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Transgenic expression of full-length 2′,5′-oligoadenylate synthetase 1b confers to BALB/c mice resistance against West Nile virus-induced encephalitis

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ABSTRACT

Susceptibility of inbred strains to infection with West Nile virus (WNV) has been genetically associated with an arginine-to-a nonsense codon substitution at position 253 (R253X) in the predicted sequence of the murine 2′,5′-oligoadenylate synthetase 1B (OAS1B) protein. We introduced by transgenesis the *Oas1b* cDNA from MBT/Pas mice carrying the R253 codon ($Oas1b^{MBT}$) into BALB/c mice homozygous for the X253 allele ($Oas1b^{BALB/c}$). Overexpression of $Oas1b^{MBT}$ mRNA in the brain of transgenic mice prior and in the time course of infection provided protection against the neuroinvasive WNV strain IS-98-ST1. A 200-fold induction of $Oas1b^{MBT}$ mRNA in the brain of congenic BALB/c mice homozygous for a MBT/Pas segment encompassing the *Oas1b* gene was also efficient in reducing both viral growth and mortality, whereas a 200-fold induction of $Oas1b^{BALB/c}$ mRNA was unable to prevent virally-induced encephalitis, confirming the critical role of the R253X mutation on *Oas1b* activity in live mice.

Introduction

West Nile virus (WNV) is a positive-sense, single-stranded RNA flavivirus transmitted by mosquitoes that infects a wide range of vertebrate hosts and causes severe illness in humans, including encephalitis, meningitis, or flaccid paralysis. About 80% of WNV infections are asymptomatic, 20% result in self-limited West Nile fever, and <1% result in neurologic disease (Hayes and Gubler, 2006). Host genetic factors might be important to control the susceptibility and severity to WNV infection (Diamond et al., 2009). Our laboratories reported that classical inbred strains such as BALB/c, whose genome is 92% of *Mus m. domesticus* origin (Yang et al., 2007), were highly susceptible to intraperitoneal infection with the neuroinvasive and neurovirulent Israeli strain IS-98-ST1 of WNV while mouse strains derived from wild progenitors of the *M. m. musculus* (MBT/Pas) or *Mus spretus* (SEG/Pas) species were resistant. We mapped the resistance locus to a critical interval of approximately 1 Mb that contains a cluster of 10 members of the *Oas* gene family (*Oas1a* to *Oas1h*, *Oas2* and *Oas3*) encoding 2′,5′-oligoadenylate synthetases, and identified a premature stop codon within exon 4 of the *Oas1b* gene, hence encoding a truncated version of OAS1B, lacking ~30% of its C-terminal domain (Mashimo et al., 2002). There was a perfect correlation among the various inbred strains of mice between the presence of this mutation and the susceptibility phenotype, strongly supporting that the truncated *Oas1b* allele was responsible for the susceptibility of laboratory mice to infection with the IS-98-ST1 viral strain. Perelygin et al.(2002) simultaneously identified the premature stop codon in C3H/He mice susceptible to infection with WNV strain Eg101 and showed that viral growth was impaired in C3H/He fibroblasts expressing a full-length OAS1B protein. *In vitro* experiments further demonstrated that expression of full-length OAS1B protein but not the C-terminally truncated form inhibits WNV replication inside infected mouse cells (Kajaste-Rudnitski et al., 2006; Lucas et al., 2003). Together, there is mounting evidence that the OAS family may play a crucial role in antiviral host immunity to WNV mediated by type-I interferons (Kristiansen et al., 2011). Consistent with this finding, genetic variations in human and horse *OAS1* are risk factors for infection with neuropathogenic WNV (Lim et al., 2009; Rios et al., 2010). To establish that the alteration in the coding sequence of the *Oas1b* gene is causative of susceptibility to flaviviruses, Scherbik et al. (2007a) replaced exons 4 and 5 in the genome of 129/Sv mice with DNA that corresponds to the MBT/Pas allele and reported that the

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knock-in mice were resistant to lethal infection with neurotropic flavivirus via the intracranial route.

In addition to the nonsense mutation, several nucleotide sequence variants have been identified in the promoter region of the *Oas1b* gene (Mashimo et al., 2003). Although none of them was consistent with the pattern of resistance or susceptibility across the inbred strains tested, we suspected that one or more of the SNPs could alter activation of *Oas1b* transcription in response to viral infection. To gain a better understanding of the molecular basis of *Oas1b*-mediated WNV resistance *in vivo*, we took a complementary approach. A cloned cDNA corresponding to the *Oas1b* mRNA expressed in resistant MBT/Pas mice was introduced by transgenesis to induce constitutive expression of the full-length coding sequence in an otherwise susceptible genetic background (BALB/c). We report that up-regulation of a wild-type *Oas1b* cDNA confers to BALB/c mice resistance against lethal WNV-induced encephalitis.

Results and discussion

Full-length OAS1B is a key-factor for mouse resistance to WNV infection

Positional cloning identified *Oas1b* as a candidate gene accounting for the resistance of wild-derived inbred strains of mice to infection with WNV (Mashimo et al., 2002; Perelygin et al., 2002). To further assess the role of the *Oas1b* gene during lethal infection, we produced a C.MBT-*Oas1b* congenic line where the *Oas* gene cluster of the MBT/Pas resistant strain was introgressed into the BALB/c susceptible genetic background. C.MBT-*Oas1b* congenic mice survive an i.p. inoculation of 1000 FFU of the virulent IS-98-ST1 WNV strain while BALB/c mice die within 10 days post-infection (Fig. 1), indicating that the MBT/Pas segment carries one or more determinant(s) of resistance (Mashimo et al., 2002). Although this result demonstrates the importance of the *Oas* gene cluster, it does not point unambiguously to the *Oas1b* nonsense mutation.

To address this issue, a transgene consisting of the CAG promoter and *Oas1b* cDNA from MBT/Pas strain was constructed (Fig. 2) and micro-injected into (C57BL/6 J × SJL/J)F2 fertilized eggs. Eggs were implanted in pseudo pregnant females. Three resulting founder animals harboured the entire transgene (*Tg*), as shown by PCR analysis, and were crossed with BALB/c mice to generate transgenic lines in a BALB/c genetic background. After twelve generations of backcrossing, *Tg*/+ transgenic mice from each of the three lines were examined for their susceptibility to lethal infection with WNV. C.MBT-*Oas1b* congenic and BALB/c mice served as controls. Mice were inoculated i.p. with 1000 FFU of the virulent WNV IS-98-ST1 strain. All

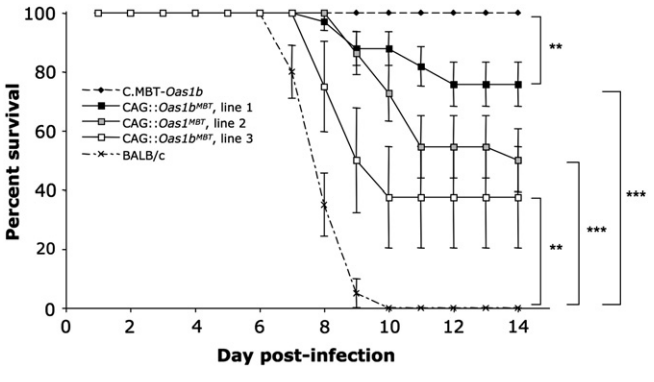

Fig. 1. Survival analysis of mice after West Nile virus infection. CAG::*Oas1b*^{MBT} transgenic mice from line 1 (N = 33), line 2 (N = 8) and line 3 (N = 22), C.MBT-*Oas1b* congenic mice (N = 20) and BALB/c control mice (N = 20) were inoculated with 1000 FFU of WNV IS-98-ST1 strain via the intraperitoneal route and monitored daily for mortality during 14 days. CAG::*Oas1b*^{MBT} transgenic mice from lines 1, 2 and 3 were significantly more resistant than BALB/c mice ($p<10^{-7}$, $p<10^{-8}$ and $p<0.005$, respectively).

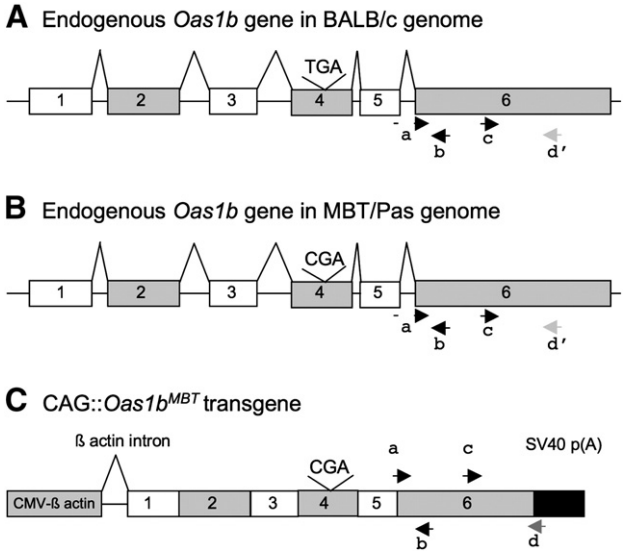

Fig. 2. Schematic structure of the endogenous *Oas1b* gene and CAG::*Oas1b*^{MBT} transgene. Structure of the endogenous *Oas1b* gene of BALB/c (A) and MBT/Pas (B) mice. Exons 1 to 6 of *Oas1b* are shown in boxes. Introns are shown by broken lines. The position of the CGA arginine codon in MBT/Pas mice that is mutated into TGA stop codon in BALB/c mice is also indicated. The positions of primers used for detecting (RT-PCR) and measuring (qRT-PCR) *Oas1b* mRNA are indicated. (C) The transgene contains the CMV/β-actin (CAG) promoter with β-actin intron and the complete MBT/Pas *Oas1b* cDNA and the SV40 polyadenylation sequence (SV40 p(A)).

BALB/c mice (N = 20) died within 10 days post-infection, whereas all C.MBT-*Oas1b* mice (N = 20) survived the infection, as expected. In CAG::*Oas1b*^{MBT} transgenic line 1, 75.7% (25/33) of the *Tg*/+ mice survived the infection (Fig. 1). There was no significant sex bias on survival. *Tg*/+ mice from transgenic lines 2 and 3 also survived the infection, though the percentage of survivors was lower (50 and 37% on average, respectively) (Fig. 1). These data indicate that the CAG::*Oas1b*^{MBT} transgene partially rescued the susceptible phenotype of BALB/c mice.

The CAG composite promoter has been shown to drive transgene expression in the whole body of adults and in embryos (Kawamoto et al., 2000; Kubo et al., 2002; Okabe et al., 1997). To assess CAG::*Oas1b*^{MBT} transgene expression *in vivo*, RT-PCR experiments were performed on various tissues of adult mice from each line. Organs were harvested and total RNAs were extracted from the heart, lung, liver, kidney, spleen and brain, RT-PCR assays were performed using primer pairs specific for the transgene (primers c–d in Fig. 2C) and the housekeeping aldolase gene, respectively. Fig. 3 shows that the transgene was expressed at detectable levels in the heart, lung, and brain in each line. However, transgene expression level was hardly detected in the liver in all lines. The CAG::*Oas1b*^{MBT} expression was not identical among the three lines suggesting that expression of the transgene was dependent on the site of integration, as commonly observed in transgenic mice (De Sepulveda et al., 1995). The more resistant transgenic line was used in subsequent investigations. To evaluate the expression of transgenic and endogenous *Oas1b* genes, we measured *Oas1b* mRNA levels in peripheral organ (spleen) and the central nervous system (brain) of BALB/c, C.MBT-*Oas1b* and CAG::*Oas1b*^{MBT} mice using primers that amplify the transcripts from the endogenous *Oas1b*^{MBT} and *Oas1b*$^{BALB/c}$ genes, and from the *Oas1b*^{MBT} transgene (primers a-b in Fig. 2). mRNAs containing a premature translation termination codon, such as *Oas1b*$^{BALB/c}$ mRNA, are generally degraded by nonsense-mediated decay (NMD) (Matsuda et al., 2008). Table 1 shows that, in the spleen, *Oas1b* mRNA level was approximately two fold higher in BALB/c than in C.MBT-*Oas1b* mice ($p<0.05$). This suggests that despite the premature translation termination codon in exon 4 of *Oas1b* in BALB/c mice, the transcript

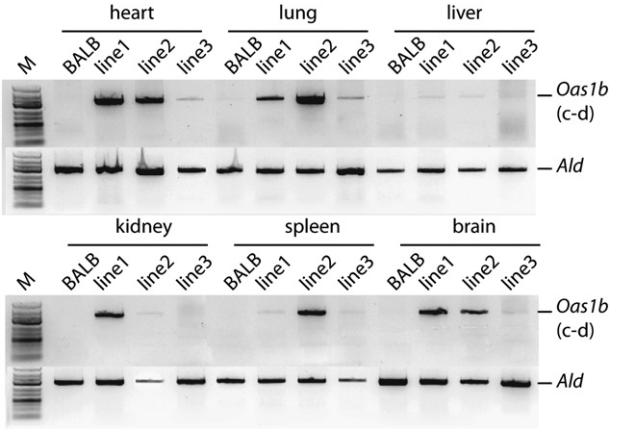

Fig. 3. Expression of the *Oas1b*^{MBT} mRNA in CAG::*Oas1b*^{MBT} transgenic mice. Agarose gel showing RT-PCR products generated from total RNA collected from the heart, lung, liver, kidney, spleen and brain of a mouse from transgenic line 1, line 2 and line 3, and BALB/c (BALB) inbred line (C). RT-PCR primers were designed to amplify fragments of the transgene *Oas1b* cDNA (343 bp) and the endogenous internal control aldolase (*Ald*) cDNA (278 bp). M (marker) denotes the low range DNA ladder, 25–700 bp (Fermentas GmbH, Courtaboeuf, France).

was not degraded by NMD. This conclusion agrees with previous detection of *Oas1b* mRNA in different tissues of BALB/c mice (Mashimo et al., 2003; Perelygin et al., 2002), and the truncated form of OAS1B in fibroblasts overexpressing *Oas1b*$^{BALB/c}$ cDNA (Kajaste-Rudnitski et al., 2006). *Oas1b* is likely an additional exception to the rule for which nonsense codons trigger NMD (Matsuda et al., 2008). In the brain of transgenic CAG::*Oas1b*^{MBT} mice, *Oas1b* mRNA levels were approximately 1,300 (i.e. 5,814/4.3) and 5,800 fold higher than in the brain of C.MBT-*Oas1b* ($p<0.001$) and BALB/c mice ($p<0.001$), respectively. Small significant difference in *Oas1b* mRNA levels was also observed in the spleen of CAG::*Oas1b*^{MBT} mice compared to C.MBT-*Oas1b* ($p<0.01$). To determine whether the expression of other *Oas* genes was affected by the increase in *Oas1b* expression, we measured *Oas1a* mRNA levels in the spleen and brain of BALB/c, C.MBT-*Oas1b* and CAG::*Oas1b*^{MBT} mice. Table 2 shows that *Oas1a* gene was expressed at similar levels in BALB/c, C.MBT-*Oas1b* and CAG::*Oas1b*^{MBT} mice, indicating that increase *Oas1b* expression was not associated with variation in *Oas1a* expression.

Table 1
Relative levels of expression of the endogenous and transgenic *Oas1b* mRNA in the spleen and brain of uninfected wild-type BALB/c, C.MBT-*Oas1b* congenic and CAG::*Oas1b*^{MBT} transgenic mice.

Mice	Expressed *Oas1b* gene(s)	Relative level of *Oas1b* mRNA expression in the spleen[a]	Relative level of *Oas1b* mRNA expression in the brain[a]
BALB/c	Endogenous *Oas1b*$^{BALB/c}$ gene	1 [0.61–1.64]	1 [0.59–1.71]
Congenic C. MBT-*Oas1b*	Endogenous *Oas1b*^{MBT} gene	0.41 [0.25–0.66][b]	4.3 [2.34–7.77]
Transgenic CAG::*Oas1b*^{MBT}	Endogenous *Oas1b*$^{BALB/c}$ gene and *Oas1b*^{MBT} transgene	1.82 [1.02–3.25]	5814 [4965–6807][c]

[a] Total RNA from the same mice and organs was analyzed for the expression of endogenous *Oas1b* mRNA (wild-type BALB/c, and C.MBT-*Oas1b* mice) and of both endogenous and transgenic *Oas1b* mRNA (CAG::*Oas1b*^{MBT} mice) by qRT-PCR. Data are expressed as the relative increase (n-fold) compared with uninfected BALB/c mice. Average values and 95% confidence intervals for the relative quantization values are from ten mice.
[b] $p<0.05$.
[c] $p<0.001$.

Table 2
Relative levels of expression of *Oas1a* mRNA in the spleen and brain of uninfected wild-type BALB/c, C.MBT-*Oas1b* congenic and CAG::*Oas1b*^{MBT} transgenic mice.

Mice	Relative level of *Oas1a* mRNA expression in the spleen [a]	Relative level of *Oas1a* mRNA expression in the brain [a]
BALB/c	1 [0.28–3.55] [b]	1 [0.27–3.65]
Congenic C.MBT-*Oas1b*	0.78 [0.26–2.37]	1.81 [0.47–7.03]
Transgenic CAG::*Oas1b*^{MBT}	1.14 [1.52–1.14]	1.27 [0.42–3.79]

[a] Total RNA from the same mice and organs was analyzed for the expression of *Oas1a* mRNA.
[b] Data are expressed as the relative increase (n-fold) compared with uninfected BALB/c mice. Average values and 95% confidence intervals for the relative quantization values are from ten mice.

Up-regulation of endogenous Oas1b in response to West Nile virus infection

Oas1b gene is induced in response to WNV infection in mouse embryonic fibroblasts (Fredericksen et al., 2008; Scherbik et al., 2007b). To determine the time course and level of endogenous *Oas1b* gene expression in susceptible and resistant mice, we measured the level of *Oas1b* mRNA in spleen and brain after lethal infection with WNV. Susceptible BALB/c, resistant C.MBT-*Oas1b*, and partially resistant CAG::*Oas1b*^{MBT} mice were infected intraperitoneally with 1000 FFU of the virulent WNV IS-98-ST1 strain and *Oas1b* mRNA was measured in samples harvested on days 1, 2, 4, 6, 8 and 10 post-infection by qRT-PCR using primers specific for the endogenous *Oas1b* transcripts (c–d′ in Fig. 2B). In susceptible BALB/c mice that carry a nonsense mutation in the *Oas1b* coding sequence, we detected no significant induction of *Oas1b*$^{BALB/c}$ mRNA in the spleen (Fig. 4A). However, in the brain, the expression of *Oas1b*$^{BALB/c}$ mRNA was increased 12 fold on day 2. *Oas1b*$^{BALB/c}$ mRNA levels peaked on day 8 in the brain (200-fold increase, $p<0.001$) (Fig. 4B), one day before the death of most infected BALB/c mice (Fig. 1). In resistant C.MBT-*Oas1b* mice, with an arginine codon replacing the stop codon in the *Oas1b* coding sequence, *Oas1b*^{MBT} mRNA levels were increased 260 to 600 fold in the spleen on days 1 and 2. At later stages, *Oas1b*^{MBT} mRNA levels in the spleen decreased gradually to reach the level seen in uninfected controls (Fig. 4C). In the brain, the peak induction of *Oas1b*^{MBT} mRNA (200-fold increase; $p<0.001$) was observed on day 4. Thereafter, *Oas1b*^{MBT} mRNA levels decreased progressively even though it still remained high on day 10 post-infection, 15 fold higher than the level measured in uninfected controls (Fig. 4D). Partially resistant CAG::*Oas1*^{MBT} transgenic mice are homozygous for the *Oas1b*$^{BALB/c}$ allele. In the spleen of CAG::*Oas1*^{MBT} mice, *Oas1b*$^{BALB/c}$ mRNA levels were moderately increased (7-fold) on day 1 and decreased progressively thereafter (Fig. 4E). In the brain of CAG::*Oas1*^{MBT} mice, the level of *Oas1b*$^{BALB/c}$ mRNA increased until day 8 (16 fold compared to uninfected controls, Fig. 4F). Altogether, resistant C.MBT-*Oas1b* mice were characterized by a significant induction of *Oas1b*^{MBT} expression in the spleen and brain at an early stage post-infection. By contrast, the expression of *Oas1b*$^{BALB/c}$ mRNA was not significantly increased in the spleen of susceptible BALB/c mice. High levels of *Oas1b*$^{BALB/c}$ mRNA in the brain at day 8 likely reflected very high viral burden at this late stage of infection preceding death from encephalitis. These data suggest that susceptibility in BALB/c mice could result from the combined effects of the nonsense mutation in the coding sequence and of one or more regulatory mutation(s) in the promoter region of the BALB/c *Oas1b* gene (Mashimo et al., 2003).

Expression of mRNA encoding full-length OAS1B in transgenic mice infected with WNV

To understand how the transgene may prevent lethal WNV infection, we analyzed the kinetics of expression of *Oas1b*^{MBT} mRNA in the spleen

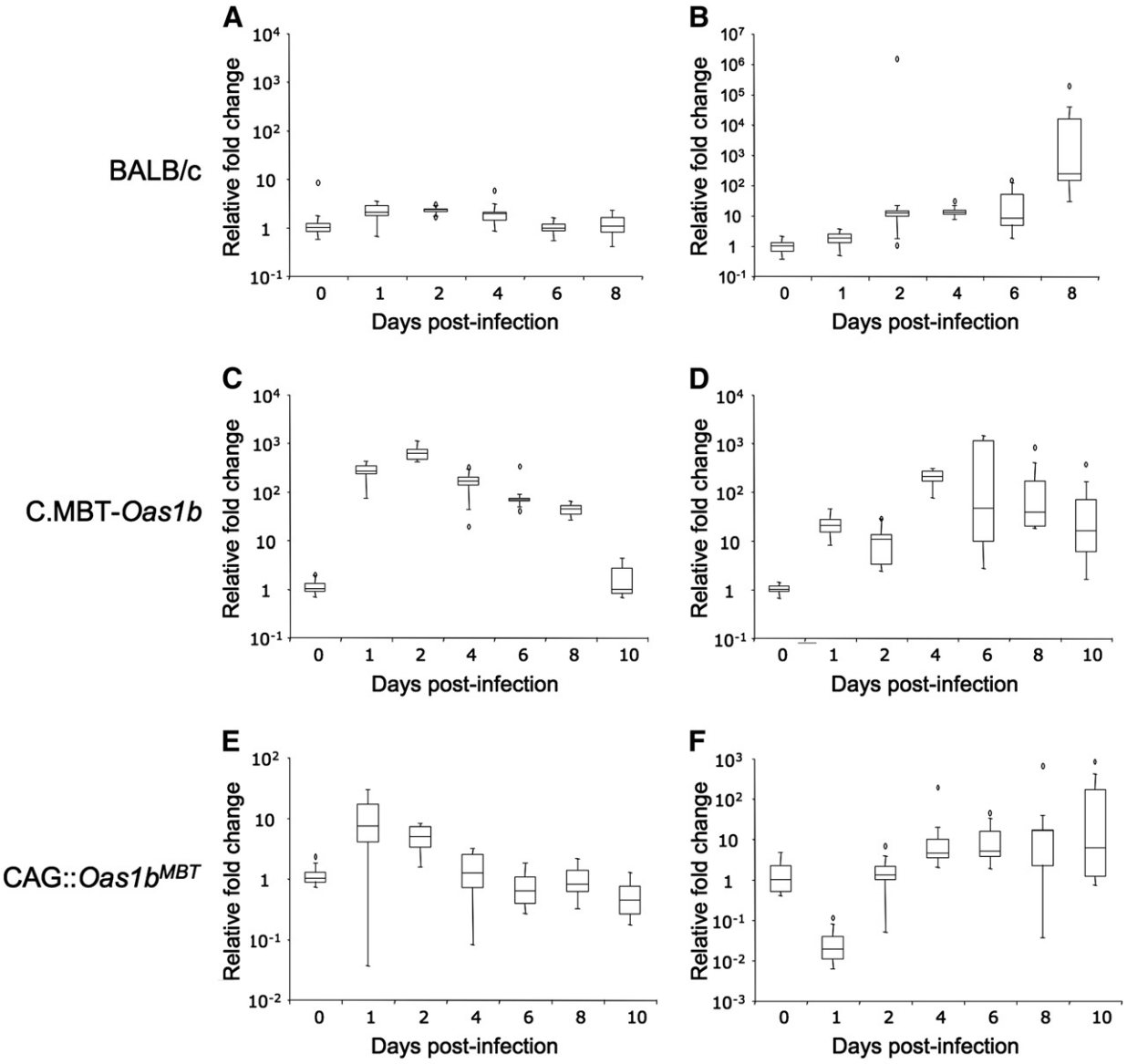

Fig. 4. Levels of endogenous *Oas1b* mRNA expression in the spleen and brain of West Nile virus-infected mice. BALB/c (A, B), C.MBT-*Oas1b* (C, D) and CAG::$Oas1b^{MBT}$ (E, F) mice were infected with 1000 FFU of the virulent IS-98-ST1 strain and euthanized on days 1, 2, 4, 6, 8 and 10. Total RNA from the spleen (A, C and E) and brain (B, D and F) was analyzed for the expression of endogenous *Oas1b* mRNA by qRT-PCR. Data are expressed as the relative increase (n-fold) compared with uninfected controls. Data from ten mice are shown as whisker-box plots. Open circles indicate outliers.

and brain of infected CAG::$Oas1b^{MBT}$ mice. CAG::$Oas1b^{MBT}$ mice were infected i.p. with 1000 FFU of WNV IS-98-ST1 strain and $Oas1b^{MBT}$ mRNA was measured from samples harvested on days 1, 2, 4, 6, 8 and 10 post-infection by qRT-PCR using primers specific for the transgenic $Oas1b^{MBT}$ mRNA (c–d in Fig. 2C). In the spleen, transgenic $Oas1b^{MBT}$ mRNA levels were decreased at an early stage, on days 1 (20 fold) and 2 (approximately 500,000 fold). On later days, $Oas1b^{MBT}$ mRNA levels were progressively restored to that of uninfected controls (Fig. 5A). In the brain, $Oas1b^{MBT}$ mRNA levels also dropped off (160 fold) on days 1 and 2, but increased significantly thereafter to peak on day 6 post-infection at 150 fold the level measured in the uninfected controls. Thereafter, $Oas1b^{MBT}$ mRNA levels decreased progressively to the level seen in the controls (Fig. 5B). Therefore, induction of the transgenic $Oas1b^{MBT}$ mRNA in the spleen may not account for the survival of transgenic BALB/c mice. These data suggest that the high expression of $Oas1b^{MBT}$ mRNA in the brain prior and after the infection is critical for the survival of CAG::$Oas1b^{MBT}$ mice.

To evaluate the effect of *Oas1b* mRNA expression on WNV infection, we analyzed viral growth kinetics in the brain of infected BALB/c, C.MBT-*Oas1b* and CAG::$Oas1b^{MBT}$ mice. Fig. 6 shows that the viral burden increased significantly in BALB/c mice between day 6 and day 8. The peak of viral burden in the brain of BALB/c mice correlated with the 200 fold increase of $Oas1b^{BALB/c}$ mRNA expression (Fig. 4B). By contrast, the viral burden did not vary between day 6 and day 8 in the brain of C.MBT-*Oas1b* and CAG::$Oas1b^{MBT}$ mice. These data suggest that the antiviral effect of $Oas1b^{MBT}$ gene expression contributes to the increased survival of C.MBT-*Oas1b* and CAG::$Oas1b^{MBT}$ mice.

In the present study, we show that inducing constitutive expression of an $Oas1b^{MBT}$ transgene in an $Oas1b^{BALB/c}$ background is able to confer resistance to an otherwise lethal infection with WNV. Our results establish the functional role played by the premature stop codon in exon 4 of *Oas1b* gene in the susceptibility of BALB/c mice but also suggest impaired activation of the *Oas1b* gene in response to viral infection in this strain. These findings are in agreement with *in vitro* studies that showed reduce viral production in WNV-infected cells overexpressing mRNA encoding the full-length OAS1B (Lucas et al., 2003; Perelygin et al., 2002). In recent studies, Scherbik et al.(2007a)

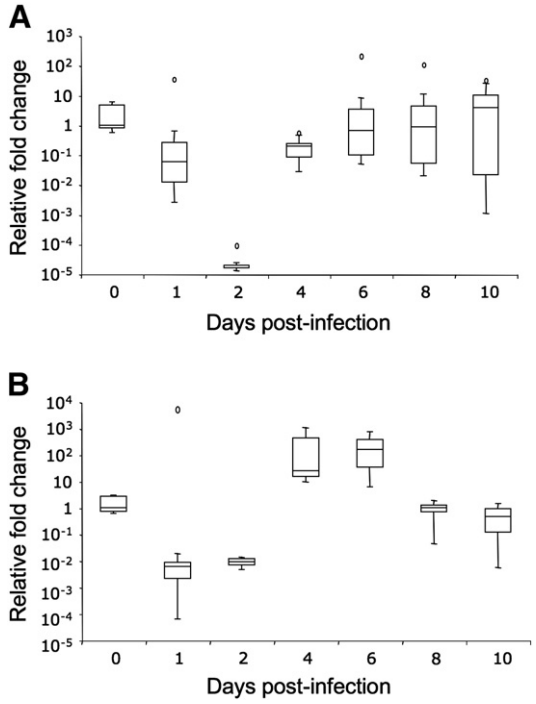

Fig. 5. Level of CAG::*Oas1b*^{MBT} mRNA expression in West Nile virus-infected transgenic mice. Mice were infected with 1000 FFU of the virulent IS-98-ST1 strain and euthanized on days 1, 2, 4, 6, 8 and 10. Total RNA from the spleen (A) and brain (B) was analyzed for the expression of transgenic *Oas1b* mRNA by qRT-PCR. Data are expressed as the relative increase (n-fold) compared with uninfected controls. Data from ten mice are shown as whisker-box plots. Open circles indicate outliers.

used a complementary experimental approach and showed that the replacement of exons 4 and 5 of *Oas1b* gene in susceptible 129/SvJ mice by the corresponding sequences from resistant mice results in the acquisition of resistance to intracerebral infection by the neurovirulent 17D strain of yellow fever virus. Together, the results of knock-in and transgenesis experiments establish that expression of a full-length OAS1B protein is a cornerstone in the resistance to infection with flaviviruses.

Important for the outcome of the disease in transgenic mice were the levels of *Oas1b*^{MBT} mRNA in the brain prior to and after infection. The 1300 fold increase in *Oas1b*^{MBT} mRNA level in the brain of transgenic mice compared to congenic mice prior to infection (Table 1) and the further 150 fold increase at day 6 post-infection (Fig. 5B) were able to control viral burden in the brain (see Fig. 6). This suggests that high levels of functional OAS1B in the brain of naive mice are protective towards later WNV infection. These data agree with recent studies showing that pre-treatment of mice *in vivo* with OAS1 protein potently inhibits viral infection (Kristiansen et al., 2010). Following infection via the peripheral route, the viral burden was not significantly lower in the brain of congenic C.MBT-*Oas1b* and CAG::*Oas1b* transgenic mice at day 6 post-infection compared to the viral burden in the brain of BALB/c mice. This suggests that the expression of full-length OAS1B protein in peripheral organs of congenic and transgenic mice had no significant effect on WNV replication in peripheral tissues and on the spreading to the brain. At day 8, a significant increase in viral burden was observed in the brains of BALB/c mice. By contrast, there was no increase in viral burden in the brain of congenic and transgenic mice. These data suggest that following infection with a neuroinvasive and neurovirulent WNV strain, OAS1B protein is crucial in the brain where it is able to limit the burden of viral genome. Despite this protection by the CAG::*Oas1b*^{MBT} transgene, approximately one-third of transgenic mice did not survive the challenge, indicating that, by contrast with the MBT/Pas chromosomal segment carried by congenic mice, the *Oas1b*^{MBT} transgene conferred only partial resistance to WNV-infected BALB/c mice. Although the reasons remain unclear, several differences between congenic and transgenic mice could account for the disparity in results. First, by contrast with endogenous *Oas1b*^{MBT} gene, the transgene expression driven by the hybrid cytomegalovirus (CMV) enhancer/chicken β-actin (CAG) promoter was down-regulated in the spleen and brain at an early stage post-infection. This decrease is most likely due to inhibition of the CMV immediate early enhancer by the cytokine response that follows the viral infection. Indeed, cytokines induced by WNV infection, such as IFN-α and TNF-α (Fredericksen et al., 2008) have been shown to reduce transgene expression from the CMV promoter (Gribaudo et al., 1995; Qin et al., 1997). Second, the activity of the transgene in the brain prior to infection varied from mouse to mouse. Lower transgene expression could be responsible for increased susceptibility in individual mice. It is interesting to note that, on average, susceptible transgenic mice died approximately two days later than BALB/c mice (10.1 ± 0.5 days for CAG::*Oas1b*^{MBT} mice and 8.2 ± 0.2 days for BALB/c mice), suggesting that transgenic *Oas1b*^{MBT} mRNA could provide some level of protection against the viral infection to all individuals. Finally, the possibility that, in congenic mice, there is a contribution to resistance by additional genetic factors within the *Oas* cluster is a critical issue that remains to be investigated.

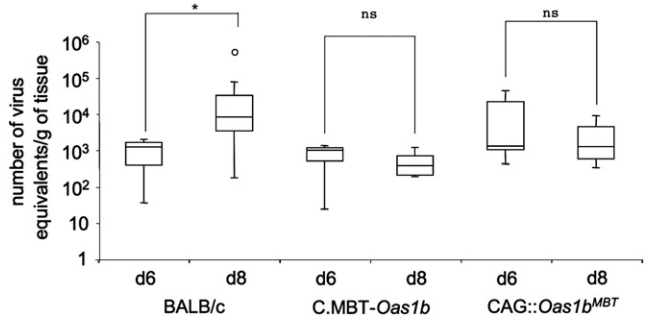

Fig. 6. Viral burden analysis in the brain of wild-type BALB/c, congenic C.MBT-*Oas1b* and transgenic CAG::Oas1b^{MBT} mice after West Nile virus infection. West Nile virus RNA in the brain was determined from samples harvested on days 6 and 8 using qRT-PCR. Data are shown as viral RNA equivalent of PFU per gram of tissue at the indicated time point. Data from ten mice are shown as whisker-box plots. *: $p<0.05$.

Materials and methods

Virus

The WNV IS-98-ST1 strain was isolated from the cerebellum of a white stork during the 1998 outbreak in Israel and passaged three times in a mosquito cell line (Malkinson et al., 2002) before its characterization in mice (Lucas et al., 2003).

Mice

All studies on animals followed the guideline on the ethical use of animals from the European Community Council Directive of 24 November 1986 (86/609/EEC). Animal experiments were approved and conducted in accordance with the Institut Pasteur Biosafety Committee. BALB/cAnNCrl (BALB/c hereafter) mice were purchased from Charles River France Laboratories (L'Arbresle, France). Congenic and transgenic mice were produced in the animal facilities at the Institut Pasteur.

Mouse infections

Eight- to ten-week-old mice were used in all experiments. Infection was performed by intraperitoneal inoculation of 1000 FFU IS-98-ST1 WNV.

Generation of congenic mice

The 1.45 Mb chromosome 5 segment from the MBT/Pas genome delimited by the *D5Mit68* and *D5Mit242* markers, that encompasses 27 genes, including the eight *Oas1* paralogs, and the *Oas2* and *Oas3* genes, was introgressed into the BALB/c background following ten successive backcrosses. Thereafter, mice heterozygous for the MBT/Pas chromosomal segment were intercrossed to produce homozygotes for the MBT/Pas segment that were maintained by sib mating. The resulting congenic line is designated C.MBT-*Oas1b*.

Production of transgenic mice

The *Oas1b* cDNA from MBT/Pas strain (GenBank: AF466823) was inserted into a pCAL3 expression vector (Niwa et al., 1991; Xu et al., 2001) containing the CMV immediate early enhancer, chicken β-actin promoter with β-actin intron and SV40 (late gene) polyadenylation sequence. The entire insert with the promoter and coding region, pCAG::*Oas1b*^{MBT}, was linearized with *Pvu*I restriction enzymes and gel-purified. The purified fragment was injected into (C57BL/6 J × SJL/J) F2 fertilized eggs. Incorporation of the transgene was assessed by PCR analyses of ear DNA. PCR primers for the transgene detection were as follows: forward primer, 5′AACCATGTTCATGCCTTCTTCT-3′; reverse primer, 5′-AAGGAACACCACCAGGTCAG-3′. Three transgenic founders were used to derive three independent congenic strains by twelve generations of backcrossing onto the BALB/c background. All transgenic mice used in this study were heterozygous for the transgene.

Quantification of Oas1a and Oas1b mRNA levels by qRT-PCR

Ten mice were used per time point. After extensive cardiac perfusion with phosphate-buffered saline (PBS), organs were harvested in 1 ml Trizol® (Invitrogen, Carlsbad, CA, USA) containing 0.4 g of glass beads (212–300 μm ø, SIGMA) and homogenized for 40 s at 6.0 in FastPrep Instrument (MP Biochemicals, Illkirch, France). Isolated RNA was repurified using an RNeasy® Plus Mini Kit (QIAGEN, Valencia, CA, USA). cDNA synthesis was performed with 5 μg RNA using RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas Inc., Glen Burnie, MD, USA). Five microliters of a 1:10 dilution of cDNA were used per qRT-PCR reaction. The cDNA obtained from organs was quantified by real-time qRT-PCR using Maxima™ SYBR Green/ROX qPCR Master Mix (Fermentas) in an ABI StepOne Plus apparatus (Applied Biosystems, Carlsbad, CA). To analyze the relative fold induction of endogenous *Oas1b* gene and CAG::*Oas1b* transgene mRNA, aldolase (*Ald*) mRNA expression levels were also determined for normalization by using the C_T method (Livak and Schmittgen, 2001). For *Ald* mRNA, the forward primer was 5′-AGCAGAATGGCATTGTACCC-3′ and reverse primer was 5′-ACAGGAAAGTGACCCCAGTG-3′. For *Oas1b* mRNAs, five primers were designed: a: 5′-GAGGTGCCGACGGAGGT-3′; b: 5′-TCCAGATGAAGTCTTCCCAAAG-3′; c: 5′-CAGGATGCTCCAGAGTCAGACG-3′; d: 5′-TGACACCAGACCAACTGGTAA-3′; and d′: 5′-GACAAGAGAAAGCCCACACC-3′. Primers a and b amplify endogenous *Oas1b* gene and CAG::*Oas1b*^{MBT} transgene mRNAs. Primers c and d′ amplify endogenous *Oas1b* mRNA. Primers c and d amplify CAG::*Oas1b*^{MBT} transgene mRNA. Therefore, primers c–d and c–d′ allow distinguishing the mRNAs from endogenous *Oas1b* gene and CAG::*Oas1b*^{MBT} transgene. To analyse *Oas1a* mRNA expression levels, the following primers were used: forward: 5′-GGAGGCGGTTGGCTGAAGAGG-3′; reverse: 5′-GAACCACCGTCGGCACATCC-3′. Reactions were quantified in triplicate and data was analyzed with StepOnePlus software version 2.1 (Applied Biosystems).

Quantification of tissue viral burden

To monitor viral spread in the brain, positive-strand viral RNA levels were measured using Taqman qRT-PCR with the Hot Pol® Probe qPCR Mix (Euromedex, Souffelweyersheim, France). The 5′-end of WNV capsid mRNA was amplified from total RNA and its expression level quantified. The following primers and probe were used: forward primer, 5′-CCTGTGTGAGCTGACAAACTTAGT-3′; reverse primer, 5′-GCGTTTTAGCATATTGACAGCC-3′; probe, 5′-FAM-CCTGGTTTCTTAGACATCGAGATCT-3′-TAMRA, as previously described (Linke et al., 2007).

Statistical analysis

Data analysis was performed using the MS Excel statistical package (Microsoft Corporation). Kaplan–Meier survival data was analyzed by the log rank test. Differences in relative expression were analyzed using one-way analysis of variance (ANOVA) or non-parametric Mann–Whitney test.

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