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Chemokine profiling of Japanese encephalitis virus-infected mouse neuroblastoma cells by microarray and real-time RT-PCR: Implication in neuropathogenesis

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Abstract

Japanese encephalitis (JE) is one of the leading causes of acute encephalopathy affecting children and adolescents in the tropics. JE virus (JEV) infection causes prominent neurological sequelae in approximately one-third of the survivors. In humans, the inflammatory response of CNS consequent to JEV induced viral encephalitis is mediated through chemokines released by various cells of CNS. In the present study, the chemokine profiles of mouse neuroblastoma cells (N2A) following JEV infection was analyzed by cDNA microarray followed by real-time RT-PCR. Eighty mRNA transcripts belonging to various functional classes exhibited significant alterations in gene expression. There was considerable induction of genes involved in apoptosis and anti-viral response. Modified levels of several transcripts involved in proinflammatory and anti-inflammatory processes exemplified the balance between opposing forces during JEV pathogenesis. Other genes displaying altered transcription included those associated with host translation, cellular metabolism, cell cycle, signal transduction, transcriptional regulation, protein trafficking, neurotransmitters, neuron maturation, protein modulators, ER stress and cytoskeletal proteins. The infection of neurons results in the synthesis of proinflammatory chemokines, which are early important mediators of leukocyte recruitment to sites of viral infection. Our results clearly suggest the implication of chemokines in neuropathogenesis of JEV infection leading to neurological sequelae. Pro- and anti-inflammatory agents targeted against chemokines such as CXCL10 may provide possible therapeutic modalities that can mitigate the morbidity associated with JEV infection of the CNS.

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this study, we demonstrated that virus infection of neuronal cells results in the strong induction of proinflammatory chemokines along with robust anti-viral response. The replicating virus within the infected host neurons alters normal gene expression profiles, which can be of significance in JEV pathogenesis at the molecular level.

A neurovirulent JEV strain, JE S982, was employed throughout this study. The propagation of virus was carried out in C6/36 cells utilizing RPMI-1640 medium containing 10% fetal bovine serum. To determine virus titers, culture media were harvested for the infected host neurons alters normal gene expression profiles, which can be of significance in JEV pathogenesis at the molecular level.

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result for an individual sample was expressed as the mean expression level of a specific gene. The relative expression between each infected sample and the uninfected control was then calculated and represented as fold upregulation/downregulation. Fold change was determined. Relative changes of gene expression were calculated by the following formula (Livak and Schmittgen, 2001), and the data are represented as fold upregulation/downregulation.fold change = $2^{-\Delta \Delta C_t}$, where $\Delta \Delta C_t = (C_t$ of gene of interest, treated – $C_t$ of gene of interest, control) – ($C_t$ of gene of interest, control – $C_t$ of HK gene, control), $C_t$ is the threshold cycle number and HK is the housekeeping gene.

The time point for microarray and PCR array study consisted of only 36 hpi of mock-infected (control) and JE virus-infected samples. For microarray and PCR array, two replicates each (control and infected) were used and the experiment is repeated once. The data of Tables 1–4 were analyzed by $t$-test and $p$-value of $\leq 0.05$.

### Table 2

| Accession no. | Gene name     | Gene description                                                                 | Fold change | $p$-Value |
|---------------|---------------|----------------------------------------------------------------------------------|-------------|-----------|
| NM_133743     | Lypld3        | GPI-anchored metastasis-associated protein homolog                               | –0.60       | 0.123     |
| NM_010317     | Gng4          | Guanine nucleotide binding protein G protein, gamma 4 subunit                    | –0.60       | 0.107     |
| NM_025681     | Lix1          | Limb expression 1 homolog                                                        | –0.60       | 0.099     |
| XM_01005167   | Sympo2        |                                                                                  | –0.60       | 0.098     |
| NM_010826     | Mtv1          | MRV integration site 1 isoform a                                                  | –0.60       | 0.093     |
| NM_027820     | 8430429K09Rik  | Hypothetical protein LOC7152                                                     | –0.60       | 0.112     |
| AK0080781     |               |                                                                                  | –0.60       | 0.105     |
| NM_144556     | Lgi4          | Leucine-rich repeat LGI family, member 4                                          | –0.60       | 0.127     |
|               |               |                                                                                  | –0.60       | 0.153     |
| NM_010585     | Itprl         | Inositol 1,4,5-triphosphate receptor 1                                            | –0.60       | 0.096     |
| XM_622260     | A1427138      |                                                                                  | –0.60       | 0.149     |
| NM_028133     | Egln3         | EGL nine homolog 3                                                               | –0.60       | 0.130     |
| NM_028940     | 4933427J24Rik  | Hypothetical protein LOC74438                                                    | –0.61       | 0.095     |
| NM_053199     | Pcdha9        | Protocadherin alpha 9                                                            | –0.61       | 0.143     |
| NM_138661     |               |                                                                                  | –0.61       | 0.036     |
| NM_011377     | St6galnac4    | ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 4 | –0.61       | 0.112     |
|               |               |                                                                                  | –0.61       | 0.102     |
| NM_011867     | Sld2a4        | Pendrin                                                                           | –0.61       | 0.096     |
| XM_125817     | 1300007009Rik |                                                                                  | –0.61       | 0.102     |
| NM_133683     | Tmem19        | Transmembrane protein 19                                                         | –0.62       | 0.096     |
| XM_204152     | Fmrpd1        |                                                                                  | –0.62       | 0.099     |
| NM_001029889  | Gm288         | Hypothetical protein LOC207806                                                   | –0.62       | 0.108     |
| NM_002786     | Pcdhh1        | Protocadherin beta 21                                                            | –0.62       | 0.931     |
| NM_007666     | Myt11         | Myelin transcription factor 1-like                                               | –0.63       | 0.088     |
| NM_000824     | Cbfa23h       | Core-binding factor, runt domain, alpha subunit 2, translocated to, 3 homolog    | –0.63       | 0.099     |
|               |               |                                                                                  | –0.63       | 0.095     |
|               |               |                                                                                  | –0.63       | 0.156     |
| NM_175383     | B3gnt6        | Beta-1,3-N-acetylgalactosaminyltransferase bGnT-6                                | –0.63       | 0.091     |
| NM_009211     | Smacrc1       | SWISS/NF related, matrix associated, actin dependent regulator of chromatin, subfamily c, member 1 | –0.63       | 0.092     |
| XM_485743     | D630002J15Rik |                                                                                  | –0.63       | 0.105     |
| NM_178444     | Egfl7         | Vascular endothelial statin isoform 1 precursor                                 | –0.63       | 0.095     |
| NM_003137     | Cstad         | CSA-conditional, T cell activation-dependent protein                             | –0.63       | 0.095     |
| NM_207298     | Ceeam1        | Cerebral endothelial cell adhesion molecule 1                                    | –0.63       | 0.109     |
| NM_198119     | Lorc24        | Leucine-rich repeat containing 24                                               | –0.64       | 0.087     |
|               |               |                                                                                  | –0.64       | 0.142     |
| NM_027865     | Tmem25        | Transmembrane protein 25                                                         | –0.64       | 0.107     |
| NM_025823     | Peyxl1        | Prelysin cytoxide 1                                                              | –0.64       | 0.089     |
| NM_010214     | Gpmln1        | G protein-regulated inducer of neurite outgrowth 1                               | –0.64       | 0.089     |
| XM_485800     | Akl1          |                                                                                  | –0.64       | 0.105     |
| XM_048146     | Col22a1       |                                                                                  | –0.64       | 0.097     |
| NM_001039079  | Prkcz         | Protein kinase C, zeta isoform b                                                 | –0.64       | 0.040     |
| NM_0025285    | Stmn2         | Stathmin-like 2                                                                  | –0.65       | 0.092     |
| NM_002043     | Nope          | Neighbor of Punc e11 protein                                                     | –0.65       | 0.106     |
| XM_283153     | Polr3g        |                                                                                  | –0.65       | 0.101     |
|               |               |                                                                                  | –0.65       | 0.131     |
| XM_130797     | Tnk           |                                                                                  | –0.65       | 0.103     |
| NM_013884     | Cspg5         | Chondroitin sulfate proteoglycan 5                                               | –0.65       | 0.104     |
| NM_172861     | Slc7a14       | Solute carrier family 7, member 13                                              | –0.65       | 0.097     |

The microarray data pertains to 36 h post-infection.
Table 3
Significant pathways of the upregulated genes at 36 h post-infection with JE virus.

| Pathways                                      | No. of genes | p-Value  |
|-----------------------------------------------|--------------|----------|
| Protein export                                | 2            | 5.46E-06 |
| Toll-like receptor signaling pathway          | 7            | 1.88E-05 |
| Circadian rhythm                              | 5            | 3.22E-05 |
| SNARE interactions in vesicular transport     | 5            | 0.00157  |
| MAPK signaling pathway                        | 14           | 0.0005   |
| Alanine and aspartate metabolism              | 3            | 0.002    |
| Aminocyl-tRNA synthetases                     | 3            | 0.002    |
| Antigen processing and presentation           | 4            | 0.004    |
| One carbon pool by folate                     | 2            | 0.005    |
| Jak-STAT signaling pathway                    | 5            | 0.008    |
| Methane metabolism                            | 1            | 0.023    |
| Novobiocin biosynthesis                       | 1            | 0.035    |
| N-glycan biosynthesis                         | 2            | 0.048    |
| Parkinson’s disease                           | 2            | 0.053    |
| Glycosylphosphatidylinositol (GPI)-anchor biosynthesis | 1           | 0.058    |

Table 4
Significant pathways of the downregulated genes at 36 h post-infection with JE virus.

| Pathway                                      | No. of genes | p-Value  |
|----------------------------------------------|--------------|----------|
| Biosynthesis of steroids                      | 5            | 0.0006   |
| Androgen and estrogen metabolism              | 1            | 0.013    |
| ECM-receptor interaction                      | 4            | 0.015    |
| Glycan structures – biosynthesis              | 2            | 0.019    |
| Diterpenoid biosynthesis                      | 1            | 0.021    |
| Focal adhesion                                | 6            | 0.024    |
| Alkaloid biosynthesis                         | 1            | 0.031    |

was considered significant. Data of time course study on chemokine synthesis was analyzed by one-way ANOVA followed Dunnet’s test for comparison between control and treatment groups. The level of significance was set at \( p \leq 0.05 \). All experiments were repeated at least thrice.

Viral replication in N2A cells infected with JE virus JaOAr S982 for 96 h duration was determined by plaque assay of the virus released in the cell culture medium (Fig. 1). The results showed that new extracellular viral progeny reached maximum at 72 h. After 72 h, a degree of cell death becomes apparent and the experiment was terminated at 96 h (Fig. 2). Transcript analysis on 36 h post-infection was thus selected to track early changes in gene expression in JE virus-infected cells. A total of 497 genes were found to be significantly upregulated after JEV infection and 223 genes were downregulated (Tables 1 and 2). Some of the pathways which show upregulated gene expression are toll-like receptor signaling, MAPK, JAK-STAT, SNARE reactions in vesicular transport, antigen processing and presentation (Table 3). Some of the significantly downregulated pathways are biosynthesis of steroids, androgen and estrogen metabolism, ECM interaction, focal adhesion, etc. (Table 4). Other differentially regulated genes that were significantly changed include those associated with cell signaling, lipid metabolism, cell cycle and vesicular transport.

In order to verify the reproducibility of the microarray results, the real-time PCR plate assay of mouse inflammatory cytokines and cytokine receptors was carried out. The results showed a set of six genes viz. Itgb, CD40, Tollip, Xcr1, CXCL1 and CXCL10 well corroborated with microarray data. GAPDH, β actin, Gusb, Hsp90 ab1 and Hprt1 were used as housekeeping genes. Out of those six genes, five genes showed congruent and significant differences in expression, while one displayed opposite expression pattern (Table 5). Upregulated genes include CXCL1 (chemokine, C–X–C motif, ligand 1), CXCL10 (chemokine, C–X–C motif, ligand 10), Itgb (integrin beta), and CD 40 (CD40 ligand). downregulated genes include Xcr1 (chemokine, C motif receptor 1). However, the qRT-PCR showed greater dynamism in fold changes than the microarray results because of the greater sensitivity of PCR compared with fluorescent detection in microarray experiment.

We found marked increase in expression of IP-10/CXCL10, which is known to play important role in the host defense against viral infection (Chen et al., 2006). CXCL10 is found to be a crucial molecule governing the protective response against various diseases like dengue, Trypanosoma cruzi, Klebsiella pneumoniae, rabies virus and corona virus infection of the CNS by enhancing innate immune responses (Hsieh et al., 2006; Hardison et al., 2006). IP-10 is an essential component in host defense by coordinating the trafficking of Th1 T lymphocytes into the CNS in response to viral infection. We demonstrated the strong induction of CXCL10 by infected neurons itself. The qRT-PCR analysis showed sustained induction of CXCL10 in JEV-infected mouse neuroblastoma cells. The results indicate that neurons may be a source of chemokine.

![Fig. 1. Kinetics of JEV growth in Mouse N2A cells. Monolayers of N2A cells were infected with the JE S982 strain of JEV at an MOI of ~5 and incubated at 37 °C. At various time intervals, samples were removed and virus titre was assayed. Values are mean ± SE of three replicates each.](image-url)
Fig. 2. Morphological pattern of neuroblastoma cells infected with JEV. Monolayers of the mouse neuroblastoma cells were adsorbed with either live JE virus (multiplicity of infection (MOI) ∼5) or mock-infected for 1 h, at 37 °C. (a) N2A cells mock-infected at 36 hpi; (b) N2A cells infected with JEV at 36 hpi; (c) N2A cells infected with JEV at 72 hpi; (d) N2A cells infected with JEV at 96 hpi.

synthesis for primary anti-viral response in JE infection (Fig. 3B). There is gradual decrease in CXCL10 expression with increased viral load in cells, suggesting its anti-viral role in JE infection. No significant fold change was detected for other chemoattractant like CXCL11 and CXCL9 (Fig. 3A–C).

Array data also showed Ccl-5 upregulation in JE infection indicating virus modulation of the host machinery in the initial stages of infection. Ccl5–Ccr5 interaction provides anti-apoptotic signals for macrophage survival during viral infection. Ccl5-responsive genes comprise a significant number of enzymes, transcription factors, and miscellaneous molecules involved in neuronal survival and differentiation, including neurite outgrowth and synaptogenesis (Valerio et al., 2004). There was significant upregulation of a number of ubiquitin–proteasome system related genes such as Usp18 during JE virus replication but it was unclear if this response was anti-viral or if the JE virus utilized components of the ubiquitin–proteasome system for its replication. We found significant upregulation in anti-viral response genes like IFN induced Isg15, Viperin, Mx 2, anti-viral genes like oligoadenylate synthetase, Parp14, Irgm, transcription factor-like STAT and ER stress regulated genes like Herpud1, ATF6 and XBP1. The marked elevation in expression of these genes at 36 h post-infection suggests a highly notable anti-viral response to JEV pathogenesis.

Downregulated genes were mainly from focal adhesion and CNS signature, as detailed and vast study is required to elaborate the importance and involvement of these significantly downregulated genes in JEV pathogenesis. Genes like Lypd3, Lgi4 are involved in protein binding, neuron maturation, myelination, etc. The real-time RT-PCR validated genes elucidate the varied transcriptional responses of JE virus-infected neuroblastoma cells. Together with other identified differentially expressed genes, these transcripts provide a better understanding of the pathogenesis of JEV at a transcriptomic level, particularly the molecular events that underpin host defense mechanism against JEV infection.

The results of the present study with mouse neuronal cells clearly show that many genes and host response pathways were upregulated during JE infection. Specific components of the response to virus such as Viperin, G1p2, Iftt3, Atf3, Irgm and CXCL10 have been implicated in JE infection for the first time to the best of our knowledge. Future research is required to explore the mechanism of JEV modulation of these genes to evade the host defense response. Our study offers an overview of the cascade of changes in host cellular expression culminating from infection with JEV. The counterbalancing of several anti-inflammatory and proinflammatory pathways together with the variable expression of apoptosis-related genes is a significant finding of the present study. The involvement of these genes indicated modulation of ini-

| Gene name | Description | Pathway | Microarray | Real-time RT-PCR |
|-----------|-------------|---------|------------|------------------|
| Itgb | Integrin beta | Focal adhesion, ECM-receptor interaction | +1 | +1.42 |
| CD40 | CD40 ligand | Toll-like receptor signaling | +1.06 | +1.6 |
| Tollip | Toll interacting protein | Toll-like receptor signaling | −0.61 | +1.40 |
| Xcr1 | Chemokine (C motif) receptor 1 | Cytokine–cytokine receptor interaction | −0.3 | −0.5 |
| Cxcl1 | Chemokine (C-X-C motif) ligand 1 | Cytokine–cytokine receptor interaction | +3.1 | +6.68 |
| Cxcl10 | Chemokine (C-X-C motif) ligand 10 | Toll-like receptor signaling | +7.7 | +35.7 |
chemokines CXCL9 and CXCL10 promote a protective immune response but do not contribute to cardiac inflammation following infection with Trypanosoma cruzi. Infect. Immun. 74, 125–134.

Hong-Lin, Su, Chung-Len, Liao, Lin, Yi-Ling, 2002. Japanese encephalitis virus infection initiates endoplasmic reticulum stress and an unfolded protein response. J. Virol. 4162–4171.

Hsieh, M.F., Lai, S.L., Chen, J.P., Sung, J.M., Lin, Y.L., Wu-Hsieh, B.A., Gerard, C., Luster, A., Liao, F., 2006. Both CXCR3 and CXCL10/IFN-inducible protein 10 are required for resistance to primary infection by dengue virus. J. Immunol. 177, 1853–1863.

Khanna, N., Agnihotri, M., Mathur, A., Chaturvedi, U.C., 1995. Neutrophil chemotactic factor produced by Japanese encephalitis virus stimulated macrophages. Clin. Exp. Immunol. 86, 209–303.

Kumar, R., Mathur, A., Kumar, A., Sethi, G.D., Sharma, S., Chaturvedi, U.C., 1990. Virological investigations of acute encephalopathy in India. Arch. Dis. Child. 65, 1227–1230.

Lin, R.J., Liao, C.L., Lin, Y.L., 2004. Replication-incompetent viros of Japanese encephalitis virus trigger neuronal cell death by oxidative stress in a culture system. J. Gen. Virol. 85, 521–533.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2^(-Delta Delta CT) method. Methods 25, 402–408.

Murali-Krishna, K., Ravi, V., Manjunath, R., 1995. Japanese encephalitis virus infection of mouse cell lines: ability to prime mice for generation of virus specific cytotoxic T lymphocytes and differences in CTL recognisable viral determinants. Arch. Virol. 140, 127–143.

Parida, M.M., Dash, P.K., Tripathi, N.K., Ambuj, Santhosh, S.R., Saxena, P.S., Agarwal, S., Sahni, A.K., Singh, S.P., Rathi, A.K., Bhargava, R., Abhyankar, A., Verma, S.K., Rao, P.V.L., Sekhar, K., 2006a. Japanese encephalitis outbreak, India, 2005. Emerg. Infect. Dis. 12, 1427–1430.

Parida, M.M., Santhosh, S.R., Dash, P.K., Tripathi, N.K., Saxena, P., Ambuj, Sahni, A.K., Rao, P.V.L., Morita, K., 2006b. Development and evaluation of reverse transcription Loop mediated isothermal amplification assay for rapid and real-time detection of Japanese encephalitis virus. J. Clin. Microbiol. 44 (11), 4172–4187.

Ravi, V., Parida, S., Desai, A., Chandramuki, A., Courie-Divi, M., Grau, G.E., 1997. Correlation of tumor necrosis factor levels in the serum and cerebrospinal fluid with clinical outcome in Japanese encephalitis patients. J. Med. Virol. 51, 132–136.

Santhosh, S.R., Parida, M.M., Dash, P.K., Patra, A., Pattanaik, B., Pradhan, H.K., Tripathi, N.K., Ambuj, S., Gupta, N., Saxena, P., Rao, P.V.L., 2007. Development and evaluation of SYBR Green I-based one-step real time RT-PCR assay for detection and quantification of Japanese encephalitis virus. J. Virol Methods 143, 73–80.

Singh, A., Kulshreshtha, R., Mathur, A., 2000. Secretion of the chemokine interleukin-8 during Japanese encephalitis virus infection. J. Med. Microbiol. 49, 607–612.

Solomon, T., Ni, H., Beasley, D.W., Ekkekennik, M., Cardosa, M., Barrett, A.D., 2003. Origin and evolution of Japanese encephalitis virus in Southeast Asia. J. Virol. 77, 3091–3098.

Tsai, T.F., 2000. New initiatives for the control of Japanese encephalitis by vaccination: minutes of a WHO/CVI meeting, Bangkok, Thailand. Vaccine 26, 1–25.

Valerio, A., Ferrari, M., Martinez, F., Locati, O.M., Ghisi, V., Bresciani, L.C., Mantovani, A., Spagnuolo, A.P., 2004. Gene expression profile activated by the chemokine CCL5/RANTES in human neuronal cells. J. Neurosci. Res. 78, 371–382.

Yang, K.D., Yeh, W.T., Chen, R.F., Chou, H.L., Tsai, H.P., Yao, C.W., Shiao, M.F., 2004. A model to study neutropenia and persistency of Japanese encephalitis virus infection in human neuroblastoma cells and leukocytes. J. Gen. Virol. 85, 635–642.