

Analysis of CCL5 expression in classical Hodgkin's lymphoma L428 cell line

FANRONG LIU^{1,2*}, YAN ZHANG^{2*}, ZI-QING WU^{1,2} and TONG ZHAO^{1,2}

¹Department of Pathology, Nanfang Hospital Affiliated to Southern Medical University; ²Key Laboratory of Molecular Tumor Pathology of Guangdong Province, Southern Medical University, Guangzhou 510515, P.R. China

Received February 25, 2011; Accepted June 17, 2011

DOI: 10.3892/mmr.2011.515

Abstract. CCL5 is one of the chemoattractant cytokines involved in inflammatory observed in both diffuse large B-cell lymphoma (DLBCL) and classical Hodgkin's lymphoma (CHL). However, the pathological effects of CCL5 remain unclear. To gain a better understanding of the role of CCL5 in CHL and DLBCL, we examined the expression of CCL5 in the CHL cell line L428 and the DLBCL cell lines Ly1 and Ly8, as well as its chemotactic effect on CD4⁺ T cells. CCL5 mRNA expression was detected by real-time quantitative RT-PCR. Intracellular CCL5 protein expression was analyzed using confocal microscopy, and CCL5 protein secretion was detected by ELISA. The chemotactic function of CCL5 was assessed using a Transwell coculture system, and the number of migrated CD4⁺ T cells was counted. Moreover, the p- $\text{I}\kappa\text{B}\alpha$ and p65 levels of NF- κB signaling molecules in these lymphoma cell lines were detected by Western blotting. The results showed that CCL5 mRNA and protein expression in the L428 cells was significantly higher than in Ly1 and Ly8 cells ($p < 0.05$). L428 cells secreted more CCL5 than the Ly1 or Ly8 cells, and the secreted CCL5 was capable of inducing CD4⁺ T cell migration. The expression levels of the NF- κB transcription factors p65 and p- $\text{I}\kappa\text{B}\alpha$ were examined in these lymphoma cells. L428, Ly1 and Ly8 cells expressed similar levels of p65, while p- $\text{I}\kappa\text{B}\alpha$ expression was higher in the L428 cells than in the Ly1 or Ly8 cells, indicating that a high CCL5 expression may be related to the increased activity of the NF- κB signaling pathway in L428 cells.

Introduction

Classical Hodgkin's lymphoma (CHL) is a lymphoid malignancy characterized by the presence of a minority of neoplastic

cells, the mononucleated Hodgkin and the multinucleated Reed-Sternberg (HRS) cells, which are surrounded by abundant CD4⁺ T lymphocytes. Diffuse large B-cell lymphoma (DLBCL) is a common type of non-Hodgkin's lymphoma and consists of homogeneous large malignant B-cells. Although tumor cells of both CHL and DLBCL are derived from B-lymphocytes, the pathological features are different (1,2). CCL5 is one of the chemoattractant cytokines. Previous studies have demonstrated the expression of CCL5 in CHL and DLBCL. The expression of CCL5 in DLBCL B-cells is up-regulated by monocytes, whereas in HRS cells it is stimulated by CD4⁺ T cells (3-5). Buri *et al* found that CCL5 was only expressed in the infiltrated non-neoplastic leukocytes of CHL but not in HRS cells (6). However, subsequent studies demonstrated the expression of CCL5 in HRS cells and its potential role in the recruitment of inflammatory cells into lymphoma tissue (7,8). Emmerich *et al* found that NF- κB transcription factor $\text{I}\kappa\text{B}\alpha$ is overexpressed in RS cells (9). Compagno *et al* have shown that there is deregulation of NF- κB in diffuse large B-cell lymphoma (10). However, it is unclear whether CCL5 expression is correlated with the NF- κB signaling pathway.

In this study, the expression of CCL5 was examined in the CHL cell line L428 and the DLBCL cell lines Ly1 and Ly8. The p65 and p- $\text{I}\kappa\text{B}\alpha$ protein expression in these cells was also studied. The results showed that, compared with Ly1 cells, L428 cells showed an increased NF- κB activity and CCL5 expression, and a greater ability to attract CD4⁺ T cells.

Materials and methods

Cell lines. The CHL cell line L428 was obtained from Professor K.C. Chan (Medical Center, Nebraska University, USA) (11). DLBCL cell lines Ly1 and Ly8 were kindly provided by Professor Zhou Xiaoyan (Affiliated Tumour Hospital, FuDan University, Shanghai, China) (12). L428, Ly1, Ly8 cells, as well as L428 cells pretreated for 24 h with BAY 11-7082 (Beyotime, China) (L428-bay) were maintained in RPMI-1640 (Life Technologies, Gaithersburg, MD, USA) with 100 IU/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 10% heat-inactivated fetal bovine serum (FBS) at 37°C in a 5% CO₂ humidified atmosphere.

Real-time quantitative RT-PCR. Total RNA was extracted from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA)

Correspondence to: Dr Zi-Qing Wu and Dr Tong Zhao, Department of Pathology, Nanfang Hospital Affiliated to Southern Medical University, Guangzhou, Guangdong 510515, P.R. China
E-mail: zhaotongketizu@163.com

*Contributed equally

Key words: CCL5, chemokine, classical Hodgkin's lymphoma, CD4⁺ T cells, NF- κB

Table I. Primer sequences used for the amplification of CCL5 and GAPDH.

Gene	Forward primer	Reverse primer	PCR product size (bp)
CCL5	CCCTCACCATCATCCTCACT	CTTCTTCTCTGGGTGTCAC	148
GAPDH	ACAGTCAGCCGCATCTTCTT	GACAAGCTTCCCGTTCTCAG	256

according to the manufacturer's instructions. The cDNA synthesis was performed using a RT-PCR kit (DRRO37s, Takara, Otsu, Shiga, Japan) according to the manufacturer's instructions. Primer sequences used for amplification are shown in Table I.

SYBR real-time quantitative RT-PCR (qRT-PCR) was performed with the SYBR PrimeScript RT-PCR kit (DRRO41s, Takara) according to the manufacturer's instructions (13). Triplicates were performed for all qRT-PCR reactions with an ABI 7500 real-time PCR system (Applied Biosystems, China). The annealing temperature was 60°C for 30 sec. The relative quantification was obtained by the C_t values, determining the reactions for each target gene and the internal control gene (GAPDH) in cells. ΔC_t [$\Delta C_t = C_t$ (CCL5 gene) - C_t (GAPDH gene)] values were calculated for each cell line. Relative expression level was determined as $2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t = \Delta C_t$ (L428 cell or Ly8 cell) - ΔC_t (Ly1 cell).

Examining CCL5 expression by confocal microscopy. L428, Ly1 and Ly8 cells (2.0×10^5 /ml) were cultured in 6-well plates (Costar, Corning, NY, USA) in complete medium for 48 h, then in serum-free medium for another 24 h. Supernatants were collected and frozen at -20°C for ELISA assay. Cells were fixated and permeabilized as previously described (14). The cells were then labeled with rabbit anti-human CCL5 mAb (2 μ g/ml, ab9679, Abcam), followed by washing and incubation with PE-conjugated goat anti-rabbit IgG (15 μ g/ml, ZF-0311, ZSGB-BIO, China). Negative controls included samples in which the primary antibodies were replaced by PBS. The cells were counterstained with DAPI (Beyotime, China) prior to being examined with an Olympus FluoView FV10i confocal laser-scanning microscope.

Peripheral blood mononuclear cell preparation and purification of CD4⁺ T cells. Peripheral blood mononuclear cells (PBMC) from healthy donors were separated by Ficoll-Hypaque (Saichi, Beijing, China) density gradient centrifugation and subsequently incubated with ammonium chloride (0.83%) for 5 min at 4°C to lyse the remaining red blood cells. PBMCs were resuspended in complete medium at a final concentration of 3.0×10^6 /ml. The CD4⁺ T cells were isolated from PBMC using anti CD4⁺ conjugated immunomagnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions and described by Fischer (7,8). The purity of CD4⁺ T cells always exceeded 95% when used in the migration assays.

ELISA. CCL5 concentration in cell supernatant was measured using commercially available kits (KHC1031, Invitrogen) according to the manufacturer's instructions. The range of sensitivity for human CCL5 was 0-2,000 pg/ml. The assay

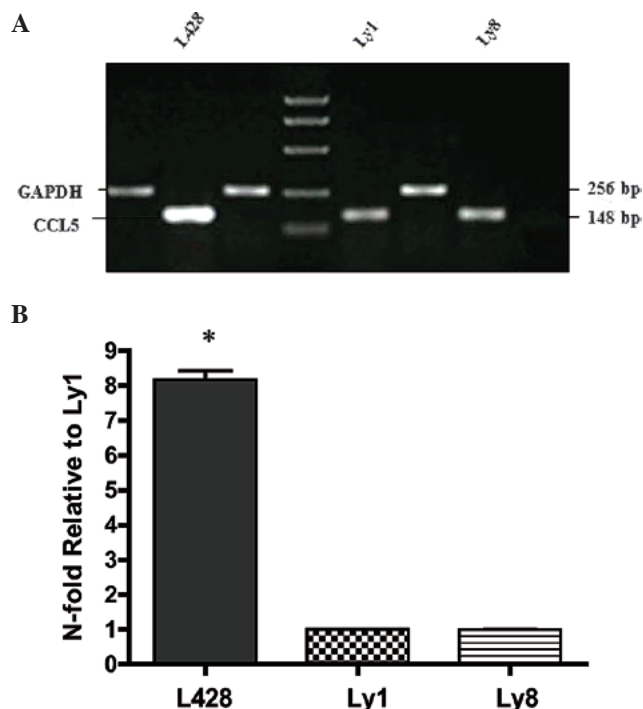


Figure 1. Expression analysis of CCL5 mRNA in lymphoma cell lines by real-time quantitative RT-PCR. Values of columns are folds of CCL5 mRNA expression in comparison with Ly1 cell line. Data are presented as the mean \pm SD. * $P < 0.05$ vs. Ly1 by one-way ANOVA. All assays were performed in triplicate.

was performed according to the manufacturer's instructions. ELISA plates were read at 450 nm.

Western blotting analysis. Cell lysates were prepared and equal amounts of protein (50 μ g) were separated on 10% SDS-PAGE. Proteins were transferred to PVDF membranes (Bio-Rad, Hercules, CA, USA) and residual binding sites were blocked by incubation for 2 h in 5% skimmed milk powder dissolved in TBS with 0.1% Tween 20. Membranes were then incubated for 2 h with rabbit anti-human p-I κ B- α (1:500, Bioworld Technology, Inc., St. Louis Park, MN, USA) and rabbit anti-human NF κ B p65 (1:1000, Abcam, Inc.). Specific binding was revealed by mouse HRP-conjugated anti-rabbit IgG (Santa Cruz, CA, USA) and an enhanced chemiluminescence system (ECL Plus, Amersham, TN, USA).

Chemotaxis assay. The migration of human *in vitro*-developed CD4⁺ T cells was tested using a modified 24-well Boyden chamber (Neuroprobe, Cabin John, MD, USA) as previously described by Fischer *et al* (7,8). Nitrocellulose filters (Millipore/Continental Water Systems, Bedford, MA, USA) with a pore size of 8 μ m were coated with human plasma

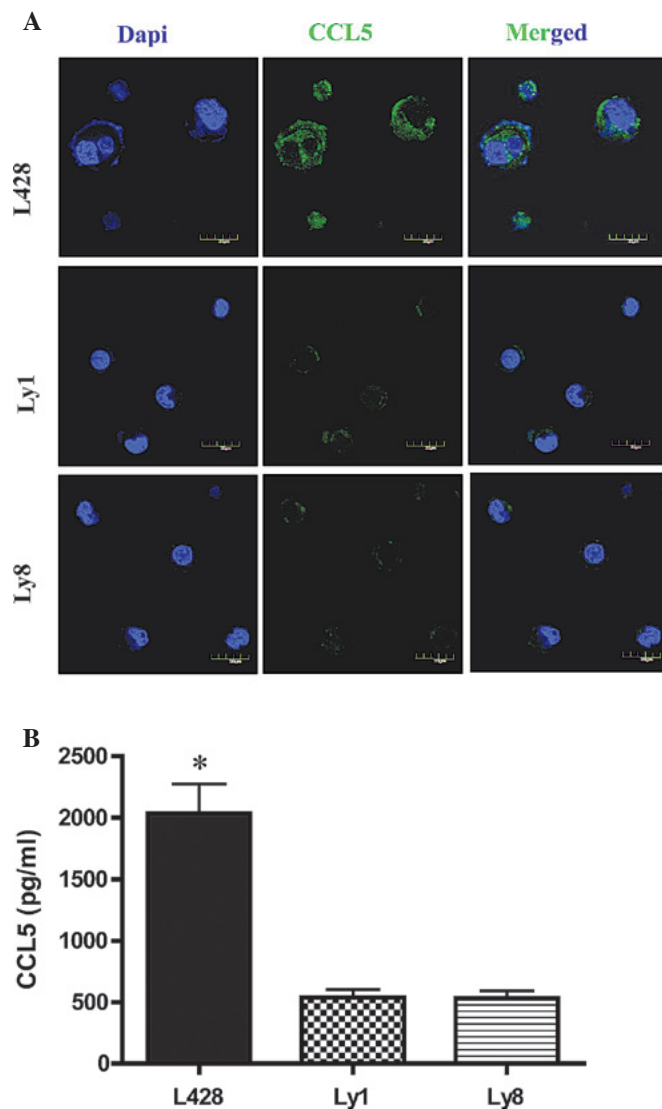


Figure 2. Intracellular and extracellular expression of CCL5 protein. (A) Confocal microscopy images showing the cellular expression of CCL5. Cells were stained with a CCL5 specific monoclonal antibody (green) and counterstained with DAPI (blue) (distance bar is equal to 20 μ m). The isotype controls are not shown. (B) Secreted soluble CCL5 protein in the culture supernatants of different cell lines. L428, Ly1 and Ly8 cells (2.0×10^5 /ml) were grown in 6-well cell culture cluster (Costar, Corning, NY, USA) with complete medium for 48 h followed by incubation in serum-free medium for another 24 h. Cell-free supernatants were analyzed using CCL5 ELISA assay. Data are presented as the mean \pm SD (n=3). * $P < 0.005$ by one-way ANOVA. All samples were performed in triplicate.

fibronectin (Life Technologies) at a concentration of 10 μ g/ml and maintained at room temperature overnight. Filters were air-dried for at least 60 min before use. In brief, supernatants from 1×10^5 L428 or Ly1 cell culture were added to 24-well Boyden chamber T at a final volume of 600 μ l. Under certain conditions, L428 cell supernatant was pretreated with human recombinant CCL5 (rCCL5, 100 ng/ml) or a neutralizing anti-CCL5 mAb (100 ng/ml) (R&D Systems). $CD4^+$ T cells (1×10^5) were added to the upper chamber at a final volume of 100 μ l. The cells were incubated at 37°C and 5% CO_2 for 2.5 h. Filters were then fixed, stained with Giemsa solution and mounted.

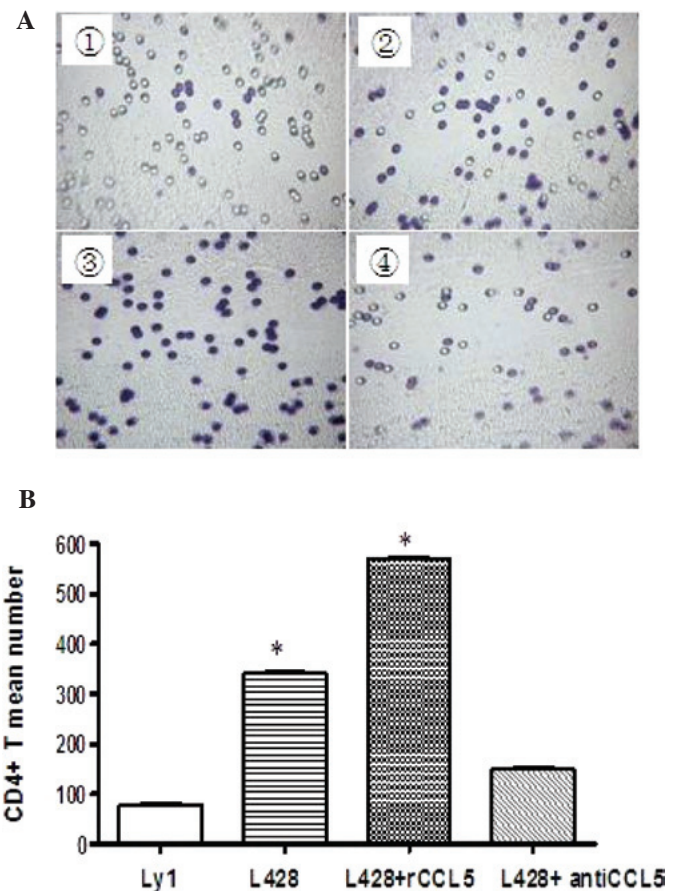


Figure 3. Chemotaxis assay and migrated $CD4^+$ T cells. (A) $CD4^+$ T cells migrated were by L428 (340.556 ± 15.852), which was significant to 79.333 ± 10.782 $CD4^+$ T cells by Ly1 ($p < 0.05$). (B) The amount of $CD4^+$ T cells migrated was increased by adding rCCL5 (10 μ g/ml) to L428 cells, and decreased by adding anti-CCL5 (10 μ g/ml) to L428 cells (571.111 ± 14.049 vs. 149.222 ± 15.171 , $p < 0.05$). Results are the mean values of 3 independent experiments and errors are shown as the SD.

Statistical analysis. Data are expressed as the mean \pm standard deviation (SD). Statistical analysis was carried out using SPSS 13.0 for Windows. The comparisons between groups were performed by least significant difference (LSD) of one-way ANOVA. $P < 0.05$ was considered to be statistically significant.

Results

CCL5 mRNA expression in different cell lines. The CCL5 mRNA expression in different CHL and DLBCL cell lines was examined by RT-PCR. All three cell lines, L428, Ly1 and Ly8 expressed CCL5 mRNA (Fig. 1A). CCL5 mRNA expression was then quantitated in these cell lines by real-time PCR. Compared with that in Ly1 cells, CCL5 mRNA expression in L428 cells was significantly increased by 8.1 ± 0.8 fold ($p < 0.05$) (Fig. 1B). In contrast, Ly8 cells showed a similar CCL5 mRNA level to that of Ly1 cells.

Intracellular and extracellular expression of CCL5 protein. Cellular expression of CCL5 protein in the three cell lines was determined by confocal microscopy. A prominent intracellular vesicular localization of CCL5 was observed in the L428

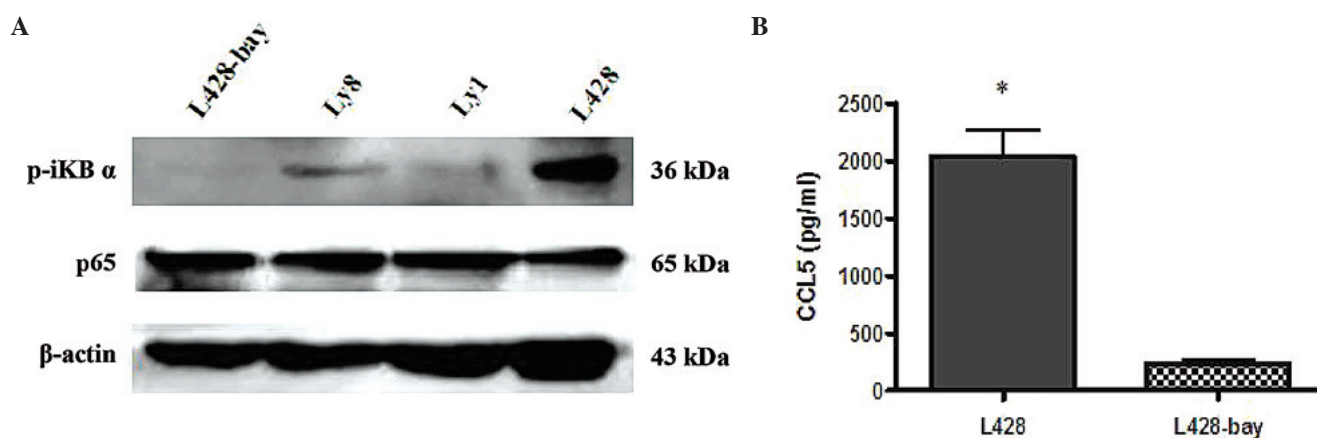


Figure 4. The relation of NF- κ B signaling pathways and CCL5 secretion. (A) Western blotting analysis of p-IkB α proteins in lymphoma cell lines. Whole-cell extracts were analyzed using specific antibodies against the p-IkB α , p65 or β -actin. L428 cells were pretreated with or without 10 μ M Bay for 24 h. (B) Secreted soluble CCL5 protein in the culture supernatants of L428 and L428-bay cells (2.0×10^5 /ml) were grown in 6-well cell culture cluster (Costar, Corning, NY, USA) with complete medium for 48 h followed by incubation in serum-free medium for another 24 h. Cell-free supernatants were analyzed using CCL5 ELISA assay. Data are presented as the mean \pm SD (n=3). *P<0.005 by one-way ANOVA. All assays were performed in triplicate.

cells (Fig. 2A), whereas the Ly1 and Ly8 cells showed weak or negative staining for CCL5. In addition, the extracellular expression of CCL5 protein in the cell culture supernatant was also measured by ELISA. Results showed that the two L428 cells secreted significantly larger amounts of soluble CCL5 protein than Ly1 or Ly8 cells (2261 ± 234 vs. 597 ± 70 and 588 ± 57 pg/ml, respectively, $p < 0.01$) (Fig. 2B).

CCL5 secreted by cell lines induced migration of CD4⁺ T cells. To verify that CCL5 produced by L428 cells has a chemotactic impact on CD4⁺ T cells, L428 (or Ly1) cell supernatant was used with CD4⁺ T cells in a Transwell system with 8- μ m pores, which allowed T cells to completely transmigrate (Fig. 3) (7,8). An increased percentage of migrated CD4⁺ T cells attracted by L428 supernatant was noted as compared to that of the Ly1 supernatant. CD4⁺ T cell migration was increased with the addition of rCCL5 and was decreased with pretreatment with the anti-CCL5 antibody (Fig. 4), confirming that the CD4⁺ T cell migration was mainly mediated by CCL5. Taken together, these data demonstrate that CCL5 secreted by the CHL cell line L428 was sufficient to attract the migration of CD4⁺ T cells, whereas the DLBCL cell line Ly1 failed to release sufficient CCL5.

p-IkB α and p65 expression in cell lines. The p65 expression level was similar among L428, Ly1 and Ly8 cells. L428 cells expressed the highest p-IkB α level. However, this level was completely eliminated when L428 cells were pretreated with the p-IkB α inhibitor BAY 11-7082 (10 μ M) for 24 h (Fig. 4). Moreover, the high level of secreted soluble CCL5 protein in the culture supernatants of L428 cells was also found to be markedly decreased dramatically by pretreatment with BAY 11-7082 (2261 ± 234 vs. 264 ± 45 , $p < 0.01$) (Fig. 4B).

Discussion

The chemoattractant cytokines are subdivided into 4 major groups on the basis of the relative positions of the first two Cys residues (C, CC, CXC and CXXC). The chemokine CCL5 is

usually thought to play a regulatory role in inflammatory processes (15). CHL and DLBCL are both B-cell-derived lymphomas but each has a distinct pathogenesis. Previous studies have shown that CCL5 plays an important role in the formation of CHL and DLBCL; however, no comparison of these two types of lymphoma has been carried out. Thus, in this study CCL5 expression and function were compared in the CHL cell line (L428) and the DLBCL cell lines (Ly1 and Ly8).

The results showed that the CHL cell line (L428) produced and secreted elevated amounts of CCL5 when compared to the DLBCL cell lines. The secreted CCL5 was functional and attracted more CD4⁺ T cells to migrate through a Transwell system compared with the supernatant of Ly1 cells. The presence of anti-CCL5 neutralizing mAb strongly inhibited the migratory response of CD4⁺ T cells, indicating that CCL5 played a significant role as a chemoattractant. Thus, compared with the DLBCL cells, CHL cells exhibit a greater ability to attract CD4⁺ T cells to infiltrate into the lymphoid tissues.

We also investigated the cause of the high expression of CCL5 in L428. A high expression level of p-IkB α was found in L428, but not in Ly1 or Ly8, and inhibition of p-IkB α with BAY 11-7082 not only decreased the level of p-IkB α , but also eliminated the secretion of CCL5 by L428 cells, indicating that a high CCL5 expression may be correlated with an increased activity of the NF- κ B signaling pathway in L428 cells.

In conclusion, CCL5 expression in CHL cells (L428 cell line) was found to be significantly higher than that in the DLBCL cells (Ly1 and Ly8). Additionally, CCL5 secreted by CHL cells was capable of inducing CD4⁺ T cell migration, correlating with the activity of the NF- κ B signaling pathways. Thus, a high expression of CCL5 may contribute to the pathogenesis of typical CHL.

Acknowledgements

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

References

1. Rooney N, Foster L and Byers R: Nodal B-cell lymphoma and Hodgkin lymphoma: current WHO classification and differential diagnostic dilemmas. *Diagnostic Histopathology* 16: 54-68, 2010.
2. Küppers R: The biology of Hodgkin's lymphoma. *Nat Rev Cancer* 9: 15-27, 2009.
3. Alizadeh AA, Eisen MB, Davis R, *et al*: Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. *Nature* 403: 503-511, 2000.
4. Mueller CG, Boix C, Kwan WH, *et al*: Critical role of monocytes to support normal B cell and diffuse large B cell lymphoma survival and proliferation. *J Leukoc Biol* 82: 567-575, 2007.
5. Poppema S: The nature of the lymphocytes surrounding Reed-Sternberg cells in nodular lymphocyte predominance and in other types of Hodgkin's disease. *A J Pathol* 35: 351-357, 1989.
6. Buri C, Korner M, Scharli P, *et al*: CC chemokines and the receptors CCR3 and CCR5 are differentially expressed in the nonneoplastic leukocytic infiltrates of Hodgkin disease. *Blood* 97: 1543-1548, 2001.
7. Fischer M, Juremalm M, Olsson N, *et al*: Expression of CCL5/RANTES by Hodgkin and Reed-Sternberg cells and its possible role in the recruitment of mast cells into lymphomatous tissue. *Int J Cancer* 107: 197-201, 2003.
8. Aldinucci D, Lorenzon D, Cattaruzza L, *et al*: Expression of CCR5 receptors on Reed-Sternberg cells and Hodgkin lymphoma cell lines: Involvement of CCL5/Rantes in tumor cell growth and microenvironmental interactions. *Int J Cancer* 122: 769-776, 2008.
9. Emmerich F, Meiser M, Hummel M, *et al*: Overexpression of I kappa B alpha without inhibition of NF-kappa B activity and mutations in the I kappa B alpha gene in Reed-Sternberg cells. *Blood* 94: 3129-3134, 1999.
10. Compagno M, Lim WK, Grunn A, *et al*: Mutations of multiple genes cause deregulation of NF-kB in diffuse large B-cell lymphoma. *Nature* 459: 717-721, 2009.
11. Höpken UE, Foss HD, Meyer D, *et al*: Up-regulation of the chemokine receptor CCR7 in classical but not in lymphocyte-predominant Hodgkin disease correlates with distinct dissemination of neoplastic cells in lymphoid organs. *Blood* 99: 1109-1116, 2002.
12. Rai D, Karanti S, Jung I, *et al*: Coordinated expression of microRNA-155 and predicted target genes in diffuse large B-cell lymphoma. *Cancer Genet Cytogenet* 181: 8-15, 2008.
13. Yang J, Jin LY, Ding JW, *et al*: Expression of toll-like receptor 4 on peripheral blood mononuclear cells and its effects on patients with acute myocardial infarction treated with thrombolysis. *Arch Med Res* 41: 423-429, 2010.
14. Feniger-Barish R, Yron I, Meshel T, *et al*: IL-8-induced migratory responses through CXCR1 and CXCR2: association with phosphorylation and cellular redistribution of focal adhesion kinase. *Biochemistry* 42: 2874-2886, 2003.
15. Luettichau IV, Nelson PJ, Pattison JM, *et al*: Rantes chemokine expression in diseased and normal human tissues. *Cytokine* 8: 89-98, 1996.