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Keratinocytes play a role in the immunity to Herpes simplex virus type 2 infection

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Summary. – Herpes simplex virus type 2 (HSV-2) infection is the most common cause of genital ulcerative disease in the developed world. Keratinocytes are the primary cells involved in clinical lesions caused by HSV-2. In our study, we intensively examined cytokine expression in the HSV-2-infected keratinocytes. We observed upregulation of a series of cytokines including early-induced antiviral cytokines as interferons α, β (IFN-α, β), tumor necrosis factor α (TNF-α), colony stimulating factors (CSFs) as G-CSF, GM-CSF, interleukin 3 (IL-3), growth factors (EGF, KGF, and IGF-β1), defensins, selectins, leukocyte function-associated antigens (LFAs,) and toll-like receptors (TLR-2, 3, 4, and 9). More importantly, we found that HSV-2-infected keratinocytes stimulated the proliferation of lymphocytes in co-cultivation system. These data suggest that keratinocytes participate in the immune response to HSV-2 infection in two ways. They secrete inflammatory cytokines to resist the HSV-2 infection directly and recruit the immune cells to eliminate the primary infection indirectly and enhance the adaptive immunity to prevent subsequent infections.

Keywords: Herpes simplex virus type 2; keratinocytes; cytokines; immunity

Introduction

HSV-2 (the subfamily *Alphaherpesvirinae*) infection is currently the most common cause of genital ulcerative disease in the developed world. In USA the prevalence of HSV-2-seropositive individuals has been increasing at a staggering rate, rising 30% from 1976 to 1994 (Fleming *et al*., 1997). HSV-2 can also cause severe disseminated disease in immunocompromised individuals or when transmitted from infected mothers to the neonates during birth. A particularly important category of HSV-associated disease is the neonatal herpes frequently resulting in fatal encephalitis. In USA, the neonatal herpes occurs in approximately 1 in 2500 births (Whitley, 2004; Kimberlin, 2005).

HSV-2 consists of a spherical enveloped virion with a capsid enclosing its linear, double-stranded DNA genome of ~154 kb in length. HSV-2 infects mucosal surfaces or damaged skin and causes most cases of genital herpes (Nahmias *et al*., 1990). The primary infection is usually asymptomatic and depends on the immunological status of the host. HSV-1, 2 are usually transmitted through several routes. Innate response is the first line of defense against viral replication in both naïve and previously infected hosts. It is subsequently integrated with and supported by an adaptive immune response that is either primary or memory in character and is required for ultimate clearance of the acute infection. In response to the initial chemokine and cytokine production, cellular components of the innate immune system are activated and recruited to the site of infection. These include neutrophils followed by the monocytes and natural

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Abbreviations: HSV-2 = Herpes simplex virus type 2; PBMCs = peripheral blood mononuclear cells; IFN-α, β = interferon α, β; CSFs = colony stimulating factors; LFAs = leukocyte function-associated antigens; TLR = toll-like receptor; IL-3 = interleukin 3; LPA = lymphocyte proliferation assay; p.i. = post infection; TNF-α = tumor necrosis factor α

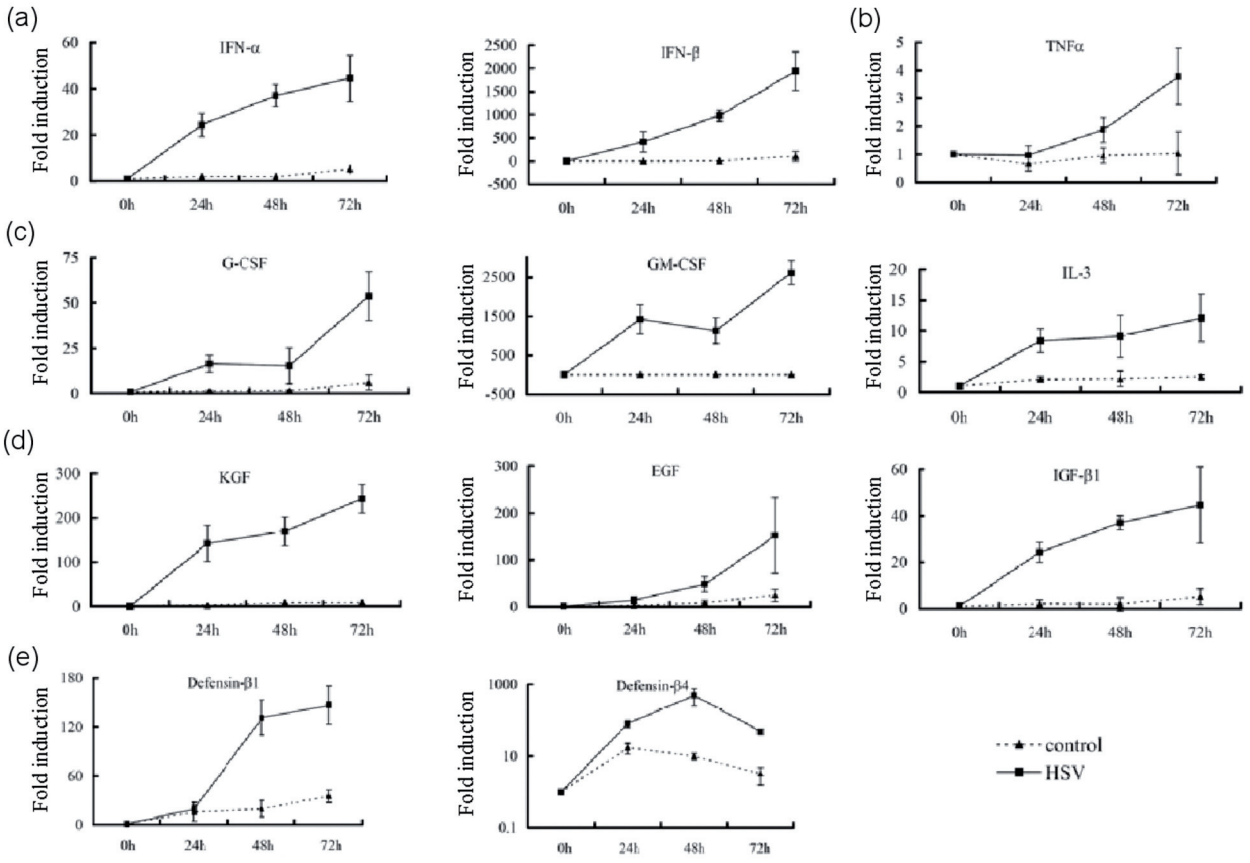

Fig. 1

Upregulation of cytokines in the HSV-2-infected keratinocytes

(a–e) Real-time RT-PCR.

killer cells. These cells and their products provide various means of limiting the virus replication and eliminating virus-infected cells in an effort to check virus spread until the appearance of adaptive immune effectors. Thus far, there is only a limited knowledge about the role of epithelium cells in the HSV-2 infection.

In this study, we used a cell line of human keratinocytes as a model of epithelial cells and addressed the question of the role of epithelial cells in innate and adaptive immune responses to the HSV-2 infection. We examined the effect of HSV-2 infection on the expression of cytokines such as IFNs, TNF-α, CSFs, IL-3, growth factors, defensins, selectins, LFAs and TLRs, as well as that of HSV-2–infected keratinocytes on the proliferation of lymphocytes.

Materials and Methods

Virus. HSV-2 was generously provided by Prof. S. Duan, Institute for Virus Diseases, Chinese Center for Disease Control and Prevention, Chinese Academy of Medical Science. The pooled virus was stored at -80°C with the approximate titer of 1 x 10^7 PFU/ml.

Cells. Vero cells were kindly supplied by Prof. S. Duan and used for the propagation and titration of the HSV-2. HaCaT cells were purchased from ATCC. Both cell lines were maintained in DMEM with additional supplements of 10% FBS, 100 IU/ml penicillin, and 50 μg/ml streptomycin.

Infection of cells. 3 x 10^6 HaCaT cells were seeded with 80% density in a 100 mm dish. After overnight incubation, 0.1 PFU/cell of HSV-2 was added. For control infection, the same batch of virus was inactivated at 56°C for 2 hrs and the same volume as the active virus was added to cells. Infected cultures were vigorously washed three times at 3 hrs post infection (p.i.) and the fresh growth medium was added to the culture at different time points (24, 48, and 72 hrs p.i.).

Real time RT-PCR. Total RNA was isolated from HSV-2-infected HaCaT cells using Trizol reagent (Invitrogen). Briefly, each plate of cells was lysed in 1 ml of Trizol and phase separated by addition of 200 μl of chloroform and centrifuged at 12,000 x *g* for 15 mins (4°C). Aqueous phase was collected and RNA was precipitated with 500 μl of isopropanol and pelleted by centrifugation for 10 mins at 12,000 x *g* (4°C). After washing in 75% ethanol, the isolated RNA was dissolved in DEPC water. Five μg of RNA was

Table 1. RT-PCR primers

Forward primer	Reverse primer	Cytokine
acccacagcctggataacag	actggttgccatcaaactcc	IFN-α
cagcagttccagaaggagga	agccaggaggttctcaacaa	IFN-α
actgaggactcaggcaccac	tgtcgatttcccacaaacaa	TNF-α
ctttgcctttgctggacttc	tctcaattgctgatgcgttc	IL-3
tctcttctacctggcgctgt	cacgaactgaagagcatcca	IGF-β1
agacagggaagagca-gaacg	atgggaggacaggagctttt	G-CSF
cagccactacaagcagcact	aaaggggatgacaagcagaa	GM-CSF
cctgagcgacacacaagaag	ttgggtccctttttactttgc	KGF
cactgaggatgggatgtcct	ctgcctccatgaagttggtt	EGF
ggtggtaactttctcaca	ggtgccttgaattttggta	Defensin-β1
ttaagagtggagccata	atcagccacagcagcttc	Defensin-β4
cagcctcaagatcatcagca	tgtggtcatgagtccttcca	GAPDH
tattgtcccctagcaagg	ctctccaattctaccatg	E-selectin
catctgggaagatttcta	tctcctcagaaaagaca	L-selectin
gatcaatctgctctttcc	gtcctgcttggcaggttg	P-selectin
agcaaatgtgacctgtg	agggtctcattttgcagg	LFA-1
ttgacaacctgtatccca	ccattcatatacagcac	LFA-3
ctgtctttgtgctttctg	gaagaatgagaatggcagc	TLR-2
catgggttcccagtgagact	agccctcaaagtggatgaga	TLR-3
cggaggccattatgctatgt	ttctcccttcctccttttcc	TLR-4
tcagcatctttgcacaggac	ggtggaagcagtaccagagg	TLR-9

subjected to the first strand cDNA synthesis with reverse transcription kit (Fermantas). Quantitative PCR was performed with Biorad MX3000P real-time PCR system. The real-time PCR primers used are shown in Table 1.

Lymphocyte proliferation assay (LPA). 10 ml of normal blood sample was collected from a healthy donor. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation in Ficoll-Paque Plus (GE Healthcare). Briefly, 10 ml of blood was added to 20 ml of Ficoll-Paque and centrifuged at 1000 x g for 30 mins. The cell layer containing PBMCs was collected, washed with PBS for three times and resuspended to the concentration of 1 x 10^6 cells/ml in RPMI 1640 medium containing 10% FBS, 100 IU/ml penicillin, and 50 μg/ml streptomycin. 100 μl of PBMCs was seeded into each well of a 96-well plate. After incubation at 37°C for 30 mins, 100 μl of HaCaT cell medium with or without cells taken at different stages of incubation were added to the PBMCs and co-incubated. After 72 hrs of incubation, cell viability was quantified by WST-1 assay (Cell proliferation reagent WST-1, Roche Applied Science) according to the manufacturer's instruction. Briefly, 20 μl of the reagent WST-1 was added to each well followed by the incubation at 37°C for 4 hrs. A_{460} and A_{480} were measured with a microplate reader (Model 680, BioRad). WST-1 is taken only into the viable cells. Thus, the cell viability was expressed as a fold of the absorbances obtained with control cultures.

Statistical analysis. Data representing the means of values from 3 independent experiments are presented as means + standard deviations. The statistical significance of differences was tested by Student's *t*-test. A difference $P \leq 0.05$ was considered as significant.

Results

Upregulation of cytokines

When we infected keratinocytes with HSV-2, levels of IFN-α and IFN-β significantly increased in a time-dependent manner (Fig. 1a). In contrast, levels of IFN-α, the type II IFN that is mainly secreted by the inflammatory cells, did not change significantly (data not shown). In our study, we also found that HSV-2-infected keratinocytes expressed high level of TNF-α suggesting that keratinocytes played the role as a first barrier against the virus infection (Fig. 1b). Some CSFs (G-CSF, GM-CSF, IL-3) and growth factors (EGF, KGF, and IGF-β1) were also found to be upregulated in the keratinocytes after HSV-2 infection (Fig. 1c, d). Unexpectedly, we did not observe a significant increase for most of the interleukins (data not shown). In addition, we examined the expression of defensin-β1 and β4 in the HSV-2-infected keratinocytes. Both defensins reached a high level at 48 hrs p.i. and persisted to 72 hrs p.i. indicating a self-defensive mechanism of the HSV-2-infected keratinocytes (Fig 1e).

Upregulation of selectins, LFAs, and some TLRs

We found by using regular RT-PCR that all three types of selectins were upregulated, when keratinocytes were infected with HSV-2 (Fig. 2a). Furthermore, the levels of LFA-1 were significantly upregulated at 48 hrs p.i., while levels of LFA-3 were increased at 72 hrs p.i. in the HSV-2-infected keratinocytes (Fig. 2b). We also found that TLR-2, 3, 4, and 9 were upregulated in the keratinocytes upon HSV-2 infection (Fig. 3). Interestingly, expression of most of the other TLRs did not change significantly (data not shown)

Promotion of lymphocyte proliferation

Lymphocyte proliferation assay measures the ability of lymphocytes placed in short-term tissue culture to undergo a clonal proliferation, when stimulated *in vitro* by foreign molecules, antigens, or mitogens. We isolated human PBMCs by density gradient centrifugation and co-cultured them with HSV-2-infected keratinocytes or with the medium containing cytokines secreted by the infected keratinocytes. When the infected medium was applied to the PBMCs, a significant difference of growth was not observed until 72 hrs of co-cultivation (Fig. 4a). If we applied both HSV-2-infected keratinocytes and infected medium to the PBMCs, an accelerated proliferation of PBMCs could be observed at 24 hrs of co-cultivation that became quite obvious at 48 hrs, suggesting that more stimuli existed in the co-cultivation system (Fig. 4b).

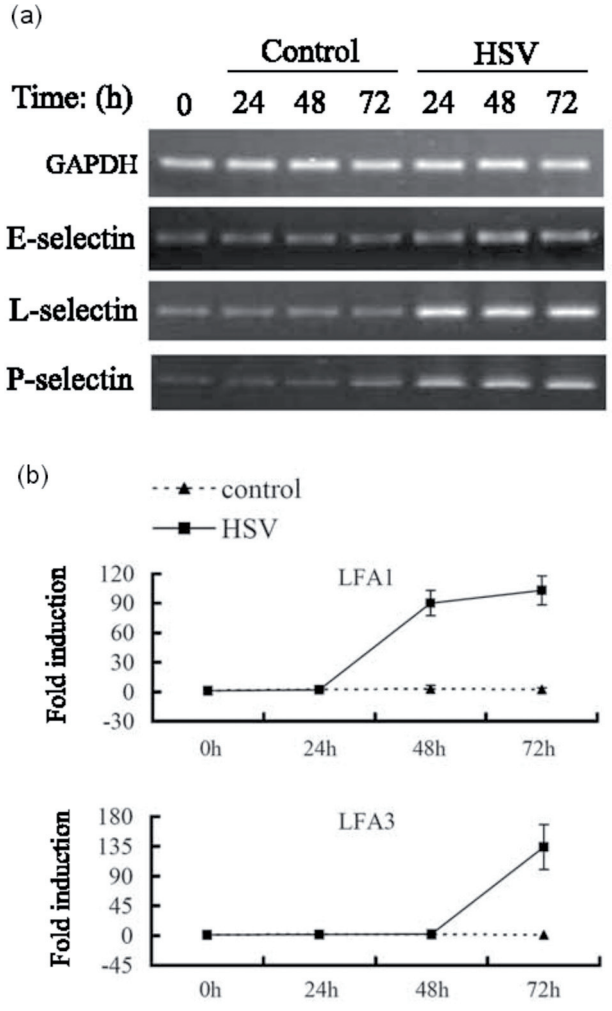

Fig. 2

Upregulation of selectins and LFAs in the HSV-2-infected keratinocytes

(a) Agarose gel electrophoresis of PCR products. (b) Real-time PCR.

Discussion

The induction of cytokines in keratinocytes infected with HSV-2 has not been fully investigated, although this cell type is capable of producing a variety of mediators in response to the viral infection (Sprecher and Becker, 1992; Su *et al*., 1996; Mikloska *et al*., 1998). Keratinocytes are the primary cells involved in clinical lesions caused by HSV-1, 2. Human keratinocytes have been successfully grown in serum-free culture medium (Boyce and Ham, 1983; Wille *et al*., 1984; Schafer *et al*., 1991). So far, most of the studies about HSV-2 have been focused on the immune cells (Duerst and Morrison, 2003). However, studies about the progression of cellular effects of HSV-1, 2 infection and the patterns of viral growth in cultured normal human keratinocytes have rarely been published.

In this study, we intensively examined the expression of cytokines in the HSV-2-infected keratinocytes. The upregulation of type I IFNs, TNF-α and defensins indicated that keratinocytes underwent a series of self-defensive processes upon HSV-2 infection. The IFN-α, β response is the prominent innate antiviral response that protects against viral replication. The importance of the IFN-α, β response in reduction of HSV-1, 2 replication is well established. The resistance or susceptibility of inbred mouse strains to HSV-1, 2 infection correlated with the amount of IFN-α, β produced (Ellermann-Eriksen *et al*., 1986), and administration of anti-IFN-α, β neutralizing antibodies during infection significantly increased the virus replication, disease, and mortality (Hendricks *et al*., 1991; Lausch *et al*., 1991; Su *et al*., 1990; Zawatzky *et al*., 1982). Treatment with IFN-α, β protected mice against infection with HSV-1 (Gangemi *et al*., 1989; Pinto *et al*., 1990; Kunder *et al*., 1993), and administration of IFN-α encoding plasmid DNA significantly increased the resistance of mice to HSV-1, 2 infections in the cornea and vagina, respectively (Noisakran *et al*., 1999; Harle *et al*., 2001). Previous reports showed that TNF-α acted directly against HSV-1, 2 by causing lysis of infected cells (Koff and Fann, 1986) and indirectly by inhibiting the viral replication (Mestan *et al*., 1986; Wong and Goeddel, 1986). TNF-α also synergized with IFN-α and/or IFN-α, β in its antiviral activity, reducing HSV-2 yield by 1000-fold in pretreated epithelial cells (Feduchi *et al*., 1989; Feduchi and Carrasco, 1991; Chen *et al*., 1994). Furthermore, in our current study, the induction of various CSFs, growth factors, selectins, and LFAs suggested that HSV-2-infected keratinocytes recruited, bound to, and promoted the proliferation and survival of inflammatory cells. This event can be easily understood, since inflammatory cells play a pivotal role in both innate immunity by clearing the microbial and infected cells, and adaptive immunity as antigen-presenting cells.

The involvement of keratinocytes in adaptive immunity was further supported by the expression of TLRs after HSV-2 infection. We observed upregulation of TLR-2, 3, 4, and 9 in the HSV-2-infected cells. Human TLRs numbered 1–10 are found on a variety of different cell types and can recognize various components of microorganisms, subsequently initiating signaling pathways important in the generation of cytokines, chemokines, antimicrobial peptides, and upregulation of adhesion and co-stimulatory molecules involved in the innate and acquired immune response (Kaisho and Akira, 2006). Previous studies demonstrated that human keratinocytes expressed TLR 1–6 and 9 (Kawai *et al*., 2002; Baker *et al*., 2003; Mempel *et al*., 2003; Pivarcsi *et al*., 2003; Miller *et al*., 2005; Kollisch *et al*., 2005). In addition, some of these studies demonstrated that TLRs on keratinocytes were functional and responded to their respective ligands

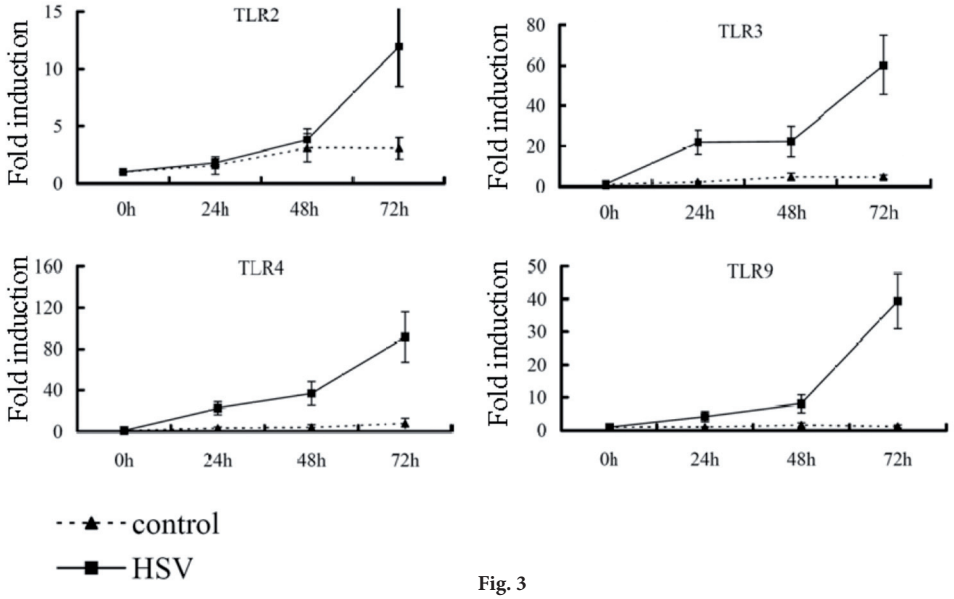

Fig. 3

Upregulation of TLRs in the HSV-2-infected keratinocytes

Real-time PCR.

by production of the cytokines, chemokines, and activation of nuclear factor κB (NF-κB). For example, several studies reported that TLR-2 and TLR-4 were expressed by the human keratinocytes and could be activated by their ligands, bacterial lipopeptides and lipopolysaccharides (Kawai *et al*., 2002; Mempel *et al*., 2003; Pivarcsi *et al*., 2003; Kollisch *et al*., 2005). Furthermore, additional studies demonstrated that TLR-3 and TLR-5 were also expressed by the human keratinocytes and could be activated by their ligands as double-stranded RNA (poly-I:C) and bacterial flagellin (Baker *et al*., 2003; Kollisch *et al*., 2005; Miller *et al*., 2005; Dai *et al*., 2006). Previous studies also demonstrated that human keratinocytes expressed TLR-9 and could respond to the CpG motifs of bacterial DNA (Mempel *et al*., 2003; Miller *et al*., 2005). Our results obtained with HSV-2-infected keratinocytes showed consistency with that obtained with HSV-1-infected cells (Lokensgard *et al*., 2002; Zhang *et al*., 2007), suggesting that expression of TLRs might be a normal response to the HSV-1, 2 infection.

Besides examination of the cytokines expression, a functional assay is needed to prove the association of keratinocytes with the immune cells. The strongest evidence in our study came from the LPA. Although the cytokines-containing medium did not potently promote a growth of PBMCs, co-cultivation of HSV-2-infected keratinocytes with PBMCs significantly enhanced the proliferation of PBMCs as early as 24 hrs of co-cultivation. We speculated that the presence of various CSFs (G-CSF and GM-CSF) and growth factors (IGF-β1), as well as some other cytokines, might contribute to the proliferation of PBMCs. Thus, our work addressed

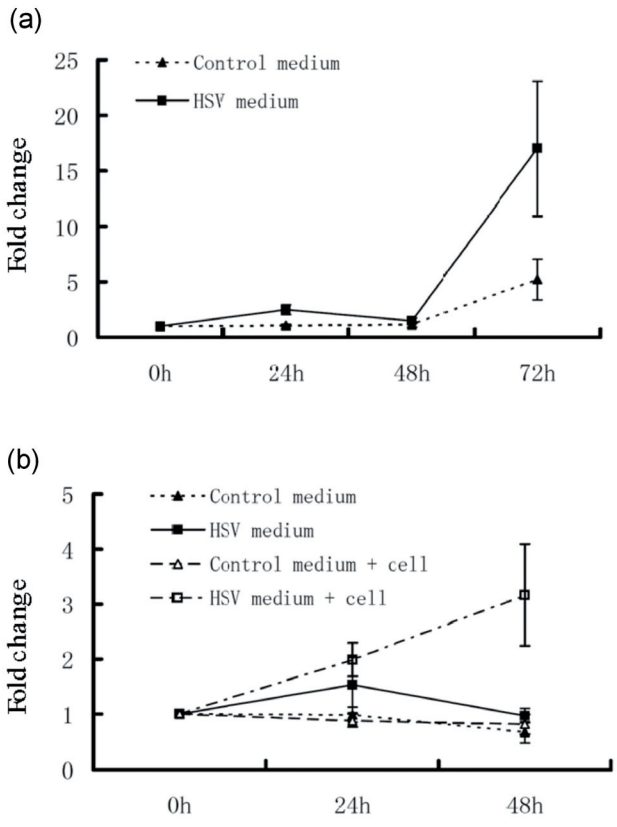

Fig. 4

Promotion of lymphocyte proliferation by the HSV-2-infected keratinocytes in co-culture

Axis y: fold change in viable lymphocyte count after 0–72 hrs of co-cultivation with HSV-2-infected keratinocytes or respective infected medium.

two aspects for the anti-microbial function of keratinocytes: (i) keratinocytes secreted inflammatory cytokines, such as IFN-α, β and TNF-α to resist HSV-1, 2 infections directly and (ii) keratinocytes recruited immune cells to eliminate the primary infection indirectly and to enhance the adaptive immunity to prevent subsequent infections.

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