Biochemical and Biophysical Research Communications 453 (2014) 321–325

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Human cytomegalovirus replication supported by virus-induced activation of CCL2–CCR2 interactions

Tsugiya Murayama [a,*], Marie Kikuchi [a], Takaki Miita [a], Rie Yamada [a], Keiko Matsubara [a], Hidetaka Sadanari [a], Naofumi Mukaida [b]

[a] Department of Microbiology and Immunology, Faculty of Pharmaceutical Sciences, Hokuriku University, Ho-3 Kanagawa-machi, Kanazawa 920-1181, Japan
[b] Division of Molecular Bioregulation, Cancer Research Institute, Kanazawa University, Kakuma-machi, Kanazawa 920-1192, Japan

ARTICLE INFO

Article history:
Received 26 August 2014
Available online 26 September 2014

Keywords:
Cytomegalovirus replication
CCL2
CCR2
siRNA
Chemokine

ABSTRACT

We previously revealed that human cytomegalovirus (HCMV) infection can cause aberrant expression of the chemokine IL-8/CXCL8. We first examined the effects of HCMV infection on the expression of another chemokine, CCL2. HCMV infection induced CCL2 expression at the mRNA and protein levels in human embryonic lung fibroblasts cells (HEL). Moreover, HCMV induced the mRNA expression of CCR2, a specific receptor for CCL2. CCL2 siRNA treatment reduced HCMV virion production, and this reduction was reversed by the addition of CCL2. We further observed that CCL2 siRNA, but not control siRNA, reduced the expression of HCMV immediate early gene (IE1) and HCMV UL54 gene (DNA polymerase) in a dose-dependent manner. Thus, HCMV infection is able to activate the CCL2–CCR2 interactions to further enhance HCMV infection and/or replication.

1. Introduction

Several lines of evidence indicate the aberrant expression of chemokines in human cytomegalovirus (HCMV) infection. HCMV, herpes simplex virus and influenza A virus dampen the expression of chemokines against dendritic cells to evade immune response [1–4]. HCMV further enhances the expression of the CXC chemokine IL-8/CXCL8, and the CC chemokines CCL2 and CCL5 [5–7]. Moreover, HCMV is able to increase CCL2 expression indirectly by inducing the expression of tumor necrosis factor (TNF)-α and IL-1β [8,9]. Furthermore, CCL2 levels are increased in the cerebrospinal fluid of HCMV encephalitis patients co-infected with HIV [10,11].

HCMV is a ubiquitous betaherpesvirus, which is present in a majority of the population worldwide [12–14]. HCMV infection is presumed to contribute to atherosclerosis, in which chemokines may have a pathogenic role. The HCMV-encoded CC chemokine receptor US28 can exacerbate atherosclerosis in the presence of CCL2 or CCL5, by inducing smooth muscle cell migration [15]. Elevated levels of CCL2 are observed in atherosclerotic plaques, where macrophages showing expression of a specific receptor for CCL2, CCR2, abundantly infiltrate [11,16]. Thus, HCMV and CCL2 may cooperatively contribute to atherosclerosis.

It still remains elusive on the effects of CCL2 on HCMV gene expression and replication. Hence, we addressed this question and confirmed that HCMV-induced CCL2 expression can further augment HCMV infection.

2. Materials and methods

2.1. Cell line and virus

Human embryonic lung fibroblast cells (HEL) were grown in Dulbecco's modified Eagle's minimal essential medium (DMEM; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) as described previously [17]. The Towne strain of HCMV was used throughout these experiments. The history of this strain has been described elsewhere [18,19]. HCMV was propagated in HEL cells. Viral infectivity was determined by plaque assay as described previously [19]. In some experiments, the stock virus suspensions were filtered through a 0.1-μm or 0.45-μm filter (Millex, syringe-driven filter unit #SLVV033RS, #SLHV033RS; Millipore Corporation, Billerica, MA) to remove virions. In other experiments, 2 ml of stock virus suspension was placed in a 3-cm dish (Falcon #3001; Becton

Abbreviations: HCMV, human cytomegalovirus; HIV, human immunodeficiency virus; HEL, human embryonic lung fibroblasts cells; IE, immediate early gene; TNF, tumor necrosis factor; DMEM, Dulbecco's modified Eagle's minimal essential medium.

* Corresponding author. Fax: +81 76 229 2781.
E-mail address: t-murayama@hokuriku-u.ac.jp (T. Murayama).

http://dx.doi.org/10.1016/j.bbrc.2014.09.071
0006-291X/

Dickinson, Franklin Lakes, NJ) and was irradiated with ultraviolet (UV) light for 5 min at 1.2 J/cm^2 to inactivate virions.

2.2. Compounds

siRNAs targeting CCL2 were purchased from Ambion through Life Technologies (Carlsbad, CA). Recombinant human CCL2 (MCP-1) was purchased from R&D Systems Inc. (Minneapolis, MN).

2.3. Viral production assay

The conditions used for viral production assay were essentially those described previously [20]. Plaque reduction rates were calculated based on the mean plaque numbers in the control cells.

2.4. Western blot analysis

The conditions used for Western blot analysis were essentially those described previously [17]. Primary antibodies were rabbit polyclonal anti-human CCL2 antibodies (ab9669; Abcam Inc., Tokyo, Japan) and rabbit polyclonal anti-b-actin (C4; Chemicon International Inc., Temecula, CA). Anti-CCL2 and anti-b-actin antibodies were diluted at 1:5000 and 1:2000 in TBS-T, respectively. Membranes were washed 3 times in TBS-T and incubated with peroxidase-conjugated secondary antibody diluted 1:10,000 in TBS-T for 1 h at room temperature. After washing 3 times in TBS-T, immune complexes were detected using the ECL system (Amersham Pharmacia Biotech AB) according to the manufacturer's instructions.

2.5. Transient transfection of siRNA

The conditions used for transient transfection of siRNA were essentially those described previously [17]. Double-stranded siRNAs targeting CCL2 sequences were prepared (siRNA ID# s12567; Ambion through Life Technologies, Carlsbad, CA). A non-targeting siRNA (Ambion through Life Technologies) was used as a negative control, and was confirmed not to affect CCL2 expression when compared with non-transfected controls. Sequences were as follows: s12567 sense, UGU UAU AAU UUC ACC AAU Att, antisense, UAU UGG UGA AGU UAU AAC Agc. At 24 h after transfection with CCL2 siRNA, cells were infected with HCMV at an MOI of 1.0. Total RNAs were extracted at 72 h after infection, and were subjected to quantitative RT-PCR, in order to assess CCL2 mRNA expression.

2.6. Analysis of gene expression

The conditions used for analysis of gene expression were essentially those described previously [17]. PCR primers were as follows: CCL2 primers (forward: 5′-GAC CAG GAA AGA ATG TGA AAG-3′, reverse: 5′-GCT CTG CCA ATT GAC TTT CCT T-3′) [21,22]; CCR2 primers (forward: 5′-GAC CAG GAA AGA ATG TGA AAG TGA-3′, reverse: 5′-GCT CTG CCA ATT GAC TTT CCT T-3′) [21]; HCMV IE1 primers (forward: 5′-TAT ACC CAG ACG GAA GAG AAA TTC-3′, reverse: 5′-CTG CAG TGC ACC CCC CAA CTT G-3′) [23]; HCMV UL54 primers (forward: 5′-TTG CGG GTT CGG TGG TTA-3′, reverse: 5′-CGG CCA TAG TGT TGA GCT TAT AGT T-3′) [24]; and b-actin primers (forward: 5′-ATC ATG TTT GAG ACC TTC AAC-3′, reverse: 5′-CAG GAA GGA AGG CTG GAA GAG-3′) [25]. Results were normalized against b-actin mRNA levels.

2.7. Statistical analysis

After means and SD were calculated, all data were analyzed using Student's *t*-test.

3. Results

3.1. Gene expression analysis in HCMV-infected cells

We first examined the effects of HCMV infection on the expression of CCL2 and its specific receptor, CCR2. HCMV infection increased the mRNA expression of CCL2 and CCR2, starting from 24 h after infection, reaching a maximal level at 72 h and decreasing thereafter (Fig. 1A). HCMV infection enhanced CCL2 and CCR2 mRNA expression in a dose-dependent manner, reaching a peak after infection at an MOI of 0.5 (Fig. 1B). Moreover, HCMV infection markedly increased CCL2 protein expression beyond 24 h after infection (Fig. 2A). Furthermore, UV-irradiated HVMV infection increased CCL2 protein expression (Fig. 2B). Filtration at 0.1 μm removed HCMV particles (unpublished data) and abrogated enhanced CCL2 protein expression, while filtration at 0.45 μm did not reduce enhanced CCL2 protein expression (Fig. 2B). These observations indicate that HCMV-induced CCL2 protein expression is directly mediated by viral particles.

3.2. Effects of CCL2 siRNA on HCMV infection

In order to examine the roles of CCL2 expression in HCMV infection, we transfected HCMV-infected cells with specific siRNA

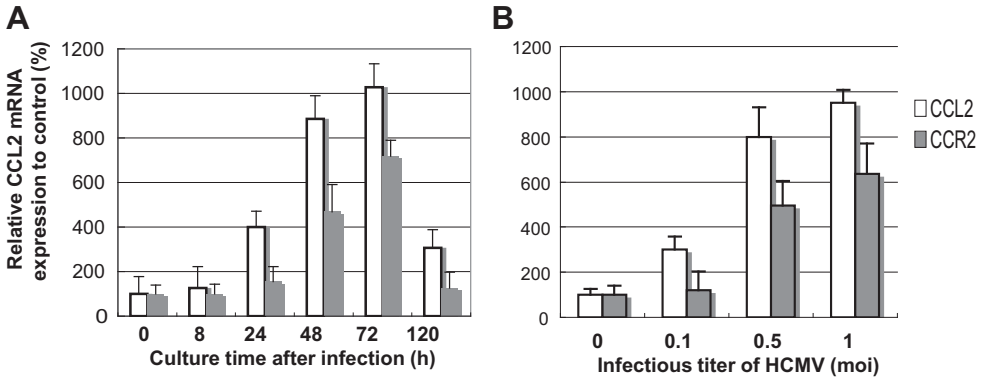

Fig. 1. Induction of CCL2 or CCR2 gene expression by HCMV infection. (A) HEL cells were infected with HCMV at an MOI of 1 and were incubated for the indicated time intervals. Total RNA was then extracted for qRT-PCR to detect CCL2 or CCR2 mRNA expression, as described in Section 2. Means ± SD was calculated from three independent experiments and are shown here. (B) HEL cells were infected with HCMV at the indicated MOI and were incubated for 72 h. Total RNA was then extracted for qRT-PCR to detect CCL2 or CCR2 mRNA expression, as described in Section 2. Means ± SD of three independent experiments were calculated and are shown here.

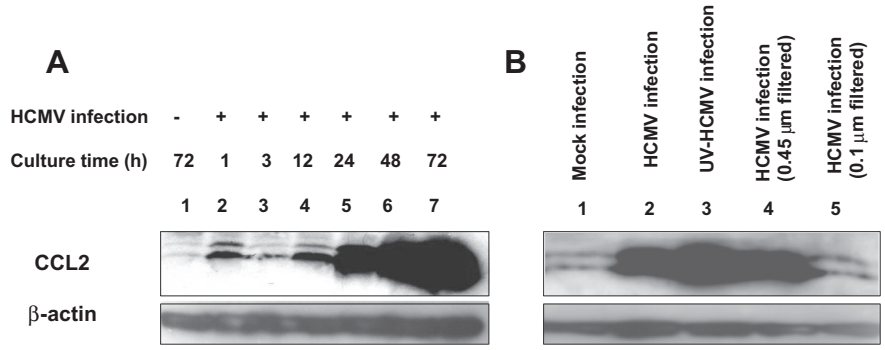

Fig. 2. HCMV infection-induced CCL2 protein synthesis in HEL cells. (A) HEL cells were infected with HCMV at an MOI of 1 and were incubated for the indicated time intervals. Proteins were then extracted for Western blotting analysis to detect CCL2 protein as described in Section 2. β-actin was determined as a control. Representative results from three independent experiments are shown here. (B) HEL cells were subjected to mock infection, or infection with HCMV, UV-irradiated HCMV or filtered (0.45 or 0.1 μm) HCMV, and were further incubated for 72 h. Proteins were then extracted for Western blotting analysis to detect CCL2 protein, as described in Section 2. β-actin was determined as a control. Representative results from three independent experiments are shown here.

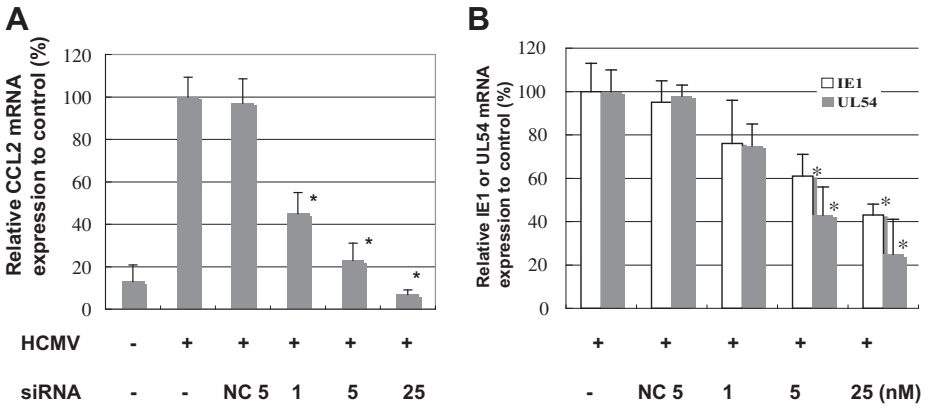

Fig. 3. Effects of CCL2 siRNA on HCMV IE1 and UL54 expression. HEL cells were infected with HCMV at 24 h after cells were transfected with CCL2 siRNA or negative control siRNA (NC). Total RNA was extracted at 72 h after HCMV infection and was subjected to qRT-PCR to detect CCL2 mRNA (A) or IE1 or UL54 mRNA (B) as described in Section 2. Means ± SD were calculated from three independent experiments and are shown here. $^{*}p < 0.05$.

against CCL2. CCL2 siRNA, but not control siRNA, reduced HCMV-induced CCL2 expression in a dose-dependent manner (Fig. 3A). Similarly, CCL2 siRNA, but not control siRNA, reduced IE1 and UL54 expression in a dose-dependent manner (Fig. 3B). Moreover, CCL2 siRNA reduced HCMV infectious virion production in a dose- and time-dependent manner (Fig. 4A and B). To exclude the possibility that the effects of CCL2 siRNA are off-target effects, we further examined the effects of CCL2 on CCL2 siRNA-treated cells. CCL2 reversed CCL2 siRNA-mediated inhibition of HCMV virion production in a dose-dependent manner, but heat-treated and inactivated CCL2 failed to reverse the effects of siCCL2 (Fig. 4C). These observations indicate that HCMV infection enhances the expression of CCL2, which can further augment HCMV infectious virion production.

4. Discussion

Several groups have demonstrated that HCMV is able to induce the expression of CCL2 [26,27]. In contrast, other groups have observed that HCMV infection decreased CCL2 mRNA expression [7]. Hence, we first examined the effects of HCMV infection on CCL2 expression. We demonstrated that HCMV infection augmented CCL2 expression at the mRNA and protein levels in HEL cells, consistent with these reports [26,27]. Moreover, heat-inactivated virus or viral inocula filtered at 0.1 μm lost the ability to enhance CCL2 expression in HEL cells. Thus, enhanced CCL2 expression requires infection with viable virus. These discrepancies may be explained by differences in host cell subpopulation, viral strain or virus stock concentration. However, considering that CCL2 expression was aberrantly augmented in various pathological conditions with HCMV infection, HCMV infection is able to increase CCL2 expression in most cases.

We revealed that CCL2 siRNA treatment significantly reduced HCMV infection in HEL cells. These observations suggest that CCL2 is able to directly support HCMV infection and/or replication. By acting on its specific receptor, CCR2, CCL2 is able to induce the migration of target cells such as monocytes/macrophages and dendritic cells [28,29]. These CCR2-expressing cells are able to sustain HCMV infection and replication [3,30,31]. Thus, enhanced expression of CCL2 may help to facilitate the spread of the virus, particularly during the initial stages of reactivation from latent infection, indirectly by recruiting these target cells.

The importance of IE function prompted us to speculate [17,25,32,33]. It is necessary, therefore, to identify novel anti-cytomegaloviral agents that can block HCMV gene expression in very early stages without causing major adverse side effects. The

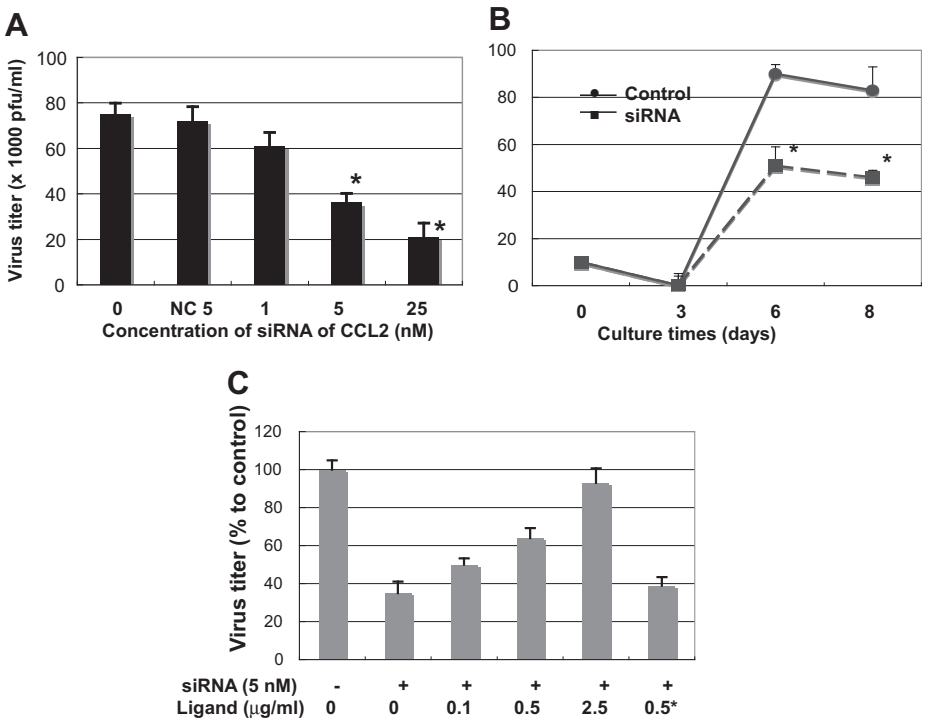

Fig. 4. Effects of CCL2 siRNA on HCMV virion production. (A) HEL cells were transfected with either the indicated concentrations of CCL2 siRNA or negative control siRNA (NC). Cells were then infected with HCMV at an MOI of 1. At 6 days after infection, supernatants were collected to determine virus titer by using plaque assay. Means ± SD were calculated from three independent experiments and are shown here. $^{*}p < 0.05$. (B) HEL cells were transfected with either CCL2 siRNA or negative control siRNA (NC). Cells were then infected with HCMV at an MOI of 1. At the indicated time intervals after infection, supernatants were collected in order to determine virus titer by plaque assay. Means ± SD were calculated from three independent experiments and are shown here. $^{*}p < 0.05$. (C) Effects of CCL2 on HCMV replication in HEL cells transfected with CCL2 siRNA. HEL cells were infected with HCMV at 24 h after CCL2 siRNA transfection. Cells were then treated in the absence or presence of the indicated concentrations of CCL2 or heat-inactivated CCL2 (100 °C, 5 min, indicated by *). Viral titers in culture supernatants were determined by plaque assay on day 6 after infection. Means ± SD were calculated from three independent experiments and are shown here.

present observations raise the possibility that CCL2 represents an additional candidate molecule targeting HCMV infection, particularly at early stages.

Acknowledgments

We are grateful to all the members of the Murayama lab for helpful discussion and constructive advice. This work was supported in part by JSPS KAKENHI Grant Number 21390306.

References

[1] M. Moutaftsi, P. Brennan, S.A. Spector, Z. Tabi, Impaired lymphoid chemokine-mediated migration due to a block on the chemokine receptor switch in human cytomegalovirus-infected dendritic cells, J. Virol. 78 (2004) 3046–3054.
[2] A.T. Prechtel, N.M. Turza, D.J. Kobelt, J.I. Eisemann, R.S. Coffin, Y. McGrath, C. Hacker, X. Ju, M. Zenke, A. Steinkasserer, Infection of mature dendritic cells with herpes simplex virus type 1 dramatically reduces lymphoid chemokine-mediated migration, J. Gen. Virol. 86 (2005) 1645–1657.
[3] S. Varani, G. Frascaroli, M. Homman-Loudiyi, S. Feld, M.P. Landini, C. Soderberg-Naucler, Human cytomegalovirus inhibits the migration of immature dendritic cells by down-regulating cell-surface CCR1 and CCR5, J. Leukoc. Biol. 77 (2005) 219–228.
[4] R. Salentin, D. Gemsa, H. Sprenger, A. Kaufmann, Chemokine receptor expression and chemotactic responsiveness of human monocytes after influenza A virus infection, J. Leukoc. Biol. 74 (2003) 252–259.
[5] T. Murayama, Y. Ohara, M. Obuchi, K.S. Khabar, H. Higashi, N. Mukaida, K. Matsushima, Human cytomegalovirus induces interleukin-8 production by a human monocytic cell line, THP-1, through acting concurrently on AP-1- and NF-kappaB-binding sites of the interleukin-8 gene, J. Virol. 71 (1997) 5692–5695.
[6] S. Michelson, P. Dal Monte, D. Zipeto, B. Bodaghi, L. Laurent, E. Oberlin, F. Arenzana-Seisdedos, J.L. Virelizier, M.P. Landini, Modulation of RANTES production by human cytomegalovirus infection of fibroblasts, J. Virol. 71 (1997) 6495–6500.
[7] A.J. Hirsch, T. Shenk, Human cytomegalovirus inhibits transcription of the CC chemokine MCP-1 gene, J. Virol. 73 (1999) 404–410.
[8] K.L. Hokeness, W.A. Kuziel, C.A. Biron, T.P. Salazar-Mather, Monocyte chemoattractant protein-1 and CCR2 interactions are required for IFN-alpha/beta-induced inflammatory responses and antiviral defense in liver, J. Immunol. 174 (2005) 1549–1556.
[9] O. Umland, H. Heine, M. Miehe, K. Marienfeld, K.H. Staubach, A.J. Ulmer, Induction of various immune modulatory molecules in CD34(+) hematopoietic cells, J. Leukoc. Biol. 75 (2004) 671–679.
[10] S. Bernasconi, P. Cinque, G. Peri, S. Sozzani, A. Crociati, W. Torri, E. Vicenzi, L. Vago, A. Lazzarin, G. Poli, A. Mantovani, Selective elevation of monocyte chemotactic protein-1 in the cerebrospinal fluid of AIDS patients with cytomegalovirus encephalitis, J. Infect. Dis. 174 (1996) 1098–1101.
[11] I.F. Charo, M.B. Taubman, Chemokines in the pathogenesis of vascular disease, Circ. Res. 95 (2004) 858–866.
[12] J.G. Sissons, A.J. Carmichael, Clinical aspects and management of cytomegalovirus infection, J. Infect. 44 (2002) 78–83.
[13] S.C. Dollard, S.D. Grosse, D.S. Ross, New estimates of the prevalence of neurological and sensory sequelae and mortality associated with congenital cytomegalovirus infection, Rev. Med. Virol. 17 (2007) 355–363.
[14] W. Britt, Manifestations of human cytomegalovirus infection: proposed mechanisms of acute and chronic disease, Curr. Top. Microbiol. Immunol. 325 (2008) 417–470.
[15] D.N. Streblow, C. Soderberg-Naucler, J. Vieira, P. Smith, E. Wakabayashi, F. Ruchti, K. Mattison, Y. Altschuler, J.A. Nelson, The human cytomegalovirus chemokine receptor US28 mediates vascular smooth muscle cell migration, Cell 99 (1999) 511–520.
[16] C. Gerard, B.J. Rollins, Chemokines and disease, Nat. Immunol. 2 (2001) 108–115.
[17] K. Akuzawa, R. Yamada, Z. Li, Y. Li, H. Sadanari, K. Matsubara, K. Watanabe, M. Koketsu, Y. Tuchida, T. Murayama, Inhibitory effects of tricin derivative from Sasa albo-marginata on replication of human cytomegalovirus, Antiviral Res. 91 (2011) 296–303.
[18] T. Furukawa, A. Fioretti, S. Plotkin, Growth characteristics of cytomegalovirus in human fibroblasts with demonstration of protein synthesis early in viral replication, J. Virol. 11 (1973) 991–997.
[19] B.B. Wentworth, L. French, Plaque assay of cytomegalovirus strains of human origin, Proc. Soc. Exp. Biol. Med. 135 (1970) 253–258.
[20] T. Murayama, Y. Li, T. Takahashi, R. Yamada, K. Matsubara, Y. Tuchida, Z. Li, H. Sadanari, Anti-cytomegalovirus effects of tricin are dependent on CXCL11, Microbes Infect. 14 (2012) 1086–1092.
[21] Y. Lu, Z. Cai, G. Xiao, Y. Liu, E.T. Keller, Z. Yao, J. Zhang, CCR2 expression correlates with prostate cancer progression, J. Cell. Biochem. 101 (2007) 676–685.

[22] N. Ogawa, T. Kawanami, K. Shimoyama, L. Ping, S. Sugai, Expression of interferon-inducible T cell alpha chemoattractant (CXCL11) in the salivary glands of patients with Sjogren's syndrome, Clin. Immunol. 112 (2004) 235–238.
[23] M. Nishiwaki, M. Fujimuro, Y. Teishikata, H. Inoue, H. Sasajima, K. Nakaso, K. Nakashima, H. Sadanari, T. Yamamoto, Y. Fujiwara, N. Ogawa, H. Yokosawa, Epidemiology of Epstein–Barr virus, cytomegalovirus, and Kaposi's sarcoma-associated herpesvirus infections in peripheral blood leukocytes revealed by a multiplex PCR assay, J. Med. Virol. 78 (2006) 1635–1642.
[24] D.T. Petrik, K.P. Schmitt, M.F. Stinski, Inhibition of cellular DNA synthesis by the human cytomegalovirus IE86 protein is necessary for efficient virus replication, J. Virol. 80 (2006) 3872–3883.
[25] S.A. Jassim, M.A. Naji, Novel antiviral agents: a medicinal plant perspective, J. Appl. Microbiol. 95 (2003) 412–427.
[26] J.L. Stern, B. Slobedman, Human cytomegalovirus latent infection of myeloid cells directs monocyte migration by up-regulating monocyte chemotactic protein-1, J. Immunol. 180 (2008) 6577–6585.
[27] S.T. Hamilton, G. Scott, Z. Naing, W.D. Rawlinson, Human cytomegalovirus directly modulates expression of chemokine CCL2 (MCP-1) during viral replication, J. Gen. Virol. 91 (2013) 2495–2503.
[28] P.T. Coates, B.L. Colvin, A. Ranganathan, F.J. Duncan, Y.Y. Lan, W.J. Shufesky, A.F. Zahorchak, A.E. Morelli, A.W. Thomson, CCR and CC chemokine expression in relation to Flt3 ligand-induced renal dendritic cell mobilization, Kidney Int. 66 (2004) 1907–1917.
[29] S. Sozzani, Dendritic cell trafficking: more than just chemokines, Cytokine Growth Factor Rev. 16 (2005) 581–592.
[30] M.S. Smith, G.L. Bentz, J.S. Alexander, A.D. Yurochko, Human cytomegalovirus induces monocyte differentiation and migration as a strategy for dissemination and persistence, J. Virol. 78 (2004) 4444–4453.
[31] G. Gerna, E. Percivalle, D. Lilleri, L. Lozza, C. Fornara, G. Hahn, F. Baldanti, M.G. Revello, Dendritic-cell infection by human cytomegalovirus is restricted to strains carrying functional UL131-128 genes and mediates efficient viral antigen presentation to CD8+ T cells, J. Gen. Virol. 86 (2005) 275–284.
[32] M. Scholz, H.W. Doerr, J. Cinatl, Inhibition of cytomegalovirus immediate early gene expression: a therapeutic option?, Antiviral Res 49 (2001) 129–145.
[33] T. Murayama, N. Mukaida, H. Sadanari, N. Yamaguchi, K.S. Khabar, J. Tanaka, K. Matsushima, S. Mori, Y. Eizuru, The immediate early gene 1 product of human cytomegalovirus is sufficient for up-regulation of interleukin-8 gene expression, Biochem. Biophys. Res. Commun. 279 (2000) 298–304.