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Inhibition of ERK and proliferation in NK cell lines by soluble HLA-E released from Japanese encephalitis virus infected cells

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ABSTRACT

Productive infection of human endothelial cells with Japanese encephalitis virus (JEV), a single stranded RNA virus induces shedding of sHLA-E. We show here that sHLA-E that is released upon infection with this flavivirus can inhibit IL-2 and PMA mediated ERK 1/2 phosphorylation in two NK cell lines, Nishi and NKL. Virus infected or IFN-γ treated cell culture supernatants containing sHLA-E were found to partially inhibit IL-2 mediated induction of CD25 molecules on NKL cells. It was also found that sHLA-E could inhibit IL-2 induced [^{3}H]-thymidine incorporation suggesting that, similar to cell surface expressed HLA-E, sHLA-E could also inhibit NK cell responses. Hence JEV-induced shedding of sHLA-E needs further investigation to better understand immune responses in JEV infections since it may have a role in viral evasion of NK cell responses.

1. Introduction

JEV is a positive single stranded RNA virus that belongs to the *Flavivirus* genus of the family *Flaviviridae* [1]. This neurotropic virus [2–4] as well as its ability to cause encephalitis [5,6] has been well studied. We have shown earlier that immortalized human brain microvascular endothelial cells (HBMEC) and the bladder epithelial cell line, ECV304 (ECV) can be productively infected with JEV which results in increased expression of HLA-A, and -B antigens as well as HLA-E, a nonclassical MHC antigen. More importantly, JEV infection or IFN-γ treatment led to the release of soluble HLA-E (sHLA-E) from HBMEC and ECV cells that could be partially blocked by matrix metalloproteinase (MMP) inhibition [7].

Cell surface HLA-E has been shown to inhibit NK cells by binding to CD94/NKG2A receptors [8,9]. However, the signaling pathways involved in this inhibition are still unclear. ERK 1/2 phosphorylation is induced upon treatment of NK cells with IL-2 leading to upregulation of CD25, a component of the high affinity IL-2 receptor and NK activation. This IL-2 mediated upregulation of CD25 could be inhibited by ERK inhibition [10] suggesting that ERK 1/2 phosphorylation may play an important role in NK activation. Cell surface expression of HLA-E on target cells was also found to inhibit ERK 1/2 phosphorylation and recruitment of cytotoxic granules in NK cells at the immunological synapse [11].

While soluble classical HLA-Class I antigens (sHLA-I) such as sHLA-A and -B have been well studied for its ability to inhibit NK cell cytotoxicity [12,13], the functional significance of soluble non-classical HLA-Class I such as sHLA-E molecules is still poorly understood especially in the context of viral evasion of immune responses. We show here that sHLA-E released from JEV-infected cells has the ability to block IL-2 mediated phosphorylation of ERK1/2 in the human NK cell lines, NKL and Nishi. We further found that treatment of the NKL cell line with IL-2 in the presence of sHLA-E containing cell culture supernatants leads to partial inhibition of [^{3}H]-thymidine incorporation and CD25 cell surface expression.

2. Materials and methods

2.1. Media, antibodies, and cell lines

Media and reagents were purchased from Sigma–Aldrich, India. FCS was obtained from Gibco, USA and Nu-Serum from BD

Abbreviations: JEV, Japanese encephalitis virus; sHLA-E, soluble HLA-E; HBMEC, human brain microvascular endothelial cells; ECV, ECV 304 cell line.

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Biosciences, USA. Anti HLA-E antibodies MEM-E/07 and MEM-E/02 were obtained from Abcam, USA. Goat anti human β-tubulin for Western blotting and donkey anti goat IgG-HRP conjugate was obtained from Santa Cruz biotech, USA. Goat anti mouse IgG-HRP and goat anti rabbit IgG-HRP was from Bangalore Genei, India. Leupeptin and PMSF were obtained from Sigma–Aldrich, India. Human IFN-γ was from Peprotech, Israel. [γ-^{32}P] ATP and tritiated thymidine ([^{3}H]-T) was obtained from BRIT, India. Tissue culture and other plasticwares were obtained from TPP, Switzerland and Corning, Germany. Nitrocellulose membranes used for sterile filtration were obtained from Sartorius, Germany. Polyvinylidene fluoride (PVDF) membranes for Western blots were obtained from Millipore, India.

Mouse anti-human CD25 antibody was obtained from Biolegend, USA. Mouse anti-human MICA, MICB, ULBP-1, ULBP-2, ULBP-3, ULBP-4 and NKG2A monoclonal antibodies were a kind gift from Amgen, USA. p-MAPK Western blot kit containing rabbit anti-human phospho-p38, phospho-ERK1/2, phospho-JNK1/2 and MAPK Western blot kit containing rabbit anti-human p38, ERK1/2, JNK1/2 and anti-rabbit HRP conjugate was obtained from Cell Signaling Technologies, USA.

ECV304 (ECV), a bladder carcinoma-derived epithelial cell line [14,15] has been used as an endothelial-like cell line along with human brain microvascular endothelial cells (HBMEC) in several *in vitro* model studies as an endothelial component of the human blood–brain barrier [16,17]. Both cell lines could be productively infected with JEV leading to the production of IFN-β and TNF-α as well as release of active virus and sHLA-E [7]. Hence the ECV cell line was selected for harvesting sHLA-E containing culture supernatants based on its ability to be cultured easily through many cell passages. The cell line was a gift from Dr. M. Jaggi, Dabar Research Center, Ghaziabad, India and was grown in DMEM containing 10% FCS. NK cell lines, NKL and Nishi were grown in IMDM medium containing 10% FCS, 3% human AB serum, non-essential amino acids, Na-pyruvate, β-mercaptoethanol and 200 U/ml IL-2. For the purpose of IL-2 starvation, cells were cultured in the same medium without IL-2.

2.2. Virus infection of cells and collection of cell-free culture supernatants

Virus titration, infection and propagation of JEV strain P20778 was done as published [7]. This strain was first isolated in Vellore, South India in 1958 and causes death of mice in 7–9 days when injected intracerebrally. For the purpose of collecting sHLA-E containing culture supernatants, ECV cells (7.5×10^5 cells/ml) were infected with JEV at MOI 10 in 6 well plates and the infected culture supernatants were collected after 24 h of infection. Culture supernatants were also collected independently from ECV cultures that were treated with IFN-γ (500 U/ml) for 24 h. They were spun at $10{,}000 \times g$ for 15 min and stored at −70 °C. Immediately before use, all supernatants were clarified by spinning at $8000 \times g$ for 15 min and subjected to UV irradiation to inactivate virus in case of infected culture supernatants. The presence of sHLA-E in these supernatants was confirmed by Western blotting with MEM-E/02 anti-HLA-E antibody.

2.3. Flow cytometry.

Viable cells (0.3×10^6) were stained and analyzed [18] using a FACSCalibur Cytometer (Becton Dickinson, USA). Data for 10,000 events was analyzed using WinMDI software (Version 2.9). NKL cells were first blocked with human serum and stained with mouse anti-human CD25 or anti-human NKG2A monoclonal antibody and appropriate FITC labeled secondary antibody. Mean fluorescence intensity (MFI) values were obtained using WinMDI (Version 2.9) software and plotted in the bar diagram as mean ± SEM of three independent experiments. Data was analyzed by Unpaired *T* test (Mann–Whitney) and P values were calculated using Unpaired *T* test using GraphPad Prism (Version 5.00) software.

2.4. Detection of ERK 1/2 phosphorylation by Western blot

NKL and Nishi cells were starved of IL-2 for 4 h and 5×10^5 cells were added into wells containing 1 ml of either DMEM containing 2.5% FCS or sHLA-E containing media supernatants (1×) that were harvested as described from JEV infected and IFN-γ treated ECV cell cultures. Cells were either left untreated or treated with 100 U of IL-2 for 10 min and lysed in 90 μl lysis buffer (50 mM Tris–HCl, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM aprotinin, 1 mM leupeptin, 1 mM NaF) at 4 °C for 30 min. The suspension was spun at $12{,}000 \times g$ for 15 min and 30 μg protein aliquots were electrophoresed on 12% SDS-PAGE gels and blotted onto Millipore Immobilon-P PVDF membranes. Blots were blocked for 2 h with 5% skimmed milk in TBS containing 0.2% Tween-20 and probed with phospho-ERK1/2 antibodies and corresponding secondary antibodies according to manufacturer's instructions. These blots were later stripped and reprobed with antibodies recognizing total ERK1/2. The blot was developed using Immobilon Western chemiluminescent HRP substrate (Millipore, India) and visualized using a Luminescent Image Analyzer (LAS 3000, Fuji Film, Japan).

2.5. Immunodepletion and elution of sHLA-E from culture media supernatant

2 μg of MEM-E/07, a conformation specific anti-HLA-E monoclonal antibody [19] was bound to protein A sepharose beads (binding capacity: 16 mg/ml) for 30 min on ice in PBS containing 0.1 M Tris pH 8. JEV infected media supernatant (1 ml) was then added to this mixture and incubated in the cold overnight on an end to end rocker. Isotype antibody was bound to beads as controls. The beads were spun down at 1000 rpm for 1 min and the supernatant was recovered for further analysis. The pellet containing the beads was washed twice with 1 ml PBS, pH 7.2. The bound sHLA-E was eluted with 30 μl glycine buffer pH 2.0 and the pH of the eluate was neutralized immediately with 1 M Tris pH 8. It was diluted to 1 ml with 2.5% FCS containing DMEM before addition to cells.

To ascertain the depletion of sHLA-E from culture supernatants by MEM/07 antibody, Western blotting was performed with anti-HLA-E, MEM-E/02 antibody on culture supernatants that were concentrated 10×. MEM/02 recognizes only the denatured HLA-E heavy chain [20]. As the isotype control antibody and anti-HLA-E antibody used for immunodepletion (MEM/07) were raised in mice, the presence of residual antibodies in the supernatant could show reactivity with secondary antibody (Anti-mouse IgG-HRP conjugate) in Western blots. Hence only a region of the blot between 45 kDa and 30 kDa confirmed to contain sHLA-E (37 kDa) by the use of markers was developed with the anti-HLA-E, MEM/02 monoclonal antibody in order to eliminate the interference due to residual antibodies.

2.6. [^{3}H]-thymidine incorporation assay in NKL cell line

Similar to the proliferation assays reported earlier [21], NKL cells were incubated without IL-2 for 12 h. 5×10^4 starved cells were added in each of 96 wells containing 250 μl of DMEM medium with 2.5% FCS or sHLA-E containing media supernatants as stated earlier. 100 U/ml of IL-2 was then added to each well. After 30 h of incubation at 37 °C, serum percentage in medium was raised to 10% and

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