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Modulation of TLR9 response in a mouse model of herpes simplex virus encephalitis

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ABSTRACT

We evaluated the effects of agonists and antagonist of toll-like receptor (TLR) 9 in comparison with a TLR3 agonist in a mouse model of herpes simplex virus type 1 (HSV-1) encephalitis (HSE). BALB/c mice received a single intranasal dose of either a TLR3 agonist (polyinosinic:polycytidylic acid; PIC), TLR9 agonists (oligodeoxynucleotides (ODNs) 1585, 1826 or 2395) or a TLR9 antagonist (ODN 2088), 1 day before and, for selected groups, 3 days after infection with HSV-1. Mice that received the pre-treatment with vehicle, PIC, ODNs 1585, 1826, 2395 and 2088 before infection had survival rates of 25%, 65%, 55%, 40%, 55% and 30%, respectively ($P < 0.05$ for PIC and ODNs 1585 and 2395 *versus* vehicle). Infected mice subsequently treated with vehicle, ODNs 2395 and 2088 had survival rates of 9%, 0% and 30%, respectively ($P < 0.05$, ODN 2088 *versus* other groups). The pre-treatment of mice with ODN 2395 reduced both the viral load ($P < 0.05$ at day 5) and the production of CCL2, IL-6 and CCL5 at days 3, 4 and 5 ($P < 0.05$ for IL-6 at day 3 and $P < 0.05$ for CCL2 and CCL5 at day 4). Treatment of infected mice with ODN 2088 reduced the production of the same cytokines ($P = 0.07$ for CCL2 and $P = 0.09$ for IL-6 at day 5). Pre-treatment of mice with TLR9 agonists before infection reduces brain viral load and cytokine levels resulting in increased HSE survival rates. On the other hand, TLR9 antagonists can be helpful to control the inflammatory response that could be detrimental after infection.

1. Introduction

Human herpesvirus 1 (HSV-1) is a common human pathogen, most often associated with orolabial, genital and ocular infections and is the most frequent cause of sporadic and potentially fatal viral encephalitis in Western countries (Tyler, 2004; Whitley and Kimberlin, 2005). Although intravenous acyclovir blocks viral replication and significantly reduces the mortality associated with HSV-1 encephalitis (HSE), many infected patients still suffer from severe neurological sequelae. The mechanisms responsible for the neuronal damage and the mortality attributable to HSE could involve both virus and immune-related processes. A recent study from our laboratory has evaluated the effect of treatment with glucocorticoids (Sergerie et al., 2007) in a mouse model of HSE and revealed that modulating the innate immune response in a timely manner could prevent neurological damage and mortality.

Toll-like receptors (TLRs) are innate immune sensors implicated in the control of infections through the recognition of pathogen-associated molecular patterns. Several TLRs, especially TLRs 2, 3 and 9 (Krug et al., 2004; Kurt-Jones et al., 2004; Lund et al., 2003; Melchjorsen, 2012; Sergerie et al., 2007), are involved in early recognition of HSV components. TLR2, which is expressed on the cell surface, is the first TLR to recognize the virus through its surface glycoproteins (Aravalli et al., 2005; Kurt-Jones et al., 2004; Sarangi et al., 2007). TLR9 and TLR3, which are expressed in the endosomes (Ahmad-Nejad et al., 2002; Latz et al., 2004; Matsumoto et al., 2003; Rutz et al., 2004), are then stimulated by HSV DNA which is rich in CpG sequences (Krug et al., 2004; Lund et al., 2003) and by self-hybridization with dsRNA from viral genes, respectively (Alexopoulou et al., 2001; Jacquemont and Roizman, 1975). The natural ligands of these TLRs, and their agonists, use different signaling pathways and induce the production of different pro-inflammatory molecules. TLR9 uses the myeloid differentiation primary response gene 88 (MyD88)-dependent pathway that induces NF-κB activation (Ashkar and Rosenthal, 2002; Janssens and Beyaert, 2002; Takeuchi and Akira, 2002) whereas TLR3 not only uses the MyD88-dependent pathway but also the TIR-domain-containing adapter-inducing interferon-β (TRIF)-dependent pathway that leads to the activation of the IFN-β promoter (Doyle et al., 2003). TLR3 and TLR9 are both able to stimulate the production of IFN-α and/or IFN-β (Takeda and Akira, 2005). Moreover, TLR9 agonists may produce high levels of IFN-α through IFN regulatory factor 7 (IRF7) (Honda et al., 2005) whereas TLR3 agonists activate IRF3 which mediates a specific gene program responsible for innate antiviral response (Doyle et al., 2002).

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TLR9 agonists consist of oligodeoxynucleotides (ODNs) which are classified into three types, namely A, B and C relative to their immunostimulatory activities. Type A induces high level of IFN-α from plasmacytoid dendritic cells (Krug et al., 2001). Type B, which stimulates B cells, induces strong humoral responses and could be potentially used as therapeutic agent or vaccine adjuvant (Klinman, 2004; Krieg, 2006). Type C, which possesses both properties, induces IFN-α production and stimulates B cells (Marshall et al., 2005). A fourth type (type S), also known as "suppressive" ODNs or TLR9 antagonists, was designed to interfere with the binding of CpG DNA and TLR9 in endosomal vesicles thus neutralizing their stimulatory effect (Krieg et al., 1998; Peter et al., 2008; Stunz et al., 2002).

Agonists of TLRs 3, 7, 8 and 9 have shown benefits in preventing initial infection and recurrences in several animal models of genital herpes (Ashkar et al., 2003, 2004; Bernstein et al., 2001; Gill et al., 2006; Herbst-Kralovetz and Pyles, 2006; McCluskie et al., 2006; Pyles et al., 2002). Our group also demonstrated that pre-treatment of mice with polyinosinic:polycytidylic acid (PIC), a TLR3 agonist, before intranasal inoculation of HSV-1 decreased HSE severity and mortality probably through an early production of IFN-β (Boivin et al., 2008). Conversely, administration of PIC after the infection resulted in increased disease severity and mortality compared to control mice (unpublished result). We also showed that pre-treatment of mice with a TLR9 agonist (type B ODN 1826) increased the survival rate of HSV-infected mice but less than PIC probably because it does not stimulate type 1 IFN production (Boivin et al., 2008). Thus, it could be interesting to evaluate the effect of types A and C ODNs that induce IFN production as well as a TLR9 antagonist that may reduce inflammation post-infection (Krug et al., 2004).

In the present study, we evaluated the effect of stimulating or blocking the TLR9 response through the use of different agonists [ODN 1585 (type A), ODN 1826 (type B) or ODN 2395 (type C)] and an antagonist (ODN 2088) administered prior to or 3 days after infection in a well-characterized mouse model of HSE.

2. Materials and methods

2.1. Agonists and antagonist of TLRs

The agonists of TLR3 (PIC; size of 1.5–8 kb) and TLR9 [ODN 1585 (type A), ODN 1826 (type B), ODN 2395 (type C)] and the antagonist of TLR9 [ODN 2088 (type S)] were obtained from InvivoGen (San Diego, CA).

2.2. Animals and experimental procedures

Four-week-old BALB/c mice were purchased from Charles River Canada (St-Constant, Quebec, Canada). In both the pre- and post-infection experiments, a single intranasal (I.N.) dose of 50 μg/mouse of either PIC, ODN type A, B, C or S was administered in a volume of 20 μl of vehicle (0.9% saline). A negative control of 20 μl of vehicle alone was also included. In the first experiment (pre-infection), six groups of 20–34 mice were pre-treated and then infected intranasally one day later with 3800 plaque forming units (PFUs) of a neurovirulent HSV-1 clinical strain (H25 grown and passaged 5 times on Vero cells) described elsewhere (Sergerie et al., 2007). Following virus stock production, the titer of the viral suspension was determined on Vero cells and a back titration was performed at the end of each experiment to confirm the inoculum administered to mice. In the second experiment (post-infection), three groups of 29–31 mice received a single I.N. dose of ODN type C or S or the vehicle 3 days following the infection with HSV-1 (4200 PFUs) as described above. In a third confirmatory experiment, 6 groups of 30–35 mice were infected with 4050 PFUs of HSV-1 to evaluate simultaneously the effect of pre- and post-infection treatments with a single I.N. dose of ODN type C or S or the vehicle. In all experiments, four to five mice per group were sacrificed on days 3, 4 and 5 post-infection for brain viral load and/or cytokine quantification. In the third experiment, brains were separated in three different parts, namely the olfactory bulb, the pons/medulla/cerebellum region and the cortex/hypothalamus region. Mice were monitored during 25 days for appearance of HSE-related symptoms, namely ruffled fur, ocular swelling, reduced mobility and shaking movements as well as for mortality. Animals were sacrificed when a $\geqslant$ 20% weight loss was observed or a combination of two clinical signs. All experimental procedures were approved by the Animal Care Ethics Committee of Laval University.

2.3. Viral load and cytokines measurements

Mouse brains were rapidly removed, dissected (when indicated) and homogenized in phosphate-buffered saline. For viral load quantification, DNA was extracted from brain homogenates with the Magnapure LC total nucleic acid isolation kit (Roche Molecular Systems, Laval, Quebec, Canada) and eluted in 100 μl of elution buffer. Real-time polymerase chain reaction (PCR) was performed using 5 μl of extracted DNA and external standards were run in parallel as previously described (Boivin et al., 2006). Viral titers were also assessed by plaque assays.

For cytokine quantification, aliquots of 500 μl of brain homogenates were mixed with protease inhibitor cocktail (Sigma–Aldrich, Oakville, Ontario, Canada) and 200 μl of KPO_4 containing 0.4% CHAPS. Samples were centrifuged and the supernatants were stored at −20 °C until use. Quantification of IL-6, CCL2 (MCP-1) and CCL5 (RANTES) was performed by magnetic bead-based immunoassay using the Bio-Rad Bio-Plex mouse cytokine group 1 3-plex assay (Bio-Rad, Mississauga, Ontario, Canada). Results were analyzed with the Bioplex system equipped with the Bioplex manager 5.0 software (Bio-Rad, Mississauga, Ontario, Canada).

2.4. Statistical analysis

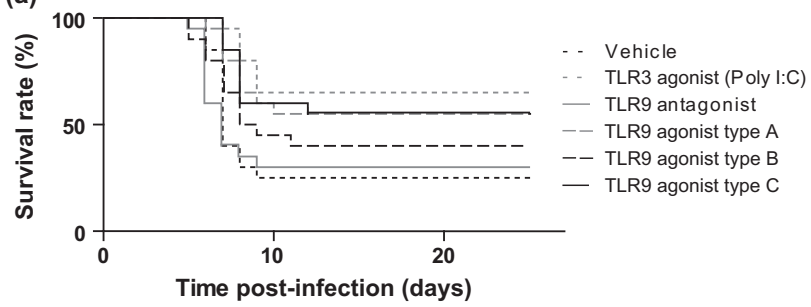
Life expectancy for each group of mice was estimated by the Kaplan–Meier method and differences between groups were compared using a log rank (Mantel-Cox) test. Viral loads and chemokine/cytokine levels were analyzed by a one-way analysis of variance (ANOVA) with the Tukey's multiple comparisons post-test or a *t*-test with Welch's correction. All statistical analyses were carried out using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA).

3. Results

3.1. Effect of pre-infection administration of TLR3/TLR9 agonists and TLR9 antagonist on HSE survival

The impact of stimulating or neutralizing TLR9 response before infection of mice was first investigated. We also used PIC, a TLR3 agonist, as a positive control. Mice that received ODN types A and C and PIC had increased survival rates, delayed appearance of clinical signs (by 1–2 days) and statistically significant longer mean life expectancies. On the other hand, administration of ODN 1826 did not significantly increase survival rate or life expectancy (Fig. 1). These results were confirmed in a subsequent experiment for selected groups (Fig. 3).

(a)

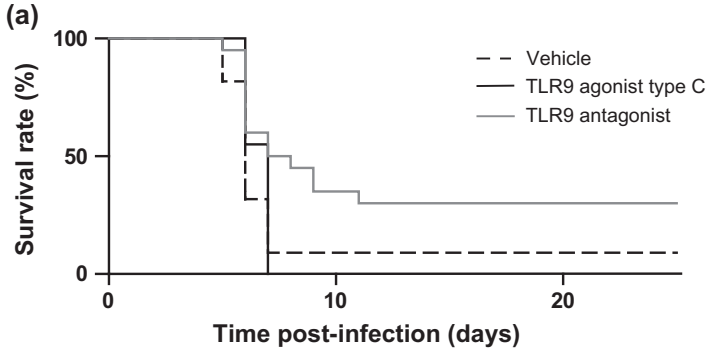


(b)

Group	Mean life expectancy (days)	number of death/n	Survival (%)
Vehicle	11.6 ± 1.8	15/20	25
TLR3 agonist (Poly I:C)	19.2 ± 1.8 *	7/20	65
TLR9 antagonist	12.1 ± 1.9	14/20	30
TLR9 agonist type A	17.3 ± 2.0 *	9/20	55
TLR9 agonist type B	14.4 ± 2.0	12/20	40
TLR9 agonist type C	17.4 ± 1.9 *	9/20	55

Fig. 1. Pre-infection treatment effect on mice. Impact of pre-treatment with TLR9/TLR3 agonists and TLR9 antagonist before infection with HSV-1 on survival rate (a) and mean life expectancy (b). Groups of 20 mice received an intranasal (I.N.) pre-treatment with ODN 1585 (TLR9 agonist type A), ODN 1826 (TLR9 agonist type B), ODN 2395 (TLR9 agonist type C), ODN 2088 (TLR9 antagonist), PIC (TLR3 agonist) or the vehicle and were then infected with 3800 plaque forming units (PFUs) of HSV-1 one day later. Mice were carefully examined once a day and obvious sickness signs (more than 20% weight loss or a combination of two clinical signs) or mortality were considered as end points for mean life expectancy. Mean life expectancies between groups were compared using a log rank test. *Significantly different ($P < 0.05$) from HSV-1-infected mice that received the vehicle.

(a)



(b)

Group	Mean life expectancy (days)	number of death/n	Survival (%)
Vehicle	7.8 ± 1.2	20/22	9
TLR9 agonist type C	6.6 ± 0.1	20/20	0
TLR9 antagonist	12.4 ± 1.9 *	14/20	30

Fig. 2. Post-infection treatment effect on mice. Impact of post-infection treatment of HSV-1 infected mice with TLR9 agonist type C or TLR9 antagonist on survival rate (a) and mean life expectancy (b). Groups of 20–22 mice were inoculated with 4200 PFUs of HSV-1 and received 3 days later an I.N. administration of ODN 2395 (TLR9 agonist type C), ODN 2088 (TLR9 antagonist) or the vehicle. Mice were carefully examined once a day and obvious sickness signs (more than 20% weight loss or a combination of two clinical signs) or mortality were considered as end points for mean life expectancy. Mean life expectancies between groups were compared using a log rank test. *Significantly different ($P < 0.05$) from HSV-1-infected mice that received the vehicle or the TLR9 type C agonist.

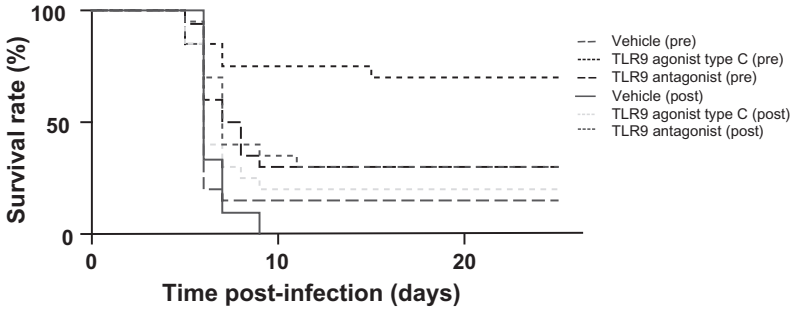
3.2. Effect of post-infection administration of TLR9 agonist and antagonist on HSE survival

For this experiment, the TLR9 agonist that resulted in the best survival rate in the pre-infection experiment (ODN 2395, type C) and the TLR9 antagonist were administered to mice along with the vehicle 3 days following HSV-1 infection. We did not use PIC here as its administration 2 days post-infection previously resulted in 100% mortality (unpublished data). Mice that received the TLR9 antagonist had an increased survival rate, a delay in mortality and a statistically significant longer mean life expectancy compared to the vehicle and the TLR9 agonist ODN 2395 (Fig. 2). These results were confirmed in a third experiment, except that the difference between the antagonist and the agonist was only significant at day 6 (Fig.3).

3.3. Effect of administration of TLR9 agonist and antagonist on brain viral load

The positive effect observed on survival rate after pre-treatment of mice with the TLR9 type C agonist was accompanied by a significant reduction of the brain viral loads determined by PCR at day 5 post-infection (Fig. 4(a and b)), and of viral titers at day 4 compared to the vehicle and the antagonist groups (0.2 *versus* 61 and 10 PFUs/μl, respectively). Also, the TLR9

(a)



(b)

Group	Mean life expectancy (days)	number of death/n	Survival (%)
Vehicle (pre)	8.8 ± 1.6	17/20	15
TLR9 agonist type C (pre)	19.7 ± 1.9 *	6/20	70
TLR9 antagonist (pre)	12.2 ± 1.9	14/20	30
Vehicle (post)	6.6 ± 0.2	20/20	0
TLR9 agonist type C (post)	10.0 ± 1.7	16/20	20
TLR9 antagonist (post)	12.3 ± 1.9 *	14/20	30

Fig. 3. Pre- and post-infection treatment effect on mice. Impact of pre- and post-treatment with TLR9 agonist type C and TLR9 antagonist before and after infection with HSV-1 on survival rate (a) and mean life expectancy (b). Groups of 20 mice received an intranasal (I.N.) pre- or post-treatment with ODN 2395 (TLR9 agonist type C), ODN 2088 (TLR9 antagonist) or the vehicle. Mice were infected with 4050 plaque forming units (PFUs) of HSV-1 and were carefully examined once a day and obvious sickness signs (more than 20% weight loss or a combination of two clinical signs) or mortality were considered as end points for mean life expectancy. Mean life expectancies between groups were compared using a log rank test. *Significantly different ($P < 0.05$) from the corresponding vehicle group. The difference between the agonist and the antagonist is significant for the pre-infection treatment and also for the post-infection treatment at day 6.

antagonist resulted in higher viral DNA load at day 4 in comparison to the agonist. As for the post-treatment, no significant differences were observed in the whole brain determined by PCR (Fig. 5(a)) or quantitative culture (8, 1 and 17 PFUs/μl for vehicle, TLR9 agonist type C and TLR9 antagonist groups). In specific brain sections, the antagonist reduced the viral DNA load at day 4 whereas the agonist significantly increased viral load at day 5 in the olfactory bulb (Fig. 5(b)).

3.4. Effect of administration of TLR9 agonist or antagonist on chemokine/cytokine production

CCL2 , CCL5 and IL-6 were measured on days 3, 4 and 5 post-infection in brain homogenates of mice that were pre-treated with the TLR9 type C agonist or the vehicle (Fig. 6). Cytokine production increased more rapidly in mice that received the vehicle when compared to the TLR9 agonist. Differences were significant at days 3 (IL-6) and 4 (CCL2 and CCL5), but not at day 5 post-infection due to the high variability observed in the vehicle group. In the TLR9 type C agonist group, levels of IL-6 at day 3 and levels of IL-6 and CCL5 at day 4 post-infection were below the limit of detection of the assay and were extrapolated by the software.

The levels of the same chemokines/cytokines were also measured on days 4 and 5 post-infection in mice that received the TLR9 antagonist and the vehicle 3 days post-infection (Fig. 7). We detected lower levels of IL-6, CCL2 and CCL5 on day 5 post-infection, that were almost significant, in the group that received the TLR9 antagonist compared to the vehicle.

4. Discussion

Herein, we report that stimulation of TLR9 response prior to the infection through the administration of type C agonist protected BALB/c mice against HSE by reducing brain viral load and, consequently, cytokine production. Conversely, its neutralization through the administration of an antagonist after the infection was also beneficial by decreasing mortality likely by a control of the exaggerated inflammatory response in the brain.

We already demonstrated that the administration of an agonist of TLR3 (PIC) before the infection in our mouse model of HSE induced a hostile pro-inflammatory environment in the brain that prevented viral replication leading to decreased HSE severity and mortality (Boivin et al., 2008). Recent data suggest that the antiviral effect induced by PIC could be also mediated through the melanoma differentiation-associated gene 5 (MDA-5) signaling pathway (Gaajetaan et al., 2012). Indeed, MDA-5 is a cytoplasmic sensor present in most cells which has been shown to recognize 1.5–8 kb PIC (Gitlin et al., 2006; Kato et al., 2006; Kawai and Akira, 2010; Wilkins and Gale, 2010). In parallel, we also showed that pre-treatment of mice with a TLR9 type B agonist (ODN 1826) protected mice from HSE, although the effect was not as pronounced as with the TLR3 agonist (Boivin et al., 2008) probably because it does not stimulate type 1 IFN production. At this time, TLR9 type B agonists were the first to be discovered and to be evaluated in HSV-2 animal models (Krieg et al., 1995). Since then, TLR9 types A and C agonists, which stimulate the production of type 1 IFN, as well as new antagonists that can reduce the inflammation have been developed.

In the present study, we analyzed in more details the effect of activating or neutralizing TLR9 response through the use of types A, B and C agonists or an antagonist in our mouse model of HSE. We showed that pre-treatment of mice with TLR9 types A or C agonists confer significantly increased survival rates, while treatment with an antagonist resulted in an improved outcome when administration was delayed after the infection. The effect observed with the TLR9 type B agonist administered before the infection on the survival rate was lower than that reported in our previous study (i.e., 40% *versus* 60%) (Boivin et al., 2008). This difference can be explained by a change in the definition of the endpoint used for the sacrifice of mice between these two studies (i.e., a weight loss of $\geqslant$20% in combination with 1 neurological sign in our previous study *versus* a weight loss of $\geqslant$20% or a combination of two clinical signs in the present study). With the new endpoint, the survival rate in our initial study would have been 46.7%, which is similar to the data obtained in this study. The brain viral load reduction in the TLR9 type C pre-treatment group is supported by a recent study that analyzed the role of different TLRs *in vitro* and showed

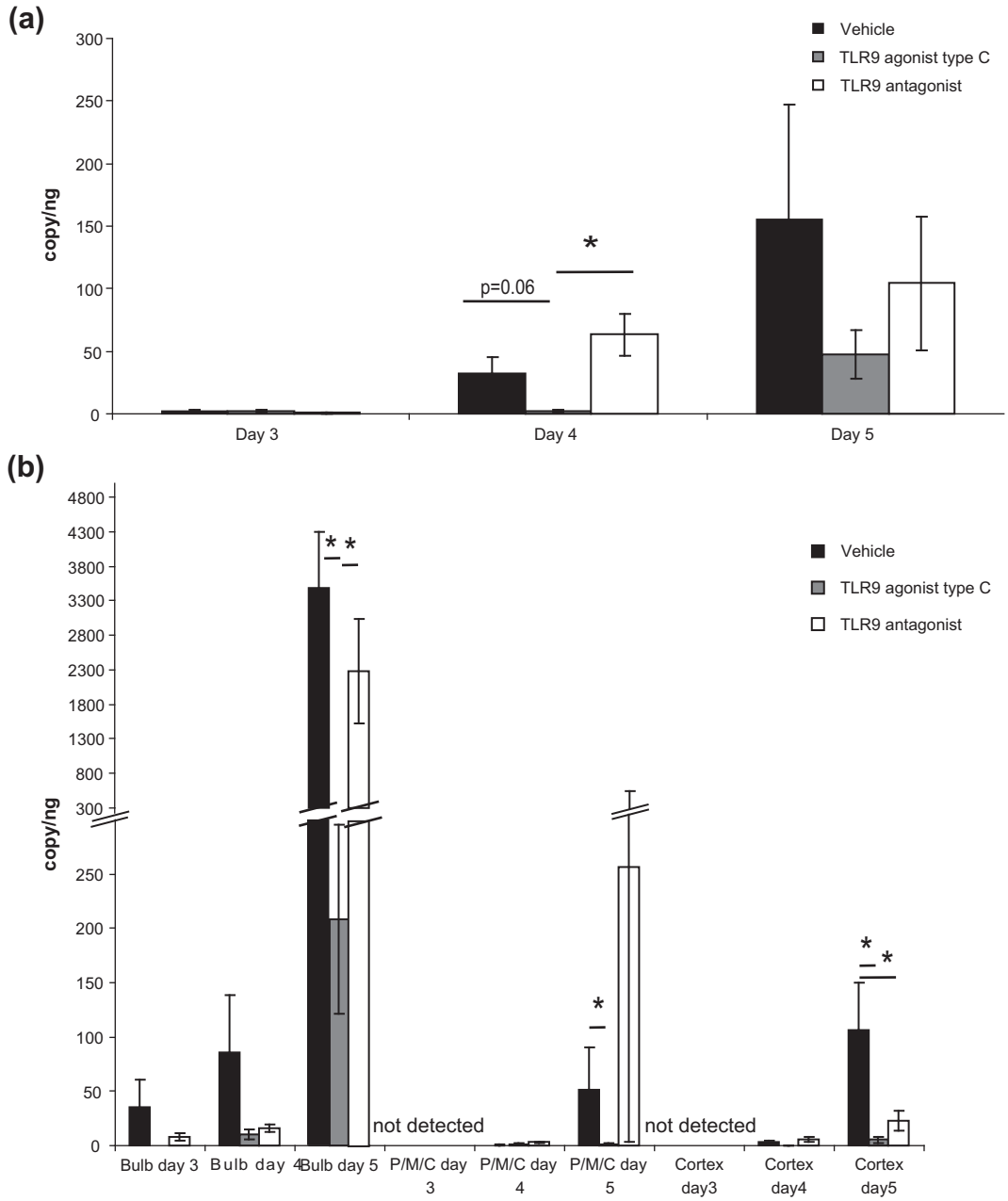


Fig. 4. Pre-infection treatment effect on viral load. Impact of pre-treatment with TLR9 agonist type C or TLR9 antagonist before infection with HSV-1 on brain viral DNA loads in the first (a) and the third (b) experiments. Groups of mice received an I.N. pre-treatment with ODN 2395 (TLR9 agonist type C), ODN 2088 (TLR9 antagonist) or the vehicle and were then infected with HSV-1 one day later. Entire brains or brain sections (4–5 mice per group) were rapidly removed and homogenized on days 3, 4 and 5 after the infection. Brain viral loads were quantified by real-time PCR with specific primers targeting the HSV-1 DNA polymerase gene on brain homogenates taken on days 3, 4 and 5. Bulb stands for olfactory bulb. P/M/C stands for pons, medulla and cerebellum. Cortex stands for the cortex/hypothalamus region. Bars show means ± SEM. *Statistically significant difference ($P < 0.05$, one-way ANOVA and *t*-test).

that stimulation of dendritic cells and fibroblasts with ODN (type A) and PIC limits HSV infection (Gaajetaan et al., 2012). The authors reported that increasing the concentration of the ODN for cell stimulation reduced the viral load and increased IFN-β production.

We have previously shown that the early induction of different pro-inflammatory cytokines resulting from the administration of PIC before the infection might limit HSV-1 replication in the CNS (Boivin et al., 2008). In this study, we chose to evaluate the levels of CCL2, IL-6 and CCL5 in the brain of mouse groups that had the best outcome during HSE and compared them with the vehicle. Indeed, these chemokines/cytokines have known implications in leukocyte recruitment and clearance of the virus and the modulation of their production is important to properly control the dissemination of different viruses in the CNS. CCL2 has been implicated in many inflammatory diseases, is highly expressed during HSV encephalitis and can cause a blood–brain barrier breakdown (Getts et al., 2008; Marques et al., 2006; Stamatovic et al., 2005). In a TLR9 knockout mouse model of HSE, the higher mortality rate compared to wild type (C57BL/6) was ascribed to slightly increased levels of CCL2 in the CNS (Lima et al., 2010). Although IL-6 is protective against lethal HSV ocular infection (LeBlanc et al., 1999), elevated levels may be neurotoxic and also increase the permeability of the blood-brain barrier (Klinman et al., 1996; LeBlanc et al., 1999; Paul et al., 2003; Takeda et al., 2011). Similarly, even if

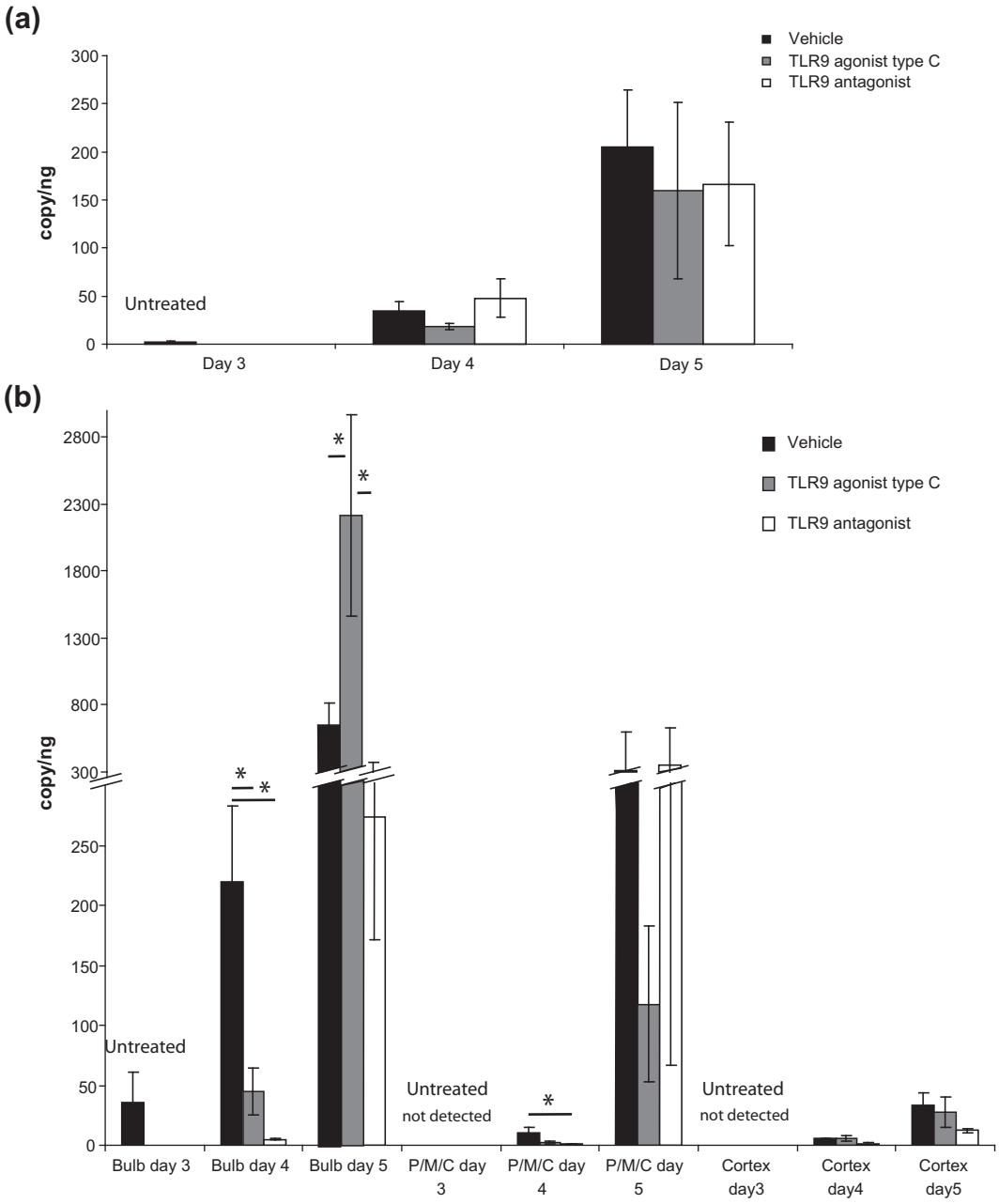


Fig. 5. Post-infection treatment effect on viral load. Impact of treatment of HSV-1 infected mice with TLR9 agonist type C or TLR9 antagonist on brain viral DNA loads in the second (a) and third (b) experiments. Groups of mice were infected with HSV-1 and received 3 days later an I.N. administration of ODN 2395 (TLR9 agonist type C), ODN 2088 (TLR9 antagonist) or the vehicle. Entire brains or brain sections (4–5 mice per group) were rapidly removed and homogenized on days 3 (untreated mice) and 4 and 5 after the infection (i.e., which correspond to days 1 and 2 post-treatment, respectively). Brain viral loads were quantified by real-time PCR with specific primers targeting the HSV-1 DNA polymerase gene on brain homogenates taken on days 3, 4 and 5. Bulb stands for olfactory bulb. P/M/C stands for pons, medulla and cerebellum. Cortex stands for the cortex/hypothalamus region. Bars show means ± SEM. *Statistically significant difference ($P < 0.05$, one-way ANOVA and *t*-test).

CCL5 is essential for leukocyte adhesion and recruitment (Malmgaard et al., 2004; Vilela et al., 2009), mice treated with an antibody targeting CCL5 had diminished leukocyte infiltration into the CNS and reduced neurological symptoms in a mouse viral model of multiple sclerosis (Glass et al., 2004). The administration of TLR9 type C agonist prior to the infection and of TLR9 antagonist following the infection reduced cytokine production in our mouse model of HSE. Although the post-infection treatment results were not statistically significant, the trends observed in our study on cytokine levels are similar to those reported by several studies evaluating the effect of TLR9 antagonists on the innate immune response. *In vitro*, the neutralization of TLR9 response with ODN 2088 was examined in corneal endothelial cells after HSV infection and resulted in a decrease level of IL-6 (Takeda et al., 2011). Moreover, blocking the TLR9 response with an antagonist also reduced the early expression of TNF-α and CCL5 induced by HSV-2 (Malmgaard et al., 2004). In light of these results, we suggest that the timing of activation of the TLR9 response following HSV-1 infection and, its subsequent modulation, are crucial for a well-controlled immune response during HSE.

In summary, we showed that the administration of the TLR9 type C agonist before infection or of a TLR9 antagonist on day 3

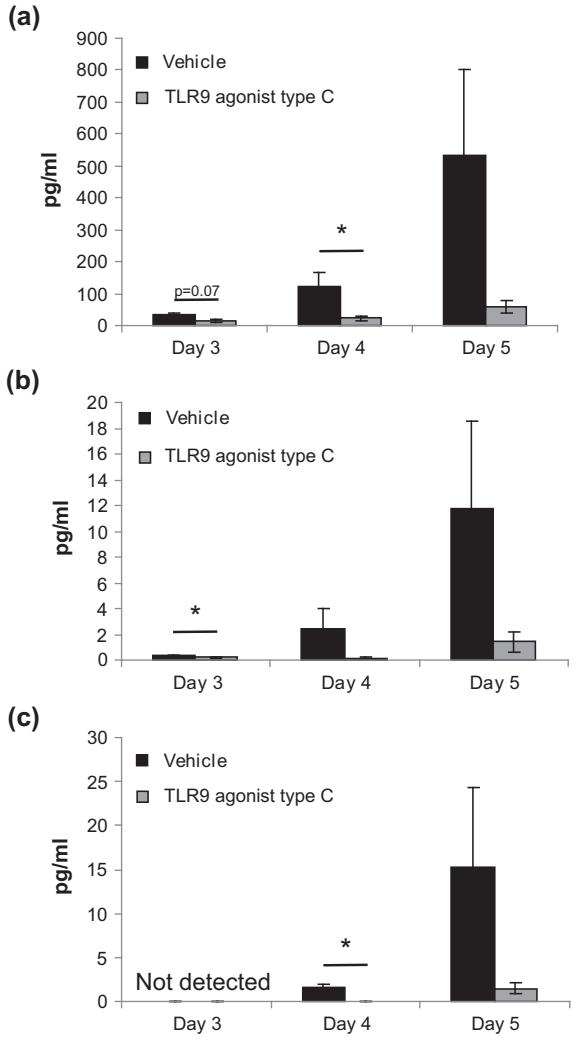


Fig. 6. Pre-infection treatment effect on cytokine production. Impact of pre-treatment with TLR9 agonist type C before infection with HSV-1 on brain levels of CCL2 (a), IL-6 (b) and CCL5 (c). Groups of mice received an I.N. pre-treatment with ODN 2395 (TLR9 agonist type C) or the vehicle and were then infected with HSV-1 one day later. Entire brains (3–5 mice per group) were rapidly removed and homogenized on days 3, 4 and 5 after the infection. Cytokine/chemokine levels were determined in brain homogenates by magnetic bead-based immunoassay. Bars show means ± SEM. *Statistically significant difference ($P < 0.05$, one-way ANOVA and *t*-test).

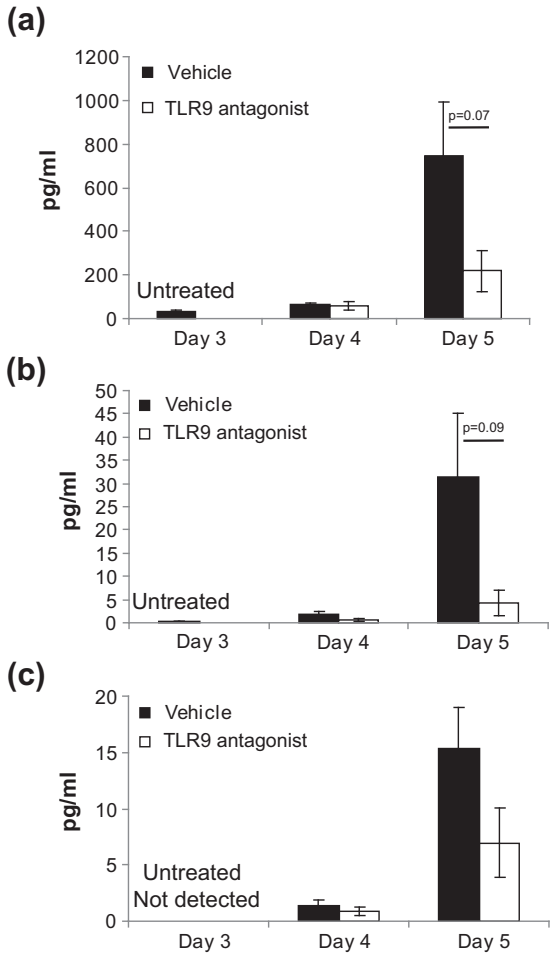


Fig. 7. Post-infection treatment effect on cytokine production. Impact of treatment of HSV-1 infected mice with a TLR9 antagonist on brain levels of CCL2 (a), IL-6 (b) and CCL5 (c). Groups of mice were infected with HSV-1 and received 3 days later an I.N. administration of ODN 2088 (TLR9 antagonist) or the vehicle. Entire brains (3–5 mice per group) were rapidly removed and homogenized on days 3 (untreated mice) and 4 and 5 after the infection (i.e., which correspond to days 1 and 2 post-treatment, respectively). Cytokine/chemokine levels were determined in brain homogenates by magnetic bead-based immunoassay. Bars show means ± SEM. There was no statistical difference between groups.

post-infection was beneficial and improve outcome in mice with HSE. To our knowledge, this is the first *in vivo* study that compared the impact of different types of ODNs which stimulate or neutralize the TLR9 response in a mouse model of HSE. Additional studies should be performed to validate the time at which such treatment should be optimally initiated as well as the dose and schedule of administration of the antagonist. To better understand the effect of modulating TLR9 and TLR3 responses during HSE, we should also evaluate the production of type 1 IFNs and perform additional studies in mouse embryonic fibroblasts. Other potential molecules such as novel human immunosuppressive ODNs (Sun et al., 2010; Yang et al., 2010) or siRNA should be also tested to evaluate their implication in the pathogenesis and management of HSE. It would be also interesting to combine an antiviral, such as acyclovir, with a TLR antagonist or an anti-inflammatory molecule, to evaluate their potential additive or synergistic effects on morbidity and mortality. This could open the door to the development of more effective and rational therapeutic regimens that could be potentially useful in humans.

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