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Veterinary Microbiology 163 (2013) 223–234

Contents lists available at SciVerse ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Production of IL-8, IL-17, IFN-gamma and IP-10 in human astrocytes correlates with alphavirus attenuation

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ARTICLE INFO

Article history:
Received 13 June 2011
Received in revised form 6 November 2012
Accepted 22 November 2012

Keywords:
Alphavirus pathogenesis
Viral encephalitis
Equine encephalitis

ABSTRACT

Venezuelan equine encephalitis virus (VEEV) is an important, naturally emerging zoonotic pathogen. Recent outbreaks in Venezuela and Colombia in 1995 indicate that VEEV still poses a serious public health threat. Astrocytes may be target cells in human and mouse infection and they play an important role in repair through gliosis. In this study, we report that virulent VEEV efficiently infects cultured normal human astrocytes, three different murine astrocyte cell lines and astrocytes in the mouse brain. The attenuation of virus replication positively correlates with the increased levels of production of IL-8, IL-17, IFN-gamma and IP-10. In addition, VEEV infection induces release of basic fibroblast growth factor and production of potent chemokines such as RANTES and MIP-1-beta from cultured human astrocytes. This growth factor and cytokine profile modeled by astrocytes *in vitro* may contribute to both neuroprotection and repair and may play a role in leukocyte recruitment *in vivo*.

1. Introduction

Venezuelan equine encephalitis virus (VEEV) is an enveloped virus with a non-segmented, positive-sense RNA genome of approximately 11.4 kb that belongs to the genus *Alphavirus* in the *Togaviridae* family. The 5′ two-thirds of the genome encodes four nonstructural proteins (nsP1–nsP4) that form an enzyme complex required for viral replication (Strauss and Strauss, 1986, 1994; Strauss et al., 1995). The approximately 4-kb-long, subgenomic RNA corresponds to the 3′ one-third of the viral genome and is translated into a structural polyprotein that is proteolytically cleaved into the capsid protein and the envelope glycoproteins E2 and E1 (Rice and Strauss, 1981).

VEEV is a zoonotic pathogen and a member of the VEE serocomplex, which is divided into six distinct antigenic subtypes (Walton and Grayson, 1988; Young, 1972; Young and Johnson, 1969). Subtypes IAB and IC were previously associated with major epidemics and equine epizootics. During the most recent major outbreak in Venezuela and Colombia in 1995 involving subtype IC VEEV, about 100,000 human cases occurred, with over 300 clinical encephalitis cases estimated (Rivas et al., 1997). Other recent epidemics indicate that VEEV still represents a serious public health problem (Weaver et al., 1996).

The murine model of Venezuelan equine encephalitis (VEE), in which C57BL/6 mice are infected with the virulent ZPC738 strain of VEEV, is a well-established model of the pathogenesis of neurological disease characterized by the development of encephalitis, paralysis and, subsequently, death (Paessler et al., 2003, 2006, 2007). To develop an

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http://dx.doi.org/10.1016/j.vetmic.2012.11.021

attenuated, immunogenic vaccine, we have previously generated chimeric viruses that encode the replicative machinery from a relatively apathogenic member of the genus, Sindbis virus (SINV), and structural genes derived from VEEV (strain ZPC738, subtype ID) (Paessler et al., 2003). The chimeric vaccine candidate SIN/ZPC was selected and extensively tested for its safety and immunogenicity in hamsters and in a variety of mouse strains with selected immune deficiencies (Ni et al., 2007; Paessler et al., 2006). It has been shown that SIN/ZPC induces a high level of protection in animals with functional αβ T cells and that the passive transfer of $CD3^+$ T cells from vaccinated immunocompetent donors protects mice lacking αβ T cells (Paessler et al., 2007).

To establish an *in vitro* model of human infection that would allow us to study the characteristics of attenuation of different VEEV viruses, we have performed studies in cultured normal (derived from primary tissue culture) human astrocytes (NHA). Among the potential targets of VEEV infection in the CNS, *e.g.*, neurons, astrocytes and glial cells, it appears from examination of the brains of VEEV infected mice that neurons are the major target, but astrocytes may be also infected in lower numbers (16). In order to allow comparison of the results with the mouse model, we also performed experiments with 3 different astrocyte cell lines of murine origin (Alliot and Pessac, 1984). While the neuronal cell population is the major target for VEEV infection, it is unclear to what degree astrocytes may be involved in amplifying the virus and/or orchestrating the inflammatory response (Schoneboom et al., 2000, 1999). It is known, however, that reactive hyperplasia and hypertrophy of astrocytes (gliosis) may be detected in infected brains (Audouy et al., 1999; Caccuri et al., 2003).

Astrocytes play a central role in the CNS in several aspects. First, astrocytes improve neuronal survival in a variety of injury models by taking up toxic amino acids and/or by producing factors such as neurotrophic factors needed for neuronal survival (Rosenberg and Aizenman, 1989; Zhao et al., 2004). For example, molecules that are secreted by astrocytes protect neurons against a variety of insults such as hypoglycemic damage (Cheng and Mattson, 1991), excitotoxicity (Mattson and Rychlik, 1990) and anoxia (Vibulsreth et al., 1987). Second, astrocytes have a role in neuronal differentiation and maturation (Chamak et al., 1987). A variety of growth factors produced by astrocytes, such as epidermal growth factor (EGF), insulin-like growth factor-1 and basic fibroblast growth factor (bFGF) modulate neuron–glia interactions and have mitogenic effects on astrocytes (Stitt and Hatten, 1990). Based on these important functions within the CNS, it is clear that the infection of astrocytes by VEEV may have an impact on neuronal survival either by direct "killing" of astrocytes or by changing their physiological profiles and indirectly influencing their neuroprotective function and their ability to proliferate.

In this study, we demonstrate that normal human astrocytes (NHA) in culture are highly susceptible to infection with VEEV. Additionally, the infection of human and mouse astrocytes by virulent VEEV results in: (1) high infectious virus yield; (2) low-level proinflammatory/innate response; (3) chemokine induction; (4) increased release of bFGF and (5) accumulation of viral nucleocapsids in the nucleus of human astrocytes. Additionally, the infection of murine astrocytes in cell culture and in the brain is demonstrated. We have also performed a more detailed investigation of the immune response to attenuated chimeric virus, which replicates at lower levels but induces a robust proinflammatory/innate response *in vitro*. This model may be useful for determining the potential mechanisms responsible for attenuation of chimeric alphaviruses and to study the role of early proinflammatory response in controlling viral infection in human cells targeted by VEEV.

2. Results

2.1. Growth curves

2.1.1. Susceptibility of cultured human and murine astrocytes to VEEV

To evaluate the validity of *in vitro* studies of VEEV attenuation in selected CNS cell lines as a parallel model of human infection, we infected cultured normal human astrocytes with three strains of VEEV that exhibit differential virulence characteristics in the murine model.

Human astrocytes were susceptible to infection with all the VEEV strains tested (Fig. 1A). ZPC738, the virulent

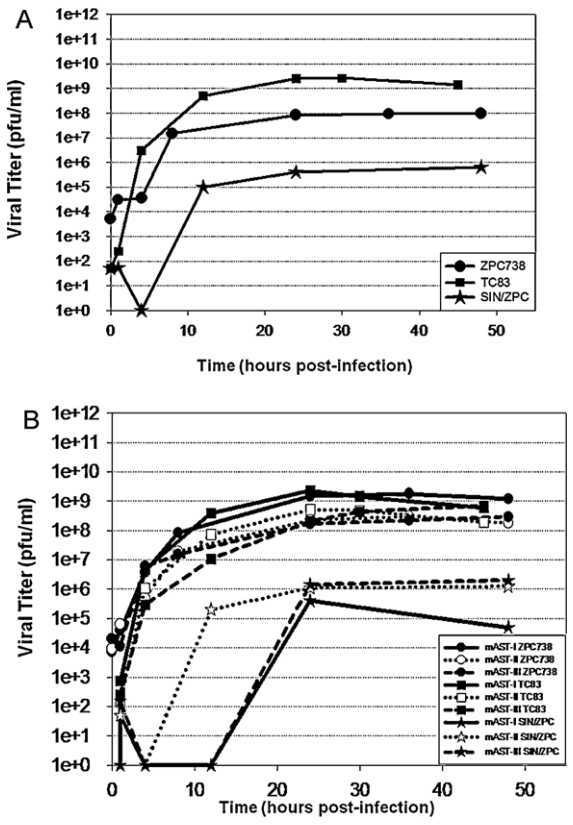

Fig. 1. VEEV can replicate in cultured astrocytes. Human (A) or murine (B) astrocytes infected with virulent ZPC738 (1 moi), vaccine strain TC83 (0.1 moi) or chimeric strain SIN/ZPC (0.1 moi) of VEEV. Viral replication was determined by plaque assay using supernatants collected 0–48 h post infection.

enzootic strain of VEEV, replicated to titers up to 7.5 $\log_{10}$ PFU/ml after 48 h (1 moi). Similarly, TC83, the cell culture adapted derivative of the TRD strain of VEEV, replicated up to titers of 9 $\log_{10}$ PFU/ml over the same period (0.1 moi). SIN/ZPC, a recombinant strain of VEEV that is highly attenuated in the murine model, replicated overall at a lower rate than the other two strains, with titers up to 5.6 $\log_{10}$ maximally (0.1 moi). Levels for infection titers of 10 moi were similar for all strains except that maximal titers occurred earlier.

To dissect the multitude of factors that may contribute to the attenuation of VEEV in the murine model, we cultivated three distinct mouse astrocyte cell lines that differ in morphology (mASTI, II and III) and examined VEEV growth and the cytokine and chemokine profiles they elicited *in vitro*. These established murine astrocyte cell lines (Fig. 1B) are highly susceptible to VEEV infection, and virus replicated to titers of 3–9 $\log_{10}$ PFU/ml after 48 h period (0.1 moi).

2.1.2. Electron microscopic analysis confirms that VEEV infects and replicates in human astrocytes

To confirm that astrocytes are productively infected with VEEV, NHA were infected with TC83 (1 moi) and examined at 24 hpi by electron microscopy. By twenty-four hours post-infection, cytoplasmic aggregates of virus protein (P) were visible, and were observed to be surrounded by virus cores (~20 nm in diameter) (Fig. 2A and B). Mature (budding) virions (~42 nm in diameter) were also present at the cell surface. In some cells, viral cores could be observed inside nuclei (Fig. 2B), presumably surrounding masses of viral protein.

2.1.3. Infected astrocytes can be detected in brain of VEEV infected mice and cytopathic effects are demonstrable in infected astrocytes in vitro

Double immunofluorescence staining of brain sections from VEEV (ZPC738) infected mice demonstrates immunoreactivity to viral antigen in cells coexpressing the astrocyte marker GFAP (Fig. 3A), indicating that astrocytes can be a target of infection *in vivo*. Morphologic changes due to VEEV infection were similar in cultured human and murine astrocytes, and consisted of extensive cell death after 48 h post infection manifested by rounding and detachment of cells (Fig. 3B). Especially for NHA, surviving cells became hypertrophic and elaborated more extensive processes.

Morphologic changes observed in cultured astrocytes surviving infection resemble those observed in brain sections after certain brain injuries (Fig. 4). The figure shows immunofluorescent labeling of viral antigens expressed by astrocytes infected with TC83 that are identified by coexpression of the astrocyte-specific marker GFAP. Astrogliosis is a pattern of reaction of astrocytes to brain injuries which includes increased complexity of processes, cell size (hypertrophy) and/or cell number (hyperplasia). Hypertrophic astrocytes can be recognized in either H&E stained sections (Fig. 4B-a) or by immunofluorescence staining (Fig. 4B-b). H&E staining shows an enlarged cell body with ample eosinophilic cytoplasm that is not usually apparent in normal, non-reactive astrocytes.

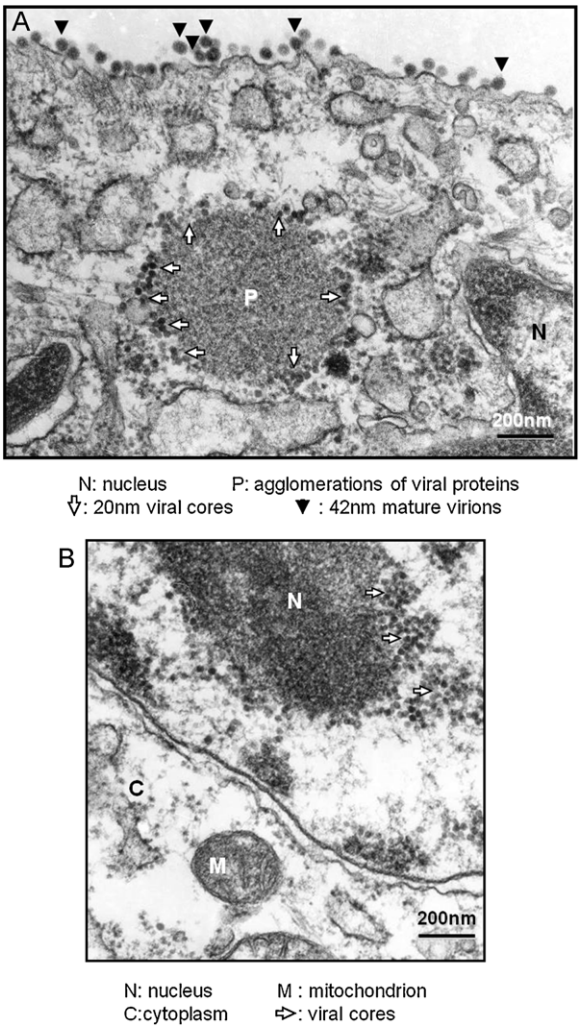

Fig. 2. Ultrastructure of VEEV in NHA. Electron micrographic image shows budding of mature viral particles on the cell surface, and agglomerations of viral proteins and viral cores in the cytoplasm (A) of NHA infected with TC83 (1 moi) at 24 h post infection. In addition, viral cores surrounding masses of viral protein are observed inside the nuclei (B).

2.2. Bio-Plex

2.2.1. Cytokine and chemokine production in VEEV infected astrocytes

To evaluate the production of soluble factors as a correlate of virulence, supernatants were collected from the three mouse astrocyte cell lines (mAST I, II and III) and from normal human astrocytes (NHA). These supernatants were examined for the production of an array of soluble factors, *e.g.*, chemokines, cytokines and growth factors (Fig. 5) by Bio-Plex analysis. A summary of the differences in cytokine secretion at 48 h post infection between uninfected and viral infected (0.1 moi) astrocytes is shown in Table 1. For NHA, of 27 cytokines, 24 show increased levels of secretion with SIN/ZPC infection, 7 with TC83 and 2 with ZPC738. bFGF and IP-10 are the only factors with increased levels in HNA with all three viral infections. For mouse cell lines, mASTI shows similar results to those for

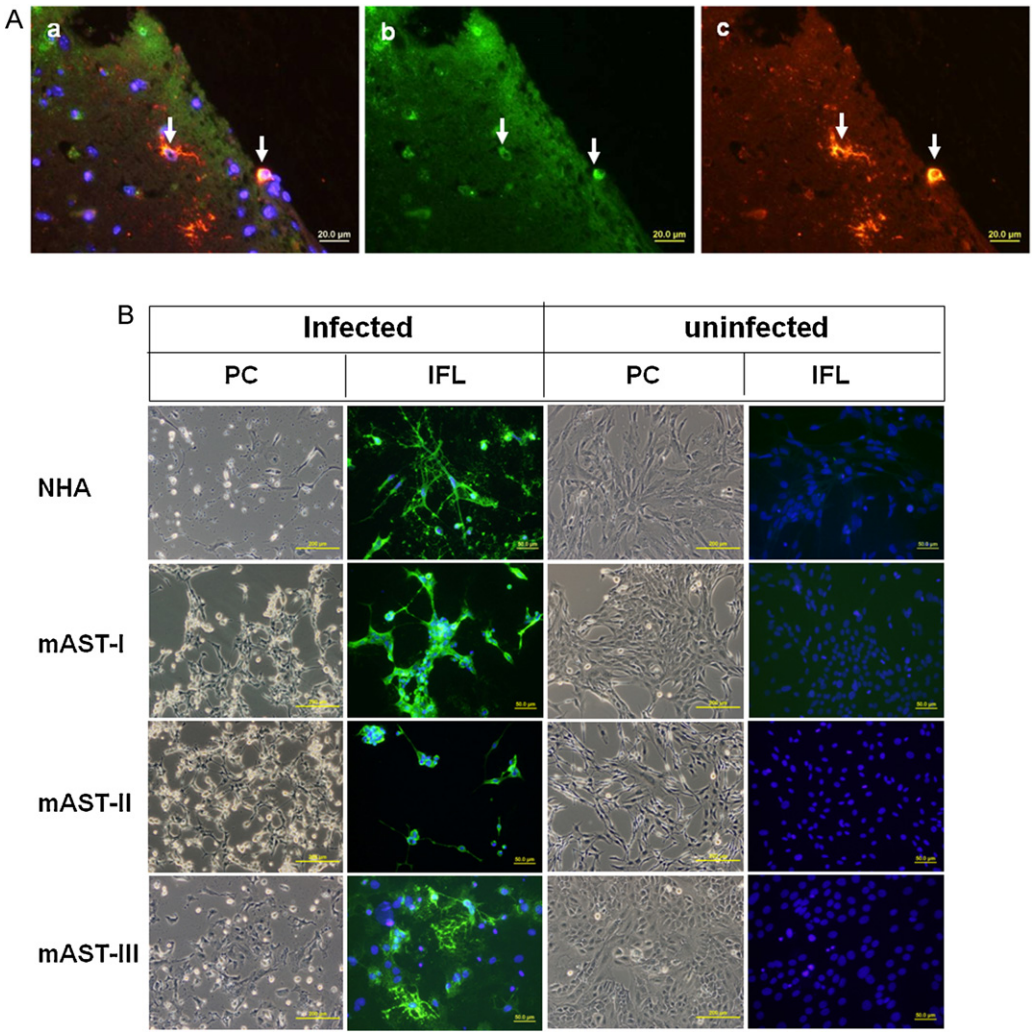

Fig. 3. VEEV infection in astrocytes in culture and in mouse brain. (A) Immuno-fluorescence labeling of viral antigens (green) and the specific astrocyte marker GFAP (orange) in a paraffin section from mouse infected with ZPC738 (5 days post infection). Panel (a) represents a combined image, with co-localization of both markers in cells indicated by arrows. Panels (b) and (c) are the individual images depicting only viral antigens or GFAP, respectively. (B) Anti-VEEV immunofluorescence (IFL) and phase contrast (PC) morphology for infected (0.1 moi TC83, 45 hpi) and uninfected astrocytes. Cytopathic effect of this viral infection is shown as rounding and detachment of cells (first column), and viral infection of cells as demonstrated by fluorescence-conjugated antibody against the TC83 antigens is shown in green (second column). The three types of murine astrocytes have been identified with fibrous (I), Bergmann (II) and protoplasmic (III) astrocyte types based on morphology, seen here in the PC images of the uninfected cells (third column). (For interpretation of the references to color in this figure caption, the reader is referred to the web version of the article.)

NHA, and the other two cell lines show increased levels for more cytokines with TC83 than with ZPC738 or SIN/ZPC infected cells. Factors showing increased levels in all three types of murine astrocytes include IL-6, IL-13, eotaxin, MIP-1β and RANTES.

2.3. VEEV (ZPC738)

2.3.1. Human

The most striking result was that in ZPC738 infected NHA, basic fibroblast growth factor (bFGF) was increased substantially from an undetectable level to over 9 pg/ml at 48 hpi, whereas no detectable bFGF was secreted in uninfected control NHA at all time points. Little to no IL-1β, IL-2, IL-4, IL-5, IL-17, eotaxin, GM-CSF, MIP-1β, or TNF-α was secreted by ZPC738 infected or uninfected NHA.

2.3.2. Mouse

MCP-1 and RANTES were the predominant soluble factors showing differential secretion in ZPC738 infected astrocytes. MCP-1 secretion was decreased in infected mouse astrocytes relative to the uninfected control, with peak decreases at 48 hpi for mASTI (50 fold), at 8 hpi for mASTII (120 fold) and at 24 hpi for mASTIII (6 fold). RANTES was also decreased relative to uninfected controls for mASTI (~6 fold), mASTII (2.5 fold) and mASTIII (12 fold) at 48 hpi. The overall profiles for this panel, which includes

19 factors in common between mouse and human, were similar for experiments with mouse cell lines and NHA. Infected and uninfected mouse astrocytes (mASTI, II and III) analyzed in parallel showed minimal secretion for the following factors: IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-17, G-CSF, GM-CSF, eotaxin, IFNγ, KC, MIP-1β and TNF-α.

2.4. TC83

The cytokine profiles for NHA and mAST cell lines were examined at 0–45 h following infection with TC83, which has been shown to retain virulence in the murine model and in other susceptible animals (Fine et al., 2008; Julander et al., 2008; Paessler et al., 2003; Steele et al., 1998).

2.4.1. Human

Most soluble factors assayed were secreted at low or undetectable levels. Eight factors showing increased levels in infected NHA include IL-1rα, IL-9, bFGF, G-CSF, IFN-γ, IP-10, MCP-1 and RANTES. bFGF, IP-10 and RANTES are particularly interesting. As in ZPC738-infected NHA, secretion of bFGF by TC83 infected NHA was increased from an undetectable level early post infection (<24 hpi) to over 7 pg/ml by 48 hpi in infected NHA. Similarly, IP-10 was undetectable in uninfected and at early time points (0, 1, 4, and 12 hpi) of infected NHA, but was increased up to 20 pg/ml at later time points (24, 30 and 48 hpi). RANTES was also undetectable before 24 hpi, but gradually increased to 70-fold over uninfected NHA.

2.4.2. Mouse

Overall, more assayed factors increased in TC83 infections than for ZPC738. Most of the remaining factors not showing significant increases were present at very low or undetectable levels.

mASTI: IL-6, MIP-1α, MIP-1β and RANTES were increased in infected cells. MCP-1 was expressed at levels greater than 200 pg/ml in uninfected mASTI, and is the only factor that was consistently decreased (up to 30 fold at 45 hpi) post infection.

mASTII: The overall secretion profile of infected mASTII was similar to that described for mASTI. IL-6, eotaxin and RANTES levels were increased in infected mASTII, while KC and MCP-1 were higher in the uninfected control *versus* infected cells between 24 and 45 hpi.

mASTIII: The overall secretion profile for infected mASTIII was similar to those of mASTI and mASTII, with relative increases in IL-6, MIP-1β, eotaxin and RANTES.

2.5. SIN/ZPC

The cytokine profiles for NHA and mAST cell lines were examined at 0–48 h following infection with SIN/ZPC.

2.5.1. Human

For SIN/ZPC infections of NHA, supernatants were collected at 0, 1, 4, 12, 24, and 48 hpi. Factors showing more than 3-fold increases in secretion post infection included: IL-1rα, IL-1β, IL-4. IL7, IL-8, IL17, G-CSF, GM-CSF, IFN-γ, MIP-1α, RANTES and TNF-α. In particular, IL-17 increased by 17-fold and RANTES by 85-fold. Additionally, IP-10 increased from undetectable levels in uninfected NHA to over 750 pg/ml by 48 hpi.

2.5.2. Mouse

For SIN/ZPC infections of mouse cells, supernatants were collected similarly to NHA.

mASTI: IL-1β, IL-6, KC, MIP-1β, RANTES and TNF-α were increased over 3-fold by 48 hpi. Specifically, IL-6 increased by up to 38-fold and MIP-1β by up to 12-fold.

mASTII: Only RANTES increased significantly (more than 200-fold) post infection. All other factors either increased by less than 3-fold or were unchanged or decreased.

mASTIII: RANTES was increased by nearly 3-fold by 48 hpi. All other factors were unchanged or decreased.

3. Discussion

3.1. Role of astrocytes in VEEV neurovirulence

VEEV is a highly neurovirulent virus in a variety of animal species and is capable of causing encephalitis in a small proportion of human cases (Weaver et al., 2004). In general, the virus causes systemic infection – initially by targeting the lymphoid tissue and visceral organs, and infecting the CNS at a later stage of disease. When introduced intranasally, the virus penetrates rapidly and grows to high titers in the brains of experimentally infected mice within a few days (Paessler et al., 2007). Our experimental data shown here, along with previously published *in vitro* (Schoneboom et al., 1999) and *in vivo* data (Jackson and Rossiter, 1997), indicate that astrocytes may be an important target in the infection. Two potentially devastating impacts of astrocyte death induced by VEEV infection in the brain may be related to decline in neuronal survival and disruption of the integrity and function of the blood brain barrier (BBB). No published information has previously been available about the capability of VEEV to efficiently infect human or murine astrocytes in cell culture models.

In this study, we show that the wild type VEEV (ZPC738) as well as the TC83 vaccine strain grow to very high titers and cause cytopathic effects in human and mouse astrocytes. Contrary to the results with VEEV, the attenuation of the chimeric SIN/ZPC vaccine candidate described previously in the murine (increasing virulence: SIN/ZPC < TC83 < ZPC738) model was also confirmed for human and murine astrocytes, and is characterized in both cell types by reduced virus growth and induction of a strong proinflammatory innate immune response. This has important implications in evaluating the safety of vaccine candidates in general, and specifically in determining the safety of the SIN/ZPC in cellular targets of human origin, *e.g.*, in human astrocytes.

3.2. Gliosis in virus infected brain

Astrocyte responses to inflammation have been studied extensively in multiple sclerosis (MS) in humans and in the mouse in experimental autoimmune encephalomyelitis

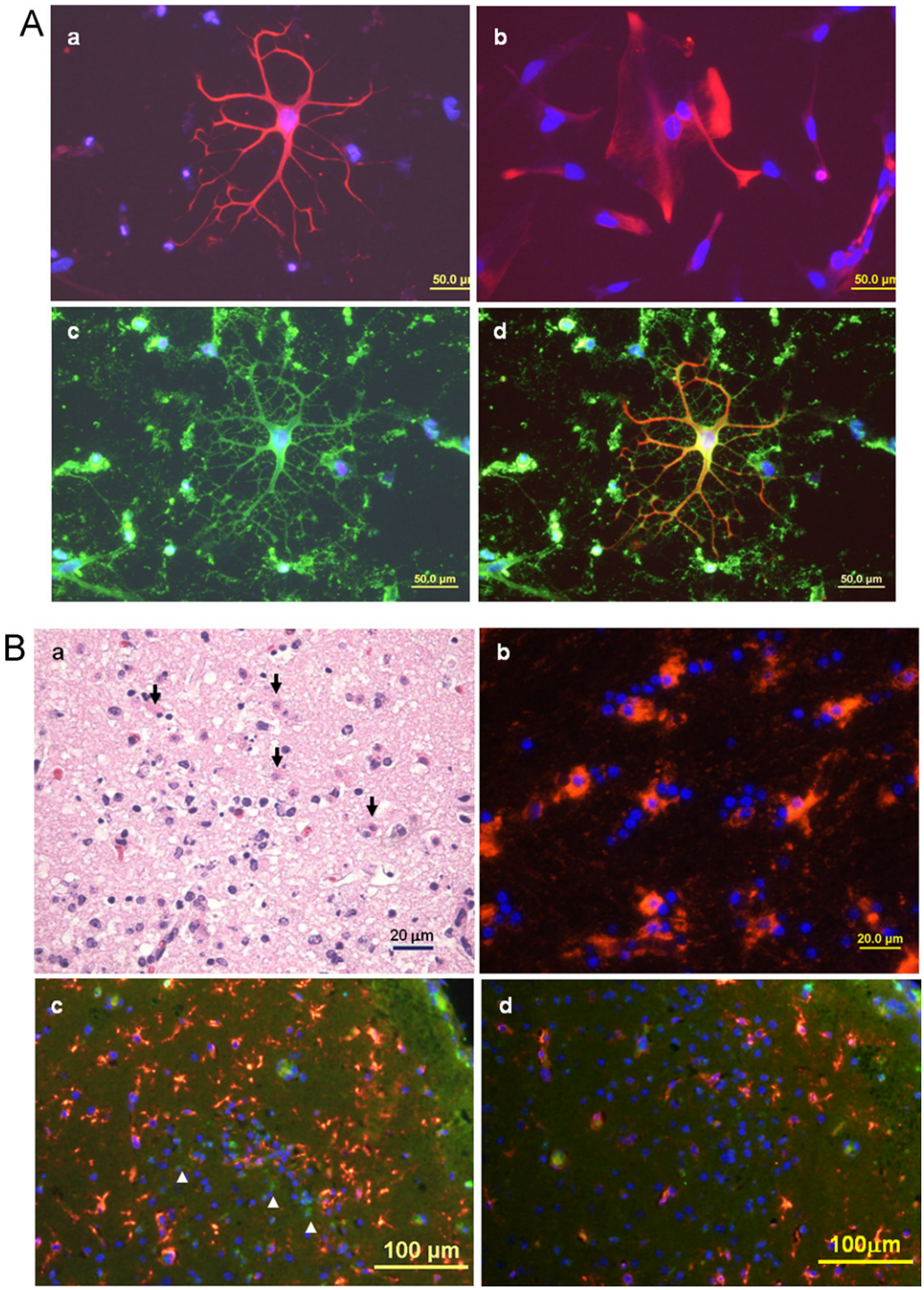

Fig. 4. VEEV infection-induced changes in astrocytes. (A) Immunofluorescence labeling of TC83-infected (a) and uninfected (b) NHA with GFAP (red). Morphologic changes observed in an astrocyte surviving infection (a) compared to the uninfected control (b) resemble those observed in astrogliosis *in vivo*. That this astrocyte is also infected with TC83 is shown by immunofluorescence labeling of viral antigens (green, c). Panel d is the combined image of a and c. (B). Increased complexity of processes in infected cells is shown in cultured NHA in (A). Hypertrophic astrocytes can be recognized in H&E stained sections (a) and by immunofluorescence staining (b). Both images (a and b) are from adjacent brain sections from the same mouse which was infected with ZPC738 (11 days post infection). Proliferation (hyperplasia) of astrocytes (GFAP, red) is demonstrated in panel c with adjacent (unidentified) cells that are immunoreactive for viral antigens (green, arrowheads). This section was from cerebral cortex of a mouse infected with ZPC738 (5 days post infection). A microscopic field from an adjacent area of cortex that is not involved by this process shows normal astrocytes (GFAP, red) and no reactivity for the viral antigen (d). Plasma in blood vessels in both c and d shows nonspecific staining with the anti-viral antibodies due to the mouse origin of the primary antibody. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of the article.)

(EAE) (De Keyser et al., 2003; Miljkovic et al., 2007; Williams et al., 2007). However, the astroglial response to infection with virulent encephalitic alphaviruses is much less well characterized.

In the setting of brain injury, astrocytes undergo a so called "astrocyte-stress-response" characterized by reactive astrocytosis or gliosis, which consists of both hypertrophic and hyperplastic/proliferative responses (Malhotra et al., 1990). A variety of molecular and cellular events may lead to the final resolution of a lesion (glial scar), and astrocytes are usually the predominant cell type involved (Fawcett and Asher, 1999). The production of the scar is potentially aimed at isolating the necrotic tissue and non-neuronal cells from the CNS parenchyma (Eddleston and Mucke, 1993), with the final goal of protecting surviving neurons (Silver and Miller, 2004). However, one of the negative aspects of the scar formation is the inability of the host to proceed with axonal regeneration in the injured area and to fully repair the damage (Silver and Miller, 2004).

3.3. The contribution of chemokine, cytokines and growth factors to gliosis

EGF, IGF-I and bFGF have been reported to be secreted by astrocytes and to modulate cell proliferation and differentiation of cultured astrocytes and neurons, as well as to be required for glial proliferation *in vivo* (Eng and Ghirnikar, 1994; McKeon et al., 1999). Our data show that VEEV infection leads to a similar "stress-response" in infected human astrocytes characterized by the release of bFGF, which is a prototype growth factor of the FGF family that includes some 22 members in vertebrates (Ornitz and Itoh, 2001). Induction of bFGF in human astrocytes also correlated with the peak of virus replication and development of cythopathic changes, suggesting a damage-dependent secretion. This observation is in contrast with previously described results obtained with rat astrocytes, in which apparently upregulation of potentially neurotoxic immunomodulators such as TNF-α and iNOS was reported, leading to the speculation that astrocytes may contribute in a negative way to pathology and apoptosis of neuronal cells in VEEV infected brains (Schoneboom et al., 1999). The late glial scar formation in survivors may also limit the ability of the host to repair the axonal damage and thus may be a factor contributing to the high rate of occurrence of neurological sequelae in people surviving alphaviral encephalitis. We suggest that the production of growth factors in the VEEV infected brain may be of considerable importance, previously poorly recognized, and more detailed characterization of this response will contribute to better understanding of the CNS phase of the disease.

3.4. Pro-inflammatory cytokines and interferon pathway

Additionally, astrocytes have been shown to play a key role in the stimulation of IL-17 and IFN-γ secretion in the context of immune-mediated injury in EAE (Miljkovic et al., 2007). Direct IFN-γ secretion by NHA was demonstrated in our study with a 4-fold increase post exposure to the SIN/ZPC chimera over uninfected controls and an approximately 2-fold increase with TC83. In contrast, the wild type VEEV replicated to high levels without increasing the production of IFN-γ. It is well-known that IFN-γ has very important antiviral functions and, while some overlap with defense pathways induced by type I interferons, others appear to be more strongly activated by IFN-γ. One of the best characterized pathways is the induction of the enzyme nitric oxid synthetase 2 (NOS2), described in the literature as inducible NOS (MacMicking et al., 1997). The activation of NOS2 may have a direct impact on the function of viral proteins and therefore negatively impact virus amplification. Additionally, there is a long and still growing list of various interferon-inducible genes that may encode for proteins with GTPase activity (Carlow et al., 1998; Taylor et al., 1996, 2000). It is well known that early IFN-γ production, which may depend on induction of IL-12 secretion, is present in many human and animal viral infections (Biron, 1999).

Additional proinflammatory marker cytokines and chemokines were produced by NHA in response to SIN/ZPC infection but not to infection by wild-type virus or other less attenuated strains of VEEV (TC83). These included IL-8, RANTES, G-CSF and MIP-1α/β. The results differ somewhat for mouse astrocytes, with RANTES, IL-1β, IL-6 and MIP-1β induced by both SIN/ZPC and TC83.

Astrocytes may play a significant role in neutrophil recruitment into the CNS in viral infection. Elevated IL-8 levels have also been demonstrated in cerebrospinal fluid of children with both bacterial and viral meningitis (Shapiro et al., 2003). IL-8 release by NHA was markedly upregulated by 48 h (3.2-fold increase at the secreted protein level), and differences between treatments (virulent *versus* attenuated virus) were noted as early as 24 h. IL-1β is a potent agonist for upregulation of CNS expression of both KC and MIP-2, which also mediate recruitment of neutrophils to sites of inflammation (Anthony et al., 1998). Transgenic expression of IL-1β in the hippocampus of mouse brain results in marked upregulation of KC (Shaftel et al., 2007). In this model, KC was found to be linked to expression in astrocytes but not microglial cells. Increased pathogenicity of coronavirus infection in the CNS has been associated with neutrophil recruitment and proinflammatory and inflammatory cytokine and chemokine expression (TNF-α, IL-6, CCL-2, and IFN-γ) (Ireland et al., 2008), and similar influx has been noted in the mouse model of VEEV (Paessler et al., 2006, 2007). Toll-like receptor 3 (TLR3) mediated signaling in response to dsRNA results in upregulation of IL-8 and IP-10 in astrocytes (Park et al., 2006). These results are similar to the cytokine profiles that we obtained for NHA following exposure to SIN/VEEV.

Macrophage inflammatory protein-1α (MIP-1α) and macrophage inflammatory protein 1β (MIP1-β) activate various cell types including monocytes and lymphocytes, and are also involved in recruitment of inflammatory cells to sites of infection (Cook et al., 1995; Domachowske et al., 2000; Trifilo and Lane, 2004). Both of these chemokines were upregulated in NHA by the attenuated SIN/VEEV strain but not TC83 or virulent strains of VEEV. Of the resident cells in the CNS, microglial cells have shown constitutive expression of MIP-1α in mouse (Rezaie et al.,

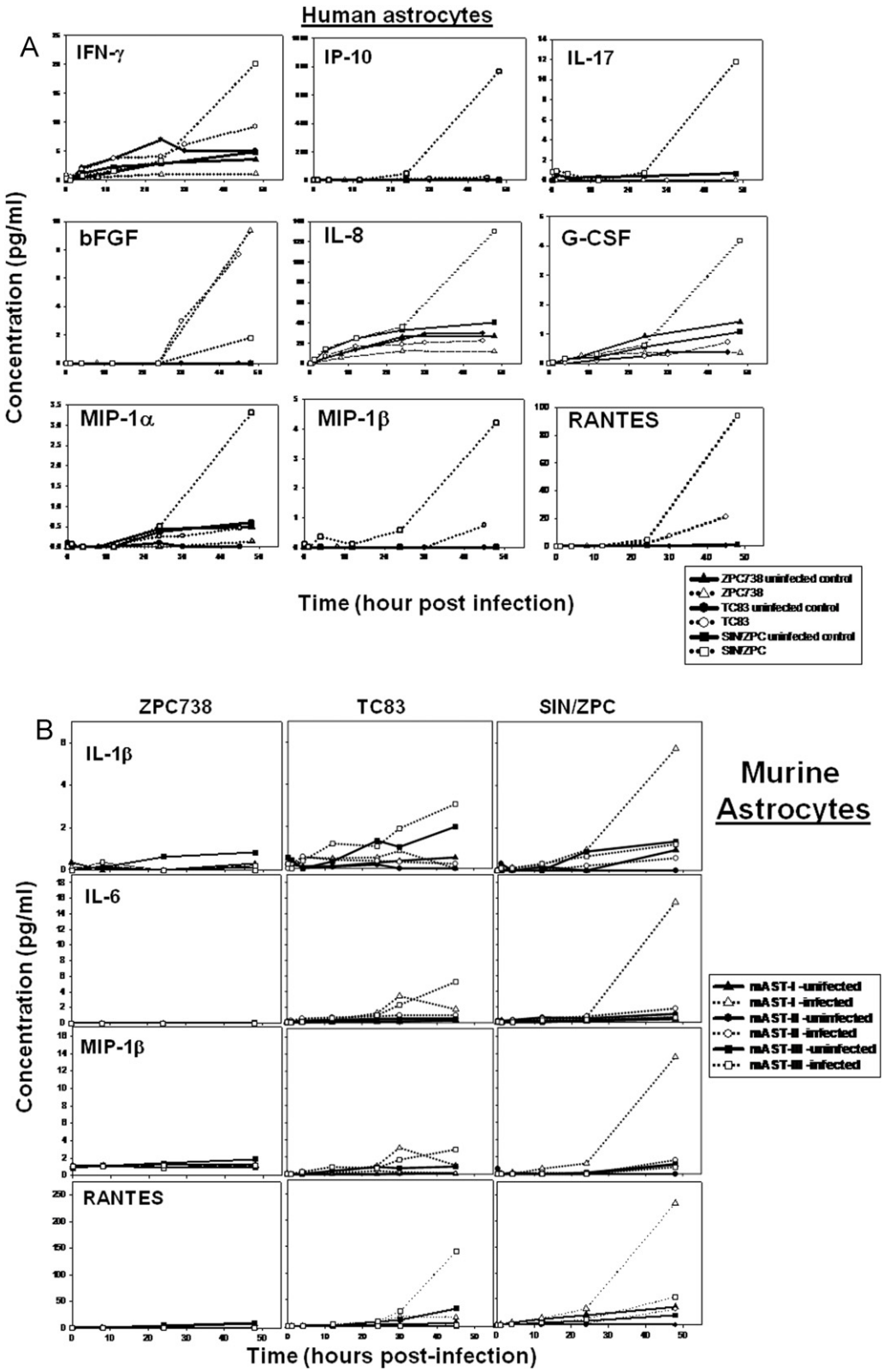

Fig. 5. Increased cytokine secretion in VEEV-infected astrocytes. Secretion of cytokines in both human (A) and murine (B) astrocytes was measured in supernatants of cell cultures infected with ZPC738, TC83 or SIN/ZPC (0–48 h post infection.). Panel A shows increased levels of several cytokines in human astrocytes (NHA) infected with the highly attenuated chimeric strain SIN/ZPC compared to uninfected controls. Secretion of bFGF in particular is positively

Table 1
Changes of cytokine levels in culture supernatants of VEEV-infected astrocytes.

Human-27	Mouse-23	NHA			mAST-I			mAST-II			mAST-III		
		ZPC	TC83	SinZPC	ZPC	TC83	SinZPC	ZPC	TC83	SinZPC	ZPC	TC83	SinZPC
	IL-1α	NA	NA	NA	*	*	(0.23)	*	3	*	0.63	1.33	*
IL-1ra		0.47	1.6	3.34	NA	NA	NA	NA	NA	NA	NA	NA	NA
IL-1β	IL-1β	*	*	4	*	*	5.4	*	(0.22)	(0.58)	0.26	1.55	0.9
IL-2	IL-2	*	*	4.8	*	*	(0.17)	*	*	*	*	2.5	*
	IL-3	NA	NA	NA	*	0.25	1	*	1	1	*	1	1
IL-4	IL-4	*	(0.09)	4.57	*	1	1	*	1	1	*	1	1
IL-5	IL-5	*	*	*	*	*	*	*	*	*	*	*	*
IL-6	IL-6	0.4	0.52	1.26	*	5.73	38.6	*	1.78	1.6	*	11.67	1
IL-7		0.47	*	3.5	NA	NA	NA	NA	NA	NA	NA	NA	NA
IL-8		0.44	0.76	3.2	NA	NA	NA	NA	NA	NA	NA	NA	NA
IL-9	IL-9	1	2.44	2.6	*	*	1.9	*	-0.1	1	*	0.87	1
IL-10	IL-10	0.22	*	2.4	0.65	*	2.7	(0.04)	*	*	0.2	0.84	0.24
	IL-12(p40)	NA	NA	NA	0.14	0.25	2.5	*	1	*	0.14	0.75	0.5
IL-12(p70)	IL-12(p70)	0.31	*	1.8	(0.1)	*	2.86	*	*	1	0.5	1	0.5
IL-13	IL-13	0.36	*	1	(1.31)	*	1.5	(0.05)	1.4	1	*	1.32	0.7
IL-15		0.15	*	3.04	NA	NA	NA	NA	NA	NA	NA	NA	NA
IL-17	IL-17	*	*	17.62	*	*	1	*	*	*	*	1	0.14
Eotaxin	Eotaxin	*	(0.1)	2.24	*	*	(7.28)	*	(6.34)	*	*	(7.96)	*
bFGF		(9.38)	(7.68)	(1.81)	NA	NA	NA	NA	NA	NA	NA	NA	NA
G-CSF	G-CSF	0.26	1.9	3.9	0.37	*	1.5	2.6	2	1	0.4	0.86	0.5
GM-CSF	GM-CSF	*	*	7.47	0.89	*	1.6	0.81	*	*	0.63	1.47	*
IFN-γ	IFN-γ	0.3	1.8	4.13	0.96	*	2.4	1.1	1	*	0.89	1.08	*
	KC	NA	NA	NA	*	*	3.6	*	0.22	0.5	*	0.74	0.23
IP-10		1.8	(20.7)	(763.8)	NA	NA	NA	NA	NA	NA	NA	NA	NA
MCP-1	MCP-1	0.41	1.1	1.17	0.02	0.03	1.2	0.13	0.06	0.5	~ 0	0.8	0.38
MIP-1α	MIP-1α	0.26	(0.46)	5.5	NA	(3.16)	NA	NA	1	NA	NA	2.39	NA
MIP-1β	MIP-1β	*	(0.74)	(4.22)	0.86	25.25	12.6	1.12	1	(1.61)	0.64	3.26	0.08
PDGFbb		0.55	*	2.82	NA	NA	NA	NA	NA	NA	NA	NA	NA
RANTES	RANTES	0.88	70.8	85.71	0.15	2.33	6.6	0.39	12.53	214	0.08	4.24	2.89
TNF-α	TNF-α	*	*	7.4	*	*	3.5	*	(0.03)	*	0.16	0.74	0.3
VEGF		0.26	*	0.87	NA	NA	NA	NA	NA	NA	NA	NA	NA

Supernatants were collected from cultured astrocytes (NHA, mAST-I, mAST-II and mAST-III) infected with 0.1 moi of ZPC738, TC83 or SINZPC at 48 h post infection. Secreted cytokines were then measured using the Bio-Plex system. Levels are expressed in fold compared to uninfected supernatants, except where the cytokine level of the uninfected sample was not detectable, in which case the actual measured concentration of the infected sample is given in pg/ml (in parentheses). Asterisk (*) indicates the level of cytokine is too low to be detected. NA, not tested.

2002). MIP-1α has been implicated in dendritic cell activation in the CNS with downstream stimulation of T cells in MHV infection (Trifilo and Lane, 2004).

Granulocyte colony stimulating factor (G-CSF) was upregulated post exposure to the SIN/ZPC chimera, beginning at approximately 30 h, to as much as 3.9-fold elevation above controls by 48 h. G-CSF is a growth factor with a role in systemic homeostasis by regulating the production of neutrophils from stem cells in the bone marrow (Demetri and Griffin, 1991). More recent studies have shown a neuroprotective effect following treatment with G-CSF in response to various injuries or other stimuli such as ischemia (Gibson et al., 2005; Komine-Kobayashi et al., 2006; Park et al., 2005; Solaroglu et al., 2006b; Schneider et al., 2005; Sheibani et al., 2004). G-CSF has been proposed to be down-modulatory with respect to inflammation in the CNS and may have a similar role in the post-exposure response by astrocytes and other cells types after viral exposure (Solaroglu et al., 2006a). Inflammation and the recruitment of autoreactive T cells have been shown to be reduced in G-CSF treated animals in an EAE model of disease (Zavala et al., 2002). Specifically, G-CSF treated mice demonstrated reduced demyelination and reduced CNS cytokine and chemokine expression. This effect was countered in the periphery by increased production of MIP-1α and MCP-1, both pro-type 1 and type 2 factors. These effects were also correlated with reduced production of TNF-α, which corresponded with a reduced inflammatory response. Other potential activities for G-CSF involve modulation of antigen-presenting cell activities (Rutella et al., 1999). RANTES, IP-10, MIP-1α and MIP-1b are upregulated by 30 h post exposure to the chimeric SIN/ZPC strain with peak levels by 50 h post exposure. Chemokines direct the trafficking of specific subsets of inflammatory cells, and potentially play key roles in the pathogenesis of viral CNS disease, repair and cellular communication. Regarding flavivirus infections, RANTES is expressed by primary mouse cells infected with Japanese Encephalitis Virus (JEV). Several cell types are involved including primary neurons with glia, mixed glia, microglia, and astrocytes, but not pure neuronal cultures (Chen et al., 2004).

correlated with the degree of virulence, with the highest level observed in ZPC738 infection. Similarly, relatively high levels of secretion of certain cytokines were observed in murine astrocytes (mAST I) with SIN/ZPC infection (B). MIP-1β and RANTES, of the common factors available for analysis, show consistent results between human and murine astrocytes.

In summary, we show that VEEV efficiently infects both human and murine astrocytes in cell culture as well as in the murine brain. The astrocyte response to VEEV infection *in vitro* appears to be aimed more toward promoting neuronal protection rather than activating antiviral innate immune response, which may allow the virus to grow more efficiently in brain. In contrast, the attenuation of virus growth of the chimeric vaccine candidate correlates positively with the induction of a strong proinflammatory innate response in astrocytes. It appears that the VEEV vaccine strain TC83 is similar to the wild-type VEEV in efficiently infecting human and murine astrocytes while inducing a host response comparable to the wild-type virus. This correlates with the fact that TC83 is virulent for mice if introduced directly into the brain as previously reported by others (Fine et al., 2008; Julander et al., 2008; Paessler et al., 2003; Steele et al., 1998) and our group (Paessler et al., 2003). Future studies are needed to identify how the virulent VEEV suppresses (or avoids inducing) the proinflammatory response in astrocytes.

4. Materials and methods

4.1. Cell culture

Normal human astrocytes (NHA; Lonza, Allendale, NJ) were maintained in the supplied astrocyte growth medium (AGM), which contains the proprietory basal medium (ABM) with added supplements (fetal bovine serum, insulin, ascorbic acid, L-glutamin, rhEGF, gentamycin and amphotericin-B). Murine astrocyte (mAST) cell lines (ref, type I, type II and type III, denoted mASTI, mASTII or mASTIII, respectively) were purchased from the American Type Culture Collection (ATCC; Manassas, VA), and were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. All cells were maintained in a humidified CO_2 (5%) incubator at 37 °C.

4.2. Viruses

All work with VEEV was approved by institutional (University of Texas Medical Branch, UTMB, Galveston, Texas) and federal agencies (US Centers for Disease Control and Prevention/US Department of Agriculture/Department of Defense). Work with TC83 (Berge et al., 1961; Kinney et al., 1989) (Acc. No. L01442), the attenuated vaccine derivative of the Trinidad Donkey (TRD) strain and SIN/ZPC (Ni et al., 2007; Paessler et al., 2006, 2007) was performed at Biosafety Level (BSL)-2, and with ZPC738 (Roehrig and Bolin, 1997; Wang et al., 1999) (Acc. No. AF100566), at BSL-3 at UTMB in accordance with all UTMB Health and Safety guidelines.

4.3. Virus infections

NHA (3×10^5 cells/well) or mAST cell lines (3×10^5 cells/well) were infected with the ZPC738 strain of VEEV at a multiplicity of infection (moi) of 1 or 10 or with the vaccine strains, TC83 and SIN/ZPC at moi of 0.1 or 10. Uninfected NHA or mAST were cultured in parallel. Culture supernatants were collected at time points between 0 and 48 h post-infection for the measurement of viral growth by plaque assay and chemokine secretion by Bio-Plex assay.

4.4. Virus titrations

To quantify the level of infectious virus in all supernatant samples collected, plaque assays were performed on Vero cell monolayers using 6-well culture plates. Briefly, serial ten-fold dilutions (200 ml/well) of supernatant sample was added to each well after the removal of medium, and then incubated for 1 h (5% CO_2/37 °C). Free virus was removed and 2 ml of agarose was layered on top of the Vero monolayer. The plates were incubated for 48 h (5% CO_2/37 °C) to allow plaques to form. Cell monolayers were stained with 0.5% crystal violet after fixation in concentrated formaldehyde (37%) solution (1 ml/well) for 30 min in the chemical hood. The numbers of plaque forming units were counted after plates were washed and air-dried.

4.5. Cytokine analysis

Supernatants collected for virus growth curves were analyzed in parallel for the protein levels of a selected set of soluble factors, including chemokines, cytokines and growth factors, using the Bio-Plex suspension array system (human cytokine 27-plex and mouse cytokine 23 plex; Bio-Rad, Hercules, CA), according to the manufacturer's recommendations. Briefly, the culture supernatant was incubated with antibody-coupled beads for 30 min at room temperature and subsequently washed to remove unbound proteins. Antibody-bound proteins were then detected by addition of the biotinylated detection antibody, followed by incubation with streptavidin–phycoerythrin. The cytokine levels were measured on a Bio-Plex workstation (model 200). The Bio-Plex Mouse Cytokine 23-Plex Panel (Cat. No. 171F11241, Bio-Rad Laboratories, Hercules, CA) was used to measure IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-17, eotaxin, G-CSF, GM-CSF, IFN-γ, KC, MCP-1, MIP-1α, MIP-1β, RANTES, and TNF-α; the Bio-Plex Human Cytokine 27-Plex Panel (Cat. No. 171A11127, Bio-Rad) was used to measure IL-1β, IL-1rα, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12(p70), IL-13, IL-15, IL-17, eotaxin, bFGF, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1, MIP-1α, MIP-1β, PDGF, RANTES, TNF-α, and VEGF.

4.6. Electron microscopy

Confluent monolayers of NHA were infected with TC83 (1 moi). At 24 h post infection, cell monolayers were fixed in a mixture of 2.5% formaldehyde, 0.1% glutaraldehyde, 0.03% trinitrophenol and 0.03% $CaCl_2$ in 0.05 M cacodylate buffer at pH 7.3, rinsed with 0.1 M cacodylate buffer, scraped off the plastic and pelleted by centrifugation for further processing, as follows. The pellets were post-fixed in 1% OsO_4 in 0.1 M cacodylate buffer, stained en bloc with 2% aqueous uranyl acetate, dehydrated in ethanol and embedded in Poly/Bed 812 (Polysciences, Warrington, PA). Ultrathin sections were cut on Reichert-Leica Ultracut S ultramicrotome, stained with lead citrate and examined using a Philips 201 electron microscope at 60 kV.

4.7. Immunofluorescence

All procedures for immunofluorescence were performed at room temperature. Briefly, cultured NHA and mAST were fixed in 10% buffered formalin for 15 min after the last time point (48 hpi). Cell monolayers were incubated for 1 h with ascites fluid from TC83-infected mice (1:2000; a generous gift from Dr. Robert Tesh, University of Texas Medical Branch at Galveston). Positively stained cells were subsequently labeled with secondary antibody conjugated with Alexa 488 (1:200, Invitrogen). To co-localize viral antigen and GFAP in VEEV infected mouse brains, we prepared sections (4 μm) of paraffin blocks from previous studies in which mouse brains were removed at day 7 post infection. Sections were incubated with antibodies against VEEV (described previously) and GFAP (1:200, rabbit anti-human IgG, Sigma) for 1 h, and the positively labeled cells were then detected by Alexa 488-conjugated secondary antibody for VEEV and Alexa 555 conjugated secondary antibody for GFAP (both at 1:200). The positive cells were visualized using an inverted fluorescence microscope (model IX71, Olympus, Center Valley, PA) equipped with an Olympus digital camera (DP71).

Acknowledgments

This work was supported by a grants from the National Institute of Allergy and Infectious Diseases through the Galveston National Laboratory Operations, Advanced Veterinary Services Core (UC7 AI070083). S. Paessler was supported by National Institutes of Health K08 Award (A1059491) and faculty support provided by the Institute for Human Infections and Immunity at UTMB. We thank Jenna Linde for outstanding assistance with data entry and preparation of figures/manuscript.

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