Inhibition of infectious human herpesvirus 8 production by gamma interferon and alpha interferon in BCBL-1 cells

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Human herpesvirus-8 (HHV-8), also known as Kaposi's sarcoma herpesvirus (KSHV), is a large double-stranded DNA virus belonging to the gammaherpesvirus subfamily (Moore & Chang, 2001). HHV-8 is present in Kaposi’s sarcoma (KS) lesions in all risk groups worldwide as well as in primary effusion lymphoma and appears to be essential for the development of both diseases (Chang et al., 1996; Dictor et al., 1996). As with other herpesviruses, HHV-8 establishes two modes of infection, latent and lytic (Roizman & Philip, 2001). Production of infectious virus is dependent on completion of the lytic cascade, a process that requires expression of numerous viral proteins (Lukac et al., 1998; Sarid et al., 1998; Sun et al., 1998). In contrast, only a few viral genes are expressed during latency, and infectious virions are not produced. Replication of viral DNA nonetheless can occur during latency, with replication of viral episomes and distribution to daughter cells occurring during cellular division (Grundhoff & Ganem, 2003; Hu et al., 2002; Rainbow et al., 1997).

Interferons (IFNs) are cytokines that play a central role in host defence against viral infection. Type I IFNs (IFN-α and IFN-β) are produced and secreted by a wide variety of virally infected cells, whereas Type II IFN (IFN-γ) is produced primarily by natural killer cells, CD4+ T helper (Th1) cells and CD8+ cytotoxic T cells (Biron, 1999; Biron et al., 1999). The two types of IFNs signal through distinct receptors, but there is some overlap in the Jak-STAT signalling molecules involved in transducing responses (Doly et al., 1998; Schindler, 1999, 2002; Schindler & Brutsaert, 1999). While both types of IFNs induce expression of antiviral proteins, divergent responses to the two types of IFN have been observed in patients with KS as well as in HHV-8-infected cells in culture. Patients with KS who have adequate CD4 counts and who lack constitutional symptoms often respond to IFN-α with regression of KS lesions (Fischl et al., 1996; Krown, 1991; Krown et al., 1992; Sawyer et al., 1990), whereas clinical trials testing the efficacy of IFN-γ for the treatment of KS were halted when several patients experienced dramatic worsening of lesions (Aboulafia et al., 1989; Ganser et al., 1986; Krigel et al., 1985). IFN-α prevents HHV-8-infected BCBL-1 cells from entering the lytic phase of virus replication in response to incubation with IFN-α, whereas IFN-γ induces higher levels of IRF-1 than did IFN-α. IFN-γ induced a minor increase in lytic viral gene expression, which was not accompanied by a detectable increase in infectious virus. When lytic replication of HHV-8 was induced using TPA, high levels of infectious virus appeared in the conditioned medium. When IFN-γ was present during TPA stimulation, the production of infectious virus was reduced by at least a 60%, and IFN-α fully blocked TPA-induced production of infectious virus. The greater reduction of viral production that occurred with IFN-α is consistent with the higher levels of the antiviral proteins PKR and 2′,5′-oligoadenylate synthetase (2′,5′-OAS) induced by IFN-α than by IFN-γ. These studies indicate that the augmentation of cellular antiviral defences by IFN-γ was sufficient to prevent production of infectious virus despite IFN-γ-induced entry of some cells into the lytic phase of HHV-8 replication.

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INTRODUCTION

Human herpesvirus 8 (HHV-8), also known as Kaposi’s sarcoma herpesvirus (KSHV), is a large double-stranded DNA virus belonging to the gammaherpesvirus subfamily (Moore & Chang, 2001). HHV-8 is present in Kaposi’s sarcoma (KS) lesions in all risk groups worldwide as well as in primary effusion lymphoma and appears to be essential for the development of both diseases (Chang et al., 1994, 1996; Dictor et al., 1996). As with other herpesviruses, HHV-8 establishes two modes of infection, latent and lytic (Roizman & Philip, 2001). Production of infectious virus is dependent on completion of the lytic cascade, a process that requires expression of numerous viral proteins (Lukac et al., 1998; Sarid et al., 1998; Sun et al., 1998). In contrast, only a few viral genes are expressed during latency, and infectious virions are not produced. Replication of viral DNA nonetheless can occur during latency, with replication of viral episomes and distribution to daughter cells occurring during cellular division (Grundhoff & Ganem, 2003; Hu et al., 2002; Rainbow et al., 1997).
with TPA, thereby preventing production of infectious virus (Chang et al., 2000; Monini et al., 1999; Pozharskaya et al., 2004). This occurs in part because entry into the lytic phase of virus replication sensitizes BCBL-1 cells to the pro-apoptotic effects of IFN-α (Pozharskaya et al., 2004). In contrast, IFN-γ induces a several fold increase in the percentage of cells that express lytic viral proteins (Blackbourn et al., 2000; Chang et al., 2000; Mercader et al., 2000). IFN-γ is often expressed at elevated levels in KS lesions (Fiorelli et al., 1998). No studies have directly examined changes in infectious virus resulting from IFN-γ, but the increase in lytic viral gene expression led to the hypothesis that the expression of IFN-γ in KS lesions stimulates infectious virus production (Blackbourn et al., 2000; Chang et al., 2000). Alternatively, the expression of IFN-γ in KS lesions might not increase viral load and might merely reflect the host response to the viral infection.

Some IFN-induced changes directly affect cellular responses to viral infection, whereas other responses to IFN are of an immunological nature. Proteins induced by IFNs that directly contribute to cellular antiviral responses include the double-stranded RNA activated protein kinase (PKR), 2′,5′-oligoadenylate synthetase (OAS) and Mx (Barber, 2001; Ronni et al., 1995; Samuel, 2001; Williams, 1999; Yaffe et al., 1996). IFNs also induce the transcription factor IRF-1 that regulates expression of mRNAs for immunomodulatory and growth regulatory proteins (Kimura et al., 1994; Kirchhoff et al., 1995; Taniguchi et al., 2001). Both types of IFN can also augment acquired immunity by inducing higher levels of expression of major histocompatibility complex (MHC) class I, a protein that is important in the presentation of viral antigens to cytotoxic T lymphocytes (Kessler et al., 2002). IFN-γ affects the host response by favouring Th1 differentiation over Th2 (Lohoff et al., 1997; Novelli et al., 1997), thereby favouring cell-mediated immune responses.

Despite studies exploring responses to IFN-γ in vitro and in vivo, it is not known whether IFN-γ affects the production of infectious HHV-8. In this report, we demonstrate that both IFN-α and IFN-γ induce expression of multiple IFN-responsive genes in the primary effusion lymphoma cell line, BCBL-1, but the patterns of induction are distinct for the two types of IFN. Neither type of IFN induces a detectable increase in the production of infectious virus, and both types function as antiviral cytokines to decrease the amount of infectious virus that results from TPA-induced entry into the lytic phase.

**METHODS**

**Cell culture.** BCBL-1 cells (HHV-8-positive, EBV-negative PEL cell line) were obtained from NIH AIDS Research and Reference Program, Manassas, VA and were cultured in RPMI 1640 medium (Cellgro) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U penicillin ml⁻¹ and 10 U streptomycin ml⁻¹. A reporter cell line for quantification analysis of infectious HHV-8, T1H6 (Inoue et al., 2003), was maintained in Dulbecco’s modification of Eagle’s medium (Cellgro) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 200 U penicillin ml⁻¹, 10 U streptomycin ml⁻¹ and 50 μg hygromycin B ml⁻¹ (Invitrogen), and split 1:10 every 3–4 days.

**Northern blot analysis.** Total cellular RNA was isolated from BCBL-1 cells using Trizol (GibcoBRL) and was size-fractionated on a 1% agarose formaldehyde gel in the presence of 1 μg ethidium bromide ml⁻¹. RNA was transferred to nitrocellulose and covalently linked by ultraviolet irradiation using a Stratalinker (Stratagene) and by baking in vacuo for 2 h at 80 °C. DNA probes included a 1-2 kb EcoRI fragment from a cDNA of 2′,5′-OAS (Benech et al., 1985), a 1-2 kb cDNA insert containing IRF-1 from pCcriF1 (Whiteside et al., 1994) and a 1-2 kb EcoRI cDNA fragment of GAPDH from clone HHVMC32 (Adams et al., 1992). The MHC I probe was a 6 kb EcoRI fragment of the human genomic HLA-B7 gene. The probe for PKR was a 1043 nt PCR fragment from the cDNA (Meurs et al., 1990). The probes for vIRF-1 (Zimring et al., 1998) and vIL-6 (Yu et al., 1999) consisted of the entire coding regions. All probes were labelled with [³²P]dCTP using an oligolabelling kit (Stratagene) in accordance with the manufacturer's recommendations. Hybridization was performed at 42 °C in 5 x SSC, 1% SDS, 5 x Denhart's, 100 μg denatured salmon sperm DNA ml⁻¹, 50% formamide and 10% dextran sulfate. Nitrocellulose was washed with a final stringency of 0-2 x SSC in 0-1% SDS at 35 °C. Blots were serially probed for the indicated sequences, and the nitrocellulose was stripped using boiling water prior to rehybridization with other probes. Densitometry was used to quantify relative differences in mRNA levels.

**Western blot analysis.** BCBL-1 cells were pelleted by centrifugation and lysed in cell lysis buffer (Cell Signalling Technology) to which protease inhibitor cocktail (BD Pharmingen) was added. Total protein was size fractionated by electrophoresis using 12-5% acrylamide SDS-PAGE. Proteins were transferred from the gel to PVDF membranes. Membranes were blocked in 5% dry non-fat milk and probed with antibodies against vIRF-1 at vIRF-1 and actin (I-19; Santa Cruz Biotechnology) at 1:1000 dilution. After the secondary antibody reaction, the membranes were washed in PBS-Tween (0-02 M Tris, pH 7-6, 0-1 M sodium chloride, 0-05% Tween-20) and visualized using enhanced chemiluminescence reaction (Amershams Pharmacia).

**Assay for extracellular infectious HHV-8 production.** The T1H6 reporter cell line contains the lacZ gene under control of the PAN promoter and responds to infection with HHV-8 in a sensitive and quantitative manner that accurately assesses the amount of infectious HHV-8 present (Inoue et al., 2003; Krug et al., 2004; Pozharskaya et al., 2004). Briefly, 8 x 10⁴ T1H6 cells per well were seeded in 48-well plates in triplicate. The next day BCBL-1 cell medium, filtered through 0-45 μm filter, was added to T1H6 cells to the presence of 8 μg polybrene (Sigma) ml⁻¹. Plates with T1H6 cells were centrifuged at 400 g for 30 min at room temperature and incubated at 37 °C for 1-5 h. Then the medium was changed, and cells were incubated for 2 days at 37 °C. After three freeze-thaw cycles with 50 μl PBS, cell lysisates were harvested, and their β-galactosidase activities were measured by luminescent β-galactosidase assay (Clontech Lab) using LUMStar Galaxy luminometer (BMG LabTechnologies). For the standard curve, dilution series of infectious virus were used as well as serial dilutions of the β-galactosidase.

**Cell viability.** Viable BCBL-1 cells were detected by direct counting with trypan blue exclusion using a haemocytometer. Mean and standard deviations (SD) from replicates were determined.
Flow cytometry. BCBL-1 cells (1 x 10⁶) were washed in staining buffer (Dulbecco’s PBS with 2% fetal bovine serum without Mg²⁺ or Ca²⁺, pH 7.4) and incubated with anti-IFN-γ receptor β-rabbit polyclonal antibody (1:50, C-20; Santa Cruz Biotechnology) or anti-IFN-α/β receptor rabbit polyclonal antibody (1:50, C-18; Santa Cruz Biotechnology) for 30 min at 4°C. After cells were washed twice in staining buffer, secondary goat anti-rabbit antibody conjugated to Alexa Fluor 488 (1:500; Molecular Probes) was applied for 30 min at 4°C. BCBL-1 cells were washed again in staining buffer and fixed in Cytofix/Cytoperm (BD PharMingen) solution for 20 min at 4°C. As a negative control, BCBL-1 cells were also incubated with secondary antibody without primary antibody. For each sample, 10,000 events were acquired on a FACScan flow cytometer (Becton Dickinson).

RESULTS

The functional consequences of incubation with IFN-γ and IFN-α are partly due to changes in expression of IFN-responsive genes. Northern blot analysis demonstrated that incubation with IFN-α induced much higher levels of mRNA levels for PKR and 2’5’-OAS than did incubation with IFN-γ (Fig. 1). The mRNA levels for 2’5’-OAS were at least 20-fold higher in response to IFN-α than in response to IFN-γ, whereas PKR levels were at least fourfold higher in response to IFN-α than in response to IFN-γ. In contrast, IFN-γ induced much higher levels of IRF-1 than did IFN-α, indicating that not all genes were more effectively stimulated by IFN-α than by IFN-γ. There were high basal levels of MHC-I mRNA in untreated (control) cells that increased in response to incubation with either IFN-α or IFN-γ, but the increase was much less than the increase of IRF-1, 2’5’-OAS or PKR. Changes also occurred in the level of expression of some mRNAs that reflect HHV-8 lytic phase. There was a low level of expression of the lytic genes vIRF-1 and vIL-6 under control conditions, and levels increased slightly during 48 h incubation, most likely reflecting spontaneous entry into the lytic phase of virus replication. Incubation with IFN-α reduced the level of expression of both of these lytic-associated mRNAs, with the maximum reduction seen at 18 and 24 h and a return to levels seen in the 48 h control when incubation with IFN-α was continued for 48 h. A transient decrease in vIRF-1 and vIL-6 mRNA expression was also noted in response to incubation with IFN-γ for 18 h, but it did not persist, with levels back to levels seen under control levels by 24 h and several fold higher than control levels when examined after 48 h incubation with IFN-γ. Examination of mRNA levels for GAPDH demonstrated only minor variations in its expression.

Characterization at the protein level using Western blot analysis confirmed that both types of IFN induced PKR expression and that incubation with IFN-α induced higher levels of PKR than IFN-γ (Fig. 2). The expression of vIRF-1 protein was low under control conditions and in cells incubated with IFN-α or IFN-γ, whereas treatment with TPA induced high levels of vIRF-1 expression. The levels of expression of the HHV-8 latent protein, LANA, showed minimal change in response to incubation with IFN-α, IFN-γ or TPA for 24 and/or 48 h. Analysis of actin expression demonstrated minor variations in protein loading, and correction for these variations confirmed that IFN-α induced higher levels of PKR than IFN-γ, and vIRF-1 expression was minimally increased in response to incubation with IFN-γ and strongly induced by TPA.

Flow cytometry demonstrated that the immunoreactivity for both receptors was faint in the majority of unstimulated BCBL-1 cells, and the expression of IFN-γ receptor was lower than the expression of IFN-α receptor (Fig. 3). While the majority of BCBL-1 cells displayed faint

**Fig. 1.** Expression of cellular and HHV-8 mRNA in response to incubation with IFN-α and IFN-γ. BCBL-1 cells were incubated in the absence (Control) or presence of IFN-α (1000 U ml⁻¹) or IFN-γ (100 U ml⁻¹). Cells were harvested at the indicated time points. Total cellular RNA (15 μg) was size fractionated and analysed by Northern blotting using the indicated probes.
immunoreactivity for the IFN-α receptor, a small population of cells was strongly positive, generating a second peak shifted to the right.

We examined the production of infectious virus to determine if the enhanced entry into the lytic phase that resulted from IFN-γ increased the amount of infectious HHV-8 in the medium since multiple steps need to be successfully completed. The presence of infectious virus in conditioned medium from stimulated BCBL-1 cells was assayed using the T1H6 reporter cell line. This cell line contains the promoter of HHV-8 Rta linked to a β-galactosidase reporter gene, and the expression of β-galactosidase is directly proportional to the amount of infectious virus present (Inoue et al., 2003; Krug et al., 2004; Pozharskaya et al., 2004). As expected, incubation with TPA induced production of infectious virus in time dependent manner (Fig. 4a). Infectious virus was first detected in the medium beginning 3 days after TPA stimulation, with higher levels seen on days 4 and 5. In contrast, incubation with either IFN-γ or IFN-α did not induce a detectable increase of infectious HHV-8 over the same time course.

There was a greater than fivefold increase in cell number between days 0 and 3 under control conditions, and cell number then plateaued (Fig. 4b). Incubation with TPA reduced the rate of increase in cell number between days 3 and 5 (Fig. 4b), a time when large amounts of infectious virus appeared in the conditioned medium (Fig. 4a). By day 5, there were 65% fewer cells after TPA treatment compared with the control. When cells were incubated with either IFN-γ or IFN-α in the absence of TPA, the reduction of cell number was less than occurred in response to incubation with TPA. By day 4, when cells were incubated with IFN-α alone or in combination with IFN-γ, cell numbers began to drop dramatically leading to cell numbers that were near those that resulted from incubation with TPA by day 5. Incubation with IFN-γ had less of an impact on the number of viable cells than did incubation with TPA, and infectious virus was exclusively seen in conditioned medium from TPA-stimulated cells and not in medium from cells incubated with either IFN-α or IFN-γ, even when cell number declined at later time points in response to incubation with IFN. This indicates that the suppression of BCBL-1 cell number resulting from TPA was accompanied by release of infectious virus, whereas the reduction that resulted from incubation with either IFN-α or IFN-γ was not.

A lack of a measurable increase in production of infectious virus might occur if the increase was below the level of detection by the assay. Alternatively, the increase in PKR, 2’5’-OAS and other antiviral genes induced by IFN-γ might
have restricted the ability of HHV-8 to complete its life cycle, thereby preventing production of infectious virus. To examine these possibilities, we induced entry of HHV-8 into the lytic phase using TPA and examined the ability of IFN-α and IFN-γ to modulate the production of infectious virus resulting from the TPA. Combined treatment of the cells with IFN-γ and TPA reduced the amount of infectious virus that resulted from incubation with TPA by approximately 60% when examined on days 3, 4 and 5 (Fig. 5a). The reduction was less than the complete disruption of production of infectious virus that resulted when IFN-α was present during incubation with TPA, and the combination of IFN-α and IFN-γ was comparable to incubation with IFN-α alone. We hypothesized that the reduction in TPA-stimulated production of infectious virus that resulted from incubation with IFN-γ or IFN-α was due to an increase in cell death prior to completion of virus replication. When IFN-γ was present during incubation with TPA, the number of viable cells was significantly reduced on days 4 and 5, by 43 and 50%, respectively, compared with TPA treatment alone (Fig. 5b). When IFN-α was present during incubation with TPA, cell death occurred at early time points, leading to a progressive reduction in the number of cells compared...
with the number present at time 0. This early cell death was accompanied by a complete disruption of TPA-induced production of infectious virus.

**DISCUSSION**

In this study, we demonstrated that there was no detectable increase in the production of infectious virus when BCBL-1 cells were incubated with IFN-γ for up to 5 days even though there was a slight increase in viral lytic gene expression in response to incubation with IFN-γ. Furthermore, IFN-γ functioned as an antiviral cytokine and reduced the amount of infectious virus that resulted when HHV-8 was induced into the lytic cascade using TPA. Incubation with either IFN-α or IFN-γ induced a transient decrease in the expression of lytic-associated viral mRNAs, but the suppression induced by IFN-γ was of shorter duration and was followed by an increase that yielded several fold higher levels than in the absence of IFN-γ. The increase in lytic gene expression that resulted from incubation with IFN-γ did not lead to much of an increase in vIRF-1 protein expression by Western blot analysis, but vIRF-1 is only transiently expressed during lytic replication (Pozharskaya et al., 2004), and the percentage of cells that enter the lytic cascade in response to IFN-γ is low (Chang et al., 2000). The magnitude of increased lytic gene expression induced by IFN-γ was dramatically less than by TPA, consistent with previous reports (Blackbourn et al., 2000; Chang et al., 2000; Mercader et al., 2000). Both IFN-α and IFN-γ induced expression of several known antiviral genes, including PKR and 2′,5′-OAS. The magnitude of induction was considerably greater in response to IFN-α than in response to IFN-γ, and IFN-α blocked TPA-induced production of infectious virus more effectively than IFN-γ. IFN-γ induced IRF-1 more effectively than did IFN-α, indicating that the two types of IFN preferentially induced distinct transcripts. Thus, the level of expression of PKR and 2′,5′-OAS were more closely linked to the antiviral effects of the IFNs than the expression of IRF-1. The greater induction of IRF-1 by IFN-γ than by IFN-α indicates that the differences in response to the two types of IFN were not merely due to the lower levels of expression of the IFN-γ receptor compared with the IFN-α receptor.

Incubation with TPA had a more profound impact on the number of viable BCBL-1 cells than incubation with either IFN-α or IFN-γ. Entry into the lytic phase is accompanied by exit from the cell cycle as cellular resources are shifted from cellular replication to virus replication (Pozharskaya et al., 2004; Wang et al., 2003; Wu et al., 2002). Considerable apoptosis also occurs in response to TPA-induced lytic replication of HHV-8 in BCBL-1 cells, but this does not prevent release of infectious virus (Pozharskaya et al., 2004). Multiple viral proteins are expressed during lytic replication that block apoptosis and responses to IFNs (Gao et al., 1997; Seo et al., 2002; Zmirling et al., 1998), but they are not expressed at sufficient levels or for sufficient duration to prevent many changes induced by IFN-α and IFN-γ (D’Agostino et al., 1999; Pozharskaya et al., 2004). We demonstrated that enhanced cell death and disruption of the production of infectious HHV-8 accompanied the enhanced expression of antiviral proteins that resulted from incubation with IFN-α and IFN-γ. The host response to viral infection is generally greater during lytic replication when multiple viral genes are present that can trigger an innate antiviral response. BCBL-1 cells supporting lytic replication of HHV-8 are more sensitive to IFN-α-induced apoptosis than latently infected cells (D’Agostino et al., 1999; Pozharskaya et al., 2004). The heightened expression of PKR and 2′,5′-OAS that resulted from incubation with IFN-α and IFN-γ probably contributed to the disruption of viral production by decreasing expression of viral proteins and by enhancing apoptosis. Both 2′,5′-OAS and PKR contain double-stranded binding domains that regulate enzymic activation (Clemens & Elia, 1997; Justesen et al., 2000; Tan & Katze, 1999). Activation generally occurs when sufficient double-stranded RNA is expressed during viral infection to activate these enzymes. Entry into the lytic cascade of HHV-8 occurs in response to expression of Rta, the product of ORF 50 (Lukac et al., 1998, 1999; Sun et al., 1998). Expression of Rta is accompanied by co-expression of complementary transcripts (Lukac et al., 1999), indicating the presence of viral double-stranded RNA that might activate PKR and 2′,5′-OAS. Activated 2′,5′-OAS catalyzes the formation of oligoadenylates in 2′,5′ linkage that activate a latent ribonuclease, RNase L (Castelli et al., 1998; Hartmann et al., 1998; Marie et al., 1999; Sarkar & Sen, 1998). PKR catalyzes the phosphorylation of eIF2α, a translation factor whose phosphorylation blocks the initiation of protein synthesis (Clemens & Elia, 1997; Samuel et al., 1997). Together, activated 2′,5′-OAS and PKR decrease translation of both viral and cellular proteins and contribute to apoptosis of virally infected cells (Balachandran et al., 2000; Barber, 2001; Castelli et al., 1998; Der et al., 1997).

Incubation of non-TPA stimulated BCBL-1 cells with IFN-α and IFN-γ had very little effect on cell number until late time points when there was also an increase in expression of lytic-associated mRNAs. This suggests that IFN-induced cellular antiviral proteins were not fully activated in latently infected cells, and spontaneous entry into the lytic phase of virus replication during prolonged incubation with either IFN-α or IFN-γ activated innate defences, thereby triggering cell death. The studies reported here were done in BCBL-1 cells, a primary effusion lymphoma cell line that is persistently infected with HHV-8. IFN-γ also functions as an antiviral cytokine in HHV-8-infected transformed human microvascular endothelial cells to suppress the expression of HHV-8 lytic transcripts without affecting the percentage of cells that support latent replication (Milligan et al., 2004).

These studies indicate that both IFN-γ and IFN-α induced the expression of cellular antiviral genes in HHV-8-infected BCBL-1, enhanced cell death and decreased the production of infectious virus. The ability of IFN-α and IFN-γ to block...
production of infectious HHV-8 was not dependent on changes in the immune system since no immune effector changes were present during these studies. Thus, IFN-α more effectively disrupted production of infectious HHV-8 than IFN-γ by modifying the function of innate cellular antiviral pathways.

ACKNOWLEDGEMENTS

We would like to thank Dr Naoki Inoue for providing us with the THI6 reporter cell line. This study was supported by US Public Health Service Grant RO-1 CA 79402.

REFERENCES


