing the cells (prepared as mentioned above) was placed on microscope slides, covered with a glass cover slip, and observed under a confocal microscope (Bio-Rad).

1.11 Cell proliferation

Daudi cells were washed and adjusted to a cell density of $1 \times 10^5 \text{ mL}^{-1}$ with RPMI 1640 culture medium containing 10% FCS. Approximately $100 \text{ }\mu\text{L}$ of cell suspension was incubated with IgG and CD40 mAb at concentrations of 0, 5, 10, and $20 \text{ }\mu\text{g mL}^{-1}$, respectively, in 96-well plates in an

atmosphere of 5% CO₂ at 37°C for 24 h. Tritiated thymidine of 3.7×10⁴ Bq was then added to each culture well. After incubation for 16 h, the cells were harvested to obtain the results in cycles per minute results. The experiment was carried out in triplicate for each group, and the average result was obtained.

1.12 Cell apoptosis

Four separate groups of Daudi or Raji cells were incubated with different treatments: (i) RPMI 1640, (ii) 15 μg mL⁻¹ IgG as a homotype control, (iii) 15 μg mL⁻¹ CD40 mAb, and (iv) 15 μg mL⁻¹ CD40L mAb for 24 h in six-well plates. The cells (5×10⁵) were washed three times in PBS and suspended in 500 μL of cold PI. Next, 5 μL each of FITC-Annexin V and PI were added, and the cell suspensions were incubated for another 10 min at 4°C in darkness. The cells were analyzed by FCM.

1.13 Statistical analysis

Statistical comparisons were conducted using the Student's *t*-test, and *P*<0.05 was considered significant. All statistical analyses were carried out with SPSS v11.0 (SPSS Inc., Chicago, IL).

2 Results

2.1 Coexpression of CD40 and CD40L in freshly isolated MM cells

CD138 is a labeled molecule of MM. Through the individual detection of CD138 and CD40 or of both CD138 and CD40L by double labeling, the expression of CD40 and CD40L in MM cells can be determined. The expression of CD40 and CD40L differed among the 10 specimens. CD40 and CD40L were coexpressed in specimen Nos. 5, 8, 9, and 10 (*, *P*>0.05) (Figure 1), particularly in specimen No. 5.

2.2 CD40L surface expression in EBV-infected RPMI 8226 cells at the RNA and protein levels

After *in vitro* cultivation of RPMI 8226 cells and the supernatant containing EBV, the LMP1 expression in the infected cells was detected by immunofluorescence antibody labeling and FCM (Figure 2A). The results showed that approximately 95% of the RPMI 8226 cells were infected with EBV. Western blot analysis also detected LMP1 expression and EBNA-2 expression in the infected RPMI 8226 cells (Figure 3A). RT-PCR analysis performed 24 h after infection indicated the upregulated expression of CD40L in the infected 8226 cells (Figure 3B). FCM performed 48 h after infection also indicated the upregulated expression of CD40L on the surface of the cell membranes (Figure 2B),

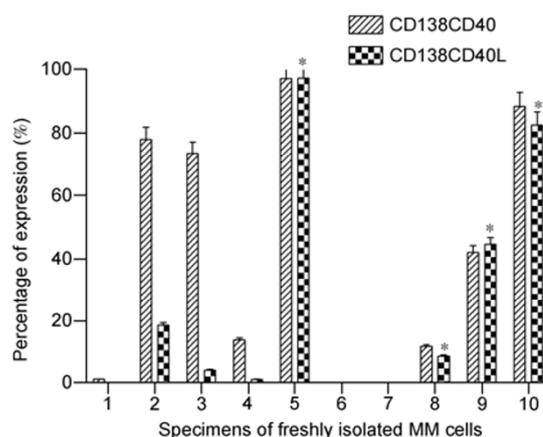


Figure 1 Coexpression of CD40 and CD40L in freshly isolated MM cells from 10 liver transplant patients with EBV infection. CD40 and CD40L were coexpressed in specimen Nos. 5, 8, 9, and 10 (*, *P*>0.05), particularly in specimen No. 5.

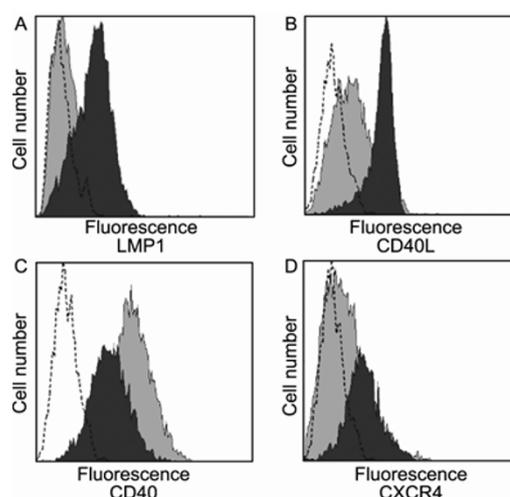


Figure 2 Phenotype detection of EBV-infected RPMI 8226 cells by FCM. Surface expression of markers on non-infected cells (light grey area), EBV-infected RPMI 8226 cells (dark grey area), or isotype controls (dashed line) 48 h after infection. The expression of LMP1, CD40L, and CXCR4 on the surface of the cell membranes was upregulated, but the expression of CD40 was downregulated.

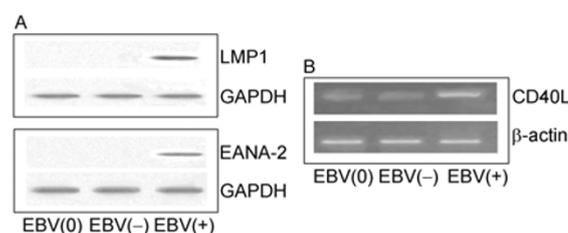


Figure 3 Biochemistry detection results. A, Western blot analysis of LMP1 and EBNA-2 expression 24 h after infection. EBV(0) indicates infected RPMI 8226 cells by UV-inactivated EBV, EBV(-) indicates non-infected RPMI 8226 cells, and EBV(+) indicates EBV-infected RPMI 8226 cells. Upper, LMP1 expression; below, EBNA-2 expression. B, RT-PCR detection of CD40L mRNA 24 h after infection. EBV(0) indicates infected RPMI 8226 cells by UV-inactivated EBV, EBV(-) indicates non-infected RPMI 8226 cells (constitutive expression of CD40L mRNA), and EBV(+) indicates EBV-infected RPMI 8226 cells (upregulation of CD40L mRNA).

whereas the expression of CD40 was downregulated (Figure 2C).

2.3 Anti-apoptosis in EBV-infected RPMI 8226 cells

No obvious change in the cell cycle of EBV-infected RPMI 8226 cells was observed 24 h after infection. However, a small apoptotic peak appeared before G₁ (Figure 4B). At 48 h after infection when the upregulated expression of CD40L was observed, the apoptotic peak disappeared (Figure 4C). The apoptosis ratio of non-infected 8226 cells was 2.35%±0.45%. The apoptosis ratio increased to 12.30%±2.10% 24 h after infection. When the expression of CD40L was upregulated 48 h after infection, the apoptosis ratio decreased to 3.92%±0.42% (Figure 5).

2.4 CXCR4 expression by the upregulation of EBV-infected RPMI 8226 cells

FCM demonstrated that the RPMI 8226 cells expressed the chemokine receptor CXCR4 48 h after infection (Figure 2D). The infected cells, under the effect of the chemokine SDF-1 α , had a significantly enhanced migration ratio

($P<0.05$) (Figure 6).

2.5 Colocation of CD40, CD40L, and LMP1 on infected cells

FCM showed green fluorescence with direct immunofluorescence antibody 3G3-FITC, which labeled the CD40 molecule on the surface of the infected cells. FCM showed red fluorescence with indirect immunofluorescence antibody 4F1 or with anti-LMP1 that labeled CD40L when combined with a secondary antibody Cy3. FCM showed yellow fluorescence if the two molecules CD40 and CD40L colocalized on the surface of the infected cells. Confocal microscopy showed that the RPMI 8226 cells became irregular after infection and that CD40 and CD40L, or EBV protein LMP1, can colocalize on the cell surface (yellow fluorescence) (Figure 7). Thus, LMP1 may be involved in CD40 signal transduction.

2.6 Effects of CD40-CD40L on Daudi cell proliferation

Daudi cell proliferation was examined after treatment with homotype IgG (the control group), CD40 mAb, or CD40L

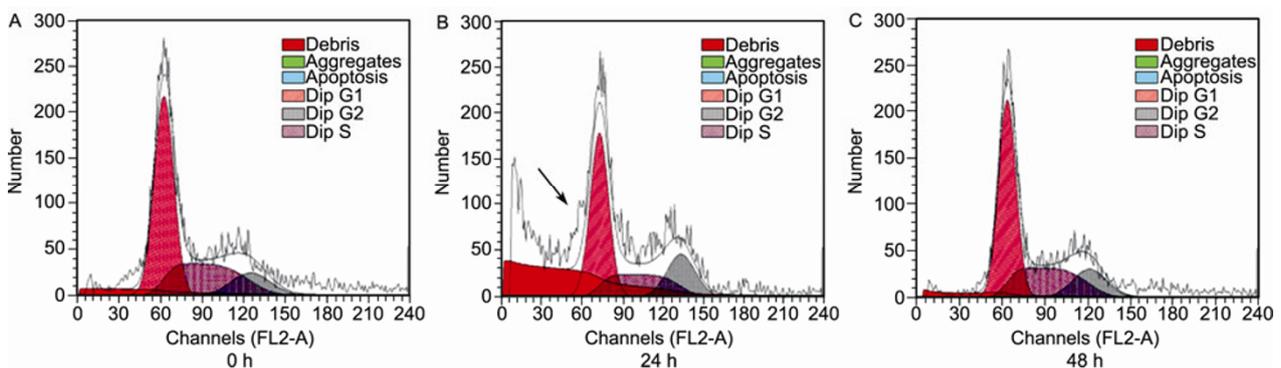


Figure 4 Cell cycle stages of EBV-infected RPMI 8226 cells with PI staining followed by flow cytometry analysis. The arrow indicates sub-G₁ 24 h after infection, which disappeared 48 h after infection when CD40L was up-regulated.

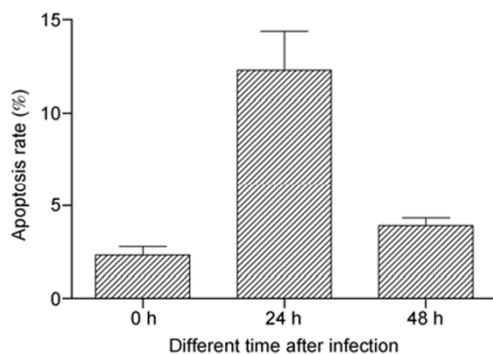


Figure 5 Apoptosis ratio of RPMI 8226 cells after EBV infection. The apoptosis index of non-infected RPMI 8226 cells was 2.35%±0.45% (0 h). The apoptosis index of EBV-infected RPMI 8226 cells 24 h after infection was 12.30%±2.10% (24 h) ($P<0.05$ vs. other groups). The apoptosis index of EBV-infected RPMI 8226 cells was 3.92%±0.42% 48 h after infection when CD40L was upregulated.

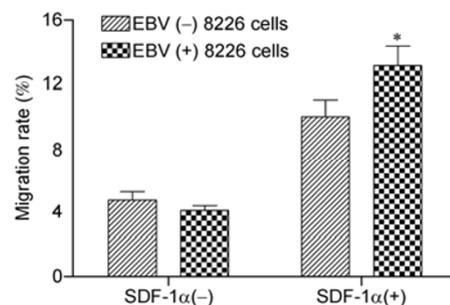


Figure 6 Migration ability of EBV-infected RPMI 8226 cells toward SDF-1 α . In the absence of SDF-1 α , 4.79%±0.52% of the non-infected RPMI 8226 cells migrated, whereas 4.14%±0.31% of the EBV-infected RPMI 8226 cells migrated. In the presence of SDF-1 α , 10.04%±1.03% of the non-infected RPMI 8226 cells migrated, whereas 13.18%±1.21% of the EBV-infected RPMI 8226 cells migrated. *, $P<0.05$ vs. infected RPMI 8226 cell migration in the absence of SDF-1 α .

mAb. The results showed that both CD40 and CD40L mAbs inhibited Daudi proliferation in a dose-dependent manner. The inhibition of CD40L mAb was stronger than that of CD40 mAb (Figure 8).

2.7 Effects of CD40-CD40L on Raji cell apoptosis

Apoptosis was examined after treatment with homotype IgG (control group), CD40 mAb, or CD40L mAb. The results showed that both CD40 and CD40L mAbs increased the rate of Raji cell apoptosis. However, only CD40L mAb increased the rate of Daudi cell apoptosis. CD40 mAb had no significant effect on Daudi cell apoptosis (Figure 8).

3 Discussion

CD40, a 45- to 50-kD membrane, belongs to the TNFR superfamily. CD40 is mainly expressed on mature B cells and may be involved in a variety of biological effects. In many

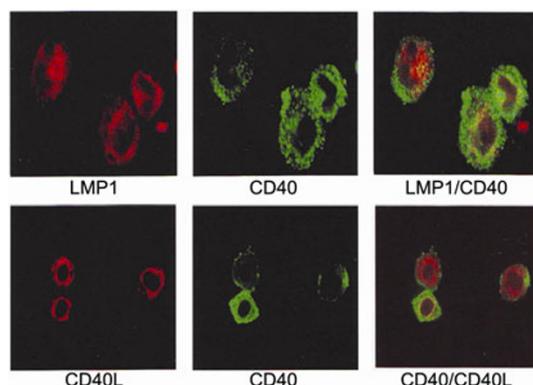


Figure 7 Colocalization of a pair of molecules of CD40, CD40L, and LMP1 on infected RPMI 8226 cells using confocal microscopy. CD40/CD40L and LMP1/CD40 are colocalized on the cell surface (yellow fluorescence).

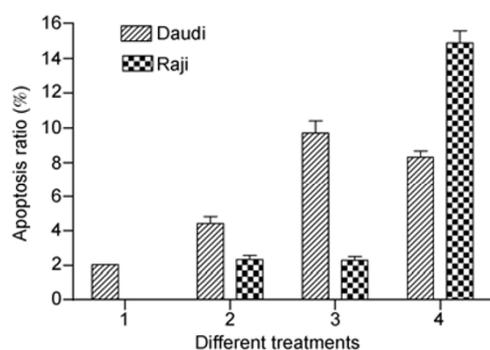


Figure 8 Apoptosis ratio of Daudi or Raji cells with different treatments. 1, RPMI 1640 medium; 2, 15 $\mu\text{g mL}^{-1}$ IgG as homotype control; 3, 15 $\mu\text{g mL}^{-1}$ CD40 mAb; 4, 15 $\mu\text{g mL}^{-1}$ CD40L mAb for 24 h in six-well plates. Both CD40 and CD40L mAbs increased the rate of Raji cell apoptosis. However, only CD40L mAb increased the rate of Daudi cell apoptosis. CD40 mAb had no significant effect on Daudi cell apoptosis.

ways, CD40 is similar to LMP1, a viral protein expressed on B cells that have been transformed by EBV. During CD40 ligation, CD40 and LMP1 on the transformed cells can be recruited to lipid rafts to create a signal transduction complex. CD40L, the ligand of CD40, also known as CD154, is a 39-kD membrane molecule from the TNF superfamily. CD40L is transiently expressed on the germinal center (GC) of B cells or abnormally expressed on B-cell-originated malignant cells. The maturation of B cells is known to depend on their binding to CD40L, which is transiently expressed on CD40⁺ T cells within the GC. This binding promotes the ligation of CD40 on B cells and results in the survival, proliferation, and differentiation of the high-affinity, non-autoreactive B cells. The CD40-CD40L interaction between the B and T cells within the GC is able to prevent the apoptosis of B cells. However, the number of T cells within the GC is limited, and the expression of CD40L on the B cells in the GC is transient. This strict selection leads to the survival of only a fraction of the functional B cells, whereas the majority of malignant or autoreactive B cells that fail to bind to T cells are destroyed. Unfortunately, many malignant cells can still escape this selective process within the GC, survive, and proliferate. Recent research has shown that receptors and ligands can be coexpressed in the same cells, particularly in malignant cells [13]. Some carcinogenic factors such as viral infection can result in ectopic or upregulated expression, which consequently allows malignant cells to avoid apoptosis or the attack of immune cells. The exact effects of the coexpression of receptors and ligands on malignant cells warrant further research.

The abnormal expression of both CD40 and CD40L on B-cell-originated malignant cells is involved in the development of tumors. The interaction of CD40 and CD40L is used in cell transformation in many carcinogens, such as in viral infections [14]. Whether the CD40L provided by these cells or the upregulation of CD40L enables the survival of malignant B cells, which normally cannot enter the T-cell-dependent pathway in the GC, remains unclear. Studies on CD40-CD40L interactions in tumors, as well as the changes in their expression and their biological effects under special conditions such as viral infections, provide important information for research on tumor development.

The present study showed that EBV can cause *ex vivo* infection of RPMI 8226 cells. FCM and Western blot analysis detected the expression of LMP1 and EBVA-2 at the protein level. FCM and RT-PCR detected the upregulated expression of CD40L after EBV infection. Other researchers reported that LMP1 plays a key role in B-cell transformation by EBV [15,16]. Although LMP1 is very similar to CD40 in terms of the signal transduction pathway and in the recruitment of TNF receptor-associated factor signaling molecules, it cannot entirely replace CD40 [17] because LMP1 and CD40 differ in some aspects. After a cell receives CD40L aglycone, LMP1 and CD40 can colocalize

and form a signal transduction complex in the form of a lipid raft that transmits the signal to the target cell [18]. B-cell proliferation *in vitro* cannot be maintained by the expression of LMP1 alone. Thus, both CD40 and CD40L may be required for this process.

FCM, RT-PCR, and Western blot analysis demonstrated the slight expression of CD40L in RPMI 8226 cells. This finding is consistent with that of a published report that showed CD40 and CD40L coexpression in B lymphomas [19].

The expression of CD40L was upregulated by EBV-infected B cells [20,21]. These CD40L molecules played an important part in the course of the transformation of B cells into immortalized lymphoblastoid cell lines. The upregulated expression of CD40L in RPMI 8226 cells may be related to the resistance of the infected cells to apoptosis [22]. Cell cycle analysis revealed an apoptotic peak of the cells 24 h after infection with a large dose of the virus. When CD40L was upregulated 48 h after infection, the apoptotic peak disappeared (Figure 4C). A similar result was found by double labeling with Annexin-V and PI to detect apoptosis (Figure 5). However, CD40 was downregulated after infection (Figure 2C). A previous report showed that CD40 induced the production of interleukin-10 at a low dose and induced the secretion of interleukin-12 at a high dose [23]. Our previous research also showed that CD40 antibody stimulated MM cell proliferation at a low dose but induced the apoptosis of myeloma cells at a high dose [24,25]. Therefore, the infected cells may be regulated through CD40L upregulation and CD40 downregulation such that a favorable signal intensity is reached to regulate cell growth.

Malignant transformation of tumor cells changes some normal physiological activities [26]. In the present study, the abnormal expression of CD40L, the coexpression of CD40 and CD40L, and their colocalization on the surface of the B lymphoma cell lines Daudi and Raji were all observed. CD40 or CD40L mAb prevented CD40-CD40L signal transduction in two ways. These antibodies can destroy the signal transduction complex on the surface of individual cells. Alternatively, the antibodies prevent the intracellular signal transduction of CD40 and CD40L, thereby inhibiting tumor cell survival and proliferation and the increased rate of apoptosis. When an inhibitory effect becomes predominant, the signal transduction complex is destroyed and the signal transduction is stopped at the level of a single molecule of the complex. Thus, the CD40 signal transduction complex can be used as a target for the clinical therapy of tumors.

In conclusion, the upregulated expression of CD40L on EBV-infected MM cells from liver transplant patients are involved in the transformation of plasma cells into MM cells. The mechanism is similar to the persistent and high-intensity expression of CD40L in the GC, which enables cells to undergo apoptosis. The expression of the chemokine receptor CXCR4 is upregulated after infection (Figure 2D) and cell

migration is significantly promoted, with an increase in the migratory ability of the cells. The findings of this study on the relationship between EBV-induced diseases and the CD40-CD40L interaction require confirmation.

The upregulated expression of CD40L on EBV-infected MM cells after liver transplantation influences plasma cell transformation to MM and the anti-apoptosis effect of MM cells after infection. CD40 and CD40L or the EBV antigen LMP1 are colocalized on the surface of EBV-infected cells. These membrane proteins are also coexpressed and colocalized on freshly isolated MM cells and on the B lymphoma cell lines Daudi and Raji.

The coexpression of CD40 and CD40L is significant because when CD40-CD40L signal transduction is inhibited by CD40 or CD40L mAb, Daudi or Raji cell growth, proliferation, and induction of cell apoptosis are remarkably inhibited. These inhibitory effects can be adjusted using different antibodies on the different molecules, and the effects of these antibodies are dose-dependent.

The relationship between EBV-induced diseases and the CD40-CD40L interaction should be further studied to discover a new approach to the prevention of myelomas through immunotherapy.

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