Induction of lytic cycle replication of Kaposi’s sarcoma-associated herpesvirus by herpes simplex virus type 1: involvement of IL-10 and IL-4

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Summary
Previously, we identified that both human herpesvirus 6 and human immunodeficiency virus type 1 Tat were important cofactors that activated lytic cycle replication of Kaposi’s sarcoma-associated herpesvirus (KSHV). Here, we further investigated the potential of herpes simplex virus type 1 (HSV-1) to influence KSHV replication. We demonstrated that HSV-1 was a potentially important factor in the pathogenesis of Kaposi’s sarcoma, as determined by production of lytic phase mRNA transcripts, viral proteins and infectious viral particles in BCBL-1 cells. These results were further confirmed by an RNA interference experiment using small interfering RNA targeting KSHV ORF50 and a luciferase reporter assay testing ORF50 promoter-driven luciferase activity. Finally, we discovered that production of human interleukin-10 (IL-10) and IL-4 partially contributed to HSV-1-induced KSHV replication. Our data present the first direct evidence that HSV-1 can activate KSHV lytic replication and suggest a role of HSV-1 in KSHV pathogenesis.

Introduction
Kaposi’s sarcoma-associated herpesvirus (KSHV, also designated human herpesvirus 8 or HHV-8) was first identified from the lesions of acquired immunodeficiency syndrome-related Kaposi’s sarcoma (AIDS-KS) (Chang et al., 1994). It has been shown to correlate to all epidemiological forms of Kaposi’s sarcoma (KS), primary effusion lymphoma (PEL) and a subset of multicentric Castleman’s disease (Dourmishev et al., 2003). All herpesviruses, including KSHV, share the ability to establish latent or lytic infections. In KS lesions, latent KSHV predominates with a low percentage of cells exhibiting lytic replication; the viral genome persists as an episome and has highly restricted expression of latent genes. The latent phase is essential for the development of KSHV-induced malignancies (Staskus et al., 1997). The lytic phase, during which the virus produces infectious virions for dissemination, modulates cellular signalling pathways through unrestricted expression of viral genes (Dourmishev et al., 2003). Regulation of viral replication is critical to disease progression as the tissue deterioration and infection progression is proportionally related to the percentage of virus-infected cells undergoing reactivation. Indeed, studies have shown that KSHV viral load is higher in KS patients than in KSHV-infected individuals without KS, and KSHV viral load also increases during progression of this disease (Decker et al., 1996; Sturzl et al., 1997). However, it remains unknown what are the specific contributions of latent and lytic gene expression to the diseases associated with KSHV (Schulz and Moore, 1999; Sun et al., 1999). In addition, KSHV appears to be necessary but not sufficient for development of KS and evidence strongly suggests other cofactors also play important roles in the pathogenesis of this disease. However, who and how to reactivate latent virus are not well defined.

The frequent occurrence of KS in patients with human immunodeficiency virus type 1 (HIV-1) and in transplant recipients strongly suggest that immunodeficiency was a significant factor contributing to the diseases associated with KSHV (Beral and Newton, 1998; Mendez et al., 1999). Besides the direct role of immune system in controlling KSHV and the proliferation of KS cells, it has been postulated that a dysfunctional immune system could contribute to the progression of KS by additional processes, including alteration of cytokine profiles that could influence the biology of endothelial and KS tumour cells (Fiorelli et al., 1995) or induce KSHV lytic replication (Monini et al., 1999; Varthakavi et al., 1999; 2002) and
failure to control infectious agents that may have an impact on KSHV or KS tumour cells. Patients with KS or AIDS-KS are often immunosuppressed and susceptible to many other infectious agents. Indeed, a couple of agents such as human cytomegalovirus (HCMV) and human herpesvirus 6 (HHV-6) have been proved to be cofactors activating KSHV (Vieira et al., 2001; Lu et al., 2005). On one hand, co-infection of herpes simplex virus type 1 (HSV-1) and KSHV were frequently detected in AIDS or KS patients (Casper et al., 2002; Chen and Hudnall, 2006; Kumar et al., 2007), recurrent aphthous ulceration patients (Lin et al., 2005), and even in healthy individuals (Miller et al., 2005). On the other hand, HSV-1 could also infect B cells and human vascular endothelial cells, the precursor of KS (Key et al., 1990; Lamontagne and Jolicoeur, 1994). Although HSV-1 and KSHV are not found in similar anatomic compartments during their latent infection, frequent reactivation of latent HSV-1 occurred in AIDS or AIDS-KS patients, leading to appearance of HSV-1 viraemia (Birek and Ficarra, 2006). Viraemia is not only present in the peripheral blood of immunocompromised adults and in neonates, but also during primary herpetic gingivostomatitis in immunocompetent children at the relatively high frequency of 34%, as tested by polymerase chain reaction (PCR) (Harel et al., 2004). HSV-1 viraemia subsequently increased opportunities for HSV-1 to contact B and/or endothelial cells, which, maybe, previously had harboured the KSHV genome. These facts led us to hypothesize that HSV-1 may play a role in the pathogenesis of KS or AIDS-KS. HSV-1 is a ubiquitous virus that infects the majority of the human population (approximately 60–90%) (van Benthem et al., 2001). After primary infection, HSV-1 persists in the host in a latent form and has the potential to cause disease upon reactivation, particularly in the immunocompromised host (Mann et al., 1984; Chen et al., 1998). Epidemiological data showed that in the USA 16–20% of AIDS patients are positive for HSV-1, while 20–57% for KSHV (Krzyzowska et al., 2000; Miller et al., 2006).

To address the role of HSV-1 in KSHV replication and KS or AIDS-KS pathogenesis, in this study we performed kinetic studies of KSHV replication induced by HSV-1. We showed that production of human interleukin-10 (IL-10) and IL-4 partially contributed to HSV-1-induced KSHV replication. These novel findings are believed to be the first report on the mechanisms of KSHV activation by HSV-1 and shed light on the understanding of AIDS-KS pathogenesis.

Results

**HSV-1 infects BCBL-1 cells in vitro**

To evaluate whether HSV-1 can affect lytic cycle replication of KSHV in BCBL-1 cells, we first determined the susceptibility of BCBL-1 to HSV-1 infection. HSV-1 was inoculated into BCBL-1 cells and cell morphologic change was observed by light microscope. As shown in Fig. 1A, BCBL-1 cells began to exhibit the cytopathic effects (CPE) since 6 h post infection. With time going, the number of infected BCBL-1 cells showing the typical CPE, such as the formation of syncytia and polykaryocytes, gradually increased. Particularly, more than 80% of BCBL-1 cells indicated the CPE at 72 h post inoculation (Fig. 1A) while only less than 5% of Mock-infected BCBL-1 cells showed CPE at this time point (data not shown). To determine whether HSV-1 can replicate in BCBL-1 cells, Western blot analysis were performed. We found that infected BCBL-1 cells started to express HSV-1 glycoprotein D (gD) (the receptor-binding glycoprotein for virus entry) (Cocchi et al., 2004) since 72 h post infection and continued to express gD at 96 h (Fig. 1B). These data indicate that HSV-1 infected BCBL-1 cells and replicated.

**HSV-1 infection of PEL cell lines results in lytic cycle replication of KSHV**

To examine whether HSV-1 can activate lytic cycle replication of KSHV, real-time quantitative PCR were performed. Analysis of data from five independent experiments between Mock and HSV-1-infected cells demonstrated that, on average, ORF50 (the molecular switch gene of KSHV) (Sun et al., 1998) mRNA expression in HSV-1-infected BCBL-1 cells was increased $2.47 \pm 0.21$-fold at 3 h, $2.49 \pm 0.23$-fold at 6 h, $7.69 \pm 0.81$-fold at 12 h, $7.13 \pm 0.78$-fold at 24 h, $6.56 \pm 0.61$-fold at 48 h and 1.5 ± 0.13-fold at 72 h, respectively, when compared with Mock-infected BCBL-1 cells (Fig. 2A). Similarly, ORF26 (viral minor capsid protein expressed only during lytic KSHV replication) (Russo et al., 1996) mRNA expression increased $1.3 \pm 0.11$-fold at 3 h, $7.59 \pm 0.72$-fold at 6 h, $8.09 \pm 0.79$-fold at 12 h, $5.38 \pm 0.56$-fold at 24 h, $2.1 \pm 0.19$-fold at 48 h and $0.94 \pm 0.08$-fold at 72 h, respectively, compared with Mock-infected BCBL-1 cells (Fig. 2B). Meanwhile, ORF29 (viral packaging protein) (Russo et al., 1996) mRNA expression in HSV-1-infected BCBL-1 cells was increased $2.09 \pm 0.19$-fold at 3 h, $2.24 \pm 0.2$-fold at 6 h, $1.95 \pm 0.19$-fold at 12 h, $1.80 \pm 0.17$-fold at 24 h, $1.72 \pm 0.16$-fold at 48 h, $0.97 \pm 0.08$-fold at 72 h, respectively, compared with the corresponding control (Fig. 2C).

To determine whether other KSHV lytic genes can be activated by HSV-1, reverse transcriptase-polymerase chain reaction (RT-PCR) analysis for ORF57, T1.1, vIRF, vFLIP and vIL-6 transcripts were performed. As shown in Fig. 2D, all mRNAs of these genes in HSV-1-infected BCBL-1 cells were significantly increased from 6 to 72 h when compared with corresponding controls. To examine
whether HSV-1-induced KSHV lytic replication is cell type specific, another KSHV latently infected cell line, BC-3, was used. After HSV-1 infection BC-3 showed similar ORF50 mRNA expression pattern to that of BCBL-1 (Fig. 2E). These data suggest that HSV-1 may activate KSHV lytic cycle RNA; this lytic cycle replication is not restricted in BCBL-1 cells and may be a common mechanism that reactivates KSHV in KS.

Induction of KSHV lytic cycle RNA also results in induction of lytic cycle protein

To determine whether induction of KSHV lytic cycle RNA by HSV-1 also resulted in the induction of lytic cycle proteins, immunofluorescence assay (IFA) were performed to detect KSHV lytic cycle proteins K8.1 and ORF59 in BCBL-1 cells. After infection of cells with HSV-1, both ORF K8.1 and ORF59 proteins expression in BCBL-1 cells were significantly increased from 24 to 72 h compared with that of Mock-infected BCBL-1 cells (Fig. 3A). Theoretically, KSHV lytic cycle replication by HSV-1 would subsequently produce infectious progeny virions. To test this hypothesis, experiments were designed to detect KSHV virion in the supernatants from HSV-1-infected BCBL-1 cell cultures. The supernatants of HSV-1-infected BCBL-1 cells were collected to infect HEK293 cells that have been previously shown to be permissive cells for KSHV (Foreman et al., 1997). After 21 h infection the total RNA was isolated and RT-PCR was performed to amplify KSHV-specific sequence, ORF26. The results demonstrated that ORF26 mRNA was readily expressed in HEK293 cells infected with the supernatants from HSV-1-infected BCBL-1 cells, while not in HEK293 cells infected with the supernatants from Mock-infected BCBL-1 cells (Fig. 3B). To more quantitatively measure the level of ORF26 expression, real-time quantitative PCR was performed. We found that ORF26 expression in HEK293 cells infected with the supernatants from HSV-1-infected BCBL-1 cells increased 1141-fold compared with that of HEK293 cells infected with the supernatants from Mock-infected BCBL-1 cells (data not shown). Together these data suggest that the induction of KSHV lytic cycle RNA by HSV-1 results in not only the induction of lytic cycle proteins, but also the production of infectious progeny virions.

HSV-1 activates KSHV lytic cycle replication through activation of ORF50

To explore whether HSV-1 infection induces KSHV lytic replication by direct binding to ORF50 promoter, we examined the effect of HSV-1 infection on ORF50 promoter activity in several cells. KSHV ORF50 encodes a replication and transcription activator homologous to the
Fig. 2. Expression of KSHV lytic cycle RNA in PEL cell lines infected with HSV-1.

A. ORF50 mRNA expressed in BCBL-1 cells following infection with HSV-1. ORF50 mRNA expression in BCBL-1 infected with Mock (white bar, Mock) or HSV-1 (black bar, HSV-1) for 3, 6, 12, 24, 48 and 72 h was quantified by real-time quantitative PCR. Relative quantities of ORF50 expression are represented on the y-axis. Results shown are from five independent experiments performed in triplicate.

B. ORF26 mRNA expressed in BCBL-1 cells following infection with HSV-1. ORF26 mRNA expression in BCBL-1 infected with Mock (white bar, Mock) or HSV-1 (black bar, HSV-1) for 3, 6, 12, 24, 48 and 72 h was quantified by real-time quantitative PCR. Relative quantities of ORF26 expression are represented on the y-axis. Results shown are from five independent experiments performed in triplicate.

C. ORF29 mRNA expressed in BCBL-1 cells following infection with HSV-1. ORF29 mRNA expression in BCBL-1 infected with Mock (white bar, Mock) or HSV-1 (black bar, HSV-1) for 3, 6, 12, 24, 48 and 72 h was quantified by real-time quantitative PCR. Relative quantities of ORF29 expression are represented on the y-axis. Results shown are from five independent experiments performed in triplicate.

D. RT-PCR analysis for ORF57, T1.1, vIRF, vFLIP and vIL-6 mRNA in BCBL-1 cells infected with HSV-1. ORF57, T1.1, vIRF, vFLIP and vIL-6 mRNA expression in BCBL-1 infected with Mock (Mock) or HSV-1 (HSV-1) for 6, 12, 24, 48 and 72 h was detected by RT-PCR. M represents DNA molecular marker and β-actin was readily detectable in all samples indicating the presence of amplifiable cDNA.

E. ORF50 mRNA expressed in BC-3 cells following infection with HSV-1. ORF50 mRNA expression in BC-3 infected with Mock (white bar, Mock) or HSV-1 (black bar, HSV-1) for 3, 6, 12, 24, 48 and 72 h was quantified by real-time quantitative PCR. Relative quantities of ORF50 expression are represented on the y-axis. Results shown are from five independent experiments performed in triplicate.

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Epstein-Barr virus Rta, which has been shown to be both necessary and sufficient to activate the KSHV lytic cycle from latency (Lukac et al., 1998; Sun et al., 1998). In this assay a 655 bp fragment 5' to the ORF50 transcriptional start site was used to drive luciferase reporter gene expression (p50-Luc construct). We (Lu et al., 2002) and Seaman et al. (1999) have previously shown that this construct has promoter activity in BCBL-1 cells. Cells infected with Mock and subsequently transfected with p50-Luc showed low baseline levels of luciferase expres-
sion (used as a control). However, cells transfected with p50-Luc demonstrated enhanced levels of luciferase expression (1.95-, 4.61-, 1.62-, 2.79- and 9.67-fold increase in BCBL-1, BC-3, Vero, B95-8 and HEK293 cells respectively) by treatment with TPA (used as a control; Fig. 4A). Notably, infection of the cells with HSV-1 resulted in a significant increase in luciferase expression (2.43-, 5.76-, 29.74-, 2.81- and 2.93-fold increase in BCBL-1, BC-3, Vero, B95-8 and HEK293 cells respectively) compared with the corresponding control (Fig. 4A).

To further confirm and extend these results, three small interfering RNA (siRNA) constructs, which were designed to silence KSHV ORF50, were introduced into BCBL-1 cells. To determine whether or which constructs can efficiently inhibit ORF50 mRNA expression in HSV-1-infected BCBL-1 cells, RT-PCR were performed. As shown in Fig. S1A, ORF50 mRNA in si-2-transfected BCBL-1 cells further infected with HSV-1 was significantly decreased at 12 h when compared with other two constructs, si-1 and si-3, and pRNAT-U6.1/Neo vector (named si-vector; as a control)-transfected or phosphate-buffered saline (PBS)-treated BCBL-1 cells further infected with HSV-1. Subsequently, Western blot was used to confirm the data from above RT-PCR. It was demonstrated that ORF50 protein in si-2-transfected BCBL-1 cells further infected with HSV-1 was also significantly decreased at 24 h when compared with the corresponding controls (Fig. 4B). As ORF50 was the molecular switch for KSHV replication and si-2 construct efficiently silenced ORF50 protein during HSV-1-induced KSHV replication, we asked whether si-2 construct could also silence other structural gene expression, such as ORF26 and ORF59, via inhibiting ORF50 protein during HSV-1-induced KSHV replication. To address this issue, real-time quantitative PCR and IFA were performed to detect ORF26 mRNA and ORF59 protein in BCBL-1 cells respectively.

Fig. 4. HSV-1 activates KSHV lytic cycle replication via ORF50.
A. Infection of PEL cell lines with HSV-1 promotes induction of KSHV ORF50 promoter activity. BCBL-1, BC-3, Vero, B95-8, and HEK293 cells were infected with Mock (Mock+p50-Luc) and HSV-1 (HSV-1+p50-Luc), or treated with TPA (TPA+p50-Luc) then further transfected with p50-Luc. Luciferase activities were measured as induction (n-fold). All data points were the averages of four independent experiments performed in triplicate.
B. Western blot analysis for ORF50 protein in si-constructs-transfected BCBL-1 cells infected with HSV-1. BCBL-1 cells were transfected with si-2 (si-2), si-1 (si-1), si-3 (si-3), si-vector (si-v) or treated with PBS (PBS) and further infected with HSV-1 for 24 h. Lysates were subjected to SDS-PAGE, transferred to membrane, then immunoblotted with the indicated anti-ORF50 antibody. The membrane wasstripped and reprobed with anti-actin to confirm the equal amounts of protein in each sample. The results shown are a representative experiment of three independent experiments with similar results.
C. IFA staining for ORF59 in si-constructs-transfected BCBL-1 cells infected with HSV-1 (original magnification, ×40). KSHV lytic protein ORF59 expression in BCBL-1 cells transfected with si-vector and further infected with Mock (Mock+si-vector), si-vector and further infected with HSV-1 (HSV-1+si-vector) or si-2 and further infected with HSV-1 (HSV-1+si-2) for 24 and 48 h was detected by IFA staining with ORF59 mAbs.
Fig. S1B, ORF26 mRNA in si-2-transfected BCBL-1 cells further infected with HSV-1 consistently decreased from 3 to 72 h compared with si-vector-transfected BCBL-1 cells infected with HSV-1. Interestingly, ORF59 protein expression in si-2-transfected BCBL-1 cells further infected with HSV-1 was significantly decreased at both 24 and 48 h compared with si-vector-transfected BCBL-1 cells infected with HSV-1 (Fig. 4C). Because other two siRNA constructs including si-1 and si-3 could partially inhibit ORF50 mRNA and protein expression in HSV-1-infected BCBL-1 cells (Fig. S1A and Fig. 4B), BCBL-1 cells were also transfected with these two constructs to determine whether they can inhibit ORF26 mRNA in HSV-1-infected BCBL-1 cells. Analysis of ORF26 mRNA expression by real-time quantitative PCR demonstrated that both si-1 and si-3 constructs failed to effectively silence HSV-1-induced KSHV replication (data not shown). These observations collectively suggest that HSV-1 activates KSHV lytic cycle replication through activation of ORF50.

Th-2 cytokines, IL-10 and IL-4, partially contribute to HSV-1-induced KSHV replication

Our previous study showed that cytokines like IFN-gamma (IFN-γ) produced by HHV-6-infected BCBL-1 cells played an important role in induction of KSHV replication (Lu et al., 2005). We reason that some inflammatory cytokines may also contribute to the KSHV replication induced by HSV-1. We first detected cytokine protein expression profile changes affected by HSV-1 infection by using cytokine antibody array technique. The cytokine antibody array V was used to screen the potential inflammatory cytokine(s) involved in this process. As shown in Fig. S2 and Table 2, expressions of many inflammatory cytokines in HSV-1-infected BCBL-1 cells were altered to some degree at different time points. Notably, Th-1 cytokines, such as IFN-γ, IL-2 and TNF-alpha in HSV-1-infected BCBL-1 cells consistently increased more than twofold at both 4 and 8 h compared with that in Mock-infected BCBL-1 cells. Similarly, Th-2 cytokines, such as IL-10 and IL-4 in HSV-1-infected BCBL-1 cells also consistently increased more than twofold (particularly, IL-10 were more than fourfold) at both 4 and 8 h compared with the corresponding control (Fig. S2 and Table 2). Interestingly, we also found that both angiogenin (a type of angiogenic factors) and eotaxin-3 (a type of chemokines) in HSV-1-infected BCBL-1 cells were increased fourfold at both 4 and 8 h and reached to 22-fold for both at 8 h when compared with the corresponding controls (Fig. S2 and Table 2). However, GM-CSF in HSV-1-infected BCBL-1 cells consistently decreased 6.23-fold at 4 h and 2.06-fold at 8 h compared with the corresponding control (Fig. S2 and Table 2). These data suggest that infection of HSV-1 may result in changes of inflammatory cytokine expressing profiles in BCBL-1 cells.

To determine whether overexpression of these cytokines may modulate KSHV lytic replication, we neutralized cytokines including IL-4, IL-10, IFN-γ, angiogenin and eotaxin-3 and monitored KSHV replication by real-time quantitative PCR. As shown in Fig. 5A, after addition of anti-IL-10 polyclonal antibody (pAb) to the culture of HSV-1-infected BCBL-1 cells, ORF26 mRNA was decreased 4.79-fold at 3 h, 2.07-fold at 6 h, and 2.95-fold at 12 h, respectively, compared with HSV-1-infected BCBL-1 cells treated with the control IgG (Fig. 5A). To further confirm these data, Western blot analysis for KSHV vIL-6 was performed. It was demonstrated that after addition of anti-IL-10 pAbs to HSV-1-infected BCBL-1 cells, vIL-6 protein expression was readily decreased at both 12 and 24 h (Fig. 5B). Meanwhile, after addition of anti-IL-4 pAb to the culture of HSV-1-infected BCBL-1 cells, ORF26 mRNA was decreased 1.6-fold at 3 h and 1.55-fold at 6 h compared with HSV-1-infected BCBL-1 cells treated with the control IgG (Fig. S3). Similarly, ORF26 mRNA in HSV-1-infected BCBL-1 cells treated with anti-eotaxin-3 pAb was decreased only at 3 and 6 h (1.85 and 1.56-fold respectively) compared with HSV-1-infected BCBL-1 cells treated with the control IgG (Fig. S4). Neutralizing antibodies against IFN-γ and angiogenin were also added to the culture of HSV-1-infected BCBL-1 cells respectively; however, no inhibiting effect on HSV-1-induced KSHV replication was observed (data not shown).

Experiments were further designed to determine whether recombinant cytokines could also induce KSHV lytic replication. BCBL-1 cells were stimulated with recombinant human IL-10 (rhIL-10) and rhIL-4 at the final concentration of 100 and 50 ng ml\(^{-1}\) and KSHV replication was examined. Using real-time quantitative PCR, studies demonstrated that ORF26 mRNA in BCBL-1 cells stimulated with rhIL-10 was increased 1.9-fold at 24 h, 1.5-fold at 48 h and 1.7-fold at 72 h, respectively, compared with PBS-treated BCBL-1 cells as a control (Fig. 5C). However, no significant difference of ORF26 mRNA was observed between PBS and rhIL-4-treated BCBL-1 cells (Fig. 5C). These data suggest that overexpression of IL-10 may play a direct role in induction of KSHV replication by HSV-1 infection.

As IL-10 and IL-4 in HSV-1-infected BCBL-1 cells consistently increased (particularly for IL-10 more than fourfold) at both 4 and 8 h compared with the corresponding control (Fig. S2 and Table 2) and neutralizing antibodies against IL-10 and IL-4 could partially inhibit HSV-1-induced KSHV replication (Fig. 5A and B; Fig. S3), we then asked whether HSV-1 infection of BCBL-1 cells can also modulate expressions of IL-10Rα and/or IL-4R. To address this issue, RT-PCR analysis for mRNAs of IL-10Rα and IL-4R
was performed. We found that both IL-10Rα and IL-4R mRNA were significantly increased at both 3 and 8 h during the early of HSV-1 infection of BCBL-1 cells (Fig. 5D). To determine whether HSV-1 upregulates expression of IL-10, IL-10Rα, IL-4 and IL-4R via directly activating their promoters, we performed a luciferase assay. We found that HSV-1-infected BCBL-1 cells further transfected with pIL-10-Luc or pIL-10R-Luc also resulted in a statistic increase in luciferase expression with the presence of TPA (2.1-fold increase, respectively, compared with the corresponding control, \( P < 0.05; \) Fig. 5E). Furthermore, HSV-1-infected BCBL-1 cells transfected with pIL-4-Luc or pIL-4R-Luc also resulted in a statistic increase in luciferase expression with the presence of TPA (2.1-fold increase of IL-4 promoter compared with the corresponding control, \( P < 0.05; \) Fig. 5F) or absence of TPA (2.48-fold increase of IL-4R promoter compared with the corresponding control, \( P < 0.05; \) Fig. 5F) respectively. These data suggest that in BCBL-1 cells HSV-1 upregulates expressions of IL-4, IL-10, and their receptors via directly activating their promoters.

**Discussion**

Some cofactors that induce KSHV from latency to lytic replication may play an important role in the pathogenesis of KS or AIDS-KS. On one hand, the lytic replication is required for the initial spread of KSHV to target B lymphocytes, monocytes and the endothelial cell progenitors to form the spindle cells that are characteristic components of KS tumour after exposure to KSHV. On the other hand, most spindle cells, which harbour KSHV in the latent phase replication, undergoing lytic replication are uniformly present in tumours and have been postulated to play a critical role in the maintenance of the tumour. Therefore, the identification of cofactors that enhance KSHV lytic replication at both early and late time points following infection is very important in understanding KS or AIDS-KS pathogenesis.

In this study we investigated the kinetic of KSHV replication by HSV-1 and explored the possible mechanisms by which HSV-1 activates KSHV cycle replication. Our experiments provide the direct experimental evidence that HSV-1 can be a potential cofactor of KS. Several factors have been previously shown to be cofactors of KS (Varthakavi et al., 1999; 2002; Vieira et al., 2001; Lu et al., 2005; Zeng et al., 2007). For instance, Vieira et al. showed infection of HCMV increased the production of KSHV in endothelial cells and activated lytic cycle gene expression in keratinocytes, suggesting that HCMV could influence KSHV pathogenesis (Vieira et al., 2001). Furthermore, by coculturing HHV-6-infected T cells with KSHV-latent BCBL-1 cell line, infecting BCBL-1 cells with HHV-6 virions, and generating heterokaryons between HHV-6-infected T cells and BCBL-1 cells, our group indicated that HHV-6 played an important role in induction of KSHV replication, implying that HHV-6 may also participate in KS pathogenesis by promoting KSHV replication.
and increasing KSHV viral load. Here we have further demonstrated that HSV-1 is also a critical factor that is responsible for the induction of KSHV lytic replication, suggesting that HSV-1 may promote KS progression by reactivating KSHV lytic replication.

During the KSHV replication, the molecular switch that controls the transition from KSHV latency to lytic replication is the product of the ORF50 gene. ORF50 is an immediate-early KSHV gene product whose expression is detectable prior to that of early lytic gene products. Previous studies indicated that during KSHV replication detectable prior to that of early lytic gene products. Previous studies indicated that during KSHV replication induced by HHV-6, HHV-6 could directly bind to ORF50 promoter (Lu et al., 2005). In current study, by using a siRNA targeting ORF50 and a luciferase reporter assay, we showed that HSV-1 activates KSHV lytic cycle replication also through induction of KSHV ORF50, which was consistent with previous reports (Varthakavi et al., 2002; Lu et al., 2005). Of course, our results did not eliminate the possibility that other immediate-early KSHV genes and their promoters or soluble factors produced by or in response to HSV-1-infected PEL cell lines may also be involved in this process.

In the current study, we found that HSV-1 not only elevated the productions of IL-4 and IL-10, but also promoted mRNAs expression of their receptors in BCBL-1 cells. This enhancement was mediated by HSV-1 production binding to promoters of IL-4, IL-10, and their receptors. IL-10 has been shown to only serve as a growth factor for AIDS-related B-cell lymphoma (Benjamin et al., 1992; Masood et al., 1995), but also be released and used by PEL cells for autonomous proliferation and was critical to the development and progression of PEL (Drexler et al., 1999; Jones et al., 1999; Oksenhendler et al., 2000). Similarly, IL-4 and its receptor have been found expressed by KS or AIDS-KS tumour cells. IL-4, a cytokine with pleiotropic effects, suppressed IL-6 in vivo, inhibited AIDS-KS-derived cells in vitro, and upregulated IL-4R, suggesting that IL-4 and its receptor may play a critical role in growth control of PEL cell lines (Sirianni et al., 1998; Husain et al., 1999; Puri, 2000; Miles et al., 2002). Both IL-10 and IL-4 are Th-2 cytokines and important regulators of the production of proinflammatory cytokines. Once HSV-1 infects individuals of KS or AIDS-KS, it can elevate production of Th-2 cytokines, such as IL-10 and IL-4. On one hand, IL-10 and IL-4 can prevent deletion of HSV-1 and/or KSHV from body by inhibiting Th-1 cytokines-mediated cell immune response. On the other hand, activation of IL-10 and IL-4 can induce KSHV replication, leading to release of many mature KSHV particles, which subsequently infect neighbour B lymphocytes, monocytes and the endothelial cells. Here, by adding rHIL-10 to the culture, we provided direct experimental evidence that IL-10, at least in part, contributed to KSHV lytic replication in BCBL-1 cells, although we did not find that rHIL-4 induced KSHV replication in BCBL-1 cells. Previous studies have shown that Th-1 cytokine IFN-γ from HIV-1-infected T cells or produced by HHV-6-infected BCBL-1 cells was partially responsible for KSHV reactivation (Mercader et al., 2000; Lu et al., 2005). In this study, although IFN-γ was significantly elevated in HSV-1-infected BCBL-1 cells, the results from antibodies blocking assay indicated that it did not contribute to KSHV replication, suggesting a major role in mediating cell immune response against HSV-1 and/or reactivated KSHV, rather than in viral replication. However, whether other cytokines, growth factors, or their soluble receptors produced by or in response to HSV-1-infected PEL cell lines may also be involved in this process is still unknown.

In summary, we have showed that HSV-1 was a potentially important factor in the pathogenesis of KS and discovered that production of IL-10 and IL-4 partially contributed to HSV-1-induced KSHV replication. As HSV-1 can alter many cytokines profiles and induce multiple signalling pathways, further studies are needed to better understand whether other cytokines and their signals by HSV-1 are also involved in KSHV replication in KS.

**Experimental procedures**

**Cell culture and virus infection**

The BCBL-1 and BC-3 cells, both of which are EBV-negative and KSHV-positive PEL cell lines, were obtained through the AIDS Research and Reference Reagent Program, National Institutes of Health. B95-8, which were KSHV-negative and EBV-positive marmoset B-cell lines, HEK293, Vero (African green monkey kidney fibroblasts), and HSB2 (a type of T cell lines) cells were obtained from American Type Culture Collection (ATCC, Rockville, MD). BCBL-1, B95-8 and HSB2 cells were maintained in RPMI-1640 containing 10% heat-inactivated fetal bovine serum (FBS), 2 mmol L⁻¹-glutamine, 100 U ml⁻¹ penicillin, and 100 µg ml⁻¹ streptomycin at 37°C in a humidified, 5% CO₂ atmosphere. BC-3 cells were grown in RPMI + 20% FBS. HEK293 and Vero cells were maintained in DMEM + 10% FBS. Before infection and/or transfection, both BCBL-1 and BC-3 cells were incubated in serum-free RPMI-1640 medium for a maximum inducibility of KSHV replication as described (McAllister et al., 2005).

HSV-1 (McKrae strain) was propagated in Vero cells as described elsewhere (Halford et al., 1996). Briefly, virus was adsorbed for 1 h at 37°C, then removed and DMEM + 5% FBS was added to the Vero monolayers. When 75% of the cells showed CPE, as determined by light microscope, cell-free culture fluid was harvested and filtered through a 0.45-µm-pore-size filter. Viral titers were determined by plaque assay in Vero cells and expressed as plaque forming units (PFU) per ml. The virus stock had a titer of 10⁶ PFU ml⁻¹ and was stored at −70°C till use. BCBL-1 or BC-3 cells (3 x 10⁶) were pelleted and infected with HSV-1 (0.01 PFU/cell) for 1 h at 37°C. After 1 h adsorption, the virus was removed and cells were suspended in 4 ml of culture medium. The supernatants from normal Vero cells culture were collected and used as a control (Mock) to infect target cells.
Table 1. Sets of primers used in RT-PCR. 

| mRNA   | Oligonucleotides    | Accession no. | Expected size (bp) | Annealling temperature (°C) | Cycles |
|--------|---------------------|---------------|--------------------|-----------------------------|--------|
| ORF57b | F: 5’-GCA TGA TAA TTG ACG GTG AG-3’ 
R: 5’-TGC ATG CCT GGG ATA GTT-3’ | U93872 | 622 | 56 | 20 |
| T1.1b  | F: 5’-CGT CCT ACT TTT CCC ACA TT-3’ 
R: 5’-TAC ACA ACG CCT TCA CCT ACA A-3’ | KSU66521 | 716 | 56 | 17 |
| vIRFb  | F: 5’-CGG AGT AGA GTG GGA AGA CGA AG-3’ 
R: 5’-AGG GTG CTG GTA AGC GAG AA-3’ | AF145700 | 860 | 56 | 20 |
| vFLIPb | F: 5’-TGG CCA CCT AGG TGA TTA T-3’ 
R: 5’-TTG CCG GTG TAC GTG TAC T-3’ | KSU90534 | 497 | 56 | 20 |
| vIL-6b | F: 5’-GCC ACC TCT GTT ACC GTA-3’ 
R: 5’-GCA AAG TGT TCC ACC CAA TAC-3’ | KSU67774 | 364 | 56 | 22 |
| ORF26b | F: 5’-AGC CGA AAG GAT TCC ACC AT-3’ 
R: 5’-TCC GTG TTG TCT ACG TCC AG-3’ | DQ984816 | 263 | 56 | 29 |
| ORF50b | F: 5’-TAA GAA GCT TCG GGC GTC C-3’ 
R: 5’-GGT AAT TGG CCG GCT TTG C-3’ | AF091350 | 700 | 56 | 27 |
| IL-10Rx | F: 5’TGC TGT GCC ATT CCA GGA TT-3’ 
R: 5’-GCC GGA TGA ATG GTG AGT TA-3’ | NM_001558 | 389 | 56 | 40 |
| IL-4Rx | F: 5’TTC GTA CAC GGC ACG GGT GA-3’ 
R: 5’-CAG GCC ATG TGA GCA CTC GTA TTT C-3’ | NM_000418 | 750 | 63 | 29 |
| β-Actin | F: 5’TGA CGG GGT CAC CCA CAC TGT GCC CAT CTG-3’ 
R: 5’-CTA GAA GCC GCC GTG TAC GAT GAC GGG-3’ | BC016045 | 661 | 56 | 19 |

a. The oligonucleotides were selected from the sequences with the indicated accession number. The size of each amplified product, its annealling temperature, and numbers of PCR cycles are indicated. F, forward; R, reverse.
b. These genes belong to KSHV genome.
c. These genes belong to human genome.

Antibodies and reagents

Anti-HSV-1 gD goat pAb and horseradish peroxidase (HRP)-conjugated donkey anti-goat and goat anti-mouse and -rabbit IgG were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Anti-KSHV ORF K8.1 A/B (clone 4A4, IgG1) and ORF59 (clone 11D1, IgG2b) mouse monoclonal antibody (mAb) and anti-human IL-10 and rhIL-4 were purchased from PeproTech (Rocky Hill, NJ). Both goat and rabbit IgG were obtained from Advanced Biotechnologies (Columbia, MD). The neutralizing antibodies including anti-human IL-4 goat pAb, anti-human IFN-γ goat pAb, and anti-human angiogenin goat pAb were obtained from R&D Systems (Minneapolis, MN). Both two other neutralizing antibodies including anti-human IL-10 rabbit pAb and anti-human eotaxin-3 goat pAb and two recombinant cytokines including rhIL-10 and rhIL-4 were purchased from PeproTech (Rocky Hill, NJ). Both goat and rabbit IgG were obtained from Biozyn (Beijing, China) as the control IgG for neutralization test. Anti-KSHV ORF50 peptide antibody was generated by immunization of rabbits with ORF50 peptide (aa 230–250) (Chang et al., 2005). Both FITC-conjugated goat anti-mouse antibody and mouse mAb to β-actin (used to monitor sample loading) were purchased from Boster Technologies, Wuhan, Hubei, China.

RNA isolation and real-time quantitative PCR

Total RNA was isolated from cells by using Trizol reagent (Invitrogen, Carlsbad, CA). Real-time quantitative PCR was performed in a GeneAmp 7300 sequence detection machine (Applied Biosystems, Foster City, CA) as previously described (Lu et al., 2005). The sequences of KSHV-specific primers and probes including ORF50, 26 and 29 were listed as previously described (Lu et al., 2002; 2005; Milligan et al., 2004). Efficiencies of the β-actin and target gene amplification were shown to be approximately equal using a validation experiment as described by the sequence detection system manufacturer.

Western blot analysis

Western blot was performed as previously described (Zeng et al., 2007). Briefly, after infection and/or transfection, cells were harvested and lysed in RIPA buffer containing a phosphatase inhibitor cocktail and protease inhibitors. Sixty to 80 μg of protein was loaded onto sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to an Immobilon-P (polyvinylidene difluoride) membrane, and blocked with 5% powdered milk in TBST (50 mM Tris, pH 7.5, 150 mM NaCl, 0.01% Tween-20). The membrane was then incubated with primary antibodies diluted in 5% powdered milk in TBST, washed extensively, and incubated with HRP-conjugated species-specific secondary antibodies. Proteins were visualized with ECL reagents (Amer sham Biosciences, Piscataway, NJ) according to the manufacturer's instructions.

Reverse transcriptase-polymerase chain reaction

RT-PCR were performed as described elsewhere (Lu et al., 2005). Briefly, cDNA was synthesized from isolated RNA using the SuperScript Preamplication system for the first strand cDNA synthesis (Invitrogen) and then amplified using standard PCR techniques following the manufacturer's instructions. Primers used for analysis in this study were listed in Table 1.

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Immunofluorescence assay

After infection of BCBL-1 cells with HSV-1, cells (10^7 ml^-1) were washed once with PBS and smeared on chamber slides. After desiccation, the slides were fixed with pre-cooled acetone (2–8°C) for 15 min and treated with 0.5% Triton X-100 for 30 min at 37°C. Then slides were incubated for 45 min at 37°C in a humidified environment with a 1:100 dilution of anti-KSHV ORF K8.1 A/B and ORF59 mouse mAb and subsequently washed twice with PBS. FITC-conjugated goat anti-mouse antibody (1:32 dilution) was used as a secondary antibody for detection. After a final wash in PBS, cells were viewed under a fluorescence microscope.

Plasmids, transfection and luciferase reporter assay

The KSHV ORF50 (also known as Rta) luciferase reporter construct (p50-Luc), containing the -661 to -7 promoter fragment of the ORF50 promoter region inserted upstream of the luciferase gene in the pGL3 basic vector (Promega, Madison, WI), was generated as described previously (Lu et al., 2002). The human IL-4 luciferase reporter construct (pIL-4-Luc) was generously provided by M. Li-Weber (German Cancer Research Center, Heidelberg, Germany) (Li-Weber et al., 1998). The promoter regions of human IL-10 (-1308 to +19) (Brenner et al., 2003), IL-10 receptor alpha (-966 to +76) and IL-4 receptor (-1078 to +2) were amplified using PCR, DNAs of HSB2 cells as templates, and specific primers with MulI and HindIII restriction enzyme cut sites engineered on the ends to facilitate directional cloning respectively. The PCR products were cloned into the pGL3 basic vector in sense orientation (designated as pIL-10-Luc, pIL-10Rα-Luc and pIL-4R-Luc respectively). All transfection experiments in this study were performed with Lipofectamine 2000 (Invitrogen) following the manufacturer’s instructions. Typical transfection of cells

Table 2. Dynamically regulated host cytokines in BCBL-1 cells early during HSV-1 infection.

| Cytokine name | Fold change at 4h post infection | Fold change at 8h post infection | Cytokine name | Fold change at 4h post infection | Fold change at 8h post infection |
|---------------|-------------------------------|-------------------------------|---------------|-------------------------------|-------------------------------|
| ENA-78        | 0.62                          | 1.43                          | VEGF          | 1.62                          | 2.18                          |
| GCSF          | 0.55                          | 1.08                          | PDGF-BB       | 1.98                          | 3.61                          |
| GM-CSF        | 0.16                          | 0.49                          | Leptin        | 2.30                          | 3.75                          |
| GRO           | 1.25                          | 0.93                          | BDNF          | 1.80                          | 2.55                          |
| GROα          | 4.38                          | 3.59                          | BLC           | 3.95                          | 4.68                          |
| I-309         | 0.98                          | 3.14                          | Ck b 8–1      | 2.34                          | 3.30                          |
| IL-1α         | 1.01                          | 2.14                          | Eotaxin       | 3.67                          | 2.19                          |
| IL-1β         | 1.57                          | 2.01                          | Eotaxin-2     | 2.18                          | 2.43                          |
| IL-2          | 2.59                          | 2.12                          | Eotaxin-3     | 6.35                          | 22.49                         |
| IL-3          | 2.43                          | 1.53                          | FGF-4         | 0.78                          | 1.75                          |
| IL-4          | 2.41                          | 2.81                          | FGF-6         | 2.04                          | 2.86                          |
| IL-5          | 0.81                          | 1.17                          | FGF-7         | 0.88                          | 1.55                          |
| IL-6          | 0.92                          | 1.16                          | FGF-9         | 0.96                          | 1.04                          |
| IL-7          | 1.08                          | 1.34                          | Fit-3 ligand  | 0.60                          | 1.92                          |
| IL-8          | 1.45                          | 1.70                          | Fractalkine   | 0.84                          | 3.01                          |
| IL-10         | 4.23                          | 6.28                          | GCP-2         | 1.75                          | 3.90                          |
| IL-12 p40p70  | 0.95                          | 9.86                          | GDNF          | 1.46                          | 1.57                          |
| IL-15         | 0.93                          | 7.69                          | HGF           | 1.44                          | 1.88                          |
| IL-16         | 1.91                          | 3.18                          | IGFBP-1       | 2.39                          | 3.03                          |
| IFN-γ         | 4.51                          | 2.81                          | IGFBP-2       | 4.40                          | 9.76                          |
| MCP-1         | 3.65                          | 2.13                          | IGFBP-3       | 0.82                          | 1.13                          |
| MCP-2         | 0.78                          | 1.99                          | IGFBP-4       | 1.24                          | 1.72                          |
| MCP-3         | 0.99                          | 1.44                          | IL-16         | 1.41                          | 1.62                          |
| MCSF          | 1.95                          | 1.17                          | IP-10         | 1.18                          | 1.40                          |
| MDSC          | 1.74                          | 2.57                          | LF             | 1.15                          | 1.49                          |
| MIG           | 1.71                          | 16.91                         | LIGHT         | 1.33                          | 2.24                          |
| MIP-1α        | 1.82                          | 3.61                          | MCP-4         | 2.46                          | 4.84                          |
| MIP-1β        | 0.52                          | 4.66                          | MIP-3 α       | 3.06                          | 3.23                          |
| RANTES        | 1.08                          | 1.84                          | NAP-2         | 1.75                          | 1.53                          |
| SCF           | 2.77                          | 2.57                          | NT-3          | 5.36                          | 4.48                          |
| SDF-1         | 3.94                          | 3.16                          | NT-4          | 0.71                          | 0.99                          |
| TARC          | 2.95                          | 2.38                          | Osteoprotegerin| 1.33                        | 1.41                          |
| TGF-β1        | 4.40                          | 4.63                          | PARC          | 1.42                          | 1.65                          |
| TNF-α         | 2.33                          | 1.98                          | PIGF          | 1.33                          | 1.59                          |
| TNF-β         | 3.93                          | 2.16                          | TGF-β2        | 1.02                          | 1.42                          |
| TGF-β3        | 2.17                          | 15.85                         | TGF-β3        | 1.22                          | 1.52                          |
| Angiogenin    | 4.04                          | 22.82                         | TIMP-1        | 1.32                          | 1.22                          |
| Oncostatin M  | 0.83                          | 1.28                          | TIMP-2        | 1.33                          | 1.16                          |
| Thrombopoietin| 1.73                          | 2.79                          |                |                               |                               |

a. Mean values from duplicate trials.
involved the introduction of 0.4 μg of reporter DNA (p50-Luc, pIL-4-Luc, pIL-4R-Luc, pIL-10-Luc or pIL-10R-Luc). Before transfection, cells were adsorbed with HSV-1 for 1 h. After 48 h transfection, cells were harvested for luciferase assay. As a positive control, 20 ng ml⁻¹ of TPA was added to cell culture at 4 h post transfection. Luciferase activity was assayed by using the Promega Bright-N-Glo system as previously described (Lu et al., 2002). The luciferase activity was measured using a Sirius luminometer (Berthold Detection Systems, Pforzheim, Germany). The number of cells present in each sample was also counted to normalize the luciferase activity with the total number of cells (Lu et al., 2002). All data points were the averages of at least four independent transfections.

Small interfering RNA

Three siRNA oligonucleotides were designed to target KSHV ORF50. Their sequences were as follows: the first oligonucleotide, GTC TAC CTT CCG AGG ATT A (610–628 nt of ORF50); the second oligonucleotide, GTT ACG TTG TTG CAG GTT A (1309–1327 nt of ORF50); the third oligonucleotide, TCC TTA TAA ATG ACG CAT A (101–119 nt of ORF50). The DNA sequences with BamHI and XhoI restriction enzyme cut sites engineered on the ends to facilitate directional cloning were synthesized and cloned into pRNAT-U6.1/Neo vector (GenScript Corporation, Scotch Plains, NJ) creating three siRNA constructs designated as si-1, si-2 and si-3 respectively. BCBL-1 cells were transfected with the siRNA construct. After 24 h culture, cells were transfected with the siRNA construct for the second time. Four hours later, cells were infected with HSV-1 and continued to culture for 3, 6, 12, 24, 48 and 72 h respectively.

Human cytokine antibody array

Human cytokine antibody array was performed according to the manufacturer’s directions (RayBio Human Cytokine Antibody Array V; RayBiotech, Norcross, GA). Briefly, cells were added ice-cold protein extraction reagent containing a phosphatase inhibitor cocktail and protease inhibitors. Protein concentration was determined using a Bradford Assay (Bio-Rad Laboratories, Hercules, CA). RayBio Human Cytokine Antibody membrane containing 79 cytokines antibodies was put into the provided 8 well tray and blocked. One microlitre of protein sample was added to membrane at room temperature for 1–2 h. After washing with buffer, diluted biotin-conjugated antibodies as primary antibody were added to each membrane at room temperature for 1–2 h. The membranes were washed with washing buffer and then reacted with HRP-conjugated streptavidin (1:1000 dilution) at room temperature for 2 h and washed again. After acting to detection buffer, the membranes were exposed to X-ray film, and the signal was detected using film developer. By comparing the signal intensities, relative expression levels of cytokines were made. The intensities of signals were quantified by densitometry. Positive control was used to normalize the results from different membranes being compared. Fold changes in protein expression were calculated and normalized the results from different membranes being compared. Fold changes in protein expression were calculated and normalized the results from different membranes being compared.

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Supplementary material

The following supplementary material is available for this article online:

Fig. S1. HSV-1 activates KSHV lytic cycle replication via ORF50.

Fig. S2. HSV-1 infection results in changes of cytokine protein expression profile in BCBL-1 cells.

Fig. S3. Real-time quantitative PCR analysis for ORF26 mRNA expression in blocking assay by pAbs against IL-4. Real-time quantitative PCR was used to detect relative quantities of ORF26 mRNA in Mock-infected BCBL-1 cells plus 13.32 μg ml⁻¹ of goat control IgG (Mock + Cont IgG), HSV-1-infected BCBL-1 cells plus 13.32 μg ml⁻¹ of goat control IgG (HSV-1 + Cont IgG), HSV-1-infected BCBL-1 cells plus 13.32 μg ml⁻¹ of pAb against IL-4 (HSV-1 + pAb-IL-4) for 3, 6, 12, 24 and 48 h indicated. The results from three independent experiments performed in triplicate are shown.

Fig. S4. Real-time quantitative PCR analysis for ORF26 mRNA expression in blocking assay by pAbs against eotaxin-3. Real...

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time quantitative PCR was used to detect relative quantities of ORF26 mRNA in Mock-infected BCBL-1 cells plus 3.33 μg ml⁻¹ of goat control IgG (Mock + Cont IgG), HSV-1-infected BCBL-1 cells plus 3.33 μg ml⁻¹ of goat control IgG (HSV-1 + Cont IgG), HSV-1-infected BCBL-1 cells plus 3.33 μg ml⁻¹ of pAb against eotaxin-3 (HSV-1 + pAb-IL-eota) for 3, 6, 12, 24 and 48 h indicated. The results from three independent experiments performed in triplicate are shown.

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