Toll-like receptor 7-induced immune response to cutaneous West Nile virus infection

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INTRODUCTION

West Nile virus (WNV), a single-stranded (ss)RNA flavivirus, has been endemic in Africa, Asia, Europe and, more recently, North America (Granwehr et al., 2004; Pletnev et al., 2006). The virus is maintained in an enzootic cycle that involves mosquitoes and birds. Human infection results primarily from mosquito bites, and symptoms include fever, headache, myalgia, meningitis and encephalitis. Neurological disease (including encephalitis) has been observed in >30% of confirmed WNV cases, with a higher frequency in the elderly and immunocompromised (Campbell et al., 2002; Pletnev et al., 2006). Human vaccines are not yet available. Treatment is mostly non-specific and supportive.

Murine infection via subcutaneous or intraperitoneal injection has been an effective in vivo experimental model for human infections to investigate WNV pathogenesis and host immune response (Beasley et al., 2002; Samuel & Diamond, 2006; Wang & Fikrig, 2004). Work from the murine model has demonstrated that type I interferons (IFNs), γδ T cells and humoral immunity are critical in controlling the dissemination of WNV (Anderson & Rahal, 2002; Diamond et al., 2003; Fredericksen et al., 2008; Klein et al., 2005; Lucas et al., 2003; Roehrig et al., 2001; Wang et al., 2003a). In addition, CD4+ (Kulkarni et al., 1991) and CD8+ (Shrestha et al., 2006; Wang et al., 2003b) αβ T...
cells contribute to host survival following WNV infection. Intradermal WNV infection, which presumably mimics natural infection in humans, has also been studied in mice (Johnston et al., 1996, 2000). Following intradermal WNV infection, the epidermal dendritic cells Langerhans cells (LCs), where the pathogen is naturally deposited during mosquito transmission of the virus, were shown to be important antigen-presenting cells in the skin (Byrne et al., 2001; Johnston et al., 2000). Moreover, these cells migrated from the epidermis by an interleukin (IL)-1β-dependent pathway and accumulated in local draining lymph nodes, thereby playing an important role in T-cell activation and proliferation (Byrne et al., 2001). Nevertheless, the underlying mechanism of this process remains undefined.

Toll-like receptors (TLRs), a family of 13 mammalian homologues of Drosophila Toll that recognize pathogen-associated molecular patterns (PAMPS), play an essential role in the initiation of innate immunity (Qureshi & Medzhitov, 2003). Among them, several TLRs have been shown to respond to viral PAMPS. TLR2 and TLR4 have been reported to recognize viral proteins (Bieback et al., 2002; Kurt-Jones et al., 2000; Rassa et al., 2002). TLR3 mediates host recognition of viral components such as double-stranded (ds)RNA and the dsRNA analogue poly(I:C) and intact virus (Alexopoulos et al., 2001). TLRs 7, 8 and 9 are vital for the recognition of ssRNA viruses, ssRNA and viral DNA, respectively (Diebold et al., 2004; Lund et al., 2004; Tabet et al., 2004; Tissari et al., 2005).

TLRs 3 and 7 have been reported to play important roles in host immunity, either pathogenic or protective, following systemic infection by WNV in mice. For example, we have shown previously that, upon a lethal dose of a mammalian cell-passaged WNV challenge via the intraperitoneal route, a TLR3-dependent inflammatory response was involved in brain penetration of the virus and neuronal injury, thereby playing a role in viral pathogenesis (Wang et al., 2004). Interestingly, another study suggests that, following a sublethal dose of insect cell-derived WNV infection either intraperitoneally or subcutaneously, TLR3 provides a protective effect against WNV infection, partially by restricting replication in neurons (Daffis et al., 2008). In a recent study, we have also demonstrated that TLR7-dependent responses to WNV systemic infection contribute to a vital host-defence mechanism by affecting immune-cell homing to infected target cells (Town et al., 2009). Upon cutaneous infection, WNV-permissive LCs could migrate from the skin to the lymph nodes (Byrne et al., 2001; Johnston et al., 2000). These cells might play a role in early infection; however, the exact mechanisms are not yet established. Epidermal keratinocytes have been shown to be involved in LC mobilization during contact-hypersensitivity reactions (Furue et al., 1996; Uchi et al., 2003). Increasing evidence suggests that TLR7 signalling in skin epidermis is mainly an attribute not of LCs, but of keratinocytes, which form a major component of innate immunity to skin infections (Asahina & Tamaki, 2006; Flacher et al., 2006; Kalali et al., 2008; Mitsui et al., 2004). Therefore, in this study, we investigated the role of the TLR7 response in host protective immunity during cutaneous WNV infection.

**METHODS**

**Mice.** TLR7−/− mice (Lund et al., 2004) [C57BL6 (B6) × 129 F2 background] were obtained from Regeneron Inc. and were bred to the B6 background by back-crossing for seven successive generations. All mice were kept under specific-pathogen-free conditions. Experiments were performed with 6- to 14-week-old animals. Groups were age- and sex-matched for each experiment and were housed under identical conditions. All animal experiments were approved by the Animal Care and Use Committee at Colorado State University.

**WNV infection in mice.** WNV NY99-6480 (G.-J. J. Chang, CDC, Fort Collins, CO, USA) was passaged three times in C6/36 Aedes albopictus cells to make a virus stock (3.3 × 10⁶ p.f.u. ml⁻¹) that was used for both cell culture and in vivo studies. For intradermal infection, we anaesthetized mice first with ketamine/xylazine. Mice were then injected intradermally into the dorsal side of the left ear with 2 × 10⁶ p.f.u. WNV isolate NY99 in 50 μl PBS with 5% gelatin. For mosquito feeding, 5-day-old Culex tarsalis mosquitoes (Turell et al., 2005) were given an artificial blood meal consisting of defibrinated sheep blood (Colorado Serum Company) containing 1 × 10⁶ p.f.u. WNV ml⁻¹. Infected females were separated under cold anaesthesia and maintained with sugar and water. At day 7 post-infection, six to ten mosquitoes were collected to confirm WNV infection by RT-PCR. At day 9 of mosquito infection, mice were anaesthetized with ketamine/xylazine and placed in a carton containing 15 WNV-infected C. tarsalis mosquitoes. Once approximately 10 mosquitoes had imbied a blood meal, the mouse was removed from the carton. Mosquitoes were frozen and WNV infection was confirmed individually by RT-PCR. Infected mice were monitored twice daily for morbidity, including lethargy, anorexia and ataxia.

**Quantitative PCR (Q-PCR) for determining viral load and cytokine production.** Blood, spleen and brain tissues from control and experimental mice were collected at the indicated days post-infection. For brain analysis, extensive cardiac perfusion with PBS was performed. RNA was extracted by using an RNeasy extraction kit (Qiagen) and 200 ng extracted RNA was used to synthesize cDNA by reverse transcription (qScript, Quanta Biosciences). The sequences for primers of the WNV envelope gene (WNVE), tumour necrosis factor (TNF)-α, IFN-α, IL-6 and IL-12 were described previously (Lanciotti et al., 2000; Wang et al., 2004). Q-PCR of cDNA was performed by using RT² Real-Time SYBR green/fluorescein PCR master mix (Superarray). To normalize the samples, the same amount of cDNA was used in a Q-PCR for β-actin. The ratio between the amount of test-gene cDNA and the amount of β-actin cDNA represented the relative level in each sample. The assay was performed on an iCycler (Bio-Rad).

**Plaque assay.** Vero cells were seeded in 12-well plates in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; Sigma) 24 h before infection. Serial dilutions of culture supernatant from infected cells were added and incubated for 2 h. Subsequently, DMEM containing 5% FBS and 1% low-melting-point agarose was added and the plates were incubated for 4 days. A second overlay of 2.5 ml 1% agarose medium containing 0.01% neutral red was added to visualize plaques. Virus concentrations were determined as p.f.u. ml⁻¹.
Keratinocyte and LC isolation. Keratinocytes were isolated from the tail skin of adult mice as described by Lichti et al. (2008). Tail skin was floated with the dermis side down on 0.25% trypsin (Mediatech Cellgro) at 4°C overnight. Dermis was peeled off and epidermis was minced, triturated and filtered through a 100 μm cell strainer. Single-cell suspensions were cultured in medium containing low Ca2+ [Chelex-treated FBS, as described by Lichti et al. (2008)] at 2×105 cells per well in a 24-well plate coated with collagen/fibronectin. LCs were obtained as described by Longley et al. (1995). Briefly, epidermis was prepared from the whole-body skin and from ears and floated on 0.25% trypsin with the dermis side down at 37°C. After removing the dermis, epidermal-cell suspensions were minced in 0.25% trypsin/DNase I (5×103 Dornase units ml−1; Fisher Scientific). Samples were applied to a Histopaque-1083 gradient to remove dead cells and keratinocytes. Cells were stained with phycoerythrin–Cy5-conjugated anti-MHC (major histocompatibility complex) class II (clone M5/114.15.2; eBioscience) and sorted on a MoFlo cell sorter (DakoCytomation). MHC class II+ bright cells were isolated at a purity of >95% as LCs (Salgado et al., 1999).

Co-culture of naïve LCs of wild-type mice with WNV-infected keratinocytes. After overnight coculture, keratinocytes were washed and adherent cells were infected with WNV at an m.o.i. of 0.5. For coculture of WNV-infected keratinocytes and LCs, 2×105 keratinocytes were mixed with 1×105 non-infected wild-type LCs in a 96-well plate at 37°C. At 40 h, cell supernatant was collected for cytokine analysis.

Flow cytometry. Freshly isolated skin epidermal cells were stained with antibodies specific for CD11c (clone HL3; BD Biosciences). After staining, the cells were fixed in PBS with 1% paraformaldehyde and examined by using a Cyan flow cytometer (Dako Cytomation). Dead cells were excluded on the basis of forward and side light scatter. Data were analysed by using Summit 4 software (Dako Cytomation).

Cytometric bead array (CBA). Cell-culture supernatants were collected on the indicated days after infection and analysed with a mouse inflammatory cytokine kit (IFN-γ, TNF-α, IL-6, IL-12, MCP-1; BD Biosciences) using a FACSArray analyser (BD Biosciences).

Histological examination of tissues. Mice were anaesthetized with isoflurane and perfused with 30 ml ice-cold PBS via a butterfly 23G needle inserted into the left ventricle. Paraformaldehyde (4%) was then injected to perfuse and fix the bodies, and the brains were removed and further fixed in 4% paraformaldehyde. Subsequently, specimens were processed and 10 μm paraffin sections were prepared for staining with haematoxylin and eosin.

Statistical analysis. Survival-curve comparisons were performed by using Prism software (GraphPad Software) statistical analysis, which uses the log-rank test (equivalent to the Mantel–Haenszel test). Values of P for viral burden, cytokine production, cell numbers and percentages were calculated with a non-paired Student’s t-test or Mann–Whitney test.

RESULTS

There was no difference in susceptibility between wild-type and TLR7−/− mice following cutaneous WNV challenge

TLR7−/− mice were more susceptible to WNV infection than wild-type mice following an intraperitoneal challenge with an LD50 dose of WNV 2741, suggesting a role for TLR7 in host-defence mechanisms (Town et al., 2009). In this study, we investigated the role of the TLR7-induced innate immune response in protective immunity during cutaneous WNV challenge. A dose of WNV NY99 strain of 104–105 p.f.u. by intradermal injection resulted in survival rates of 25–40% in mice (data not shown). Similar results were reported in an early study using WNV Sarafend strain (Burke et al., 2004). Therefore, we infected TLR7−/− mice (129/B6 mixed background) and their littermate controls intradermally with 2×102 p.f.u. WNV NY99 and monitored mice twice daily for survival. Surprisingly, we found no statistical differences in the survival rates between these two groups (27 versus 38%, wild-type vs TLR7−/−, P>0.05; Fig. 1a). To exclude the possible effect of genetic background on host susceptibility, TLR7−/− mice (back-crossed to B6 background) and their controls were infected intradermally with the same dose of WNV. No significant difference in host susceptibility was found between these two groups of mice (37.5 versus 27.2%, wild-type versus TLR7−/−, P>0.05; data not shown). In a separate study, we fed WNV-infected C. tarsalis mosquitoes on wild-type and TLR7−/− mice. Following a 3 week interval, 24% of TLR7−/− mice survived compared with 50% of wild-type controls (P>0.05, Fig. 1b). Furthermore, there was no

![Fig. 1. Comparison of susceptibility between wild-type (■) and TLR7−/− (▲) mice following cutaneous WNV infection. Data shown were pooled from three independent experiments. (a) Mice were injected intradermally with a dose close to the LD50 of WNV. P=0.43 (>0.05) for wild-type (n=15) versus TLR7−/− (n=16) mice. (b) Mice were infected by feeding of WNV-infected mosquitoes. P=0.14 (>0.05) for wild-type (n=14) versus TLR7−/− (n=13) mice.](https://www.microbiologyresearch.org/10.1099/vir.0.86802-0)
significant difference in the mean time of survival between wild-type (15 ± 1.7 days) and TLR7−/− (10.8 ± 1.6 days) mice (P>0.05). Overall, these data suggest that there was no difference in host susceptibility between wild-type and TLR7−/− mice following cutaneous WNV infection.

Viral load and histological analyses revealed similar levels of infection in the tissues, and neuronal death and infiltration in the central nervous system (CNS) regions, between the two groups of mice following intradermal WNV challenge

To understand viral pathogenesis during cutaneous WNV infection, we challenged wild-type and TLR7−/− mice intradermally with 2 × 10^6 p.f.u. WNV. cDNA from different tissues was used to determine viral load by Q-PCR. Blood and spleens were collected at early (day 2), mid (day 4) and late (day 6) stages of infection. Brains were harvested at days 4 and 6 post-infection. Throughout infection, the kinetics and magnitude of viral RNA levels in the tissues of TLR7−/− mice were not significantly different from those of wild-type controls (Fig. 2a, b, c; P>0.05). Histological analyses of the brain tissues of infected mice on day 6 revealed both neuronal death and infiltration in various regions of the CNS, especially apparent in the olfactory bulb and neocortex. However, no differences in the severity of cell death and infiltration were found between the wild-type and TLR7−/− mice (Supplementary Fig. S1, available in JGV Online). These data indicate that the TLR7-induced immune response might not be involved in the control of viral dissemination following intradermal WNV challenge.

There was a higher level of cytokines in blood of wild-type mice than in TLR7−/− mice at early stages of infection; however, this difference was diminished in blood and brains at later stages of infection

TLRs 7 and 8 are known to elicit antiviral effects, including type I IFN and proinflammatory cytokine production upon stimulation by viral ssRNA (Lund et al., 2004; Town et al., 2009; Wang et al., 2006). We measured cytokine production in blood and brains following intradermal WNV challenge. At day 2 post-infection, levels of IFN-α (Fig. 3a), IL-12 (Fig. 3b) and IL-6 (Fig. 3c), but not TNF-α (Fig. 3d), were significantly higher in blood of wild-type mice than in TLR7−/− mice. At day 4 post-infection, the differences in IFN-α and IL-12 levels had diminished. However, IL-6 and TNF-α expression were 30–40 % higher in wild-type mice than in TLR7−/− mice (Fig. 3a–d). In the brains, there was no significant difference in cytokine production between these groups of mice (Fig. 3e, f, h; P>0.05), except that IL-6 expression was about twice as high in wild-type mice at day 6 post-infection (Fig. 3g; P<0.05). No significant differences were detected in non-infected mice of these two groups.

Fig. 2. Viral load analysis in mice infected intradermally with WNV. Viral load was determined by Q-PCR in blood (a), spleens (b) and brains (c) of wild-type (empty columns) and TLR7−/− (filled columns) mice at the indicated days following WNV infection. The y-axis depicts the ratio of the amplified WNVE cDNA to β-actin cDNA of each sample. Data are presented as means ± SEM; n=9 or 10. Data shown are representative of two independent experiments.

WNV infection of wild-type epidermal keratinocytes induced more proinflammatory cytokine production, which might promote migration of LCs carrying WNV from skin to other peripheral tissues

Upon cutaneous infection, epidermal LCs migrate from the skin to the lymph nodes (Byrne et al., 2001; Johnston et al., 2000), a finding that provides evidence for a possible role of these cells in early infection. Keratinocytes, the predominant cells in the skin expressing high levels of TLR7, are known to be involved in LC mobilization (Furue et al., 1996; Uchi et al., 2003). We found that cultured LCs from wild-type and TLR7−/− mice showed no significant difference in WNV replication (data not shown). To study WNV infection in epidermal keratinocytes, we isolated...
these cells from wild-type and TLR7−/− mice and infected them with WNV in vitro. Plaque assays revealed 70% enhancement of WNV replication in TLR7−/− keratinocytes compared with wild-type cells at day 6 post-infection (Fig. 4a; P<0.05). Consistent with these data, we noted 50% higher IFN-α production in cultured wild-type keratinocytes at 24 h post-infection (Fig. 4b; P<0.05). LCs were previously shown to migrate from the epidermis in an IL-1β-dependent manner (Byrne et al., 2001). We found a >120% greater IL-1β production in wild-type keratinocytes than in TLR7−/− keratinocytes at 24 h post-infection (Fig. 4c; P<0.05). Further, CBA analysis showed higher levels of production of proinflammatory cytokines, including IL-6 (Fig. 4d) and IL-12 (Fig. 4e), in the supernatant of WNV-infected wild-type keratinocytes. Nevertheless, TNF-α production was not different between the two groups (Fig. 4f; P>0.05). Further, co-culture of WNV-infected keratinocytes with naive LCs of wild-type mice induced more IL-6 and IL-12 production than LCs cultured alone; this enhancement was significantly greater with WNV-infected wild-type keratinocytes than with TLR7−/− keratinocytes (Fig. 5a, b; P<0.05). Finally, at day 2 post-intradermal infection, the percentage and total number of CD11c+ LCs in the epidermis were reduced in wild-type mice by about 50 and 40%, respectively, compared with naive wild-type mice. This reduction was not observed in TLR7−/− mice (Fig. 5c, d). Collectively, these data suggest that the TLR7 response in epidermal keratinocytes might contribute to LC migration and WNV dissemination from the skin to other peripheral tissues upon intradermal WNV infection. This could further compromise the protective effect of the TLR7 response in host immunity.

Fig. 3. Cytokine production in wild-type and TLR7−/− mice following intradermal WNV challenge. Cytokine levels were determined using Q-PCR in blood (a–d) and brains (e–h) of wild-type (empty columns) and TLR7−/− (filled columns) mice at the indicated days post-infection. Data are presented as means ± SEM; n=7–13 and n=4 for WNV-infected samples and non-infected controls (NF), respectively. *P<0.05; **P<0.01, compared with wild-type mice.
DISCUSSION

Recent research has focused on understanding host immunity after parenteral (usually intraperitoneal) WNV infection. However, little is known about the protective immune responses against cutaneous WNV challenge, which is most relevant to the natural route of infection. In this study, we investigated the role of the TLR7 response following intradermal WNV infection. We demonstrated

![Fig. 4. WNV infection of cultured epidermal keratinocytes in vitro. (a) Plaque assay of culture supernatant collected at day 6 post-infection; n=8. (b, c) IFN-α (b) and IL-1β (c) production by Q-PCR analysis at 24 h post-infection. Data shown are representative of two independent experiments; n=3 or 4. (d-f) IL-6 (d), IL-12 (e) and TNF-α (f) production determined by CBA at 24 h post-infection; n=5. Empty columns, wild-type mice; filled columns, TLR7−/− mice; NF, non-infected controls; UD, undetectable. Data are presented as means ± SEM; *P<0.05 compared with wild-type.]

![Fig. 5. Keratinocytes promote LC migration following WNV infection. (a, b) IL-6 or IL-12 production in culture supernatant of naïve LCs of wild-type mice alone or those co-cultured with WNV-infected wild-type (WT) or TLR7−/− (KO) keratinocytes at 40 h. *P<0.05 for LCs alone versus LCs co-cultured with WNV-infected keratinocytes. #P<0.05 for wild-type versus TLR7−/− mice. Data are presented as means ± SEM; n=4. (c, d) Percentage of CD11c+ (c) or total number of LCs (d) in skin epidermis of wild-type or TLR7−/− mice. Empty columns, non-infected; filled columns, day 2 post-infection. Data are presented as means ± SEM; n=6. Data shown are representative of two independent experiments. **P<0.01 for non-infected versus infected mice.]

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that, upon cutaneous challenge, there was no difference in host susceptibility between TLR7−/− mice and wild-type controls. Viral load analysis by Q-PCR of WNV infection in blood, spleens and brains further supported this finding. Although there was a higher level of cytokines in the blood of wild-type mice at early stages of infection, this difference was diminished in blood and brains at later stages. These results suggest that the TLR7 response might not be involved in protective immunity following intradermal challenge.

These findings seem to contradict our recent report in which TLR7 signalling was shown to contribute to host-defence mechanisms, leading to reduced viraemia and lethality when WNV infection of mice was initiated by intraperitoneal injection (Town et al., 2009). To understand the difference between these two murine models of WNV infection, we first ruled out the possibility that the genetic background of mouse strains had an effect on host susceptibility (data not shown). WNV NY99 and other 1999 WNV isolates found in the USA, including WNV CT2741 (Anderson & Rahal, 2002), that have caused encephalitis in the elderly share high genetic similarity (99%). Two recent reports on the role of TLR3 in host immunity during WNV infection by our group and others (Daffis et al., 2008; Wang et al., 2004) suggest that virus dose (lethal versus sublethal) and passage history of the virus (Vero cell-derived versus insect cell-derived) may modulate infection outcome. Nevertheless, we have seen similar results from infection by WNV CT2741 (data not shown). Following cutaneous WNV challenge, the virus is deposited in the skin and disseminated to lymph nodes and other peripheral tissues by the migrating LCs to initiate systemic infection. DC maturation and migration upon microbial infection is an innate response that leads to adaptive immunity to foreign antigens (Bennett et al., 1998; Cella et al., 1996; Koch et al., 1996; Ridge et al., 1998). Microbial products such as lipopolysaccharide or proinflammatory cytokines, including TNF-α, IL-6 and IL-12, have been reported to promote this process (Conti et al., 2005; Dieli et al., 2004). We found that WNV infection of wild-type keratinocytes was significantly lower than that of keratinocytes from TLR7−/− mice and that a higher level of cytokines, including IFN-α, IL-1β, IL-6 and IL-12, was induced in wild-type cultures. We did not note any difference in TNF-α production between WNV-infected wild-type keratinocytes and TLR7−/− cultures, which supported previous findings by Byrne et al. (2001). Moreover, we demonstrated that co-culture of naïve wild-type LCs with infected wild-type keratinocytes induced more IL-6 and IL-12 production than with infected TLR7−/− keratinocytes or LCs cultured alone. Following intradermal infection, there was a reduced number of CD11c+ cells (LCs) in the epidermis, whereas this phenomenon was not observed in TLR7−/− mice. Overall, our findings suggest that, upon cutaneous infection, the TLR7-induced immune response plays a role in viral pathogenesis by promoting WNV dissemination from the skin to other peripheral organs to initiate systemic infection. This process might compromise its possible protective effects during the systemic-infection stage.

Animal-model studies have provided important information for development of vaccines or pharmacotherapeutic treatments for vector-borne pathogens, such as WNV and other arboviral encephalitic agents. Intradermal WNV infection in mice, which presumably mimics natural infection in humans, will be an important model to study host immunity to vector-borne pathogens (Johnston et al., 1996, 2000). A recent study (Styer et al., 2007) revealed that mean and median doses of WNV inoculated by C. tarsalis mosquitoes as they probed and fed on peripheral tissues of a mouse were 10^4.5 and 10^5 p.f.u., respectively, which were shown to result in survival rates of 25–40% following intradermal WNV challenge in our study and another (Burke et al., 2004). Although natural infection by mosquitoes is more complex than intradermal infection, due to concurrent exposure to components of mosquito saliva (Schneider et al., 2006; Skalova et al., 2008), we found that survival rates were similar after intradermal inoculation by needle or mosquito. Our findings provide the first evidence that the role of TLR7 signalling differs between intraperitoneal-infected animal models and those infected by the natural route.

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