Effect of shRNA targeting mouse CD99L2 gene in a murine B cell lymphoma in vitro and in vivo

FANG LIU$^{1,2,*}$, GONG ZHANG$^1$, FANRONG LIU$^1$, XINHUA ZHOU$^{1,3}$, XIAOYAN CHEN$^{1,2}$, XIQUAN HAN$^{1,3}$, ZIQING WU$^{1,3}$ and TONG ZHAO$^{1,3}$

$^1$Department of Pathology, School of Basic Medical Science, Southern Medical University, Guangzhou 510515; $^2$Department of Physiology and Pathophysiology, Medical School, Foshan University, Foshan 528000; $^3$Department of Pathology, Nanfang Hospital, Southern Medical University, Guangzhou 510515, P.R. China

Received November 7, 2012; Accepted December 11, 2012

DOI: 10.3892/or.2013.2244

Abstract. Mouse CD99 antigen-like 2 (mCD99L2) has previously been confirmed to be expressed in murine B lymphoma (A20) cells by our group. The present study aimed to establish a mCD99L2-downregulated A20 cell line and to investigate the effect of shRNA targeting mCD99L2 in A20 cells in vitro and in vivo. Four pLenti6/mCD99L2 expression vectors containing the mCD99L2 shRNA-expressing cassette were constructed, transplanted into A20 cells and stable mCD99L2-downregulated A20 subclones, termed A20-mCD99L2- cells, were established and identified by quantitative PCR and western blot analysis. Light and transmission electron microscopy, MTT assay, flow cytometry and immunofluorescence labeling were used to observe the morphological, biological and phenotypic characteristics in vitro. Some of the A20-mCD99L2- cells exhibited H/RS-cell like morphology, a decreased proliferative ability, a prolonged G2 phase and increased CD30 and CD15 expression. Upon injecting cells into nude or immunocompetent BALB/c mice, tumors in nude and BALB/c mice were observed. A20-mCD99L2- cells induced tumors in nude and BALB/c mice, but with less potency in the latter compared with the controls. Similar morphological, biological and phenotypic characteristics were observed in the A20-mCD99L2- cell-induced tumors as those in vitro. Several cytokines including CD30T, IL-12p40/p70, IL-3, IFN-γ, CXCL16, MIP-1α and CD40 were upregulated following mCD99L2 downregulation when detected using antibody arrays. The results from western blot analysis indicated that the regulation of mCD99L2 expression may involve the activated nuclear factor-κB pathway in the murine B lymphoma cells. The present study provides data for further investigation into the mCD99L2 gene in tumor cells.

Introduction

Human CD99 is a 32 kDa transmembrane glycoprotein encoded by a pseudoautosomal MIC2 gene located in Xp22.33-pter and Yp11-pter (1). It is expressed in several types of cells and is involved in several cellular events such as intercellular adhesion between lymphocytes and endothelial cells (2), migration of immune cells to inflammatory sites (3,4) and attenuation of graft-versus-host disease (5). Certain studies have found that CD99 is expressed in tumors, including lymphoblastic lymphoma/leukemia (6), Ewing’s sarcoma/primitive neuroectodermal tumors (ES/PNET) (7), dermatofibrosarcoma protuberans, as well as giant cell fibroblastoma (8) and hepatoblastomas (9), while others found that CD99 is expressed in benign pancreatic endocrine neoplasm and gastric adenocarcinoma (10), but not in malignant counterparts.

Mouse CD99 antigen-like2 (mCD99L2), a widely expressed antigen of unknown function with moderate sequence similarity between mCD99L2 and human CD99, was shown to have similar functions. However, to date, the majority of studies on mCD99L2 have focused mainly on its role in inflammation. Its effects on tumor cells have yet to be reported.

A20 is a murine cell line derived from a spontaneously arising tumor in an aged BALB/c mouse (14). It pathologically mimics the characteristics of human diffuse large B cell lymphoma (15). An A20-related animal model may be used in studies on the association between tumors and hosts (16). We successfully established a disseminated A20 animal model and described its immuno-characteristics (17). We also...
observed that mCD99L2 was expressed in A20 cells (18). To investigate the role of mCD99L2 in A20 cells, four pLent6/mCD99L2 expression vectors containing the mCD99L2 shRNA-expressing cassette were constructed in the present study, transfected into A20 cells using DMRIE-C2, and the stable mCD99L2-downregulated A20 cell line, termed A20-mCD99L2 cells, was established and identified using quantitative PCR and western blot analysis. The effect of shRNA targeting mCD99L2 during continuous culturing was observed. The morphological, biological and phenotypic characteristics of the A20-mCD99L2 cells were extensively investigated in vitro and in vivo, to provide data for additional functional studies of mCD99L2 in associated tumors.

Using CD99-deficient IM9 and BJAB B cell lines, investigators have confirmed that the downregulation of CD99 is a primary requirement for generating ‘Hodgkin and Reed-Sternberg’ (H/RS) cells (19,20). A previous study of ours suggested that the CD99-upregulated H/RS cell line (L428) lost its nature as H/RS cells (21). In this study, we investigated whether mCD99L2 downregulation can induce cells with an H/RS morphology and phenotypes in murine B lymphoma cells, to determine whether there is a functional similarity between human CD99 and murine CD99 genes.

Materials and methods

Cell lines and morphology observation. The BALB/c-derived mouse B lymphoma A20 cell line was kindly provided by Professor Chan of the Nebraska Medical Center, Omaha, NE, USA. The subclones of A20 cells transfected with shRNAs targeting mCD99L2 or negative control vectors were constructed and cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA).

Morphological observation of live cells was conducted with an inverted microscope. Transmission electron microcopy (TEM) was performed according to the manufacturer's instructions. Hematoxylin and eosin (H&E) staining was applied on the fixed cells previously dripped onto slides and preserved under -80°C.

Preparation of lentiviral vectors and RNA interference (RNAi). Four different sequences targeting the mCD99L2 gene were selected by BLOCK-it™ RNAi Designer (Invitrogen, Carlsbad, CA, USA). The preparation of lentiviral vectors expressing mCD99L2 short hairpin RNA (shRNA) was performed using the BLOCK-it Lentiviral RNAi Expression System (catalog no. K4944-00; Invitrogen). Four pLent6/mCD99L2 expression vectors containing the mCD99L2 shRNA-expressing cassette were constructed. The lentiviral vectors containing the human Lamin A/C shRNA-expressing cassette (sequence 5'-CTGGACCTCCAGAGAACA-3') were used as the positive control and the pLent6/U6 mock vector was used as the negative control. A20 cells were transfected with specific or negative control lentiviral vectors using DMRIE-C2 at suitable ratios and selected for stable integrants by culturing in complete medium containing blasticidin (Invitrogen). Several single blasticidin-resistant colonies were isolated using the soft agar clone formation protocol, expanded into sub-cell lines by 96-well plate limiting dilution assay.

RNA isolation and quantitative PCR. Total RNA was extracted using the Takara RNAiso plus kit and cDNA was prepared from 2 µg total RNA by PrimeScript Reverse Transcriptase (Takara Bio Co., Ltd., Shiga, Japan). Real-time PCR was performed on a 7500/7500 Fast Real-Time PCR System (Applied Biosystem, Foster City, CA, USA) using a SYBR-Green Premix Ex Taq™ kit (Takara), following the manufacturer's instructions under the conditions of 95°C for 30 sec, followed by 40 cycles of 95°C for 5 sec and 58°C for 34 sec. PCR primers were purchased from Invitrogen and were as follows: mCD99L2, forward, 5'-GCCGCAGCAACAAGCAAGCAT-3' and reverse, 5'-CCCAACCACCTAGTCTTCCG-3'; GAPDH, forward, 5'-ACAGTCTACCGCATTTCTT-3' and reverse, 5'-GACAGGCTTCCGTTTCTCAG-3'. The results were analyzed using the software installed in the 7500/7500 Fast Real-Time PCR System (Applied Biosystem) and the relative expression ratio was determined using the formula $2^{-ΔΔCt}$.

Cell proliferation. Cell proliferation was analyzed using the MTT assay (Sigma, St. Louis, MO, USA). Briefly, 1x10^5 cells were seeded into each well of a 96-well plate with quadruplicate repeats for each condition. After 24 h of incubation, cells were mixed with MTT reagent and incubated for 4 h. The formazan crystals formed by viable cells were then solubilized in dimethyl sulfoxide (DMSO) and measured at 490 nm. Each experiment was performed in triplicate.

Flow cytometry. The cultured cells were harvested at the exponential growth phase and prepared as single cell suspensions. Cells (1x10^6) were fixed in 70% ethanol in phosphate-buffered saline (PBS) on ice, pelleted, incubated with RNase A (0.1 µg/ml) for 30 min at 37°C and stained with propidium iodide (PI) (40 µg/ml) for cell cycle analysis.

Cells were stained with panels containing fluorescein isothiocyanate (FITC)-conjugated anti-mouse antibodies against CD19 and CD20, R-Phycocerythrin (PE)-conjugated anti-mouse antibodies against CD30 and CD15, and control FITC- or PE-conjugated mouse IgG1 (BD Pharmingen, San Diego, CA, USA), as indicated. CD antigen expressions were analyzed on a FACSCalibur machine (ELITE; Beckman-Coulter, Fullerton, CA, USA).

Immunofluorescence. Cells (2.0x10^5/ml) were inoculated into each well of 6-well plates (Costar, Corning, NY, USA) and cultured in complete medium for 48 h followed by in serum-free medium for another 24 h. After deposition, fixation and permeabilization, the cells were labeled with rabbit anti-mouse CD30 mAb (2 µg/ml; Abcam) followed with PE-conjugated goat anti-rabbit IgG (15 µg/ml, ZF-0311; ZSGB-BIO, Beijing, China). Negative controls were performed by replacing the primary antibodies with PBS. The cells were observed under a fluorescence microscope (Nikon, Tokyo, Japan).

Mouse cytokine antibody arrays. RayBio® Mouse Cytokine Antibody Arrays (RayBiotech, Inc., Norcross, GA, USA) were used to investigate the expression of 62 cytokines in the different cell groups, according to the manufacturer's instructions. In brief, proteins were extracted, quantified and transferred onto membranes. The membranes were then sealed and incubated with antibodies against cytokines. The diffe-
tolerance in cytokine expression were visualized, photographed and analyzed.

Western blot analysis. Cell lysates were prepared, and equal amounts of protein (50 μg) were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were incubated with 5% skim milk in TBS-0.1% Tween-20 for 2 h to block the residual binding sites followed by immunoblotting overnight at 4°C with appropriately diluted rabbit anti-human p-IκBα antibody (1:500; Bioworld Technology, Inc., St. Louis Park, MN, USA), rabbit mCD99L2 antibody (Abcam, Cambridge, MA, USA) and rabbit β-actin antibody (ZhongShan Golden Bridge Biotechnology, Beijing, China). Specific binding was revealed by mouse HRP-conjugated anti-rabbit IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and an enhanced chemiluminescence system (ECL-Plus; Amersham Biosciences Inc., Piscataway, NJ, USA).

Animals and in vivo tests. Twelve nude mice and 84 BALB/c mice (six to eight-week-old female/male) were purchased from the Central Laboratory of Animal Science of the Southern Medical University (Guangzhou, China) and randomized into an A20-mCD99L2 group and an A20-empty group of nude and BALB/c mice, respectively (Table I). Tumor cells (2x10⁶ to 2x10⁷) were injected subcutaneously into the left flank of the mice using various methods. Tumor growth was observed by calculating the tumor volume. Mice were sacrificed when the tumor volume reached a threshold value (1000 mm³).

Statistical analysis. The SPSS 13.0 software was used for statistical analysis. The results are expressed as the means ± standard deviation (SD). Where indicated, differences were compared using the Student's t-test. Statistical significance between in vitro cell growth and in vivo tumor growth were examined using analysis of variance (ANOVA) for factorial design. Proliferation assay and FACS results of antigen expression or lymphocyte percentages were examined using one-way ANOVA. Statistical analysis of tumor growth was carried out using Dunnett's multiple comparison tests. P<0.05 was considered to indicate a statistically significant difference.

Results

Establishment of A20 subclones using shRNA targeting mCD99L2 gene. Four primers were designed based on the RNAi technique, shRNA was constructed and sequencing was confirmed followed by transient transfection into A20 cells (Table II). Interference efficacy was examined using real-time RT-PCR until targeting sequences were screened. No. 2 lentivirus vector was most effective at blocking mCD99L2 expression (Table II, no. 2 shRNA). Subsequently, the pLenti6/mCD99L2 (no. 2) and pLenti6/U6 mock vector were transfected into the A20 cells and estadiolin-resistant single clones were selected, achieving permanent transfection to gain stable integrants. The transfection rate was 57% at 16 days assayed by PI staining flow cytometry. For the sake of convenience, clones transfected using pLenti6/mCD99L2 or pLenti6/U6 mock vector were termed A20-mCD99L2 and A20-empty cells, respectively. Stable integrants of low mCD99L2 expression of the A20 cells were achieved (A20-mCD99L2 cells) and confirmed in various clones. mCD99L2 expression was significantly lower in the A20-mCD99L2 cell group compared with the A20 and A20-empty groups; it decreased by 50%, as indicated by real-time PCR and western blot analysis (Fig. 1).
Morphological changes by downregulating mCD99L2 expression in vitro. Morphology was extremely significant in the present study. Some cells in the A20-mCD99L2 group demonstrated morphological changes including larger volume, abundant cytoplasm, marked pleomorphism, large and deeply stained nucleus, while some were binucleated or polynucleated (Fig. 2A). Even when cells were continuously cultured to the 20th passages (Fig. 2B), these changes in various clones of the A20-mCD99L2 group remained. TEM observation indicated that controls were naïve cells with less cytoplasm and organelles and relatively larger nucleus, while the giant cells in the A20-mCD99L2 group were much larger, with abundant cytoplasm and organelles, particularly mitochondria and endoplasmic reticulum (Fig. 2C). The unique morphology of binucleated or polynucleated appearance with larger nucleus and nucleoli exhibited by some A20-mCD99L2 cells partly mimic that of human H/RS cells.

Biological characteristics of A20-mCD99L2 cells. The growth of cultured A20-mCD99L2 cells was investigated using the MTT method. Slower kinetics of cell proliferation compared with the controls were observed in the A20-mCD99L2 group (Table III) (P<0.05), which indicated that mCD99L2 down-regulation induces a weaker proliferative ability.

To evaluate the cell cycle distribution, the DNA contents of asynchronous cultures of various cell groups were measured. The S phase of each group showed no significant difference (P>0.05, n=4), while the G2 phase was significantly prolonged in the A20-mCD99L2 cells compared to the A20 and A20-empty cells (Table IV) (P<0.05), which indicated that A20-mCD99L2 cells may be defective in cytokinesis.

Immunophenotypes of A20-mCD99L2 cells in vitro. As H/RS cells are characterized by a high expression of CD15 and CD30 (22) and previous findings have demonstrated that upreg-

Table II. Assignment of animals groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Animal (no.)</th>
<th>Methods</th>
<th>Cells inoculated (/mice)</th>
<th>Inoculation site</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Nude mouse (6)</td>
<td>Subcutaneous inoculation</td>
<td>2x10^7/0.2 ml</td>
<td>Axillary fossa</td>
</tr>
<tr>
<td>B1</td>
<td>BALB/c mouse (7)</td>
<td>Subcutaneous inoculation</td>
<td>2x10^6/0.1 ml</td>
<td>Left axillary fossa</td>
</tr>
<tr>
<td>B2</td>
<td>BALB/c mouse (7)</td>
<td>Subcutaneous inoculation</td>
<td>2x10^7/0.1 ml</td>
<td>Left axillary fossa</td>
</tr>
<tr>
<td>C1</td>
<td>BALB/c mouse (7)</td>
<td>Subcutaneous transplantation</td>
<td></td>
<td>Left axillary fossa</td>
</tr>
<tr>
<td>C2</td>
<td>BALB/c mouse (7)</td>
<td>Subcutaneous transplantation</td>
<td></td>
<td>Right axillary fossa</td>
</tr>
<tr>
<td>D1</td>
<td>BALB/c mouse (7)</td>
<td>Subcutaneous transplantation</td>
<td>5x10^6/0.2 ml</td>
<td>Caudal vein</td>
</tr>
<tr>
<td>D2</td>
<td>BALB/c mouse (7)</td>
<td>Subcutaneous transplantation</td>
<td>2x10^7/0.4 ml</td>
<td>Caudal vein</td>
</tr>
</tbody>
</table>

Figure 1. Western blot analysis of mCD99L2 protein. Western blot analysis of mCD99L2 protein (26 kDa) and the control, β-actin (43 kDa) in (A) A20-mCD99L2, (B) A20 and (C) A20-empty cells.

Figure 2. Morphological changes and immunofluorescence (IFC) labeling of CD30. (A) The morphological changes under an inverted microscope (x400). (B) H&E staining of different clones of A20-mCD99L2 cells during continuous passage of culture (x200). (C) Observation by transmission electron microscopy (x8,000). (D) IFC labeling of CD30.

[Image of Western blot analysis of mCD99L2 protein and immunofluorescence labeling of CD30]
ulated CD99 markedly downregulates the expression of CD30 and CD15 (19-21), flow cytometry was applied to examine the changes in CD antigen expression by downregulating mCD99L2. The results indicated that when compared with the controls, A20-mCD99L2- cells exhibited significantly higher CD30 and CD15 levels (P<0.01, n=3) and moderately decreased CD19 and CD20 levels (P<0.05, n=3). The enhanced expression of CD30 was also confirmed by immunofluorescence (IFC) labeling using mouse CD30 antibodies and the giant cells as well as some transformed A20-mCD99L2- cells were CD30+ (Fig. 2D).

Histology, biology and phenotypes of tumors in nude mice.
Subcutaneous tumor models in nude mice were successfully established (Table VI, Fig. 3A-E). Tumor growth of the A20-mCD99L2- group was much slower than the A20-empty group (Fig. 3F). The time frame of tumorigenesis in the A20-mCD99L2- group was much longer compared with the control group (Fig. 3G), which indicated that the proliferative ability of the A20-mCD99L2- cells was weaker due to the downregulation of mCD99L2.

Histologically, the A20-empty tumors were characterized by a diffuse homogeneous infiltrate consisting of large and cohesive tumor cells with moderate cytoplasm and pleomorphic nuclei (Fig. 3C), while the A20-mCD99L2- tumor cells showed marked pleomorphism, a diffuse distribution pattern, and some had two or more large and deeply stained nuclei (Fig. 3D and E).

Table III. Proliferation of various cell groups in vitro by MTT (n=6).

<table>
<thead>
<tr>
<th>Day</th>
<th>A20</th>
<th>A20-empty</th>
<th>A20-mCD99L2-</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.43±0.02</td>
<td>0.43±0.02</td>
<td>0.43±0.02</td>
</tr>
<tr>
<td>2</td>
<td>0.53±0.02</td>
<td>0.53±0.03</td>
<td>0.47±0.02</td>
</tr>
<tr>
<td>3</td>
<td>0.65±0.02</td>
<td>0.64±0.03</td>
<td>0.55±0.04</td>
</tr>
<tr>
<td>4</td>
<td>0.84±0.02</td>
<td>0.83±0.03</td>
<td>0.65±0.03</td>
</tr>
<tr>
<td>5</td>
<td>0.95±0.02</td>
<td>0.93±0.03</td>
<td>0.73±0.05</td>
</tr>
<tr>
<td>6</td>
<td>0.94±0.03</td>
<td>0.92±0.01</td>
<td>0.71±0.03</td>
</tr>
<tr>
<td>7</td>
<td>0.92±0.02</td>
<td>0.92±0.02</td>
<td>0.70±0.02</td>
</tr>
</tbody>
</table>

*a*P-value of time, *P<0.05; *b*P-value between groups, *P<0.05. mCD99L2, mouse CD99 antigen-like 2.

Table IV. Cell cycle analysis of various cell groups (n=4).

<table>
<thead>
<tr>
<th>Cell groups</th>
<th>G1 (%)</th>
<th>S (%)</th>
<th>G2 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A20</td>
<td>42.60±4.23</td>
<td>54.05±4.86</td>
<td>3.33±1.31</td>
</tr>
<tr>
<td>A20-empty</td>
<td>41.63±3.93</td>
<td>55.03±4.10</td>
<td>3.32±1.30</td>
</tr>
<tr>
<td>A20-mCD99L2-</td>
<td>35.90±3.13</td>
<td>53.55±5.50</td>
<td>10.58±4.97</td>
</tr>
</tbody>
</table>

*a*P<0.05 and *b*P<0.05. mCD99L2, mouse CD99 antigen-like 2.
The immunophenotypes of the primary tumor cells dissociated from the xenotransplanted tumors were examined using flow cytometry. CD30 expression was significantly upregulated, whereas CD19 expression was lower in the A20-mCD99L2 compared with the A20-empty group.

Effect of downregulation of mCD99L2 in BALB/c mice. Inoculating BALB/c mice with A20-mCD99L2 cells and A20-empty cells was conducted using various methods (Table II, Fig. 4A and B). It was difficult for the A20-mCD99L2 cells to form tumors in BALB/c mice irrespective of the methods applied (Table VI), indicating that the proliferative ability of the A20 cells with decreased mCD99L2 expression was significantly impaired in the immunocompetent BALB/c mice.

The histological characteristics of the A20-empty cell-induced tumor tissues demonstrated uniform B lymphoid cells, which were most consistent with those in human diffuse large B cell lymphoma (DLBCL) (Fig. 4C). Cells in A20-mCD99L2 tumor tissues exhibited marked pleomorphism and large and deeply stained nuclei, some were binucleated or polynucleated (Fig. 4D and E, red arrow). In addition, some lymphocytes were observed in the tumor tissues (Fig. 4D, black arrow).

CD30 was positive in A20-mCD99L2 cell-induced tumors as observed using IFC labeling of mouse CD30, particularly in the giant cells (Fig. 4F). As in Hodgkin's lymphoma, H/RS cells were accompanied by a number of background cells, including T lymphocytes. Immunohistochemistry was applied to detect the expression of CD3+ T lymphocytes in tumor tissues. The results indicated that more CD3+ lymphocytes infiltrated into the A20-mCD99L2 cell-induced tumor tissues compared with the control group (Fig. 4G), which suggested that various immune reactions were induced in vivo.

Differential cytokine profile of cells and tissues. The weak proliferative ability and pathological features exhibited in the A20-mCD99L2 groups, which partly mimicked those of Hodgkin's lymphoma, encouraged us to investigate the differential expression of cytokines/chemokines (Fig. 5A and B) induced by the downregulation of mCD99L2, as several cytokines are involved in the cross-talk between H/RS cells and the background inflammatory environment. Cytokine protein arrays showed that several cytokines including CD30T, IL-12p40/p70, IL-3, IFN-γ, CXCL16, MIP-1α and CD40, were upregulated (≥1.5-fold) in the A20-mCD99L2 cells and no cytokine was downregulated in excess of 1.5-fold (Table VII). The changes in morphology, biology and phenotypes may be directly or indirectly associated with these differentially expressed cytokines/chemokines.

mCD99L2 downregulation correlates with the activated nuclear factor-κB (NF-κB) pathway. As regards the mechanism involved, the NF-κB pathway plays a crucial role in the pathology of Hodgkin's lymphoma and is a significant factor affecting cytokines/chemokines. NF-κB is retained in the cytoplasm of inactivated cells through the interaction with members of the IκB inhibitor family, including IκBα. The phosphorylation and subsequent degradation of IκB lead to the release of NF-κB, allowing it to translocate to the nucleus and activate transcription. The level of p-IκBα, which could
The results indicated that the expression of the mCD99L2 protein in the A20-mCD99L2- cells (Fig. 5C, lane 4) was weaker compared with that in the A20 and A20-empty cells (Fig. 5C, lanes 1 and 2), which was an evidence of the effect of shRNA targeting mCD99L2. The expression of p-IκBα protein was stronger in the A20-mCD99L2- cells (Fig. 5C, lane 4) compared with that in the A20 and A20-empty cells (Fig. 5C, lanes 1 and 2), suggesting that NF-κB activity was elevated in the A20-mCD99L2- cells.

When the cells were treated with BAY, an inhibitor of the NF-κB signaling pathway, p-IκBα protein expression was significantly decreased in the A20-mCD99L2- cells, while mCD99L2 protein expression was not affected (Fig. 5C, lane 3), which suggests a potential correlation between the downregulation of mCD99L2 and the NF-κB pathway.

Discussion

Effect of shRNA targeting mCD99L2 on B cell lymphoma.

Previous reports have indicated that the overexpression of the full-length CD99 isoform (CD99wt), one of the two distinct proteins produced by the alternative splicing of the CD99 gene transcript, dramatically inhibits cancer cell proliferation, migration and metastasis, whereas the overexpression of the short CD99 isoform (CD99sh) remarkably favors these phenomena (23), while the effects of mCD99L2 on tumor cells have not yet been reported. Our previous study confirmed that mCD99L2 is expressed in the A20 cell line (18). In the present study, the effective shRNA sequence targeting mCD99L2 was selected and identified, the mCD99L2-downregulated A20 subclones (A20-mCD99L2- cell) were established and the
morphological, biological and phenotypic characteristics of the A20-mCD99L2 cells were investigated in vitro and in vivo for the first time.

Our results strongly suggest that suppressing mCD99L2 may impair the proliferative ability of murine B cell lymphoma. As the A20 cell‑induced tumors were pathologically described as DLBCL, which is a type of lymphoma with poor prognosis, our investigation on mCD99L2 may provide a potential target of CD99‑related antigens for the clinical therapy of B cell lymphoma.

**Downregulation of mCD99L2 leads to the transformation of some A20 cells into H/RS-like cells.** Using CD99‑deficient IM9 and BJAB B cell lines, investigators have confirmed that the downregulation of CD99 is a primary requirement for the generation of H/RS cells (19,20). A previous study of ours suggested that the CD99‑upregulated H/RS cell line (L428) lost its nature as an H/RS cell line (21). Although a functional similarity between CD99 and mCD99L2 in lymphoma cells has yet to be confirmed, unique morphological changes were observed in the transformed A20‑mCD99L2 cells; some giant cells similar to human H/RS cells were observed in the cultured cells, as well as in nude and BALB/c mice, which suggests that the downregulation of mCD99L2 led to the transformation of some A20 cells into H/RS-like cells.

Biologically, the suppressive effect of the downregulation of mCD99L2 on the proliferative ability of mouse B lymphoma cells was observed in the cultured cells and tumor tissues. Although the A20‑mCD99L2 cells grew at a slower rate compared with the A20 cells, cell cycle analysis showed that the S phase of each group did not vary significantly. However, the G2 phase of the A20‑mCD99L2 cells lasted for a longer period of time. The fact that some A20‑mCD99L2 cells stay in the G2 phase and exhibit difficulty in entering the M phase may be due to the fact that giant cells with two or more nuclei were induced in the transformed A20‑mCD99L2 cells. The weaker proliferative ability and prolonged G2 phase imply that A20‑mCD99L2 cells are similar to H/RS cells to a certain extent; thus defects were noted in the cell cycle regulation as one of several anti-apoptotic mechanisms (24,25).

The vast majority of classical Hodgkin’s lymphomas are thought to arise from transformed germinal center B cells due to the loss of B cell characteristics during antigen selection (26). The H/RS cells possess unique morphological and biological features and phenotypic characteristics, thus various phenotypic changes were detected in these cells. The significantly increased CD30 and CD15 expression and moderately decreased CD19 and CD20 expression indicate that the A20-mCD99L2 cells tend to lose part of their B cell characteristics and gain some phenotypic features of H/RS cells, which are characterized by the high expression of CD15 and CD30 (22). Moreover, in the BALB/c mouse tumor tissues of the A20-mCD99L2 group, typical H/RS-like cells were detected, the CD30 antigen was highly expressed and more CD3 + T lymphocytes were observed.

Our results in vitro and in vivo encouraged us to investigate the cytokine expression in various cell groups for the interaction of cytokines/chemokines, which may lead to an environment in which H/RS cells are able to proliferate, escape from apoptosis and survive host antitumor defense (27-29). The results show that several cytokines, such as CD30T, IL-12p40/p70, IL-3, IFN-γ, CXCL16, MIP-1α and CD40 were upregulated, a number of which have been associated with Hodgkin’s lymphomas or H/RS cells in previous reports (30,31). For instance, compared with other types of lymphoma, the expression of CD30 and CD40 in Hodgkin’s lymphoma is highly expressed (32). IL-3 is overexpressed in H/RS cell lines (30). CXCL16 has also been reported to be expressed in some H/RS cell lines (33). Thus, the cytokine expression profile in the A20-mCD99L2 cells exhibited some similarities to that of H/RS cells. Combining our in vitro and in vivo observation, A20-mCD99L2 cells partly mimic the characteristics of human H/RS cells.

**Effect of shRNA targeting mCD99L2 may involve NF-κB pathway.** The A20-mCD99L2 cells were observed to have difficulty in forming tumors in mice with normal immune functions. More CD3 + lymphocytes infiltrated into the A20-mCD99L2 cell-induced tumor tissues compared with the control group in the BALB/c mice, which suggested that certain immune reactions were induced by certain cell groups. Of the upregulated cytokines in the A20-mCD99L2 cells in the cytokine profile, IFN-γ, IL-12p40/p70 and MIP-1α have been reported to be involved in regulating immune func-

---

**Table VII. Upregulated cytokines in excess of 1.5-fold in A20-mCD99L2 cells compared with A20-empty cells.**

<table>
<thead>
<tr>
<th>Row</th>
<th>Col</th>
<th>Col</th>
<th>Name</th>
<th>1 Primary</th>
<th>2 Primary</th>
<th>1 Standard</th>
<th>2 Standard</th>
<th>No. 2/1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1, 2</td>
<td>9</td>
<td>j</td>
<td>CD30T</td>
<td>7199</td>
<td>12042</td>
<td>0.0564</td>
<td>0.164</td>
<td>2.914</td>
</tr>
<tr>
<td>1, 2</td>
<td>10</td>
<td>j</td>
<td>CD40</td>
<td>12714</td>
<td>16692</td>
<td>0.173</td>
<td>0.261</td>
<td>1.519</td>
</tr>
<tr>
<td>1, 2</td>
<td>13</td>
<td>m</td>
<td>CXCL16</td>
<td>11067.5</td>
<td>14359.5</td>
<td>0.137</td>
<td>0.212</td>
<td>1.548</td>
</tr>
<tr>
<td>3, 4</td>
<td>6</td>
<td>f</td>
<td>IFN-γ</td>
<td>11977.5</td>
<td>15866.5</td>
<td>0.156</td>
<td>0.243</td>
<td>1.5598</td>
</tr>
<tr>
<td>3, 4</td>
<td>13</td>
<td>m</td>
<td>IL-3</td>
<td>8380.5</td>
<td>10564</td>
<td>0.081</td>
<td>0.134</td>
<td>1.650</td>
</tr>
<tr>
<td>5, 6</td>
<td>6</td>
<td>f</td>
<td>IL-12 p40/p70</td>
<td>9328.5</td>
<td>12161</td>
<td>0.101</td>
<td>0.1677</td>
<td>1.654</td>
</tr>
<tr>
<td>7, 8</td>
<td>6</td>
<td>f</td>
<td>MIP-1α</td>
<td>10985</td>
<td>14135.5</td>
<td>0.135</td>
<td>0.208</td>
<td>1.533</td>
</tr>
</tbody>
</table>

mCD99L2, mouse CD99 antigen-like 2. 1, A20-empty cell; 2, A20-mCD99L2 cell.
tions between tumor cells and lymphocytes (34). CXCL16 has also been reported to be involved in the tumor anti-host reactions (35). Although details are yet to be elucidated, the differentially expressed cytokines may elucidate some of the mechanisms involved in the effect of the downregulation of mCD99L2 in A20 cells and may provide clues for further study.

As regards the pathways, the constitutive NF-κB activation is a striking feature and the major pathogenetic mechanism in H/RS cells (36-38); therefore, we focused on the NF-κB pathway. Our finding suggesting that p-IκBα, an indicator of the activation of the NF-κB pathway, was enhanced in the A20-mCD99L2 cells, suggested the elevated NF-κB activity by the downregulation of mCD99L2. Treatment with BAY significantly decreased the p-IκBα level in A20-mCD99L2 cells without affecting mCD99L2 protein expression, suggesting a potential regulatory role of mCD99L2 in the NF-κB pathway. Although the downregulation of mCD99L2 may trigger various pathways to regulate several cytokines, our results suggested that the downregulation of mCD99L2 in the A20 cells may correlated with the activated NF-κB pathway, which may partly contribute to the morphological, biological and phenotypic changes induced by shRNA targeting mCD99L2 in A20 cells. The regulatory mechanisms between mCD99L2 and NF-κB require extensive investigation in the future.

In conclusion, the stable mCD99L2-downregulated A20 cell line was established and identified. The effect of shRNA targeting mCD99L2 in vitro and in vivo was observed. The downregulation of mCD99L2 led to the transformation of some A20 cells into H/RS-like cells, impaired the proliferative ability of murine B cell lymphoma, changed the immunophenotypes, led to differentially expressed cytokines and tissues (34). CXCL16 is a striking feature and the major pathogenetic mechanism in H/RS cells (36-38); therefore, we focused on the NF-κB pathway. Our finding suggesting that p-IκBα, an indicator of the activation of the NF-κB pathway, was enhanced in the A20-mCD99L2 cells, suggested the elevated NF-κB activity by the downregulation of mCD99L2. Treatment with BAY significantly decreased the p-IκBα level in A20-mCD99L2 cells without affecting mCD99L2 protein expression, suggesting a potential regulatory role of mCD99L2 in the NF-κB pathway. Although the downregulation of mCD99L2 may trigger various pathways to regulate several cytokines, our results suggested that the downregulation of mCD99L2 in the A20 cells may correlated with the activated NF-κB pathway, which may partly contribute to the morphological, biological and phenotypic changes induced by shRNA targeting mCD99L2 in A20 cells. The regulatory mechanisms between mCD99L2 and NF-κB require extensive investigation in the future.

In conclusion, the stable mCD99L2-downregulated A20 cell line was established and identified. The effect of shRNA targeting mCD99L2 in vitro and in vivo was observed. The downregulation of mCD99L2 led to the transformation of some A20 cells into H/RS-like cells, impaired the proliferative ability of murine B cell lymphoma, changed the immunophenotypes, led to differentially expressed cytokines and suggested the involvement of the activated NF-κB pathway. Our study provides experimental data for additional studies on the mCD99L2 gene and protein in lymphomas.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (grant nos. 81071941, 81071659 and 81101537).

References