Anti-inflammatory activity of intravenous immunoglobulins protects against West Nile virus encephalitis

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West Nile virus (WNV), an important global human pathogen, targets neurons to cause lethal encephalitis, primarily in elderly and immunocompromised patients. Currently, there are no approved therapeutic agents or vaccines to treat WNV encephalitis. Recent studies have suggested that inflammation is a major contributor to WNV encephalitis morbidity. In this study we evaluated the use of IVIG (intravenous immunoglobulins – a clinical product comprising pooled human IgG) as an anti-inflammatory treatment in a model of lethal WNV infection. We report here that IVIG and pooled human WNV convalescent sera (WNV-IVIG) inhibited development of lethal WNV encephalitis by suppressing central nervous system (CNS) infiltration by CD45 high leukocytes. Pathogenic Ly6C high CD11b+ monocytes were the major infiltrating subset in the CNS of infected control mice, whereas IVIG profoundly reduced infiltration of these pathogenic Ly6C high monocytes into the CNS of infected mice. Interestingly, WNV-IVIG was more efficacious than IVIG in controlling CNS inflammation when mice were challenged with a high-dose inoculum (105 versus 104 p.f.u.) of WNV. Importantly, adsorption of WNV E-glycoprotein neutralizing antibodies did not abrogate IVIG protection, consistent with virus neutralization not being essential for IVIG protection. These findings confirmed the potent immunomodulatory activity of generic IVIG, and emphasized its potential as an effective immunotherapeutic drug for encephalitis and other virus induced inflammatory diseases.

INTRODUCTION

West Nile virus (WNV), a mosquito-borne enveloped virus of the genus Flavivirus, has emerged globally as a major cause of encephalitis (Nash et al., 2001; Petersen & Marfin, 2002). Initially isolated from the blood of a febrile woman in the West Nile district of Uganda in 1937, it is now endemic in Asia, Australia and Africa, and recently caused widespread epidemics in both Eastern Europe and Israel (Pauli et al., 2013; Tyler, 2001). Following its introduction in New York in 1999, it spread rapidly across North America, and numerous cases have since been reported across Canada, Mexico, the USA and the Caribbean islands (Hayes & Gubler, 2006; Pauli et al., 2013; Tyler, 2001) (ArboNET: http://www.cdc.gov/westnile/resourcpages/survResources.html). WNV is maintained in an enzootic cycle between mosquitoes and birds, but it can cause disease in humans and horses. The incubation phase for WNV infection in humans is 2–14 days (Pauli et al., 2013; Suthar et al., 2013). Whilst most WNV infections in humans are asymptomatic, infection of neurons can result in neuroinvasive disease (Diamond et al., 2003; Hayes & Gubler, 2006; Murray et al., 2006; Pauli et al., 2013; Suthar et al., 2013). Between 1999 and 2013, the Centers for Disease Control and Prevention reported that 17 463 of a total 39 557 cases (44 %) in the USA presented with neuroinvasive disease, with a cumulative mortality rate (1668 cases) of ~ 4 % (ArboNET: http://www.cdc.gov/westnile/resourcpages/survResources.html). The majority of these deaths occurred in the elderly or immunosuppressed population. Overall, incidence of neuroinvasive disease in the total population has remained low at less than one case per 100 000.

In the murine model, the initial targets of WNV infection following introduction of virus into the skin are likely Langerhan’s dendritic cells (DCs), which migrate to and replicate in regional lymph nodes, resulting in viraemia and
viral dissemination to non-lymphoid organs (Davis et al., 2006; Samuel & Diamond, 2006; Suthar et al., 2013). Following systemic spread, WNV breaches the blood–brain barrier and enters the central nervous system (CNS) ~4–5 days post-infection (p.i.). In immunocompetent mice, virus is cleared within 1–2 weeks from both peripheral tissues and the CNS (Suthar et al., 2013). In mice lacking an intact immune system, WNV persists in the CNS eventually causing encephalitis (WNV encephalitis) and death, similar to immune-compromised patients (Pauli et al., 2013; Suthar et al., 2013). The murine model has been used successfully to mimic symptoms of human disease and study parameters of viral pathogenesis.

Treatment for WNV infection is mainly supportive (Pauli et al., 2013). The antiviral drug ribavirin in high doses and IFN-αb inhibit WNV replication in vitro, but with inconsistent results in vivo (Anderson & Rahal, 2002; Chan-Tack & Forrest, 2005). Currently, there are no approved therapeutic agents or vaccines available to treat WNV encephalitis (Diamond, 2005; Pauli et al., 2013). Recent studies implicating dysregulated CNS inflammation as a major cause of WNV-induced mortality suggested that immunomodulatory drugs may be beneficial (Getts et al., 2008; King et al., 2011; Wang et al., 2011). Several reports have documented the success of IVIG (intravenous immunoglobulins) as a treatment for patients with WNV CNS infections (Agrawal & Petersen, 2003; Kumar et al., 2004; Makhou et al., 2009; Morelli et al., 2010; Rhee et al., 2011; Saqib et al., 2008; Shimoni et al., 2012; Wadei et al., 2004; Yango et al., 2014). Studies in murine models using low doses of IVIG preparations that contained high titres of WNV neutralizing antibodies confirmed robust protection from WNV encephalitis (Ben-Nathan et al., 2003, 2009). Passive transfer of WNV-specific monoclonal or murine polyclonal antibodies aborted or limited WNV infections in rodent models in a dose-, time- and complement-dependent manner (Agrawal & Petersen, 2003; Engle & Diamond, 2003; Mehlhop et al., 2005; Morrey et al., 2006).

Our earlier investigation of the protective mechanisms of IVIG in a model of herpes simplex virus type 1 (HSV1) encephalitis demonstrated that IVIG’s potent anti-inflammatory and immunomodulatory activities, rather than its neutralizing activity, were essential for protection. Understanding how IVIG protects against WNV encephalitis is vital for optimizing its use as a treatment modality. IVIG is manufactured from pooled plasma collected from thousands of donors and it is composed predominantly of IgG1. Although introduced initially as a replacement therapy for primary and secondary immunodeficiency (Cohen, 1988; Looney & Huggins, 2006), IVIG is increasingly being used off-label as a treatment for a wide array of autoimmune and inflammatory diseases (Looney & Huggins, 2006; Negi et al., 2007). IVIG has a broad repertoire of neutralizing antibodies for various pathogens and neutralization is commonly assumed to be the mechanism of protection against viral infection. However, we have shown in the HSV1 model that IVIG devoid of neutralizing antibodies is as effective as IVIG in protection against encephalitis (Ramakrishna et al., 2011) and the reports that non-neutralizing antibodies can protect against WNV infection are consistent with this (Chung et al., 2006; Mehlhop et al., 2005; Vogt et al., 2011). Studies investigating IVIG protection from WNV infection have based dosing on its neutralizing activity and have thus used IVIG at suboptimal doses (0.1–0.6 g kg⁻¹) to elicit anti-inflammatory responses. In this study, we administered IVIG at a high dose (1 g kg⁻¹) to determine whether its anti-inflammatory and immunomodulatory activities could protect against lethal WNV encephalitis, and we additionally tested IVIG adsorbed to be free of neutralizing antibodies directed to the envelope glycoprotein (E-gp). Most of the antibodies mediating protection against WNV infection are directed to E-gp (Oliphant et al., 2006, 2007); hence, it was used to deplete IVIG of neutralizing antibodies. We found that a single dose of IVIG or WNV convalescent sera (WNV-IVIG) protected all mice from lethal WNV encephalitis when administered 24 h p.i. IVIG suppressed CNS infiltration by inflammatory cells, particularly the pathogenic Ly6Chigh CD11b⁺ monocytes and CD11c⁺ TNF⁺ inducible nitric oxide synthase producing (Tip) DCs (Getts et al., 2008; King et al., 2011; Ramakrishna et al., 2011; Terry et al., 2012). Importantly, IVIG lacking E-gp-specific antibodies still conferred significant protection, which showed that virus neutralization was not the main mechanism by which IVIG protected against WNV encephalitis. These findings extend our experience with IVIG protection against HSV1 encephalitis (Ramakrishna et al., 2011), and support the use of IVIG as an effective immunotherapeutic for treatment of suspected cases of WNV and HSV1 encephalitis.

RESULTS AND DISCUSSION

Murine studies have shown that during WNV infection, viral invasion of the CNS by infiltrating Ly6C⁺ high macrophages and microglia results in lethal encephalitis (Getts et al., 2008; Terry et al., 2012). This finding suggested that optimal treatment of WNV encephalitis required both antiviral and anti-inflammatory modalities. We previously showed that IVIG could temper CNS infiltration by pathogenic Ly6C⁺ high monocytes and Ly6G⁺ neutrophils, and thereby protect mice from lethal HSV1 encephalitis (Ramakrishna et al., 2011). Additionally, earlier studies showed that high titered IVIG collected from WNV endemic areas, but not non-immune IVIG, was protective in mouse WNV challenge studies when given at low doses (0.1–0.6 g kg⁻¹) (Ben-Nathan et al., 2009; Engle & Diamond, 2003). To determine the therapeutic potential of generic IVIG as an anti-inflammatory agent, we investigated the effects of high-dose (≥1 g kg⁻¹) and low-dose (160 mg kg⁻¹) IVIG on the development of WNV encephalitis.

A single dose of IVIG is able to protect from lethal WNV infection

To determine the minimum infectious dose of WNV at which the majority of BALB/c mice succumbed to
encephalitis, mice were infected intraperitoneally with various doses, and observed for mortality and morbidity. BALB/c mice given doses of $10^2$ or $10^3$ p.f.u. WNV showed no signs of encephalitis, whereas mice infected with $\geq 10^4$ p.f.u. WNV succumbed to WNV encephalitis by days 9–11 p.i. (Fig. 1a). To determine if IVIG and WNV-IVIG would protect against a lethal challenge, mice inoculated with $10^4$ p.f.u. WNV were injected intraperitoneally with 4 or 25 mg IVIG or 4 mg WNV-IVIG 24 h p.i. and survival was compared with PBS-treated mice. Whilst all PBS-treated mice succumbed to WNV encephalitis between days 7 and 11 p.i., all IVIG- and WNV-IVIG-treated mice survived (Fig. 1b). Importantly, WNV-infected mice treated with 25 mg IVIG adsorbed to lack WNV E-gp antibodies protected $65\%$ of mice (Fig. 1c) despite being incapable of neutralizing virus infectivity (Table 1). This result showed that WNV neutralizing activity of IVIG was not critical for protection from WNV encephalitis and that alternative mechanisms could mediate protection (Vogt et al., 2011), which we anticipated based on our demonstration that IVIG protection against HSV1 encephalitis was independent of neutralizing antibodies (Ramakrishna et al., 2011).

A recently proposed mechanism for IVIG protection postulated that a minor fraction of IVIG bearing terminal sialic acid residues on Asp297 in the IgG Fc domain, resulting in increased binding affinity for the mouse C-type lectin receptor SIGNR1 (and the human orthologue DC-SIGN) compared with Fc receptors, is crucial for protection from arthritis and immune thrombocytopenia in mouse models (Anthony & Ravetch, 2010; Anthony et al., 2008). According to this anti-inflammatory mechanism, the interaction of 2,6-α-sialylated IgGs (sIgG) with SIGN-R1+ macrophages induces IL-33 that drives production of the Tgf2 helper cytokines IL-4 and IL-13 by basophils, which ultimately leads to upregulation of the inhibitory FcγRIIB receptor on macrophages (Anthony et al., 2011; Schwab & Nimmerjahn, 2013), thereby increasing the activation threshold for innate monocytes and macrophages. Although this hypothetical mechanism is highly controversial (Sharma et al., 2014; von Gunten et al., 2014), it was nevertheless important to determine if sIgG was involved in protection from WNV encephalitis. We found that there was no significant reduction in protection when WNV-inoculated mice were given 25 mg desialylated IVIG 24 h p.i. as $60\%$ mice were protected. Moreover, mice treated with either low-dose (4 mg) or high-dose (25 mg) IVIG were protected equally and as sIgG is present in limiting amounts in IVIG, this result further discredits a...
role for sIgG in protection from WNV encephalitis. Deglycosylation of IVIG abrogated protection against WNV encephalitis. As deglycosylation is known to destabilize the integrity of the IgG Fc domain (Anthony & Ravetch, 2010), this result suggested that IVIG’s protective anti-inflammatory effects were critically dependent on Fc–FcR interactions (Chung et al., 2006).

To determine IVIG protection against WNV encephalitis after high-dose infection, mice inoculated with 10^5 p.f.u. WNV were treated with either IVIG or WNV-IVIG at 24 h p.i. The generic IVIG used in this study (collected during 2010–2013) had a high WNV antibody neutralizing titre, reflecting the increased exposure of the US population, particularly in the western states, to WNV. A single dose of WNV-IVIG protected all mice inoculated with a high dose of WNV, whereas despite having an equivalent WNV antibody titre, IVIG failed to protect the mice (Fig. 1d, Table 1). The data suggested that WNV-IVIG derived by pooling sera from convalescent patients might contain WNV-specific antibodies of higher affinity or increased in vivo functionality compared with the WNV-specific antibodies in IVIG. It is notable that a single dose of IVIG markedly extended the survival of mice inoculated with high doses of WNV as the mice survived until day 20 p.i. before succumbing to WNV encephalitis (Fig. 1d), whereas the PBS-treated mice all succumbed by day 10 p.i. (Fig. 1a).

### IVIG reduces viral titres following WNV infection

To determine if unrestrained viral replication was the cause of death in mice infected with a high dose of WNV (10^5 p.f.u.), tissues from infected mice treated with PBS or IVIG collected on days 4, 6, 8, 12 and 20 p.i. were used to determine WNV RNA levels. PBS-treated mice had high levels of WNV RNA in peripheral organs such as the spleen at days 4–6 p.i., but replication was controlled by day 8 p.i. (Fig. 2a). In contrast to the high viral titres in spleen at day 4 p.i., virus was not detectable in the brains of PBS-treated mice before day 6 p.i., but thereafter high levels of WNV RNA were detected (Fig. 2b); these mice succumbed to WNV encephalitis by days 8–9 p.i. (Fig. 1a). As expected, hyperimmune WNV-IVIG-treated animals had no viral RNA expression in either the spleen or brain at any time point. Impressively, IVIG reduced virus titres by >10-fold in the spleen on days 4 and 6 p.i. In contrast to the PBS-treated mice, virus was not detected in the brains of IVIG-treated mice until day 8 p.i. (Fig. 2b). Although virus replication in the brains of IVIG- and PBS-treated mice was similar at day 8 p.i., viral titres were reduced in brains from IVIG-treated mice at day 12 p.i. and the majority of these mice had completely controlled virus replication by day 21 p.i. (Fig. 2b). This result suggested that virus replication in the brain was not the major cause of death for these mice.

### CNS inflammation correlates with mortality following high-dose infection

To determine if CNS inflammation was causally associated with fatal encephalitis in mice inoculated with a high dose of WNV (10^5 p.f.u.), we analysed cells infiltrating the CNS by flow cytometry. A schematic depicting the analysis of CD45<sup>high</sup> and other infiltrating cells is shown in Fig. S1 (available in the online Supplementary Material). Fig. 3(a) shows that CD45<sup>high</sup> cells were increased by ~8% in the brains of PBS-treated WNV-infected mice on days 6 and 8 p.i. compared with IVIG-treated mice. Conversion of percentage CD45<sup>high</sup> cells to numbers further highlights the around twofold increase in total CD45<sup>high</sup> cells infiltrating the brains of PBS-treated mice compared with IVIG-treated mice (Fig. 3a). F4/80<sup>+</sup> macrophages and CD11c<sup>+</sup> DCs were the major cell types present in the CNS infiltrates, and were slightly increased in the brains of IVIG-treated mice compared with PBS-treated mice on days 6 and 8 p.i. (Fig. 3b, c). When presented as numbers, the difference in macrophages and DCs infiltrating the brains of PBS-treated mice compared with IVIG-treated mice on days 6–8 p.i. was shown to be 1.5- to twofold (Fig. 3b, c). Very low numbers of T- and B-cells were present in the brain between days 6 and 8 p.i., suggesting that lymphocytes did not contribute to CNS inflammation at these time points (data not shown).

Prior reports implicated Ly6C<sup>high</sup> monocytes as major determinants of the pathology of WNV encephalitis (Getts et al., 2008; Terry et al., 2012). Interestingly, we found that Ly6C<sup>high</sup> monocytes were also the major inflammatory cell subset causally associated with HSV1 encephalitis and we demonstrated that IVIG treatment impeded CNS infiltration by these pathogenic cells (Ramakrishna et al., 2011). To determine if these macrophages and DCs were inflammatory, we stained the cells for several surface markers. We found that 50% of F4/80<sup>+</sup> macrophages and 66% of CD11c<sup>+</sup> DCs expressed high levels of Ly6C in the brains of PBS-treated mice (Fig. 3d, e). In contrast, only 11% of F4/80<sup>+</sup> macrophages and 13% of CD11c<sup>+</sup> DCs in the brains

### Table 1. WNV-specific antibody titres in IVIG preparations used in this study

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<tr>
<td>Adsorbed IVIG</td>
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<td>&lt;1:10</td>
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ELISA and neutralizing assays were performed to determine antibody titres as described in Methods. E-gp antibodies were removed from adsorbed IVIG as described in Methods.

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of IVIG-treated mice were Ly6C<sup>high</sup>. Similarly, at day 8 p.i., 70% of F4/80<sup>+</sup> macrophages and 40% of CD11c<sup>+</sup> DCs in the brains of PBS-treated mice expressed high levels of Ly6C (Fig. 3d, e). However, Ly6C expression was reduced to 40% on F4/80<sup>+</sup> macrophages and 7% on CD11c<sup>+</sup> DCs in the brains of IVIG-treated mice (Fig. 3d, e). These data confirmed that IVIG suppressed the generation of inflammatory Ly6C<sup>high</sup> macrophages and Tip DCs during WNV infection, similar to our observations in HSV1-infected mice (Ramakrishna et al., 2011). Unexpectedly, CD45 high infiltrates, specifically Ly6C<sup>high</sup> F4/80<sup>+</sup> macrophages (95%) and CD11c<sup>+</sup> DCs (90%), were increased in the brains of IVIG-treated mice at day 12 p.i. (Fig. 3d, e), suggesting infiltration of inflammatory cells into the CNS had resumed. This renewed inflammation correlated with increased mortality in high-dose infected mice treated with a single dose of IVIG. Thus, for mice inoculated with a high dose of WNV, IVIG affords transient protection up to day 20 p.i. and thereafter the mice succumb to encephalitis as a consequence of renewed CNS infiltration by pathogenic Ly6C<sup>high</sup> macrophages from around day 12 p.i.

**Two doses of IVIG suppresses inflammation in mice infected with 10<sup>5</sup> p.f.u. WNV**

As resurgent CNS infiltration by inflammatory Ly6C<sup>high</sup> macrophages and DCs resulted in death of all mice inoculated with a high dose of WNV that received a single IVIG dose (Fig. 3a–e), we investigated whether a second dose of IVIG would improve survival. Treatment with two doses of IVIG significantly augmented protection, resulting in survival of ~70% of mice infected with a high dose of WNV (Fig. 1c). To determine if the second IVIG dose enhanced survival by suppressing inflammation, mononuclear cells were analysed by flow cytometry for CD45<sup>high</sup> cells infiltrating the CNS of mice treated with either a single dose of IVIG or two doses of IVIG. As shown in Fig. 3(f), at day 21 p.i., CD45<sup>high</sup> cells, particularly F4/80<sup>+</sup> macrophages and CD11c<sup>+</sup> DCs, were markedly increased in the brains of WNV-infected mice given a single dose of IVIG compared with mice given a second dose of IVIG on day 9 p.i. (48 versus 16%). Flow cytometry analysis of F4/80<sup>+</sup> macrophages and CD11c<sup>+</sup> cells within the CNS revealed that >70% of F4/80<sup>+</sup> macrophages and >75% of CD11c<sup>+</sup> DCs in the CNS of mice given a single dose of IVIG expressed high levels of Ly6C (Fig. 3f). In contrast, only 10% of F4/80<sup>+</sup> macrophages and 15% of CD11c<sup>+</sup> DCs were Ly6C<sup>high</sup> in the brains of high-dose infected mice given two doses of IVIG (Fig. 3f). Intriguingly, mice that were protected by a second dose of IVIG had increased levels of CD8 T- and B-cells (~60%) infiltrating the brain at day 21 p.i., indicating that these cells might play an important role in protection (not shown). These results are consistent with two doses of IVIG enhancing survival of mice inoculated with a high dose of WNV by exerting a greater moderating effect on the massive inflammatory response arising in the brains of these mice (Fig. 1d).
A single dose of IVIG protects mice infected with $10^4$ p.f.u. WNV

Whilst two doses of IVIG were required to protect mice inoculated with $10^5$ p.f.u. WNV, a single dose sufficed for mice inoculated with $10^4$ p.f.u. (Fig. 1b). Analysis of spleens of PBS-treated mice revealed higher levels of viral RNA compared with IVIG-treated mice at day 7 p.i. (Fig. 4a), but virus was controlled in spleens of IVIG-treated mice after day 12 p.i. Viral RNA was detected in the brains of both PBS- and IVIG-treated mice at day 7 p.i., and IVIG reduced viral RNA in the brain from day 12 p.i. such that virus replication was controlled by day 20 p.i. (Fig. 4b).
Similar to mice infected at high dose, mice challenged with 10^4 p.f.u. WNV and treated with WNV-IVIG did not have viral RNA in either the spleen or brain at any of the time points examined, which shows that WNV-IVIG was more effective than IVIG in inhibiting virus replication both in the periphery and in the CNS.

Analysis of CNS inflammation in mice infected with 10^4 p.f.u. WNV showed that CD45high cells were increased in the brains of PBS-treated WNV-infected mice at day 7 p.i. compared with IVIG-treated mice (representative dot-plots shown in Fig. S1). At day 7 p.i., CD45high infiltrates in the brains of PBS-treated mice reached 28%, compared with 18 and 14% in the brains of IVIG- and WNV-IVIG-treated mice, respectively (Fig. 5a); similar values were observed for brain infiltration at days 12 and 24 p.i. The brains of PBS-treated mice comprised ~10^6 CD45high infiltrating leukocytes compared with dramatically reduced levels in the brains of IVIG (5 x 10^5)- and WNV-IVIG (1.9 x 10^5)-treated mice at day 7 p.i. (Fig. 5a). Unlike mice infected with 10^4 p.f.u. that were treated with a single dose of IVIG, reduced numbers of CD45high cells persisted in the brains of mice infected with 10^4 p.f.u. that were given a single dose of IVIG or WNV-IVIG (Fig. 5a). All PBS-treated mice died before day 12 p.i. Within the CD45high infiltrates, F4/80+ macrophages (29 versus 23%) and CD11c+ DCs (50 versus 23%) were increased in the brains of PBS compared with IVIG-treated mice (Fig. 5b, c), and this reduction was even more pronounced in the brains of WNV-IVIG-treated mice (Fig. 5b, c). Similarly, considering absolute cell numbers, there was a threefold reduction in the number of macrophages and a fivefold reduction in DCs infiltrating the brains of IVIG- and WNV-IVIG-treated mice compared with PBS-treated mice at day 7 p.i. (Fig. 5b, c). The transient increase in percentage of CD11c+ DCs infiltrating the brains of WNV-IVIG-treated mice at day 12 p.i. was minimal when analysis was based on absolute cell numbers of CD11c+ DCs (Fig. 5c).

In fact, there was a slight decrease in CD11c+ DCs from 1.5 x 10^6 cells at day 7 p.i. to 1 x 10^6 cells at day 12 p.i. in the brain (Fig. 5c). To determine the phenotype of DCs and macrophages, these cells were probed for expression of the inflammatory marker Ly6C. Flow cytometric analysis of macrophages and DCs in the brains of PBS-treated mice at day 7 p.i. revealed that 49% of F4/80+ macrophages and 44% of CD11c+ DCs expressed high levels of Ly6C (Fig. 5d, e), which contrasted with only 2% of F4/80+ macrophages and 12% of CD11c+ DCs in the brains of IVIG-treated mice expressing high levels of Ly6C. IVIG completely suppressed the generation of Ly6Chigh macrophages and DCs during the acute phase of infection (day 7 p.i.). Moreover, when based on cell numbers, there was an even more impressive seven- and 10-fold reduction in inflammatory Ly6Chigh macrophages and Tip DCs, respectively (Fig. 5d, e). However, the Ly6Chigh population within F4/80+ and CD11c+ cells increased transiently to 60 and 66%, respectively, by day 12 p.i. in IVIG-treated mice before declining to low levels by day 20 p.i.

IVIG induces many different effector mechanisms including induction of T_{h}2 cytokines such as IL-4 and IL-13 via interaction with SIGNR1, generation of IL-10-secreting regulatory T (T_{reg})-cells and FoxP3+ ICOS+ CD4+ T-cells, and downregulation of activating FcRs and upregulation of the inhibitory FcR2b to exert its potent immunomodulatory effects (Anthony et al., 2008, 2011; Engle & Diamond, 2003; Ramakrishna et al., 2011; Suthar et al., 2013). We have previously shown that IVIG induced FoxP3+ T_{reg}-cells in mice infected with HSV1 that could protect naïve adoptively transferred mice from HSV1 encephalitis (Ramakrishna et al., 2011). To determine whether IVIG-induced T_{reg}-cells were involved in protection from WNV, we analysed lymphoid organs and brain tissues of WNV-infected FoxP3-GFP reporter mice, and found very low levels of T_{reg}-cells in both PBS- and IVIG-treated mice, which did not support a role for T_{reg}-cells in IVIG-mediated protection from WNV encephalitis (data not shown). We have shown that IVIG prevents development of HSV1 encephalitis by inducing IL-10-secreting T_{reg}-cells and ICOS+ CD4+ T-cells (Ramakrishna et al., 2011). To investigate a role for IL-10 in IVIG protection from WNV encephalitis, we infected IL-10−/− mice with 10^4 p.f.u. WNV. Whilst all PBS-treated mice succumbed to WNV encephalitis between days 7 and 11 p.i., IVIG protected...
all IL-10−/− mice, which excluded a role for IL-10 in IVIG-mediated protection (Fig. S2a). Additionally, intracellular IL-10 or IL-4 production was not detected in ICOS+ CD4+ T-cells stimulated in vitro with WNV-infected cell lysate (not shown). Thus, unlike in the HSV1 model, neither Treg-cells nor IL-10 were involved in IVIG-mediated protection from WNV encephalitis. We analysed cytokines and chemokines in the sera of mice infected with 10^4 p.f.u. WNV to determine if IVIG modulated inflammatory cytokines to mitigate infiltration of inflammatory monocytes into the brain. Only marginal differences in the expression of chemokines, such as RANTES, macrophage inflammatory protein (MIP)-1a, MIP-1b, MIP-2 and MCP-1, were observed in sera from the two groups. However, CXCL1 (KC), a neutrophil chemoattractant, was increased at day 2 p.i., and CXCL9 (MIG) and CXCL10 (IP-10), that interact with CXCR3+ immune cells, were increased at day 6 p.i. in the PBS group compared with the IVIG group (Fig. S3), possibly contributing to the increased cell infiltration observed in the brains of PBS-treated mice after day 6 p.i. Similarly, there were few if any

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**Fig. 5.** IVIG suppresses CNS inflammation in BALB/c mice inoculated with a low dose (10^4 p.f.u.) of WNV. Mononuclear cells isolated at the indicated time points from the brains of PBS-, IVIG (25 mg)- or WNV-IVIG (4 mg)-treated infected mice were analysed for CNS inflammation. Bar graphs depict percentage (L: left y-axis, depicted as bars) and number (#) (R: right y-axis, depicted as symbols) of CD45<sup>hi</sup> infiltrating cells (a), F4/80<sup>+</sup> macrophages (b) and CD11c<sup>+</sup> DCs (c) within the CD45<sup>hi</sup> infiltrates; and the pathogenic Ly6C<sup>hi</sup> subset within (d) F4/80<sup>+</sup> monocytes and (e) CD11c<sup>+</sup> DCs, as determined by flow cytometry. Data representative of three experiments; n=3 mice per time point. *P<0.05, ***P<0.001).
differences between inflammatory cytokines in the sera at any time point analysed. IL-1α was slightly increased at days 2–6 p.i. in the sera of PBS-treated mice (~80 pg ml⁻¹), but not IVIG-treated mice (<30 pg ml⁻¹), reflecting the overall inflammatory status of PBS-treated mice compared with IVIG-treated mice. Consistent with data from intracellular flow analysis, there were no differences in the T½2 cytokines IL-4, IL-10 and IL-13. Whilst intracellular flow cytometric analysis revealed an increase in IFN-γ⁺ CD4 and CD8 T-cells in the spleens of IVIG-treated mice, this difference was not reflected in the ELISA analysis of sera isolated from these groups of mice. Interestingly, IL-15 was dramatically upregulated in the sera of IVIG-treated mice at day 4 p.i. (7100 pg ml⁻¹) compared with PBS-treated mice (20 pg ml⁻¹) (Fig. S2b). As IL-15 is a known regulator of NK-cells, we analysed NK-cell activity in the spleens of IVIG- and PBS-treated WNV-infected mice. Surprisingly, as determined by CD107a⁺ expression after stimulation, there were no significant differences in NK-cell IFN-γ secretion or degranulation for the two groups of mice (data not shown). We are currently further investigating a possible role for IL-15 in IVIG-mediated protection from WNV encephalitis.

Cumulatively, our data show that IVIG-mediated protection against WNV encephalitis depends primarily on its anti-inflammatory activity. Protection was independent of sIgG as desialylated IVIG protected robustly, whereas deglycosylated IVIG failed to protect, revealing a critical role for the IgG Fc domain interacting most likely with activating FcRs to mediate protection (Chung et al., 2006). Results presented here warrant testing generic IVIG clinically as a treatment that can be given in multiple doses to combat WNV CNS infections.

METHODS

Mice and viral infections. BALB/c mice were purchased from the Jackson Laboratory. BALB/c IL-10 knockout mice (Jackson Laboratory) and Fox3-GFP reporter mice (Dr Tim Ley, Washington University, St Louis, MO, USA) were bred in the City of Hope Animal Research Center. Mice at 6–8 weeks of age were anaesthetized with ketamine/xylazine mixture prior to infection with WNV NY99 strain by either the intraperitoneal or subcutaneous route. All animal procedures were performed in the University of California Irvine (UCI) Animal Biosafety Level 3 (ABSL3) facility in strict compliance with the ABSL3, UCI Institutional Animal Care and Use Committee and within the framework of the ‘Guide for the Care and Use of Laboratory Animals’. Tissues were harvested from deeply anaesthetized perfused mice for further analysis.

WNV strain NY99 (kindly provided by M. Diamond, St Louis, MO, USA) stock virus was grown in Vero cells using a previously published protocol (Diamond et al., 2003). Infectious virus titres were determined by plaque assay on Vero cells. Briefly, serially diluted viral supernatants were incubated on confluent Vero cell monolayers in six-well plates for 90 min at 37 °C under 5 % CO₂, following which they were overlaid with Dulbecco’s modified Eagle’s medium containing 2 % low-melting-point agarose. After 3–5 days the monolayer was stained with 0.1 % crystal violet for plaque visualization.

Administration of IVIG. Human serum obtained from WNV convalescent patients (WNV-IVIG) was provided by the American Red Cross, as described previously (Planitzer et al., 2007). IVIG (Carimune NF) was obtained from CSL Behring. WNV-IVIG was given at 4 mg per mouse and IVIG at 4 (low dose) or 25 mg (high dose) per mouse by intraperitoneal injection 24 h p.i. Either 25 mg desialylated, deglycosylated or WNV E-gp-adsorbed IgG (prepared as described in Ramakrishna et al., 2011) was administered to WNV-infected mice 24 h p.i. Most of the antibodies mediating protection against WNV infection are directed to E-gp (Engle & Diamond, 2003; Oliphant et al., 2006); hence, it was used to deplete IVIG of neutralizing antibodies.

WNV-specific antibody titres were determined by ELISA. Briefly, ELISA was performed using sterile 96-well flat-bottomed microplates coated with the WNV antigen or Escherichia coli produced WNV E-gp overnight at 4 °C. After blocking with a 5 % solution of skimmed milk at 4 °C overnight, various dilutions of IVIG, adsorbed IVIG (adsorbed to remove WNV E-gp-specific antibody and neutralizing antibody) or WNV-IVIG were added and plates were incubated at 37 °C for 2 h. Detection was performed using a biotinylated rabbit anti-human IgG (1:50 000, 37 °C for 2 h), streptavidin (1:5000 dilution, 30 min at room temperature) and 3,3′,5,5′-tetramethylbenzidine substrate (10 min at room temperature/dark). The A₄₅₀ was measured following addition of 1 M H₂SO₄. The neutralizing antibody titre in the IVIG preparations was determined as follows. Mixtures of WNV (200 p.f.u.) incubated with serial dilutions of IVIG, adsorbed IVG or WNV-IVIG for 1 h at 37 °C were used to infect confluent Vero cell monolayers in 24-well flat-bottom microplates at 37 °C for 90 min; thereafter, the medium was replaced with agarose, and after 3–4 days the agarose (1 %) overlay was removed and the monolayer stained using crystal violet to facilitate enumeration of plaques. Analysis of mouse cytokines and chemokines in the sera of infected mice was performed in the Pacific Southwestern Regional Center of Excellence Core at the University of California Davis using a multiplex Luminescent ELISA (Millipore MILLIPLEX mouse cytokine/chemokine MCYTOMAG-70K-PMX).

Isolation of mononuclear cells from the CNS. CNS-derived mononuclear cells were isolated as described previously (Ramakrishna et al., 2011). Briefly, brains were dissected from mice perfused with PBS, minced, and digested with a mixture of collagenase and DNase for 30 min at room temperature prior to centrifugation at 1250 g for 25 min on a one-step Percoll gradient.

Flow cytometric analysis. Single-cell suspensions isolated from either the brain or spleen were blocked with a rat anti-mouse CD16/32 mAb (2.4G2; BD Pharmingen) for 15 min prior to incubation with antibodies to determine cell surface expression of various markers. Phycoerythrin-, FITC- or allophycocyanin-conjugated antibodies specific for F4/80 (BMB, CD11b (M1/70), CD11c (N418) and Ly6C (AL-21) were obtained from eBioSciences. PerCP (peridinin chlorophyll protein)-conjugated CD45 (30-F11) was obtained from BD Pharmingen. F4/80⁺ macrophages were characteristically CD45high. Inflammatory macrophages and DCs express high levels of Ly6C and CD107a⁺, and our analysis focused on CNS infiltration by these cells specifically as we and others have previously shown that these cells are causally involved in HSV1 and WNV encephalitis. CD4⁺ Treg-cells were identified by reactivity to CD25 and Fox3-GFP expression using Fox3-GFP reporter mice. Cells fixed with 4 % paraformaldehyde were acquired on an Accuri flow cytometer and analysis was performed using CFlow Plus software.

Real-time (RT)-PCR. Viral RNA was isolated from homogenized tissues (brain or spleen) by using Maxwell 16 Tissue LEV Total RNA Purification kit (Promega). WNV NY99 E-gp-specific primers were used for amplification and a TaqMan probe was used for quantification. The TaqMan probes were labelled at the 5’ end with the reporter dye FAM and at the 3’ end with the quencher dye TAMRA. Quantitative one-step RT-PCR of the extracted viral RNA was carried
out using a TaqMan Real-Time PCR kit (Qiagen) according to the manufacturer’s instructions. WNV E gene primers and probe sequences were: WNV E-fwd, nt 1160–1180, 5’-TCAGGCGATCTCT-CCACCAAAG-3’; WNV E-rev, nt 1209–1229; 5’-GGGTCAGCACG-TTGTGACTTT-3’; and WNV E TaqMan probe, nt 1186–1207, 5’-TGCCGACCATGGGAGAAGCTC-3’. A standard curve with a dynamic range of detection in the range 10^6–10^8 copies was constructed by preparing 10-fold serial dilutions of standard WNV RNA. All experiments were performed in duplicate.

**Statistical analysis.** GraphPad Prism software was used for drawing graphs and for analysis of mortality using the log-rank (Mantel–Cox) test taking into account both the time to death and overall mortality.

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**REFERENCES**


IVIG protection against WNV encephalitis


