

Complexity of the microglial activation pathways that drive innate host responses during lethal alphavirus encephalitis in mice

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Cite this article as: Esen N, Blakely PK, Rainey-Barger EK and Irani DN (2012) Complexity of the microglial activation pathways that drive innate host responses during lethal alphavirus encephalitis in mice. ASN NEURO 4(4):art:e00088.doi:10.1042/AN20120016

ABSTRACT

Microglia express multiple TLRs (Toll-like receptors) and provide important host defence against viruses that invade the CNS (central nervous system). Although prior studies show these cells become activated during experimental alphavirus encephalitis in mice to generate cytokines and chemokines that influence virus replication, tissue inflammation and neuronal survival, the specific PRRs (pattern recognition receptors) and signalling intermediates controlling microglial activation in this setting remain unknown. To investigate these questions directly *in vivo*, mice ablated of specific TLR signalling molecules were challenged with NSV (neuroadapted Sindbis virus) and CNS viral titres, inflammatory responses and clinical outcomes followed over time. To approach this problem specifically in microglia, the effects of NSV on primary cells derived from the brains of wild-type and mutant animals were characterized *in vitro*. From the standpoint of the virus, microglial activation required viral uncoating and an intact viral genome; inactivated virus particles did not elicit measurable microglial responses. At the level of the target cell, NSV triggered multiple PRRs in microglia to produce a broad range of inflammatory mediators via non-overlapping signalling pathways. *In vivo*, disease survival was surprisingly independent of TLR-driven responses, but still required production of type-I IFN (interferon) to control CNS virus replication. Interestingly, the ER (endoplasmic reticulum) protein UNC93b1 facilitated host survival independent of its known effects on endosomal TLR

signalling. Taken together, these data show that alphaviruses activate microglia via multiple PRRs, highlighting the complexity of the signalling networks by which CNS host responses are elicited by these infections.

Key words: encephalitis, microglia, Sindbis virus, Toll-like receptors, type-I interferon, UNC93b1.

INTRODUCTION

TLRs (Toll-like receptors) are widely expressed innate immune sensors that evolved to recognize specific PAMPs (pathogen-associated molecular patterns) (Janeway and Medzhitov, 2002; Takeda et al., 2003). Within the TLR family, TLR3, TLR7, TLR8 and TLR9 are nucleotide-sensing receptors located in various intracellular compartments, including the ER (endoplasmic reticulum), endosomes and lysosomes (Saitoh and Miyake, 2009). These receptors detect common replication intermediates of most viral pathogens, including double-stranded RNA (TLR3), single-stranded RNA (TLR7 and TLR8) and unmethylated DNA (TLR9). Along with TLR2 and TLR4 that can respond to certain viral proteins, these receptors form a critical network of sensors that activate innate host responses against viruses. Indeed, six human TLRs (TLR2, TLR3, TLR4, TLR7, TLR8 and TLR9) and five murine TLRs (TLR2, TLR3, TLR4, TLR7 and TLR9) can trigger the production of type-I IFN (interferon) that is central to antiviral host defence (Zhang et al., 2007; Barbalat et al., 2009). The activation of these TLRs also drives transcription of

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Abbreviations: Baf A1, bafilomycin A1; CCL, CC motif ligand; CNS, central nervous system; CXCL13, C-X-C motif ligand 13; EAE, experimental autoimmune encephalomyelitis; ER, endoplasmic reticulum; FBS, fetal bovine serum; HBSS, Hanks balanced salt solution; HSE, herpes simplex encephalitis; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; MDA5, melanoma-differentiation-associated gene 5; MyD88, myeloid differentiation response gene 88; NSV, neuroadapted Sindbis virus; ODN, oligodeoxynucleotide; PBMC, peripheral blood mononuclear cell; pfu, plaque-forming units; poly(I:C), polyinosinic:polycytidylic acid; PRR, pattern recognition receptor; qPCR, quantitative PCR; RLR, retinoic acid-inducible gene I-like receptor; RRMS, relapsing-remitting form of multiple sclerosis; SV, Sindbis virus; TLR, Toll-like receptor; TNF, tumour necrosis factor.

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various pro-inflammatory cytokines by innate immune cells; this broadens the early host response to viral infection and facilitates adaptive antiviral immunity, but it can also accelerate tissue injury. Over the last decade, mice with defects in these various nucleotide-sensing TLRs or their downstream adaptor molecules have been shown to exhibit diverse outcomes when challenged with different viral pathogens (Akira et al., 2006; Zhang et al., 2007). Novel intracellular TLRs (e.g. TLR13) that detect and respond to viruses continue to be identified (Shi et al., 2011).

Recently, a point mutation of the ER protein, UNC93b1, was found to ablate TLR3, TLR7 and TLR9 signalling in mice (Tabeta et al., 2006; Brinkmann et al., 2007). Subsequent studies showed that the mutant protein fails to transfer these TLRs from the ER to their expected location in endosomes (Kim et al., 2008). This 3d (triple defect) causes macrophages from these mice to be profoundly impaired in their responses to ligation of the nucleic acid-sensing TLRs as measured by the production of multiple cytokines, including IFN α / β (Tabeta et al., 2006; Brinkmann et al., 2007). Such *Unc93b1*^{3d/3d} animals (herein referred to as UNC93b1-mutant mice) proved highly susceptible to a systemic viral infection that normal mice survived (Tabeta et al., 2006). Parallel studies showed that humans with similar germline mutations of *Unc93b1* can have complete loss of UNC93b1 expression, PBMCs (peripheral blood mononuclear cells) that do not produce IFN α / β in response to TLR3, TLR7 or TLR9 ligands, PBMCs with reduced IFN α / β production following exposure to different viruses, and high lethality from HSE (herpes simplex encephalitis) (Casrouge et al., 2006). Conversely, these same individuals mount effective immune responses against other viruses, verifying the presence of redundant pathways that drive type-I IFN production previously shown to exist in rodents (Casrouge et al., 2006).

Since the susceptibility of UNC93b1-mutant mice to CNS (central nervous system) viral infection has not been well studied, we challenged these animals with an NSV (neuroadapted Sindbis virus) that causes a highly lethal encephalomyelitis. Titres of infectious virus, local inflammatory responses and clinical disease outcomes were compared with wild-type hosts. To characterize how CNS-derived myeloid cells known to express the nucleotide-sensing TLRs respond to NSV, primary microglia isolated from wild-type and UNC93b1-mutant animals were exposed to virus or synthetic TLR ligands *in vitro*. Our data show that multiple PRRs (pattern recognition receptors) and downstream signalling pathways, including but not limited to the endosomal TLRs, drive the breadth of microglial cytokine and chemokine production in response to NSV. Nonetheless, host survival from infection appears largely independent of these TLR-driven responses, even though it requires production of type-I IFN to control CNS virus replication. Furthermore, the heightened susceptibility of UNC93b1-mutant mice to NSV infection is likely explained by a mechanism other than a defect in endosomal TLR signalling, in large part because redundant pathways driving type-I IFN production keep early

CNS virus replication in check. In this light, therapeutics directed at these receptors and their signalling intermediates for the treatment of acute alphavirus encephalitis in humans must be carefully considered.

MATERIALS AND METHODS

Animals

Wild-type C57BL/6 mice and TLR3-deficient mice were purchased from The Jackson Laboratory. C57BL/6-*Unc93b1*^{3d/3d} mutant mice were obtained from the Mutant Mouse Regional Resource Center at the University of California. Mice rendered genetically deficient in the TLR adaptor protein, MyD88 (myeloid differentiation response gene 88) were a gift from Dr Steven Kunkel (University of Michigan). IRF7 (IFN response factor 7)-deficient mice were a gift from Dr Gabriel Nunez (University of Michigan). All animals were bred, housed and used on-site under specific pathogen-free conditions in strict accordance with guidelines set by the National Institutes of Health and protocols approved by the University Committee on the Use and Care of Animals. Mice were housed on a 10 h light/14 h dark cycle in ventilated cages containing not more than five animals per cage. Food and water were available *ad libitum*.

Induction of experimental viral encephalitis and other animal manipulations

To induce encephalomyelitis, 5–6-week-old mice were anaesthetized with isoflurane (Abbott Laboratories) and 1000 pfu (plaque-forming units) of NSV suspended in 10 μ l of PBS were inoculated directly into the right cerebral hemisphere of each animal. Some infected mice received 0.25 ml of clodronate liposomes intraperitoneally on days 1–5 post-infection to deplete circulating monocytes as described (King et al., 2009). Clodronate liposomes and PBS liposomes were both gifts from Roche Diagnostics. In other infected cohorts, mice were injected with 0.5 mg of RB6 (anti-Gr-1) or a control IgG on days 1, 3 and 5 post-infection to deplete circulating neutrophils as described (Carlson et al., 2008; Daley et al., 2008). The RB6 and control IgG antibodies were both gifts from Dr Benjamin Segal (University of Michigan). Most cohorts of infected mice were monitored daily for survival in accordance with approved animal protocols. Some groups of animals were killed at defined intervals post-infection to collect brain and spinal cord tissue for further analysis. Following intra-cardiac perfusion with ice-cold PBS, the left cerebral hemisphere and the lower half of the spinal cord were isolated from some animals, snap-frozen on dry ice and stored at -80°C for virus titration assays (see below). Remaining brain and spinal cord tissues from these mice were frozen at -80°C and used to generate

tissue homogenates for ELISA (see below). In other groups, PBS-perfused CNS tissue was used to isolated mononuclear cells for flow cytometric analysis (see below).

Virus titration assays

Ten percent (w/v) homogenates of each tissue sample were prepared in PBS, and serial 10-fold dilutions of each homogenate were assayed for plaque formation on monolayers of BHK-21 cells, as previously described (Irani and Prow, 2007). The results are presented as the geometric means \pm S.E.M. of the \log_{10} number of pfu per gram of tissue derived from a minimum of three animals at each time point.

Cytokine and chemokine assays

Frozen tissue samples were thawed, minced and homogenized in 0.5 ml of PBS containing a protease inhibitor cocktail and an RNase inhibitor (Sigma-Aldrich). After homogenates were centrifuged to pellet all remaining tissue debris, total protein content was measured in each extract and supernatants were diluted in PBS to a normalized total protein concentration. Microglial culture supernatants were used undiluted in these assays without further manipulation. Levels of individual cytokines and chemokines were measured directly in samples using commercial sandwich ELISA kits according to the manufacturers' instructions. The results for tissue samples reflect the means \pm S.E.M. of pg of chemokine/mg of tissue extracted protein derived from a minimum of three animals at each time point, while for culture supernatants reflect the means \pm S.E.M. pg of chemokine/ml of culture supernatant derived from triplicate cell culture wells. Quantification of TNF α (tumour necrosis factor α), IL (interleukin)-12p40, and CCL (CC motif ligand) 2 levels was performed using mouse OptEIA kits (BD Biosciences). IFN α , CCL3, CCL5 and CXCL13 (C-X-C motif ligand 13) levels were measured using DuoSet mouse ELISA kits (R&D Systems). The lower limit of detection for all these assays was 5 pg/ml.

Analysis and separation of CNS-infiltrating immune cell populations by flow cytometry

Perfused brains and/or spinal cords were minced into small fragments and pressed through a 70- μ m mesh sieve into HBSS (Hanks balanced salt solution) containing 10% FBS (fetal bovine serum) before digestion with collagenase (0.2 mg/ml; Worthington Biochemicals) and DNase (28 units/ml; Sigma-Aldrich) for 40 min at 37°C. Mononuclear cells were then isolated over a 30%/70% Percoll gradient (GE Healthcare Life Sciences) and washed with HBSS. All cells were resuspended in PBS containing 2% FBS and stained with fluorescently conjugated primary antibodies followed by flow cytometric analysis on a FACSCanto II flow cytometer (BD Biosciences). To quantify individual cell populations, cell suspensions were stained with antibodies against CD3, CD4, CD8, CD11b, CD45, Ly-6C and Ly-6G (all from eBioscience). A minimum of 10000 events within a defined forward and side

scatter gate containing all CD45+ cells were analysed to determine the proportion of each cell type in each experimental sample. The total number of each cell population present in individual brain or spinal cord specimens was then calculated by multiplying the total number of gradient-isolated cells from each sample (counted on a haemocytometer) by the proportion of cells labelled with each antibody. The results presented reflect the absolute numbers of cells collected from a minimum of three animals at each experimental time point. For intracellular cytokine staining, CNS mononuclear cells were cultured for 5 h in 20 ng/ml phorbol myristate acetate, 1 μ g/ml ionomycin and 5 μ g/ml brefeldin A, fixed and permeabilized using a Cytofix/Cytoperm kit (BD Biosciences), and stained with directly conjugated anti-CD4, anti-IFN- γ and anti-IL-17 antibodies (eBiosciences). For cell sorting, CNS myeloid cells were stained with a combination of CD45 and CD11b, and then separated into CD45^{low}/CD11b+ and CD45^{high}/CD11b+ populations using a MoFlo XDP High-Speed Cell Sorter (Beckman-Coulter). Individual cell populations pooled from 10 animals were stored at -20°C in PrepProtect RNA stabilization solution (Miltenyi Biotec) until RNA isolation was performed.

qPCR (quantitative PCR) analysis of type-I IFN expression by distinct CNS-derived mononuclear cell populations

Lysates of flow sorted myeloid cell populations were thawed and carefully removed from the PrepProtect solution. Total RNA was isolated from CD45^{low}/CD11b+ cells and CD45^{high}/CD11b+ cells pooled from the brains of ten naïve or NSV-infected mice using QIAshredder Kit and the RNeasy Mini Kit according to the manufacturer's instructions (Qiagen). cDNA was then generated using the SuperScript[®] III First Strand Synthesis System for reverse transcriptase-PCR (Invitrogen). qPCR was undertaken to measure *ifn β 1* mRNA transcripts using the MyiQ Single Color Real-Time PCR Detection System (Bio-Rad) and the *ifn β 1* primer/probe set in the TaqMan[®] Gene Expression Assay (Applied Biosystems). Data were analysed using the Δ/Δ Ct calculation, and the *ifn β 1* results were normalized to the Δ/Δ Ct values for β -actin mRNA.

Preparation and use of primary microglial cultures

Primary microglia were isolated and cultured from the cortices of 2–3-day-old mice as described (Esen and Kielian, 2005). When mixed glial cultures reached confluency after 7–10 days, flasks were shaken overnight at 200 rev./min at 37°C to detach microglia from the more firmly adherent astrocytes. Cells in suspension (>95% pure CD11b+ microglia) were collected and 1×10^5 cells plated into each well of 96-well plates. The next day, microglia were stimulated for 24 h either with NSV or a known TLR ligand in a total volume of 200 μ l as follows: 1×10^6 pfu NSV (infectious virus

particle-to-cell ratio of 10:1), 100 ng/ml *Escherichia coli* LPS (lipopolysaccharide) (List Biological Laboratories) (a TLR4 stimulus), 25 mg/ml poly(I:C) (polyinosinic:polycytidylic acid; Invivogen) (a TLR3 stimulus), 1 mM Ixoriobine (Invivogen) (a TLR7 stimulus) or 3 μ M unmethylated DNA ODNs (oligodeoxynucleotides) bearing CpG motifs (CpG-ODN) (Invivogen) (a TLR9 stimulus). Some experiments were conducted in the presence of 20 nM of Baf A1 (bafilomycin A1) (Sigma-Aldrich) to prevent the acidification of endosomes and thus blocking virus uncoating by inhibiting acid-induced fusion of viral envelopes and the endosomal membrane (Jan and Griffin, 1999). Pilot studies were performed using UV light-inactivated NSV generated by exposing the virus to a germicidal lamp (wavelength=254 nm) at a distance of 5 cm for 1 h at 4°C. Heat inactivation of NSV was accomplished by maintaining the pathogen at 60°C for 15 min before use. Inactivation of viral infectivity was confirmed via the plaque titration assay described above. At the end of the 24-h microglial treatment period, culture supernatants were collected and stored at -80°C until cytokine and chemokine analyses could be performed by ELISA assays. Microglial viability was assayed using well-established MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] assay.

Statistical analysis

The Prism 5.0 software package (GraphPad Software) was used for all statistical analyses. Differences in survival among cohorts of mice were measured using a log-rank (Mantel-Cox) test. Unpaired Student's *t* test was used to assess differences between different experimental groups at single time points. A two-way ANOVA test was applied when comparing different experimental groups over time. In all cases, differences at a $P < 0.05$ level were considered significant.

RESULTS

Effects of UNC93b1 mutation on the course of NSV encephalitis in mice

Humans with germline *Unc93b1* mutations were found to exhibit endosomal TLR signalling defects and have high susceptibility to HSE even as they mounted more normal immune responses against other viruses (Casrouge et al., 2006). To further investigate how the UNC93b1 protein affects CNS viral pathogenesis, wild-type and UNC93b1-mutant mice were challenged with NSV, a neuroadapted member of the alphavirus family that causes lethal encephalomyelitis in rodents and is closely akin to the neurotropic alphaviruses that infect humans. Outcome in this model is highly dependent on an effective type-I IFN response to control early CNS virus replication (Byrnes et al., 2000;

Ryman et al., 2000). *In vivo* challenge experiments showed that UNC93b1-mutant animals were very susceptible to infection, with significantly accelerated death compared with wild-type controls (Figure 1A). Surprisingly, however, this poorer outcome was not explained by impaired type-I IFN production in the CNS (Figures 1B and 1C), or by excessive virus replication in target tissues (Figures 1D and 1E). Thus, the wild-type UNC93b1 protein must regulate some host response that protects mice from NSV encephalitis via a mechanism other than one either directly targeting the pathogen itself or the main antiviral immune effector.

CNS host responses in wild-type and UNC93b1-mutant mice during NSV encephalitis

To investigate the effects of UNC93b1 mutation on CNS host responses elicited during NSV encephalitis, brain and spinal cord leucocytes were characterized by flow cytometry. Quantification of T-cells and myeloid cells was emphasized, since these populations constitute the bulk of the parenchymal infiltrate elicited during infection (Moench and Griffin, 1984; Irani and Griffin, 1990). The total number of CD45+ leucocytes isolated from the CNS of infected animals was similar between UNC93b1-mutant mice and wild-type controls (Figure 2A). Tissue-infiltrating CD3+ T-cells were also found in comparable numbers between the two hosts (Figure 2B), and CD4+ and CD8+ T-cell subsets occurred in equal proportions (data not shown). Within the CD4+ T-cell population, cells capable of making IFN γ or IL-17 were detected with equivalent frequencies (data not shown). These data would seem to exclude a role for UNC93b1 in T-helper cell differentiation in this disease model. Conversely, fewer CD11b+ myeloid cells were isolated from both the brains and spinal cords of UNC93b1-mutant animals at later stages of NSV infection (Figure 2C). These CD11b+ cells were both CD45^{high}, likely infiltrating monocytes and neutrophils or highly activated microglia, as well as CD45^{low}, likely microglia in a more quiescent state (Figure 2D). Separation of these discrete CD11b+ populations via flow sorting followed by analysis of the mRNA content via qPCR of cells pooled from multiple animals revealed that both CD45^{low} and CD45^{high} cells were important sources of type-I IFN in the CNS during the early stages of infection (Figure 2E). The observed differences in CD11b+ cell numbers found in the CNS suggest that the accelerated mortality seen in UNC93b1-mutant mice could be due to impaired recruitment of a protective myeloid cell population from the periphery. Alternatively, the local tissue microglial response to infection could differ in some way other than type-I IFN production that influences overall host survival.

Effects of CD11b+ monocyte or neutrophil depletion during NSV encephalitis

Since higher numbers of CNS myeloid cells were associated with the improved outcome of wild-type versus

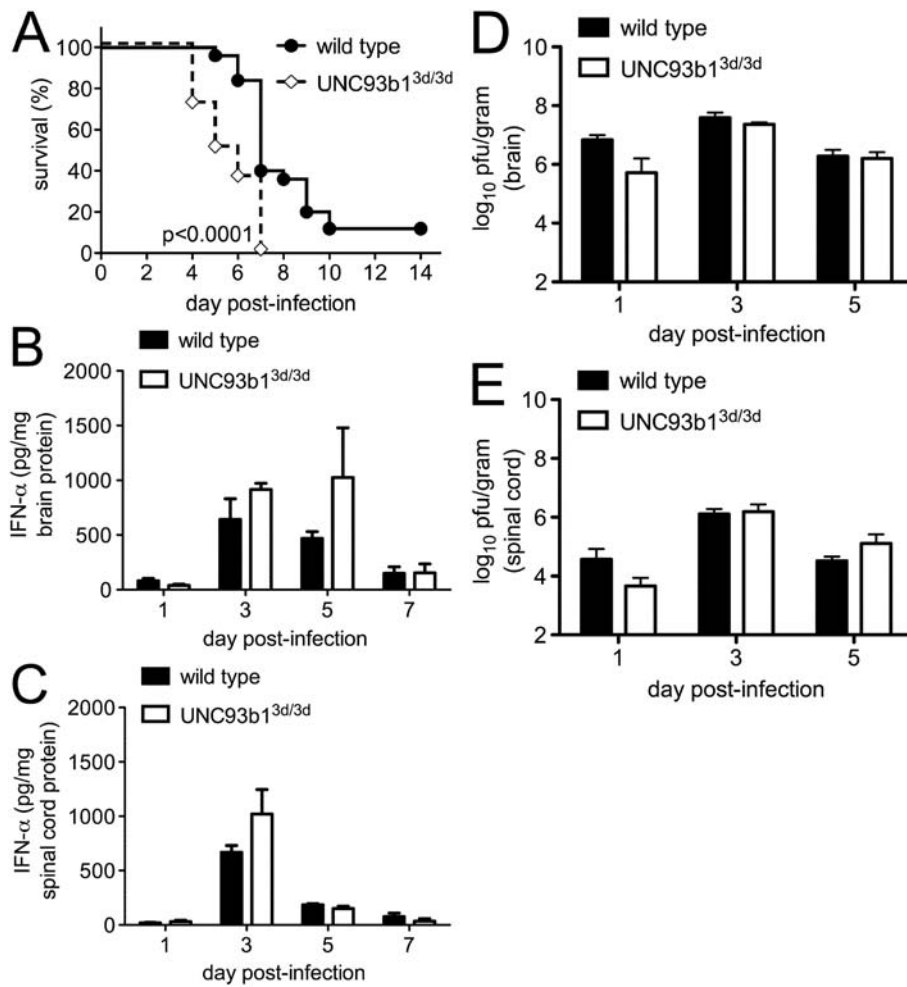


Figure 1 Mutation of the ER protein, UNC93b1, alters the pathogenesis of NSV encephalitis in mice. UNC93b1-mutant animals are more susceptible to fatal NSV infection than wild-type controls ($n=20$ mice per group) (A). This heightened disease susceptibility is not associated with altered type-I IFN levels in the brain or spinal cord as measured by ELISA (B, C), nor is it due to unrestrained viral replication in either tissue compartment as determined by plaque assay (D, E). Type-I IFN levels and viral titres in these experiments were measured in triplicate CNS samples collected at each time point from a single cohort of mice.

UNC93b1-mutant mice (Figure 2C), *in vivo* depletions were performed to investigate whether two distinct CD45^{high}/CD11b+ cell types normally present in circulation might enter the CNS during NSV encephalitis to facilitate host survival. In the first approach, wild-type animals were infected with NSV and then treated with clodronate-loaded liposomes that get phagocytosed by circulating monocytes, causing their apoptotic destruction (King et al., 2009). Flow cytometry demonstrated that CD45^{high}/CD11b+ monocytes had largely been eliminated from the CNS of clodronate liposome-treated animals compared with controls that received PBS liposomes on day 6 post-infection (Figures 3A and 3B). Likewise, Ly-6C+ monocytes were virtually undetectable in the blood of these animals 24 h after their last clodronate liposome treatment (data not shown). Nonetheless, the two cohorts of animals

showed no difference in overall disease survival (Figure 3C). In the second set of experiments, wild-type mice were treated with the monoclonal antibody, RB6, used to deplete circulating Gr-1+ neutrophils *in vivo* (Carlson et al., 2008; Daley et al., 2008). Treatment was highly effective in eliminating the desired population from the CNS on day 6 post-infection compared with animals given a control antibody (Figures 4A and 4B), but depletion of these cells also had no effect on disease outcome (Figure 4C). Anti-RB6-treated mice had almost no Ly-6G+ cells present in circulation at the same time that CNS tissues were collected (data not shown). These data exclude the involvement of the two main circulating CD45^{high}/CD11b+ cell populations in NSV pathogenesis. By extension, they suggest that wild-type UNC93b1 may act through an endogenous CNS cell-type to slow disease progression.

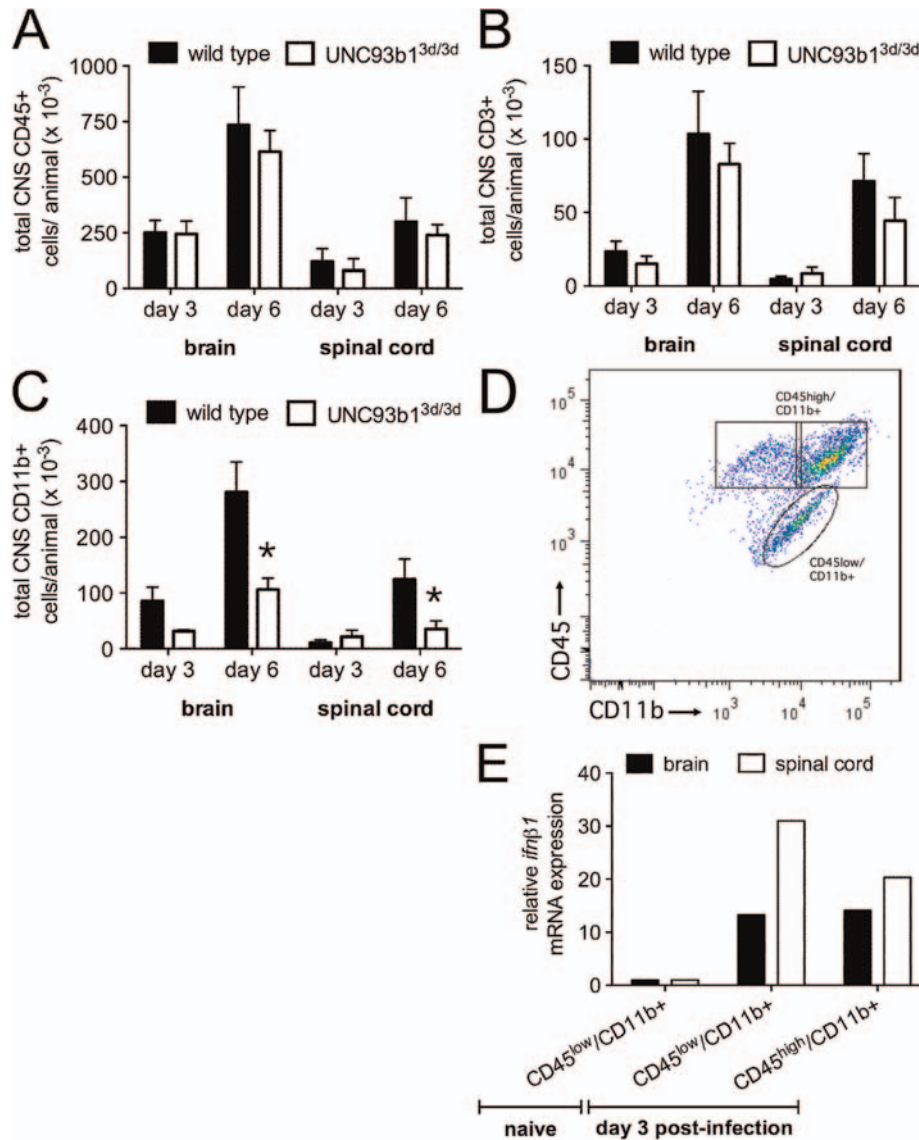


Figure 2 Analysis of CNS infiltrates in wild-type and *UNC93b1*-mutant mice during NSV encephalitis by flow cytometry and qPCR. Although the total numbers of CD45+ leucocytes (A) and CD3+ T-cells (B) present in brain and spinal cord are comparable between these two hosts during NSV infection, fewer CD11b+ myeloid cells accumulate at both CNS sites over time in *UNC93b1*-mutant mice (C). Tissue cell numbers were determined as described in Materials and Methods section in a single cohort of mice using samples collected from three animals at each time point. By flow cytometry, these CD11b+ cells in the brains of wild-type mice on day 6 post-infection include both CD45^{high} and CD45^{low} populations (D). Flow sorting of these individual cell populations pooled from brains of naive or day 3 post-infection mice shows that both CD45^{low}/CD11b+ and CD45^{high}/CD11b+ cells contain type-I IFN mRNA by qPCR analysis (E). This assay was performed once using cells pooled from 10 naive or 10 NSV-infected mice as source material for total cellular RNA extraction.

Cytokine and chemokine responses generated by *UNC93b1*-mutant microglia *in vitro*

As microglia express multiple TLRs and become activated early during NSV encephalitis (Irani and Prow, 2007; Prow and Irani, 2007), primary cultures of these cells were prepared from wild-type and *UNC93b1*-mutant animals for *in vitro* study. When directly exposed to NSV at an infectious particle-to-cell ratio of 10:1, primary microglia did not become productively infected or show any loss of cell viability over a

24-h culture interval (data not shown). Still, both NSV and various synthetic TLR ligands triggered a broad range of inflammatory mediators in wild-type cells. This included production of a subset of pro-inflammatory cytokines (Figures 5A and 5B), type-I IFN (Figure 5C) and multiple chemokines (Figure 6). Since many of these mediators are induced in the CNS during NSV encephalitis *in vivo* (Wesselingh et al., 1994; Irani and Prow, 2007), microglia may be important early integrators of the host response to infection.

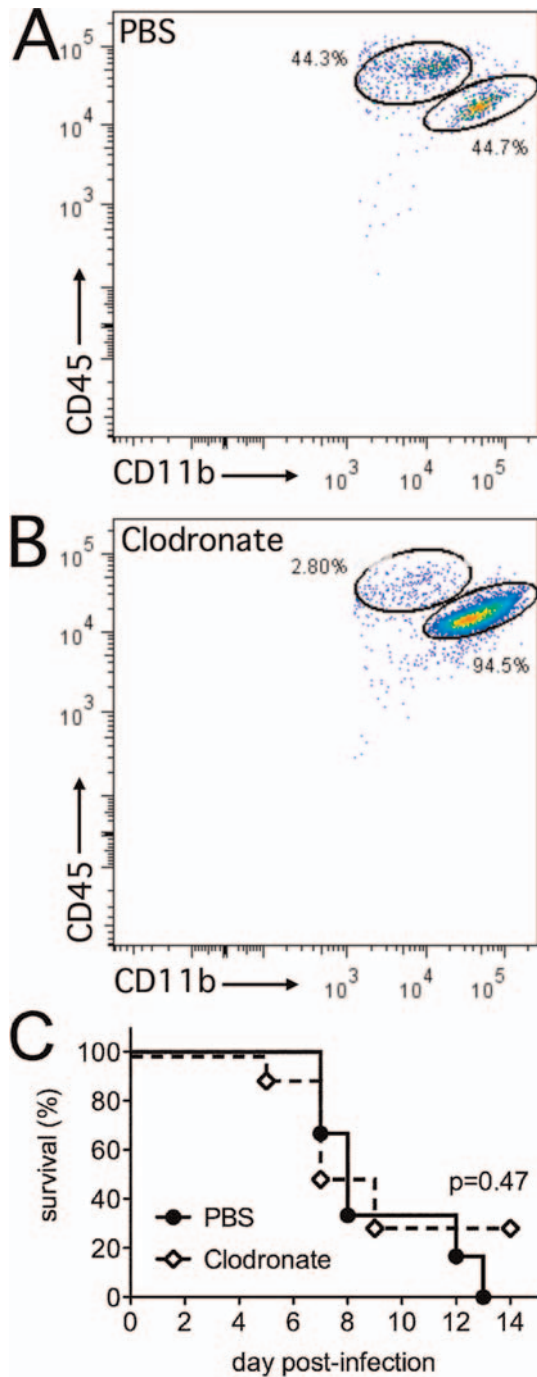


Figure 3 Effects of monocyte depletion on the course of NSV encephalitis in wild-type mice
 Treatment with clodronate-loaded liposomes effectively prevents CNS infiltration of CD45^{high} circulating monocytes on day 6 of NSV encephalitis compared with PBS-loaded liposomes (A, B). Dot plots show staining when gated on CD11b⁺ cells only. Systemic depletion of these cells has no effect on the outcome of NSV encephalitis (C). Data are representative of two separate experiments performed under identical conditions.

To better understand whether the endosomal TLRs contribute to microglial activation following exposure to NSV, cells derived from UNC93b1-mutant mice were

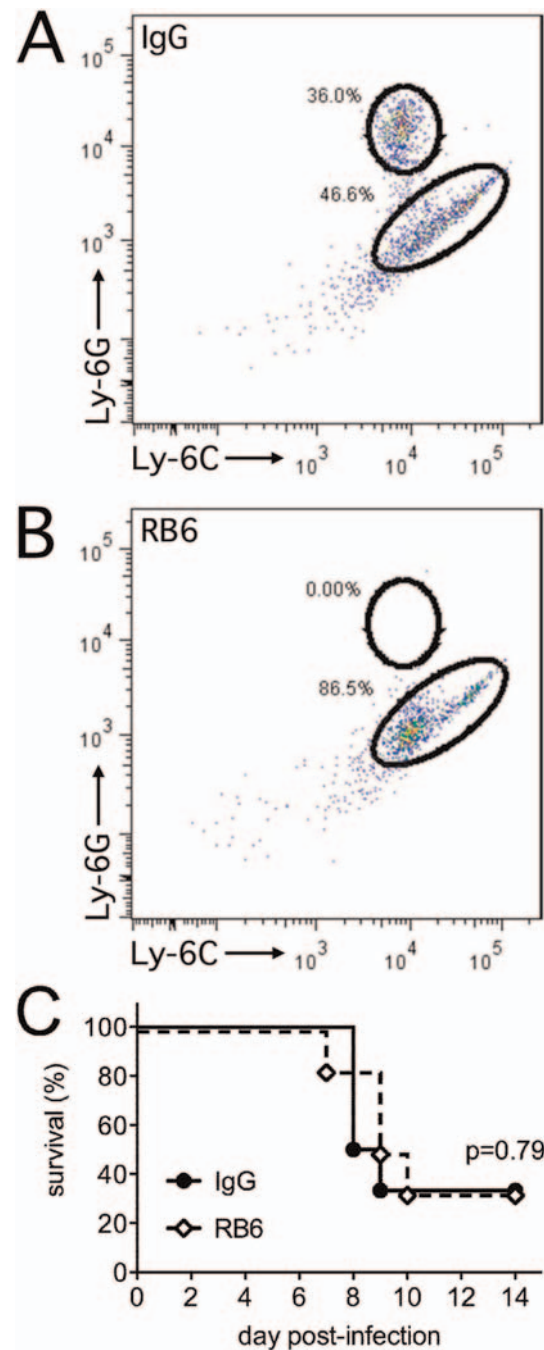


Figure 4 Effects of neutrophil depletion on the course of NSV encephalitis in wild-type mice
 RB6 antibody (anti-Gr-1) treatment effectively prevents CNS infiltration of Ly-6G⁺ circulating neutrophils on day 6 of NSV encephalitis compared with a control IgG (A, B). Dot plots show staining when gated on CD45^{high}/CD11b⁺ cells only. Systemic depletion of these cells has no effect on the outcome of NSV encephalitis (C). Data are representative of two separate experiments performed under identical conditions.

compared with those from wild-type hosts. These assays showed that pro-inflammatory cytokine responses were ablated in mutant cells following stimulation with NSV or synthetic TLR3, TLR7 or TLR9 ligands (Figures 5A and 5B), as

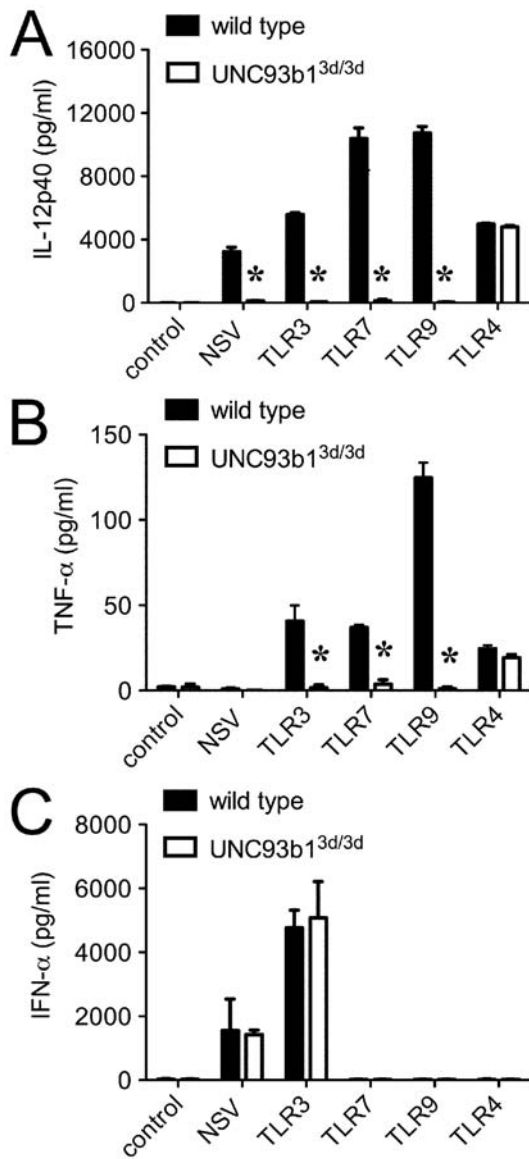


Figure 5 *In vitro* cytokine production by primary microglia cultures derived from either wild-type or UNC93b1-mutant mice. Cultures were prepared as outlined in Materials and Methods section. Three wells were left untreated, exposed to NSV or stimulated with synthetic ligands for the TLRs indicated also as described in Materials and Methods section. Cytokine concentrations were measured in culture supernatants 24 h later using ELISA-specific for IL-12p40 (A), TNF α (B) and IFN α (C). The production of these mediators was measured using primary cells prepared on two separate occasions.

were the chemokine responses triggered by TLR7 or TLR9 ligands (Figure 6). LPS, a TLR4 ligand that acts at the cell surface rather than in endosomes, stimulated equivalent production of all mediators in both cell types (Figures 5 and 6). IFN α and various CC chemokine ligands were also generated at similar levels by cells of both genotypes in response to NSV or poly(I:C) (Figures 5C, 6A and 6C), although virus-induced production of CXCL13 was significantly reduced in mutant versus wild-type cells (Figure 6D).

In addition to being a known TLR ligand, poly(I:C) also triggers the cytoplasmic RNA sensor, MDA5 (melanoma-differentiation-associated gene 5) (Kato et al., 2006). The activation of this pathway could explain why both poly(I:C) and NSV still provoked certain responses in UNC93b1-mutant cells. Nonetheless, these data show that NSV induces the production of a broad range of inflammatory mediators by primary microglia and that such responses are activated through non-overlapping pathways, only some of which are triggered by the endosomal TLRs.

Viral determinants that activate microglial cytokine and chemokine production

Pilot assays showed that NSV particles exposed to heat or UV light did not trigger microglial cytokine or chemokine production (data not shown). This suggests that damage to the viral genome prevents its recognition by these cells. At the onset of the alphavirus replication cycle, receptor-mediated endocytosis brings the virus into early endosomes where exposure to an acidic pH induces conformational changes that allow fusion of the viral envelope with the endosomal membrane and delivery of the single-stranded viral RNA genome into the cytoplasm (Strauss and Strauss, 1994). Since neurons can detect and respond to NSV at the time of cell entry (Jan and Griffin, 1999), we tested whether blockade of viral fusion had any effect on microglial cytokine and chemokine production. Baf A1, a selective inhibitor of the vacuolar proton-ATPase (Drose and Altendorf, 1997), blocks endosomal acidification and prevents NSV from uncoating in neuronal cells (Jan and Griffin, 1999). We found that wild-type microglia pretreated for 30 min with 20 nM Baf A1 prior to virus exposure were significantly impaired in their capacity to generate IL-12p40, IFN α and several CC chemokine ligands compared with untreated control cells (Figure 7). Drug treatment had no effect on cell viability or on the capacity of microglia to trigger IFN α production in response to poly(I:C) (data not shown). We conclude that NSV must uncoat itself in primary microglia in order to trigger innate immune signalling. This suggests that intact viral nucleic acids, rather than viral envelope glycoproteins, are the main stimuli that activate these cells. Unfortunately, any subsequent requirement for endosomal versus cytosolic signalling pathways cannot be inferred from these data since all events downstream of virus entry are blocked in Baf A1-treated cells.

Downstream signalling intermediates involved in the microglial response to NSV

To further understand how various microglial PRRs signal in response to NSV, cells derived from other mutant animals were examined. All TLR signalling pathways except ones downstream of TLR3 converge on the common intracellular adaptor protein, MyD88 (Akira and Takeda, 2004). Both the TLRs and the cytoplasmic RLRs (retinoic acid-inducible gene I-like receptors) also activate the IRFs, particularly IRF3 and

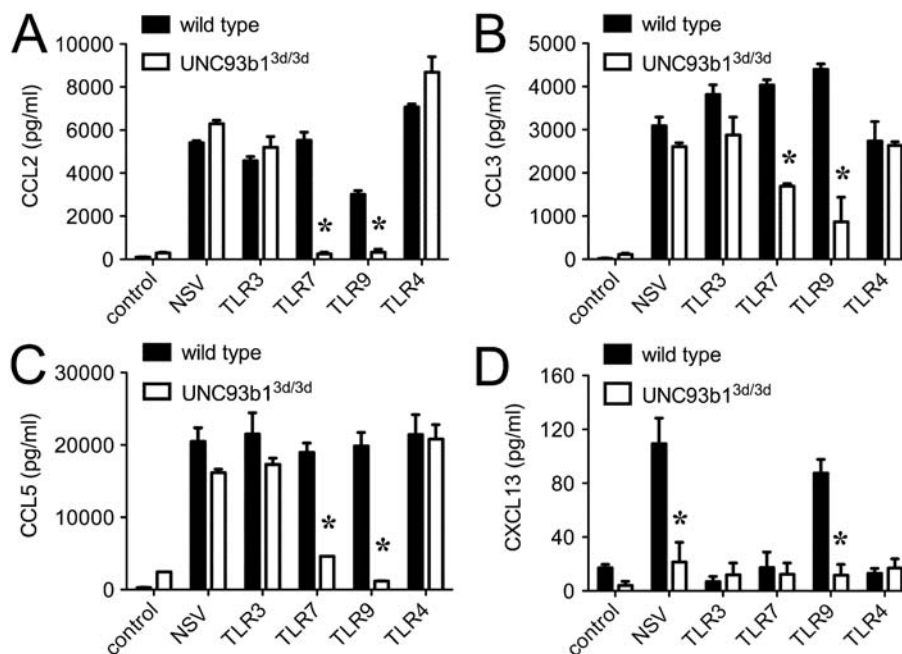


Figure 6 *In vitro* chemokine production by primary microglia cultures derived from either wild-type or UNC93b1^{3d/3d} mutant mice. Cultures were prepared as outlined in Materials and Methods section. Three wells were left untreated, exposed to NSV or stimulated with synthetic ligands for the TLRs indicated also as described in Materials and Methods section. Chemokine concentrations were measured in culture supernatants 24 h later using ELISA-specific for CCL2 (A), CCL3 (B), CCL5 (C) and CXCL13 (D). The production of these mediators was measured using primary cells prepared on two separate occasions.

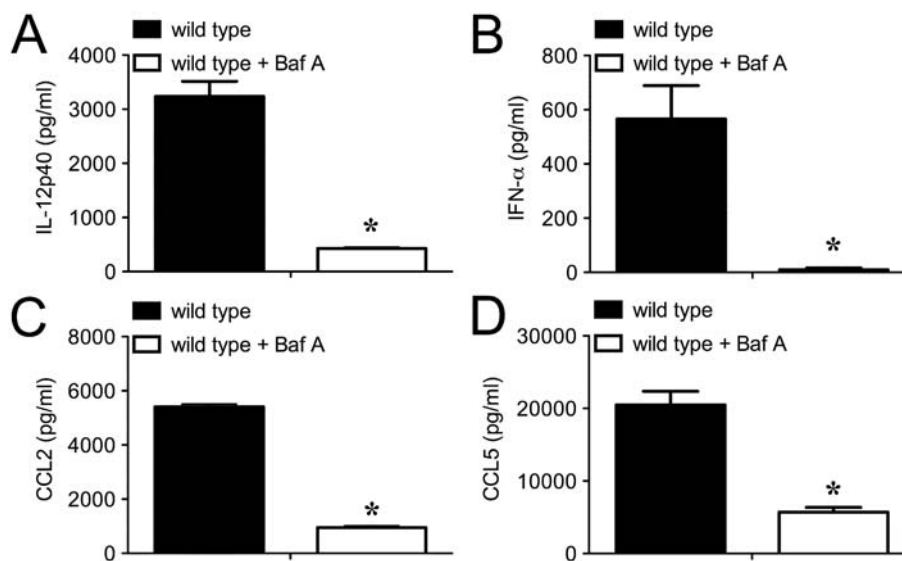


Figure 7 Effects of Baf A1, a pharmacological inhibitor of virus uncoating in endosomes, on cytokine and chemokine production by primary microglia cultures prepared from wild-type mice and then exposed to NSV. Some wells were treated with Baf A1 as outlined in Materials and Methods section. A minimum of three wells either without or with Baf A1 pretreatment were then exposed to NSV, and cytokine and chemokine concentrations measured by ELISA in culture supernatants 24 h later using ELISA-specific for IL-12p40 (A), IFN- α (B), CCL2 (C) and CCL5 (D). The production of these mediators without or with Baf A1 treatment was measured using primary cells prepared on two separate occasions.

IRF7, to drive the release of type-I IFN (Genin et al., 2009). We found that virus-induced production of IL-12p40 was fully ablated in primary microglia derived from MyD88-deficient hosts (Figure 8A). Since this defect was also observed in UNC93b1-mutant cells (Figure 5A), it is likely that TLR7 and/or TLR9 signalling stimulate release of this particular mediator in response to NSV. The production of IFN α , on the other hand, depended entirely on IRF7 activation (Figure 8B), which could be induced via upstream TLR3 and/or RLR activation. However, because TLR3-deficient cells generated significant levels of IFN α in response to both NSV and poly(I:C) (Figure 8C), cytoplasmic RLR pathways such as those triggered by MDA5 are presumably involved. Virus-induced production of CCL2 by microglia was partially MyD88-dependent (Figure 8D). Since its release was unaffected in UNC93b1-mutant cells exposed to NSV (Figure 6A), signals delivered by a non-endosomal TLR as well as some non-TLR pathway must both be involved in the induction of this mediator. Finally, NSV actually triggered augmented production of CXCL13 in the absence of IRF7 (Figure 8E), suggesting that type-I IFN might negatively regulate this chemokine. Overall, these data confirm that multiple PRRs and downstream signalling pathways drive the breadth of cytokine and chemokine production by primary microglia in response to NSV (Table 1).

Impact of specific PRR signalling defects on the outcome of NSV encephalitis

Given both the redundancy and breadth of virus-induced PRR activation in cultured microglial cells, involvement of these signalling intermediates during NSV encephalitis was examined *in vivo*. As was observed in UNC93b1-mutant mice (Figure 1A), IRF7-deficient animals were highly susceptible to fatal NSV infection (Figure 9A). In this case, however, CNS virus replication was poorly controlled; upwards of 100-fold more infectious NSV was found in the brains and spinal cords of mutants compared with wild-type animals (Figures 9B and 9C). These IRF7-deficient mice failed to generate any measurable amount of type-I IFN in the CNS over the course of infection (data not shown). In contrast, the survival of both MyD88- and TLR3-deficient mice was not significantly different from wild-type controls (Figures 9D and 9E), and neither of these mutant hosts showed any defect in type-I IFN production within the CNS over the course of NSV infection (Figure 9F). These findings reinforce the importance of type-I IFN for the control of NSV replication and host survival, as previously reported during infections caused by less virulent viral strains (Byrnes et al., 2000). Although it is possible that TLR3- and MyD88-dependent responses fully compensate for one another during NSV infection, our *in vitro* data strongly implicate the actions of a non-TLR-dependent pathway in the type-I IFN response (Figures 5C and 8C). This suggests that TLR-dependent responses do not contribute to disease outcome, and by extension, that the wild-type UNC93b1 protein confers protection via some mechanism independent of endosomal TLR signalling.

DISCUSSION

Alphaviruses infect neurons of the brain and spinal cord, and neuronal survival controls *in vivo* outcome in acute encephalitis models (Lewis et al., 1996; Kerr et al., 2002). Not only can these pathogens directly kill the target cells they infect, but they also provoke substantial bystander damage to uninfected neurons (Havert et al., 2000; Nargi-Aizenman et al., 2004; Prow and Irani, 2008). Indeed, blockade of this bystander neuronal damage greatly reduces NSV-induced mortality without affecting CNS viral replication or clearance (Nargi-Aizenman et al., 2004; Irani and Prow, 2007; Prow and Irani, 2007; 2008). Current data suggest that innate host responses arising from activated microglia trigger changes in adjacent astrocytes that drive this bystander neuronal injury (Darman et al., 2004; Irani and Prow, 2007; Prow and Irani, 2007, 2008; Carmen et al., 2009). Blockade of the type-I IFN response, however, causes unrestrained CNS virus replication early in disease that is rapidly lethal to the host (Byrnes et al., 2000). Thus, early activation of innate immunity is crucial in alphavirus pathogenesis. We sought to identify the signalling receptors triggering microglial production of type-I IFN and other inflammatory mediators that influence disease outcome. Our data show that NSV activates microglia via multiple PRRs, that these cells are an important source of type-I IFN in the CNS during early stages of disease, that type-I IFN production occurs independent of TLR signalling and that the ER protein, UNC93b1, plays an unexpected and still poorly understood protective role during infection.

Although other investigators have shown that RNA viruses can trigger microglia to produce inflammatory cytokines (Olson and Miller, 2004), our studies reveal a previously unreported breadth and complexity of this response. Not only do microglia make a wide range of cytokines and chemokines following exposure to NSV, but it is clear that such responses are driven by non-overlapping signalling pathways activated by multiple PRRs (Table 1). UNC93b1 and MyD88 are both required for generating certain pro-inflammatory cytokines such as IL-12p40 (Figures 5A and 8A), meaning that an endosomal pathway utilizing TLR7 and/or TLR9 is involved. The CC ligand chemokine responses, on the other hand, occur independently of UNC93b1 and are only partially dependent on MyD88 (Figures 6A–6C and 8D), demonstrating that signals delivered by a non-endosomal TLR and some non-TLR pathways are both required. Like IL-12p40, production of CXCL13 by microglia depends on both UNC93b1 and MyD88 (Figures 6D and 8E), but this chemokine is also negatively regulated via an IRF7-dependent mechanism (Figure 8E). Most importantly, production of type-I IFN absolutely requires IRF7 and is not activated by any of the classical endosomal TLRs (Figures 5C, 8B and 8C). This complexity presents opportunities to subvert an individual pathway for therapeutic purposes during infection. In particular, interventions that target the pro-inflammatory cascades resulting in bystander neuronal injury might be feasible without disrupting the type-I IFN response.

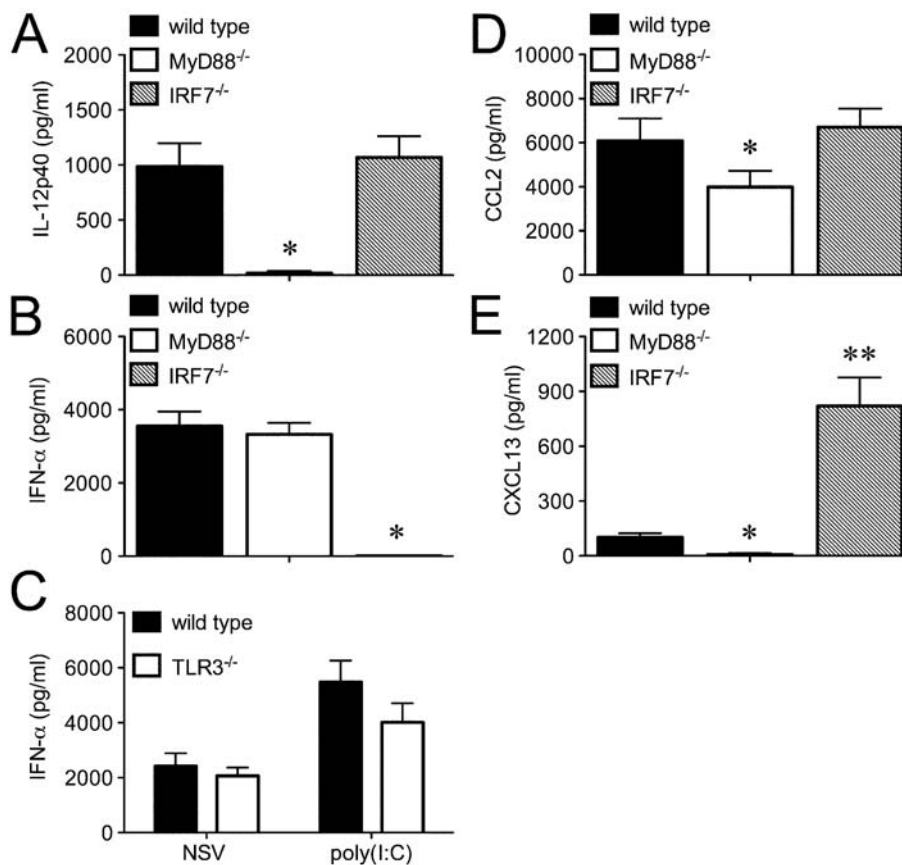


Figure 8 *In vitro* chemokine production by primary microglia cultures derived from either wild-type, MyD88^{-/-}, IRF7^{-/-} or TLR3^{-/-} mice following exposure to either NSV (A, B, D, E) or NSV or poly(I:C) (C)

Cultures were prepared as outlined in Materials and Methods section. A minimum of three wells were exposed to NSV or poly(I:C) as described in Materials and Methods section. Cytokine and chemokine concentrations were measured by ELISA in culture supernatants 24 h later using ELISA-specific for IL-12p40 (A), IFN- α (B, C), CCL2 (D) and CXCL13 (E). The production of these mediators was measured using primary cells prepared on two separate occasions.

Until now, both the source and mechanism of type-I IFN production in the CNS during SV (Sindbis virus) encephalitis has remained poorly understood. IFN α/β receptor null mice are highly susceptible to a number of viruses, including the mosquito-borne alphaviruses (Muller et al., 1994; Huang et al., 1995; Grieder and Vogel, 1999). Replication of wild-type SV is strongly inhibited by IFN α *in vitro* (Depres et al., 1996), and SV encephalitis generates large amounts of type-I IFN within the CNS *in vivo* (Vilcek, 1964; Sherman and Griffin, 1990). Mice deficient in the IFN α/β receptor rapidly succumb to attenuated strains of SV; direct intracerebral inoculation results in uncontrolled CNS virus replication and death that

normal mice survive (Byrnes et al., 2000), while systemic challenge causes fatal disease with expanded tissue tropism and high peripheral and CNS tissue viral titres not seen in receptor-sufficient hosts (Byrnes et al., 2000; Ryman et al., 2000). Our data show that for the virulent NSV strain, the type-I IFN response within the CNS is triggered via TLR-independent pathways (Figures 8B, 8C and 9F). Furthermore, early type-I IFN production arises from both microglia and infiltrating myeloid cells (Figure 2E), and is highly IRF7-dependent (Figure 8B and data not shown). Mice incapable of making type-I IFN have high lethality and uncontrolled CNS virus replication following NSV challenge (Figures 9A–9C).

Table 1 NSV triggers the production of inflammatory mediators in primary microglia via multiple non-overlapping signalling pathways

Mediator	UNC93b1-dependent	MyD88-dependent	IRF7-dependent
IL-12p40	+	+	–
CCL2	–	+/-	–
CXCL13	+	+	+*
IFN α	–	–	+

* Inhibitory.

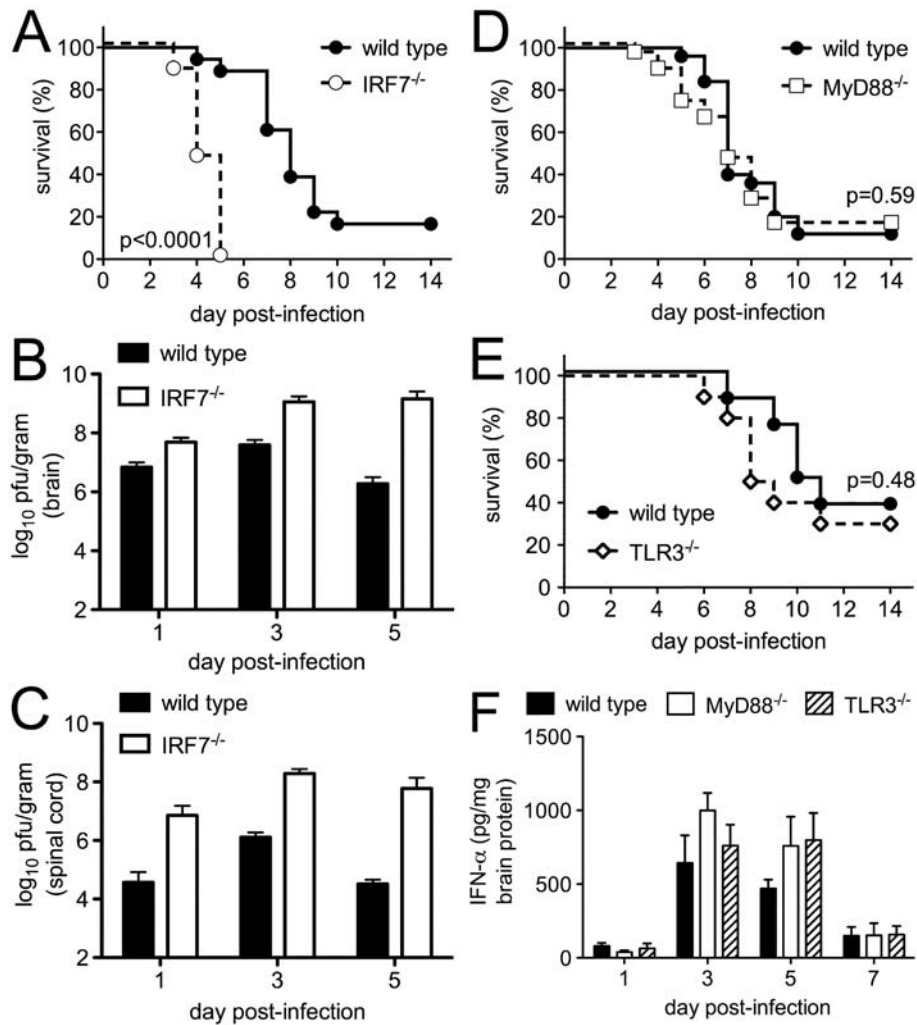


Figure 9 Pathogenesis of NSV encephalitis in IRF7^{-/-}, MyD88^{-/-} and TLR3^{-/-} mice
 IRF7-deficient mice are highly susceptible to fatal NSV infection compared with wild-type controls ($n=10$ per group) (A). This heightened disease susceptibility is associated with unrestrained viral replication in the brain (B, $P=0.0009$ by two-way ANOVA) and spinal cord (C, $P=0.0002$ by two-way ANOVA). Neither MyD88^{-/-} nor TLR3^{-/-} mice show altered disease susceptibility compared with wild-type controls ($n=12$ per group) (D, E). Both MyD88^{-/-} and TLR3^{-/-} deficient hosts generate equivalent levels of type-I IFN in the CNS over the course of infection compared with wild-type controls (F).

Since expression of IRF7 is restricted to haematopoietic cells and can be induced by various external stimuli (Ning et al., 2011), it is possible that the early type-I IFN response could be augmented by manipulating IRF7 levels. Our findings shed important light on this critical antiviral host defence pathway during alphavirus encephalitis.

Because of their capacity to detect common DNA and RNA replication intermediates of many viral pathogens, the endosomal TLRs are considered central to innate host defence against viral infections. Recently, a point mutation of the ER chaperone protein, UNC93b1, was found to ablate TLR3, TLR7 and TLR9 signalling in mice because the mutant protein fails to transfer the receptors from the ER to their natural location in endosomes (Tabeta et al., 2006; Brinkmann et al., 2007; Kim et al., 2008). Macrophages from these mice have

profoundly impaired cytokine responses, including type-I IFN production, when exposed to synthetic ligands that activate the endosomal TLRs (Tabeta et al., 2006; Brinkmann et al., 2007). Such animals are also highly susceptible to viral pathogens such as murine CMV (cytomegalovirus) due to uncontrolled systemic virus replication (Tabeta et al., 2006). Together, these data suggested that UNC93b1-mutant mice should be highly susceptible to NSV challenge because of their impaired host response to infection. Although enhanced disease susceptibility proved to be true, mutant animals mounted a surprisingly normal type-I IFN response and fully controlled NSV replication in the CNS (Figure 1). We have not yet identified a mechanism through which the wild-type UNC93b1 protein prolongs survival in this disease. Looking at other models, however, animals carrying this mutation have a

heightened susceptibility to experimental HSE without an impaired CNS type-I IFN response or altered virus replication in the brain (Wang et al., 2011). On the other hand, UNC93b1-mutant mice were recently found to have unrestrained parasite replication during experimental *Toxoplasma gondii* infection fully independent of their TLR signalling defects (Melo et al., 2010). These data, in conjunction with our findings, reveal that this chaperone protein serves some critical TLR-independent function in the setting of intracellular infection.

Another notable result among the data reported here is the apparent role of IRF7 in dampening microglial production of the lymphoid chemokine, CXCL13 (Figure 8E). This finding suggests that type-I IFNs act in either an autocrine or paracrine manner to negatively regulate the generation of this mediator within the CNS. Under normal circumstances CXCL13 helps form germinal centres in lymphoid organs (Forster et al., 1996; Legler et al., 1998; Bagaeva et al., 2006), but its ectopic expression has been reported around B-cell aggregates that develop in the inflamed meninges of mice with EAE (experimental autoimmune encephalomyelitis) and humans with multiple sclerosis (Magliozzi et al., 2004, 2007; Serafini et al., 2004; Aloisi et al., 2008; Lalor and Segal, 2010). Perivascular mononuclear cells and parenchymal microglia within active multiple sclerosis plaques also express CXCL13 (Krumbholz et al., 2006), and cerebrospinal fluid levels of the protein are elevated in samples from patients with the RRMS (relapsing-remitting form of multiple sclerosis) and decline with successful therapy (Seltebjerg et al., 2009; Piccio et al., 2010; Khademi et al., 2011; Ragheb et al., 2011). The production of CXCL13 is pathogenic during EAE by sustaining myelin-specific CD4+ T-cell responses, and *in vivo* neutralization of CXCL13 ameliorates disease (Bagaeva et al., 2006). Since subcutaneous or intramuscular administration of IFN β has been convincingly shown to reduce relapse rate, disability progression and the formation of new brain and spinal cord lesions in patients with RRMS (The IFNB Multiple Sclerosis Study Group, 1993; UBC MS/MRI Study Group and The IFNB Multiple Sclerosis Study Group, 1993; The Multiple Sclerosis Collaborative Research Group, 1996; Prevention of Relapses and Disability by IFN β 1a Subcutaneously in Multiple Sclerosis Study Group, 1998; The Multiple Sclerosis Collaborative Research Group, 1998), its capacity to regulate CXCL13 production may be an explanation for its mechanism of action in this complex neuroinflammatory disease. Further studies are underway to address this important question.

In summary, data presented here show that microglial activation in response to a neurotropic alphavirus is a complex process involving multiple, non-overlapping PRRs. Although endosomal TLR, non-endosomal TLR and non-TLR pathways all contribute to virus-induced cytokine and chemokine production by these cells, the type-I IFN response is activated in a TLR-independent manner. Microglia are an important source of type-I IFNs within the CNS during the initial stages of infection, and this response is critical for both early control of virus replication within the target tissue and

subsequent host survival. Recent studies show that activated microglia trigger a cascade of cellular events, including disruption of the homeostatic support provided by astrocytes that augment neuronal injury during NSV encephalomyelitis (Darman et al., 2004; Irani and Prow, 2007; Prow and Irani, 2007, 2008; Carmen et al., 2009). Being able to selectively target such pro-inflammatory mediators by means of disrupting the receptors and pathways that activate them, without impacting the type-I IFN response, raises hope that more precisely targeted immunotherapies can be developed for these life threatening human infections.

ACKNOWLEDGEMENT

We thank Dr Benjamin Segal for helpful advice and for critical review of our manuscript.

FUNDING

These studies were supported by a grant from the National Institutes of Health [AI057505].

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Received 2 March 2012/30 March 2012; accepted 3 April 2012

Published as Immediate Publication 3 April 2012, doi 10.1042/AN20120016
