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Miki Takahara 1), Toshihiro Nagato 1), Yuhki Komabayashi 1), Kazumi Yoshino 1), Seigo Ueda 1), Kan Kishibe 1), and Yasuaki Harabuchi 1)

1) Department of Otolaryngology-Head and Neck Surgery, Asahikawa Medical University, Asahikawa, Japan

Running title: Role of soluble ICAM1 in the nasal NK/T-cell lymphoma cells

Address correspondence and reprint requests to: Dr. Miki Takahara MD, PhD
Department of Otolaryngology-Head and Neck Surgery, Asahikawa Medical University, Midorigaoka-Higashi 2-1-1-1, Asahikawa, 078-8510, Japan
Phone: +81-166-68-2554, Fax: +81-166-68-2559
E-mail: miki@asahikawa-med.ac.jp

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Abstracts

Nasal natural killer/T-cell lymphoma (NNKTL) is associated with the Epstein-Barr virus (EBV), and has distinct histological features such as angiocentric and polymorphous lymphoreticular infiltrates that contain too many cell types, including tumour and inflammatory cells. We have previously shown that intercellular adhesion molecule (ICAM)-1 is expressed in NNKTL cells, and that soluble ICAM-1 (sICAM-1) is significantly increased in patients’ sera. However, the functional role of sICAM-1 remains unknown. In the present study, we found that EBV-positive NNKTL cell line SNK6 secreted sICAM-1 in a time-dependent manner. Moreover, exogenous sICAM-1 enhanced the growth of SNK6 cells in a dose-dependent manner. Comparatively, neutralising ICAM-1 and LFA-1 antibodies, as well as the LFA-1 blocker simvastatin, caused a dose-dependent reduction in the number of viable SNK6 cells. Double immunohistological staining of NNKTL tissues confirmed that CD56 positive lymphoma cells co-expressed LFA-1. Moreover, serum sICAM-1 levels in NNKTL patients decreased after treatment, suggesting that the levels reflected disease progression. We conclude that NNKTL cells secrete sICAM-1 that acts as an autocrine factor for lymphoma progression, and suggest that simvastatin may be a potential candidate to treat NNKTL.
Key words

Nasal natural killer (NK)/T-cell lymphoma (NNKTL)

Epstein-Barr virus (EBV)

Lymphocyte function-associated antigen-1 (LFA-1)

Soluble intercellular adhesion molecule-1 (sICAM-1)

Epstein-Barr virus latent membrane protein 1 (LMP-1)
Introduction

Nasal natural killer (NK)/T-cell lymphoma (NNKTL), has distinct epidemiological, clinical, histological and aetiological features. Clinically, NNKTL is characterized by progressive necrotic lesions in the nasal cavity and a poor prognosis caused by rapid progression [1, 2]. The original cells of NNKTL are reported to be natural killer (NK)-cells or gamma delta T-cell lineages [1, 3, 4].

We have previously indicated the presence of Epstein-Barr virus (EBV) DNA, EBV oncogenic proteins, and the clonotypic EBV genome in NNKTL [1, 5, 6]. Subsequent establishment of the EBV-positive cell lines SNK6 and SNT8 from primary lesions [4] accelerated understanding of the biological characteristics of NNKTL. We have previously shown that SNK6 cells produce several cytokines and chemokines such as interferon (IFN)-γ, interleukin (IL)-9, IL-10 and interferon-γ-inducible protein (IP)-10, which play roles in cellular proliferation and invasion in an autocrine manner [7-9]. Furthermore, monocytes attracted by IP-10 enhance the proliferation via cell-to-cell contact [10]. However, the biological characteristics of NNKTL are yet to be completely understood.

Intercellular adhesion molecule-1 (ICAM-1), a member of the immunoglobulin supergene family with a five-domain structure [11], has been characterized as one of the natural ligands to the lymphocyte function-associated
antigen-1 (LFA-1) molecule that is expressed on all leukocytes [11, 12]. ICAM-1 is constitutively expressed on a limited number of tissues, but is induced in a wide variety of normal cells such as vascular endothelial cells, leukocytes, and some epithelial tissues [13]. Soluble ICAM-1 (sICAM-1) secretion in vitro was previously reported in cell lines derived from melanoma, lymphoma, and renal carcinoma [14-18]. In addition, highly significant sICAM-1 levels were reported in the sera from patients suffering from different malignancies [18-20]. We previously revealed ICAM-1 expression on NNKTL cells [21] and SNK6 [8], as well as significantly higher levels of sICAM-1 in the sera from NNKTL patients [21]. Moreover, human peripheral blood NK-cells are reported to express LFA-1 at 2–3-fold higher levels compared to resting T-cells [22]. Binding of LFA-1 to ICAM-1 induces an activation signal that enhances the cytotoxicity and cell growth of NK-cells [23, 24]. However, the functional role of their interaction in NNKTL cells has not yet been investigated yet. In this study, we examined whether NNKTL cells express LFA-1 and secrete sICAM-1 in vivo and vitro, and whether the binding induces the cell growth in an autocrine manner.

**Material and methods**

**Patients**
Twelve Japanese patients (10 males and 2 females; aged 21–70 years; median, 53 years) participated in the serological and immunohistological study. The pertinent information was summarized in Table 1. All patients were diagnosed with NNKTL, and treated in our clinic between 2008 and 2012. The diagnosis was carried out according to the World Health Organization (WHO) classification of haematological malignancies [25]. According to the Ann Arbor classification system, ten and two patients were categorised as stage I and II, respectively. The patients underwent arterial infusion chemotherapy concomitant with radiotherapy, and are currently alive without relapse [26]. The patients signed informed consent forms for this study that were approved by the Institutional Review Board.

Cell culture

EBV-positive NK-cell lines (SNK1, SNK6, KAI3, and YT) and negative lines (KHYG1 and NKL) were used in our studies. SNK1 and SNK6 established from NNKTL patients were kindly provided by Dr. Shimizu (Tokyo Medical and Dental University) [4]. KAI-3 originated from a patient with a severe mosquito allergy [27]. The human NK leukemia cell line, YT [28] was a kind gift from Prof. Eva Klein (Department of Microbiology Tumour and Cell Biology, Karolinska Institutet). Western blot analysis was used to confirm that Epstein-Barr virus latent membrane protein 1 (LMP-1) was expressed in the SNK1, SNK6, KAI3
cell lines, but not in the YT cells [29]. The EBV-negative NK cell lines KHYG1 and NKL were established from patients with NK- cell leukemia [30, 31]. All cell lines were cultured in RPMI 1640 media supplemented with 10% fetal bovine serum (FBS), with 50 U/mL penicillin, 50 μg/mL streptomycin (Life Technologies, Inc., Gaithersburg, MD) and 250 U/mL recombinant human IL-2 (Takeda Pharmaceutical Company Limited, Osaka, Japan).

Flow cytometry

For flow cytometric analysis of surface molecules, the cell lines were washed in cold phosphate-buffered saline (PBS) containing 1% bovine serum albumin, centrifuged, and re-suspended in PBS. The cells were incubated with FITC-conjugated mouse anti-human CD11a, CD18, and CD54 monoclonal antibodies (EXBIO, Praha, Czech Republic) for 30 min at 4°C. FITC-conjugated mouse IgG1 (EXBIO) was used as the isotype control. The samples were analyzed with FACScan (BD Biosciences, Mountain View, CA).

Enzyme-linked immunosorbent assay (ELISA)

The sICAM-1 in the cell culture supernatants and sera was quantified using sICAM-1 ELISA kit (BioSource, Camarillo, CA). The supernatants of the cell culture medium were collected after 24-h and 48-h hours incubation, and were
then further incubated in 96-well flat-bottomed plates coated with anti-human ICAM-1 antibody. The supernatants were diluted with the assay diluent and added to each well. The plates were washed with wash buffer after 2 h of incubation at room temperature. The detection antibody conjugated to horseradish peroxidase was added to each well, and the mixture was incubated at room temperature for 2 h. After washing, the substrate solution was added, and the plates were incubated for 30 min in the dark. The absorbance of each well was determined at 450 nm by using a microplate reader (NJ-2300, Nalge Nunc International, Tokyo, Japan). The measurements were done in triplicate. A standard curve was generated using serial dilutions of the recombinant sICAM-1 in the kit.

MTS assay

The SNK6, KHYG1, and YT cells (5 × 10^4 cells) were cultured in 96-well plates in 200μl of RPMI 1640 medium containing 10% FBS and 50U/mL IL-2. The wells were treated with either anti-human ICAM-1 antibody (1 or 10 μg/ml, Lab Vision Corporation Fremont, CA.), anti-human LFA-1 antibody (CD11a, 1 or 10 μg/ml, EXBIO), recombinant human sICAM-1 (10 or 100μg/ml, eBioscience, Inc., San Diego, CA), or simvastatin/pravastatin (0.1 or 1 μM, LKT Laboratories, Inc., St. Paul, MN) for 48 h. To determine the number of viable cells, we used the Cell Titer 96 Aqueous One Solution Cell Proliferation assay (Promega, Madison,
The cells were incubated for 4 h at 37°C under 5% CO2 with 20μl of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium solution (MTS). The absorbance at 490 nm was measured with an ELISA plate reader. Measurements were done in duplicate, and the experiments were repeated at least three times.

**Immunohistological and in situ hybridization studies**

Immunohistological studies were performed on formalin-fixed and paraffin-embedded tissue sections by using EnVision+ peroxidase-labeled dextran polymer (DAKO, Glostrup, Denmark) for visualisation, as described elsewhere [32]. Anti-CD11a (Epitomics, Inc., Burlingame, CA), anti-CD56 (Zymed, South San Francisco, CA) and anti-LMP1 (DAKO) monoclonal antibodies were used. The slides were placed in 10 mmol/L citric acid buffer at pH 6.0 and subjected to antigen retrieval by a 10-min cycle at 750 W and 95°C in a microwave oven. Serial sections were used for single staining with each antibody. In the double immunohistological staining assay, CD56 was stained brown by diaminobenzidine tetrahydrochloride substrate solution (DAKO) for 10 min, and the CD11a was stained red by using the EnVision/alkaline phosphatase-labeled dextran polymer (DAKO) with the Fast Red substrate solution (DAKO).

EBV encoded small RNA (EBERs) on paraffin embedded sections tissue was
detected by in situ hybridization (ISH) using the fluorescein-conjugated peptide nucleic acid (PNA) probe for EBERs (DAKO) and PNA ISH Detection Kit (DAKO) according to the manufacturer’s instructions.

Statistics

Two group comparisons were tested using nonparametric test procedures such as the Mann–Whitney U-test. Transition of serum sICAM1 level through the treatment was tested using the Wilcoxon signed-rank test. Statistical tests were based on a level of significance at P<0.05.

Results

NNKTL cell line expresses both LFA-1 and ICAM-1.

Initially, we performed flow cytometric analysis to investigate whether the NNKTL cell lines express LFA-1 (composed of CD11a and CD18) and its ligand ICAM-1 (CD54). Both LFA-1 and ICAM-1 were expressed in the NNKTL cell lines (SNK1 and SNK6), the other EBV-positive NK cell line, and the EBV-negative NK cell lines (KHYG1 and NKL) also expressed them (Figure 1A). Comparatively, YT, which is EBV-positive, but LMP1-negative, expressed
ICAM-1, but not LFA-1 (Figure 1A).

NNKTL cell line secretes soluble ICAM-1

Next, we measured the sICAM-1 levels in the cell culture supernatants to investigate whether the NNKTL cells secrete sICAM-1. The SNK6 and KAI3 cells, which are positive for both EBV and LMP-1, secreted sICAM-1 in a time-dependent manner even in the absence of IL-2; and the level of secretion increased in the presence of IL-2 (Figure 1B). Comparatively, the EBV-negative NK cell lines (KHYG1 and NKL) and YT secreted very low levels of ICAM-1, irrespective of the presence or absence of IL-2 (Figure 1B).

Soluble ICAM-1 acts as an autocrine growth factor for NNKTL cell line

Both antibodies caused a dose-dependent reduction in the numbers of viable SNK-6 and KHYG1 cells (Figure 2A), but the effect was greater in the SNK6 cells than in the KHYG1 cells. Significant reduction was seen in SNK6 treated with 10μg/ml anti-human LFA-1 antibody. In contrast, neither of the antibodies affected the number of viable YT cells (Figure 2A). Next, we measured the number of viable cells following treatment with recombinant sICAM-1 in the absence of IL-2. Recombinant sICAM-1 enhanced the growth of the SNK6 and KHYG1 cells in a dose-dependent manner (Figure 2B). Significant enhancement
was seen in SNK6 and KHYG1 treated with sICAM-1. However, the YT cells were not influenced by the presence of recombinant sICAM-1 (Figure 2B). These results indicate that sICAM-1 acts as an autocrine growth factor for NNKTL (SNK6) cells.

Simvastatin inhibits cell growth of NNKTL cell line

Simvastatin and pravastatin are inhibitors of 3-hydroxy-3-methylglutaryl CoA reductase, and are used as anti-hypercholesterolemia drugs. Simvastatin, but not pravastatin, binds to the inserted domain of LFA-1, and prevents binding to ICAM-1 [33]. Therefore, we measured the number of viable cells following incubation with simvastatin to determine whether simvastatin inhibits the cell growth of NNKTL cell line. Simvastatin caused a dose-dependent reduction in the numbers of viable SNK6 and KHYG1 cells; however, at 1 μM, the effect was much greater on the SNK6 cells than on the KHYG1 cells (Figure 3A).

Significant reduction was seen in SNK6 treated with 1 μM Simvastatin.

Comparatively, pravastatin did not affect the viability of the SNK6 and KHYG1 cells, and viability of the YT cells was not altered by any treatment (Figure 3A).

LFA-1 (CD11a) and ICAM1 are expressed in lymphoma cells in NNKTL tissues, and the serum level of soluble ICAM-1 is associated with lymphoma progression.
and LMP-1 expression.

Our previous work utilizing samples from patients clearly demonstrated that ICAM-1 is expressed in lymphoma cells in NNKTL tissues, and serum levels of sICAM-1 are higher in NNKTL patients than in other lymphomas of the head and neck [21]. Immunohistological staining revealed that LFA-1-positive cells preferentially co-localized with the CD56-positive lymphoma cells in biopsy samples from the twelve NNKTL patients (Figure 3B). In addition, double immunohistological staining confirmed that CD56-positive lymphoma cells co-expressed LFA-1 on the cell surface (Figure 3C). In 12 biopsy samples from the patients, EBERs and LMP-1 was detected in 11 (92%) and 5 (42%) samples, respectively.

The sICAM-1 levels before and after treatment ranged from 160–290 (median 150) ng/ml and 15–115 (median 45) ng/ml, respectively. The levels significantly decreased after treatment (p<0.05) (Figure 3D). The results of measurements about LFA-1, EBERs, LMP-1, and sICAM-1 in the 12 patients are summarized in Table 1. The serum sICAM-1 levels from the five patients with LMP-1 positive tumor were significantly higher than those from remained seven patients (p<0.05) (Figure 3E).

**Discussion**
Previous studies have reported that ICAM-1 (CD54) is highly expressed on SNK6 [8, 34]. In this study, we showed that the EBV-positive NK-cell lines SNK6 and KAI3, which are also positive for LMP-1, expressed LFA-1 at the cell surface (Figure 1A). In addition, these cells lines secreted large amounts of sICAM-1 into the supernatant culture fluids even in the absence of IL-2 (Figure 1B) in time-dependent manner, thereby suggesting that sICAM-1 plays a role in autocrine signalling. Because LFA-1 on normal NK-cells reportedly induces cell growth signalling following binding to ICAM-1[24], we investigated whether sICAM-1 act as a growth factor. We found that the anti-ICAM-1 neutralizing antibody as well as the anti-LFA-1 blocking antibody decreased the numbers of viable SNK6 cells (Figure 2A) and that exogenous sICAM-1 dose-dependently increased the cell numbers in a dose-dependent manner (Figure 2B). These findings clearly show that sICAM-1 has a functional role as an autocrine growth factor for SNK6 cells. We think that ICAM-1 on the cell surface also has same function. However, surface ICAM-1 may be not expressed at sufficient levels to bind all of LFA-1. On the other hand, large amount of sICAM-1 produced by EBV-positive NK-cell lines seems to be able to occupy all of LFA-1. This may be the reason why anti-LFA-1 antibody has more inhibitory effect on SNK6 cells than on KHYG1 cells (Figure 2A), and has the more effect on SNK6 cells than anti-ICAM-1 antibody do (Figure 2A). The finding that exogenous sICAM-1
worked on KHYG1 cells more effectively than on SNK6 (Figure 2B) support this speculation.

In the patients, we previously showed higher levels of sICAM-1 in sera and expression of ICAM-1 on the lymphoma cells in NNKTL tissues [21]. In this study, we confirmed that LFA-1 was expressed on the CD56-positive lymphoma cells in the NNKTL tissues (Figure 3BC), and that serum sICAM-1 levels significantly decreased after the treatment (Figure 3D) suggesting that serum sICAM-1 level reflect the lymphoma progression. The findings observed in samples from the patients support the proposal that sICAM-1 acts as an autocrine growth factor for lymphoma cells in vivo.

Although previous studies have reported sICAM-1 secretion in vitro in several cell lines [14-18] and highly significant sICAM-1 levels in patients’ sera in a wide variety of malignancies [18-20], the functional role of soluble ICAM-1 is yet to be completely understood. To the best of our knowledge, the present study is the first to show that sICAM-1 has a functional role as an autocrine growth factor for neoplastic cells. Because T-cell also activated by signaling from LFA-1 [35], same autocrine growth mechanism may take place in T-cell malignancies.

Although the EBV-positive NK-cell line YT, which is negative for LMP-1[29], expressed ICAM-1 on the cell surface, the cells did not secrete
sICAM-1. Therefore, it is likely that LMP-1 expression is associated with sICAM-1 secretion. sICAM-1 is secreted by proteolytic cleavage of membrane-bound ICAM-1 [36], and IFN-γ, which is secreted by SNK6 cells [8], reportedly induces the secretion of sICAM-1 by shedding membrane-bound ICAM-1 on human melanoma cell lines [14] and renal carcinoma cell lines [17]. Actually, LMP-1 is reported to induce NF-κB-dependent IFN-γ secretion in lymphoblastoid cell lines [37]. On the basis of these findings, we suggest that LMP-1 enhance sICAM-1 secretion via IFN-γ–induced proteolytic cleavage. The increased secretion of sICAM-1 following treatment with IL-2 observed in this study (Figure 1B) is not surprising, because exogenous IL-2 enhances LMP-1 expression [8]. Because LMP-1 expression was also related to serum sICAM-1 levels in in vivo materials (Figure 3E), the relationship may be also present in the lesions of patients.

Simvastatin and pravastatin inhibit of 3-hydroxy-3- methylglutaryl CoA reductase, and are antihypercholesterolemia drugs. Simvastatin, but not pravastatin, binds to the inserted domain of LFA-1, and prevents binding to ICAM-1 [33]. Indeed, Katano et al reported that treatment with simvastatin inhibited the cell growth of EBV-transformed lymphoblastoid cell lines (LCLs) and delayed the development of EBV lymphomas in severe combined immunodeficiency (SCID) mice inoculated with EBV-transformed LCLs [38].
The growth inhibition is partial, however, simvastatin is not chemotherapeutical
drug. In this point, simvastatin may be a potential drug for the treatment including
combined use of other chemotherapeutical drugs.

Conclusion

We showed that the NNKTL (SNK6) cells, which expressed both LFA-1 and
ICAM-1, secreted sICAM-1, possibly via LMP-1-dependent mechanism. We
further demonstrated that sICAM-1 played a functional role as an autocrine
growth factor in SNK6 cells. In the NNKTL patients, we confirmed that the
lymphoma cells expressed both LFA-1 and ICAM-1, and that the serum sICAM-1
levels are related to LMP-1 expression, and decreased after the treatment. Finally,
we revealed that simvastatin, which blocks the interaction between LFA-1 and
ICAM-1, displaces cell growth of SNK6 cells. We conclude that NNKTL is a
particular lymphoma secreting soluble ICAM-1 as an autocrine factor for
lymphoma progression, and that simvastatin may be one of the potential
candidates for the treatment of NNKTL.

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**Conflict of interest disclosure**

No financial interest/relationships with financial interest relating to the topic of this article have been declared.

**References**


[22] Robertson MJ, Caligiuri MA, Manley TJ, Levine H, Ritz J. Human


**Figure legends**

**Figure 1** Expression of LFA-1 (CD11a and CD18) and ICAM-1 (CD54) in NK-cell lines, and Production of soluble ICAM-1 from NK-cell lines

(A) Expression of LFA-1 (CD11a and CD18) and ICAM-1 (CD54) in NK-cell lines was examined by flow cytometry. The bold line shows the fluorescence intensity of the cells stained with FITC-conjugated mouse anti-human CD11a,
CD18, or CD54 monoclonal antibody. The grey zone shows FITC-conjugated mouse IgG1 (isotype control). (B) The cell lines (5 × 10⁴ cells/200 μl medium) were cultured in 96-well flat-bottomed plates with or without 50U/mL IL-2, and the supernatants were collected after 24, and 48 h. Subsequently, the amount of soluble ICAM-1 was measured by ELISA. The white and grey bars show the soluble ICAM-1 levels with and without IL-2, respectively. The results represent the mean ± SD values of three independent experiments.

Figure 2  Viable cell numbers of NK-cell lines following treatment with anti-LFA-1 or ICAM-1 blocking antibody or with soluble ICAM-1.

(A) The SNK6, KHYG1, and YT cells (5 × 10⁴ cells) were cultured in 96-well plates in 200μl of RPMI 1640 medium containing 10% FBS and 50U/mL IL-2. Then, mouse-IgG (control), anti-human ICAM-1 antibody (1 or 10 μg/ml) or anti-human LFA-1 antibody (CD11a; 1 or 10 μg/ml) was added. After 48-h incubation, the viable cell number was measured by the MTS assay. The results represent the mean ± SD values of three independent experiments. Ab, antibody.

(B) SNK6, KHYG1, and YT cells (5 × 10⁴ cells) in 96-well plate were cultured in 200μl RPMI 1640 medium containing 10% FBS without IL-2. Then recombinant Human soluble-ICAM-1 (10 or 100μg/ml) was added. After 48 hours culture, a viable cell number was measured by the MTS assay. Results represent the mean
+SD of three independent experiments.

Figure 3  Altered cell viability following treatment with simvastatin or pravastatin, Localization of LFA-1 (CD11a) and CD56 positive cells in the NNKTL tissues, Transition of serum sICAM-1 levels through the treatment, and Relationship between LMP-1 expression and sICAM-1 levels.

(A) The SNK6, KHYG1, and YT cells (5 × 10⁴ cells) in 96-well plate were cultured in 200μl RPMI 1640 medium containing 10% FBS with 50U/mL IL-2. Then, simvastatin or pravastatin (0.1 or 1 μM) was added. After 48-h incubation, the viable cell number was measured by the MTS assay. The results represent the mean + SD values of three independent experiments. (B) Single staining for CD11a or CD56 on serial section. (C) Double staining for CD56 (brown) and CD11a (red). (D) Transition of serum sICAM-1 levels through the treatment. (E) Relationship between LMP-1 expression and serum sICAM-1 levels.
Figure 2

A (absorbance)

- Mouse IgG (Isotype control)
- anti-LFA-1 ab: 1ug/ml
- anti-ICAM-1 ab: 1ug/ml
- anti-ICAM-1 ab: 10ug/ml

SNK6 | KHYG1 | YT

B (absorbance)

- No treatment
- Soluble-ICAM-1 10ug/ml
- Soluble-ICAM-1 100ug/ml

SNK6 | KHYG1 | YT
Figure 3

A (absorbance)

- No treatment
- Simvastatin: 0.1 μM
- Simvastatin: 1 μM
- Pravastatin: 0.1 μM
- Pravastatin: 1 μM

SNK6 | KHYG1 | YT

B
LFA-1 (CD11a)

C

D
Before After Treatment

E
LMP-1
Table 1. Characteristics of 12 patients with nasal NK/T-cell lymphoma

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<th>Clinical stage</th>
<th>EBV status</th>
<th>Serum sICAM-1 (ng/ml)</th>
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Abbreviations: Epstein-Barr virus, EBV; EBV latent membrane protein-1, LMP-1; EBV encoded small RNA, EBER; Lymphocyte function-associated antigen-1, LFA-1; sICAM-1, soluble ICAM-1; ND, not done