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Herpes simplex type 2-mediated disease is reduced in mice lacking RNase L

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Abstract

RNase L helps mediate the antiviral state induced by type I interferons (IFNαβ). Although herpes simplex virus (HSV) encodes inhibitors of the IFNαβ-induced antiviral response, the IFNαβ system serves the body as a first line of defense against HSV. We investigated whether RNase L limits HSV-2 replication and virulence. RNase$L^{-/-}$ and wild-type C57BL/6 mice were infected intravaginally with HSV-2 strain 333. Although initial replication in the genital epithelium was similar, mice lacking RNase L developed *less* severe genital and neurologic disease than wild-type mice, survived longer, and contained lower viral titers in the nervous system. $CD4^+$ T cell infiltration into the genital tract and spinal cord of RNase $L^{-/-}$ mice was reduced, suggesting that a restricted inflammatory response may account for reduction in disease. Thus, RNase L does not play a significant role in control of HSV-2 infection in vivo; instead RNase L may regulate aspects of the inflammatory response that contribute to disease.

Introduction

The innate IFNαβ response exerts potent antiviral activity in part by causing RNA degradation and translational arrest in virus-infected cells to limit virus replication and spread. IFNαβ are produced by most cells in response to virus infection, and they initiate a signal transduction cascade by binding the IFNαβ receptor (IFNαβR) on uninfected cells to stimulate transcription of genes that prime for an antiviral state that is activated upon virus infection. Among the many antiviral pathways induced by IFNαβ, one prominent antiviral function is mediated by protein kinase R (PKR), which phosphorylates eIF2α to arrest translation. The oligoadenylate synthetase (OAS)/RNase L pathway is another of the most extensively characterized. IFNαβ signaling stimulates synthesis of OAS and RNase L (Floyd-Smith, 1988), and once the cell is infected, viral double-stranded RNA (dsRNA) activates OAS to produce 2′5′-linked oligoadenylates (2′5′A). The 2′5′A then activate RNase L to degrade RNA, resulting in translation inhibition and blockade of viral replication.

In vivo, herpes simplex virus (HSV) infections typically initiate in a moist epithelial surface. Virus quickly spreads from the epithelium into sensory nerves to establish latent infection. Innate immune responses, particularly the IFNαβ response, are essential during the first few days of infection to limit HSV replication in the epithelium and spread into the nervous system.

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In mouse models of HSV-1 ocular infection, neutralization of IFNαβ results in a 1000-fold increase in titer of HSV-1 shed from the cornea (Hendricks et al., 1991;Lausch et al., 1991). Expression of IFNαβ in the cornea via plasmid DNA transfection or adenovirus-mediated transduction limits replication and enhances survival after corneal infection with HSV-1 (Noisakran et al., 1999;Al-khatib et al., 2004;Austin et al., 2005;Al-khatib et al., 2005). IFNαβ also have a protective role against HSV-1 infection in vitro, reducing viral immediately early (α) gene expression (Klotzbucher et al., 1990;Mittnacht et al., 1988;Oberman and Panet, 1988;Oberman and Panet, 1989) and viral yield in fibroblasts (Harle et al., 2002b;Noisakran et al., 2000;Harle et al., 2002a;Khabar et al., 2000) and cells of neural origin (Al-Khatib et al., 2003;Carr et al., 2003). The IFNαβ response is an important component of host resistance during HSV-2 replication in the genital epithelium, limiting virus spread to the lumbosacral spinal cord and preventing neurologic disease (Murphy et al., 2003). Expression of IFNα1 in the vaginal epithelium prior to infection further reduces HSV-2 replication and virulence (Harle et al., 2001).

Although the IFNαβ response intercedes in the early stages of HSV-1 and HSV-2 infections, these neurotropic viruses have developed resistance mechanisms to partially overcome its effects. For example, HSV inhibits IFNαβ-induced antiviral activities mediated by PKR through the actions of ICP34.5 and Us11 (Cassady et al., 1998b;He et al., 1997). HSV-1 γ34.5 null mutants are severely attenuated in wild-type mice, but restored in $PKR^{-/-}$ mice (Leib et al., 2000), and the absence of ICP34.5 can be compensated by expression of Us11 as an immediate-early gene (Cassady, Gross et al., 1998a). Furthermore, replication and virulence of wild-type virus is not enhanced in the absence of PKR (Harle et al., 2001;Leib et al., 2000), demonstrating that HSV effectively blocks PKR-mediated antiviral activity using these proteins. However, wild-type HSV replicates more readily in mice that lack the IFNαβR (Murphy et al., 2003;Leib et al., 1999), a finding which indicates the existence of additional IFNαβ-induced antiviral pathways capable of suppressing wild-type HSV growth in vivo.

Evidence has accumulated for the activity of the OAS/RNase L pathway in protecting against HSV infection. In vitro, HSV-1 replicates to higher titers in RNase $L^{-/-}$ murine embryonic fibroblasts (MEFs) and trigeminal ganglion neurons than in wild-type cells (Harle et al., 2002b;Carr et al., 2003), suggesting a role for RNase L in countering HSV infections. In vivo, however, an antiviral function for RNase L remains controversial. Studies supporting an antagonistic role have shown an increase in replication of HSV-1 in the corneal epithelium of RNase $L^{-/-}$ mice (Austin et al., 2005), and more severe ocular disease and higher mortality (Zheng et al., 2002) compared to wild-type mice. Yet contrasting results indicate that HSV-1 replication is not enhanced in the corneal epithelium (Leib et al., 2000;Al-khatib et al., 2004) and trigeminal ganglia (Al-khatib et al., 2004;Austin et al., 2005) of RNase $L^{-/-}$ mice, and neither is overall virulence (Al-Khatib et al., 2005). This lack of effect could be explained by the purported induction by HSV-1 of 2′5′A analogs that have diminished capacity to activate RNase L (Cayley et al., 1984).

The role of the OAS/RNase L pathway in mediating IFNαβ-induced protection against HSV-2 replication and spread is not known. Although some data support a role for RNase L as an HSV-1 antagonist, differences between HSV-1 and HSV-2 have been found regarding their mechanisms of resistance to mediators of IFNαβ-induced antiviral effects (Murphy et al., 2003). Thus, the role of RNase L during HSV-2 genital infection may differ from its role in protecting the cornea against infection with HSV-1. We therefore investigated whether the OAS/RNase L pathway plays an important role in host resistance to HSV-2 by examining virus replication and disease induction during primary genital infection of wild-type and RNase $L^{-/-}$ mice.

Results

RNase L is a mediator of the IFNαβ response, and IFNαβ plays an important role in the innate antiviral response against HSV-2 *in vivo* (Murphy et al., 2003). To determine whether RNase L contributes to host defense against primary HSV-2 infection, wild-type C57BL/6 and RNase $L^{-/-}$ mice were infected by the intravaginal (i.vag.) route with 2×10^6 pfu/mouse of HSV-2 strain 333. Titers of virus shed from the genital epithelium and genital disease were assessed. 2′5′-OAS is upregulated in the genital epithelium 24 hr after HSV-2 infection (Palliser et al., 2006). We therefore expected that if activated RNase L mediates a vital antiviral response to HSV-2 in genital epithelial cells, RNase $L^{-/-}$ mice would show prolonged virus replication and more severe disease than wild-type mice. However, replication of HSV-2 in the genital epithelium of RNase $L^{-/-}$ and wild-type mice was similar at all time points (Fig. 1). Thus it appears IFNαβ-stimulated pathways other than those mediated by OAS/RNase L are important for controlling HSV-2 replication in cells in the genital epithelium. Alternatively, HSV-2 may have evolved mechanisms that effectively counteract RNase L-mediated antiviral responses in the genital mucosa, thus neutralizing RNase L-mediated effects.

Surprisingly, despite virtually identical levels of virus replication in the epithelium, signs of genital disease developed more slowly in RNase $L^{-/-}$ mice than in wild-type mice (Fig. 2A). Inflammation of the external genitalia was less pronounced in RNase $L^{-/-}$ mice than wild-type mice on days 4 to 6 post-infection ($p<0.001$). Genital lesions were present in 70% of wild-type mice on day 5 and in 100% on day 6, but RNase $L^{-/-}$ mice did not develop lesions until day 6, when only 49% of the mice were affected ($p<0.0001$ for days 5 and 6). Signs of neurologic disease were also greater in wild-type mice, with 61% developing hind-limb paralysis by day 6. In contrast, only 12% of RNase $L^{-/-}$ mice became paralyzed ($p<0.0001$). Delayed development of HSV-2-mediated disease in RNase $L^{-/-}$ mice was reflected in their slower weight loss as infection progressed (Fig. 2B; $p\leq0.0001$ on days 5 and 6 post-infection). Survival times also differed between wild-type and RNase $L^{-/-}$ mice (Fig. 3). On day 6, all RNase $L^{-/-}$ mice were alive, compared with 53% of wild-type mice ($p=0.0003$). On day 7, 59% of RNase $L^{-/-}$ mice were surviving, but only 21% of wild-type mice were still alive ($p=0.0252$). Overall, the mean day of death for RNase $L^{-/-}$ mice was 8.0±1.3, but wild-type mice succumbed by a mean of 6.8±1.0 days post-infection ($p=0.0021$). These data indicate similar kinetics of virus replication in the genital epithelium but slower development of genital inflammation and neurologic signs in mice lacking RNase L. The delayed onset of inflammation and neurologic disease in RNase $L^{-/-}$ mice corresponded with their slower demise.

Delayed development of neurologic signs and death in RNase $L^{-/-}$ mice could be due to decreased virus replication in the central nervous system (CNS), or to decreased inflammation contributing to paralysis. To address these possibilities, we first determined viral titer in the CNS of mice 6 days post-infection, when neurologic signs were apparent. Virus titers in the spinal cords of RNase $L^{-/-}$ mice were 3-fold lower than in wild-type B6 mice ($p=0.0152$) and 6-fold lower in the brainstems ($p=0.0116$; Fig. 4A). Thus, HSV-2 entered into or replicated in the nervous systems of RNase $L^{-/-}$ mice less efficiently than wild-type mice, despite replicating equally well in the genital mucosae of both mouse strains. Direct intracranial injection of virus revealed no difference in capacity of HSV-2 to replicate in brain tissue of RNase $L^{-/-}$ or wild-type mice (Fig. 4B), suggesting that a difference in neuroinvasion rather than neurovirulence accounted for the reduced titers of HSV-2 in the spinal cords and brainstems of RNase $L^{-/-}$ mice.

We examined the additional possibility that a difference in activation or recruitment of inflammatory infiltrates into infected tissues resulted in less inflammation in RNase $L^{-/-}$ mice and therefore reduced signs of disease. Six days post-infection, vaginas and lumbosacral spinal

cords were dissected, subjected to collagenase digestion, and mononuclear cells were isolated over Percoll gradients. Phenotypic staining followed by flow cytometry revealed that i) infiltration of mononuclear cells in the vaginas and spinal cords increased markedly in all infected mice relative to uninfected controls. ii) The proportion of activated ($CD69^+$) cells among $CD4^+$ and $CD8^+$ T cells infiltrating the vaginas and spinal cords of wild-type and RNase $L^{-/-}$ mice was similar. iii) The influx of $CD8^+$ T cells into tissues was relatively small, and the percentage (Fig. 5A) and number (Fig. 5B) of $CD45^+CD8^+$ lymphocytes was higher in the vaginas of infected RNase $L^{-/-}$ mice than wild-type mice but not significantly different in the spinal cords. In contrast, iv) the percentage of $CD45^+CD4^+$ lymphocytes was lower in both the vaginas and spinal cords of infected RNase $L^{-/-}$ mice (Fig. 5C), and quantification of infiltrating $CD4^+$ lymphocytes revealed similar trends (Fig. 5D). The latter observation suggests that fewer $CD4^+$ T cells had entered the infected tissues in RNase $L^{-/-}$ mice than wild-type mice, and this delayed the development of inflammation.

Discussion

Contrary to our expectation that HSV-2 would replicate more readily and cause more disease in RNase L-deficient mice after primary genital infection, we found that disease caused by HSV-2 infection is *milder* in RNase $L^{-/-}$ mice than wild-type mice. Viral replication, although similar in the vaginal epithelium, is modestly *lower* in the nervous system of RNase $L^{-/-}$ mice. Thus, RNase L is not necessary for resistance to primary HSV-2 infection in vivo, at least as assessed by genetic deletion. Although the differences in viral titers in the spinal cords and brainstems of RNase $L^{-/-}$ mice compared with wild-type mice are modest, small reductions in virus replication in the CNS may have profound effects on survival time, in that a threshold may exist for causing paralytic or lethal neurologic disease. Viral titers in the nervous systems of wild-type mice may have reached this threshold more often and more quickly than in RNase $L^{-/-}$ mice. Because a small number of wild-type mice died before CNS tissues were collected for determination of viral titer day 6 post-infection, the difference in viral titers we observed (Fig. 4A) may underestimate the true magnitude of the difference in replication of HSV-2 in the CNS of wild-type and RNase $L^{-/-}$ mice on day 6. We did not evaluate earlier time points, and thus it is possible that titers in the spinal cords of wild-type and RNase $L^{-/-}$ mice may be equivalent initially and then gradually diverge.

Certain antiviral effector molecules such as RNase L may play a more significant role in resistance to HSV infection if upregulated by IFNαβ exposure prior to infection than they do in the natural course of response to primary infection. Indeed, IFNβ pretreatment suppresses HSV-1 replication in MEFs or in cells derived from the trigeminal ganglia by a mechanism requiring RNase L (Carr et al., 2003). Primary HSV-2 infection of vaginal epithelial cells would be expected to stimulate some IFNαβ to protect neighboring cells in advance of subsequent rounds of virus replication. Thus, it would be predicted that HSV should replicate more readily in RNase L-deficient mice. Instead, replication is similar in the vaginal epithelium, indicating that RNase L activity does not limit HSV-2 replication during primary genital infection of mice with HSV-2. The role of RNase L may be usurped by other antiviral mechanisms in the vaginal epithelial cells, creating no net change in virus replication. In contrast to the vaginal epithelium, virus titer in the spinal cord and brainstem of RNase $L^{-/-}$ mice is modestly restricted, but how RNase L affects virus titer is unclear. A subtle difference in genetic background between wild-type and mutant mice cannot be ruled out but is unlikely to be the sole cause of the disparity in titers. Reduced spinal cord and brainstem titers after peripheral inoculation of virus may result from differential neuroinvasion rather than neurovirulence, as suggested by the observation that virus grew to equivalent titers after direct introduction into the nervous systems of RNase $L^{-/-}$ and wild-type mice. The lesser influx of inflammatory cells into the spinal cords of RNase $L^{-/-}$ mice may relate to neuroinvasion by delaying an increase in vascular

permeability or by restricting virus entry in association with leukocytes trafficking into the tissue.

A surprising finding was the delayed development of severe genital inflammation, genital lesions and paralysis in mice lacking RNase L, suggesting that RNase L plays a potentiating role in some aspects of HSV infection, whether directly or indirectly. At least three possibilities may be entertained to explain the mechanism underlying this observation. First, disruption of a synergistic relationship between the ribonuclease activities of the virion host shutoff (vhs) protein and host RNase L could explain the increased survival in RNase $L^{-/-}$ mice. However, Northern analysis of GAPDH mRNA revealed that mRNA degradation was equivalent in wild-type and RNase $L^{-/-}$ MEFs infected with HSV-2 (data not shown), in accord with previous observations with HSV-1 (Smith et al., 2003). Second, HSV-mediated disease may appear less severe in mice lacking RNase L because less apoptotic cell death occurs in infected tissues. Cells derived from mice lacking RNase L undergo apoptosis more slowly or show a deficiency in apoptosis induction (Castelli et al., 1998;Zhou et al., 1998). Apoptosis is not generally associated with erythema and edema, and thus an impact of RNase L on apoptotic cell death probably does not explain decreased signs of genital disease in HSV-2-infected RNase $L^{-/-}$ mice. However, this hypothesis could pertain to a possible decrease or delay in neuronal cell death, staving off paralysis and allowing longer survival time.

Finally, there is evidence that RNase L plays a role in modulating T cell responses in that RNase $L^{-/-}$ mice reject tissue grafts more slowly than wild-type mice (Silverman et al., 2002). T cells play important roles in host defense against viruses but their activities also can contribute to immunopathology. For example, IFNγ-producing $CD4^+$ T cells have been previously found in the genital tract after primary HSV-2 infection (Milligan and Bernstein, 1995) and may be important for virus clearance (Milligan et al., 2004). However, their activities may also contribute to genital inflammation associated with HSV-2 infection (Harandi et al., 2001;Murphy et al., 2003). Our finding that activated $CD4^+$ T cells enter the vaginal tracts of RNase $L^{-/-}$ mice less frequently than wild-type mice is consistent with this interpretation. Reduced $CD4^+$ T cell migration into vaginal tissue of RNase $L^{-/-}$ mice may delay development of erythema and edema characteristic of the inflammatory response to HSV-2. Similarly, subdued inflammatory influx into the spinal cords of HSV-2-infected RNase $L^{-/-}$ mice may delay the onset of paralysis. $CD4^+$ T cell influx into the CNS consonant with virus infection (Marten et al., 2001;Glass et al., 2005), and subsequent contribution to pathology (Gerety et al., 1994;Lane et al., 2000;Rowell and Griffin, 1999), has been observed in a number of virus-induced CNS diseases. Thus, delaying the pathologic consequences of an effector T cell response within the CNS could enhance the life-prolonging effect of lower viral CNS titers in RNase $L^{-/-}$ mice. A small reciprocal increase in the percentage of $CD8^+$ T cells infiltrating the genital tract of RNase $L^{-/-}$ mice was also seen. HSV-specific $CD8^+$ as well as $CD4^+$ T cells have been associated with immunopathology in the eye (Niemialtowski and Rouse, 1992;Keadle et al., 2002;Osorio et al., 2004). It is possible that $CD8^+$ T cells also contribute to genital inflammation, but this contribution is inapparent because $CD8^+$ T cells comprise a minor proportion of the tissue infiltrates. Further experiments investigating inflammatory cytokine production by T cells and/or apoptosis induction in the genital epithelium and nervous system are needed to determine the mechanism by which the lack of RNase L attenuates HSV-2-mediated disease.

Materials and Methods

Cells and viruses

Vero cells were maintained in Dulbecco's modified Eagle's medium supplemented with 3% newborn and 3% fetal calf serum. SB5, a plaque-purified clone of HSV-2 strain 333, was obtained from the American Type Culture Collection (VR-2546). A cell lysate stock was

prepared as previously described (Morrison and Knipe, 1996). Titer of the virus stock was determined by titration on Vero cell monolayers.

Mice

RNase $L^{-/-}$ mice on the C57BL/6 background (Zhou al., 1997) were generously provided by Bob Silverman. Wild-type C57BL/6 mice were purchased from the National Cancer Institute (Fredericksburg, MD), the same source as previously used by the Silverman laboratory (Silverman et al, 2002;Leitner et al., 2003). RNase $L^{-/-}$ mice were bred and maintained in the Department of Comparative Medicine, Saint Louis University School of Medicine in accordance with institutional and Public Health Service guidelines. Mice were used at 6 weeks of age. Several experiments were performed and the data from individual experiments were pooled.

Genital swab and neural tissue titers

Mice were infected by the i.vag. route with 2×10^6 pfu/mouse as previously described (Morrison et al., 1998). Two vaginal swabs per mouse were taken on days 0–4 post-infection using calcium alginate swabs (Puritan Medical Products, Guiford ME) which were placed together in vials containing phosphate-buffered saline and stored at −80°C. At 6 to 7 days post-infection, mice were sacrificed and the spinal cord, brainstem, and brain were dissected. Alternatively, groups of mice were inoculated intracranially with 5×10^3 pfu of HSV-2 in 10μl vol using a 29-gauge insulin syringe (Terumo Medical Corp., Somerset NJ). Brain tissue was dissected 1, 2 or 3 days post-infection. Tissues were placed in microcentrifuge tubes containing phosphate-buffered saline and 1-mm glass beads, and stored at −80°C. Thawed tissues were disrupted using a Mini BeadBeater 8 (Biospec Products, Bartlesville, OK). Viral titers were determined by standard plaque assay (Knipe and Spang, 1982).

Disease scores

Severity of genital and neurologic disease was assessed on days 2 through 7 post-infection using the following scale: 0, no signs; 1, mild erythema and edema of the external genitalia; 2, moderate erythema and edema; 3, severe erythema and edema.

Flow cytometry

Genital tracts from the vaginal orifice to the exocervix and lumbosacral spinal cords were dissected from wild-type and RNase $L^{-/-}$ mice 6 days post-infection. Minced tissues were digested in 1 ml of 1 mg/ml dispase/collagenase (Roche Applie Sciences, Indianapolis IN) for 40 min at 37°C. Cells released from genital tracts were incubated for 4 hr at 37°C in 12-well plates. Non-adherent cells were collected for staining. Supernatants of spinal cord digests were layered over 30%–70% discontinuous Percoll gradients (Redi-Grad, Amersham Biosciences, Piscataway NJ) and sedimented by centrifugation at 2000 rpm for 20 min. Cells at the interface were collected and washed before staining. Cells from individual mice were incubated with FcBlock (BD PharMingen, San Diego CA) and then stained with a cocktail of anti-CD45-APC, anti-CD4-FITC, anti-CD8-PE, and anti-CD69-PerCP (BD PharMingen; all used at a 1:150 dilution. Antibodies used to detect CD4 and CD8 produce a mean fluorescence intensity shift of one decade (10-fold) over background of negative cells. Isotype controls were used in initial experiments to verify authentic CD4 and CD8 staining. Stained cells were subjected to 4-color flow cytofluorometric analysis using a FACSAria (Becton Dickinson) and CellQuest analysis software.

Statistics

Significance of difference in viral titer between groups and between percentage of $CD4^+$ cells in infiltrates was determined by *t* test. The nonparametric Kruskall-Wallis test was used to determine significance of difference in disease scores between groups on individual days.

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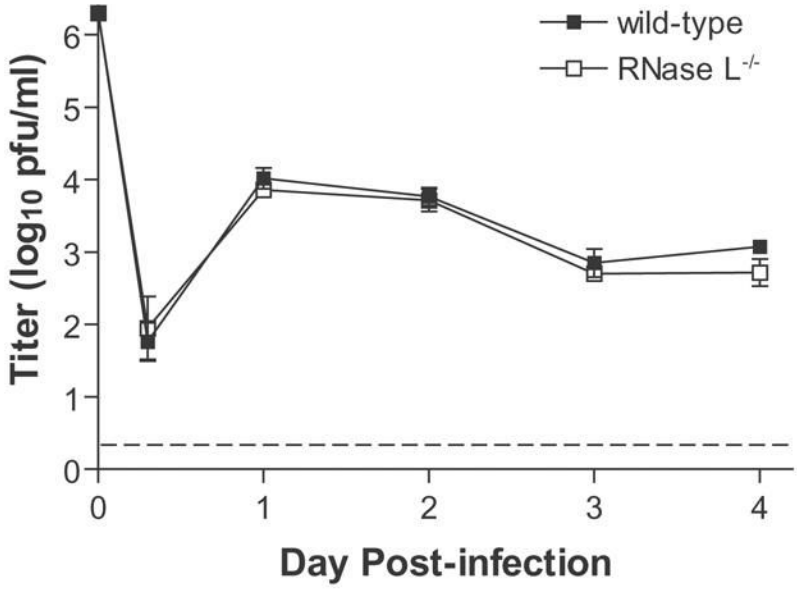

Figure 1. Replication of HSV-2 in the genital mucosa of wild-type B6 and RNase L$^{-/-}$ mice

Mice were infected i.vag. with 2×10^6 pfu/mouse of HSV-2 strain 333. A) Titer of virus in vaginal swab samples taken 9 hr to 4 days post-infection was determined by standard plaque assay on Vero cells. Data points represent the geometric mean ± standard error of the mean for 6 mice per group in one of two experiments with similar results.

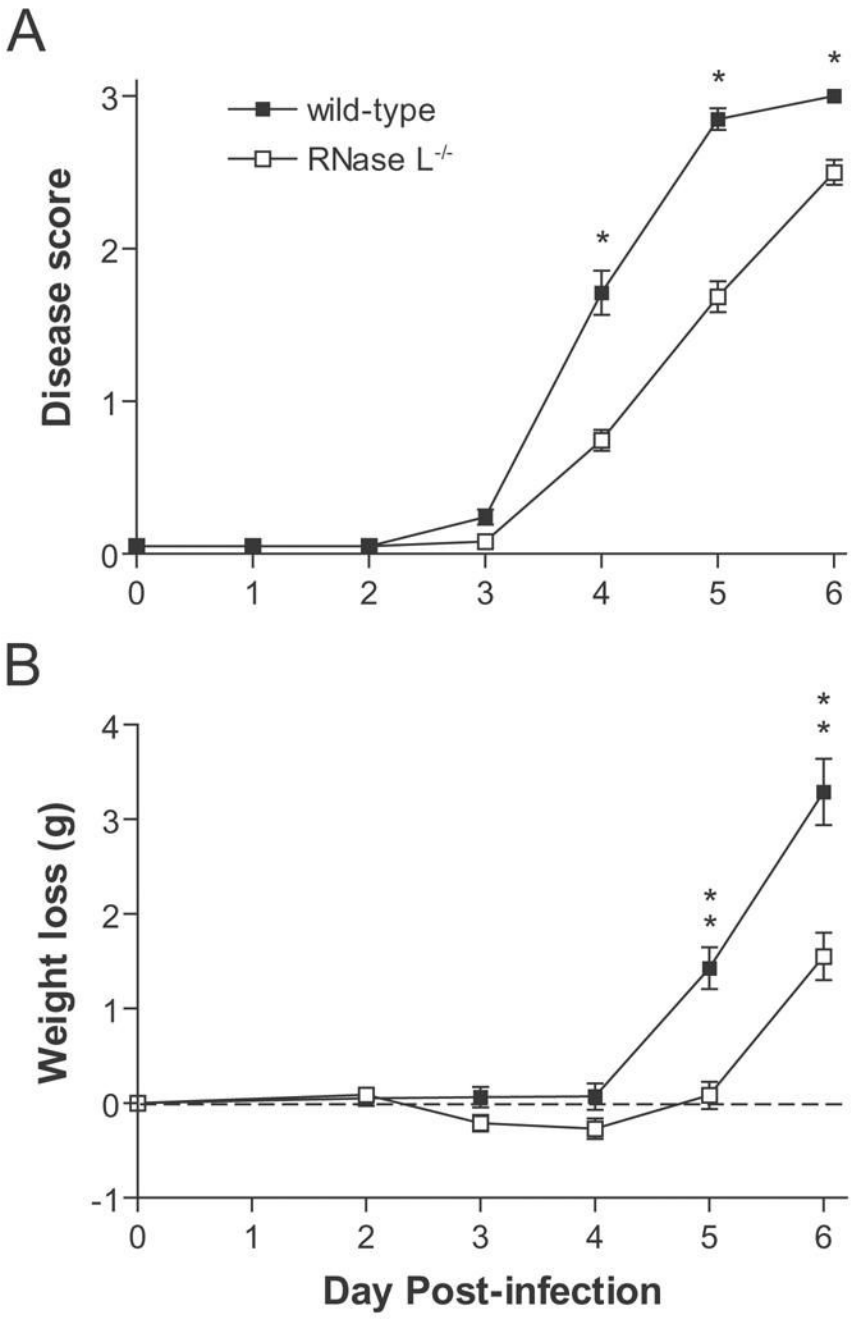

Figure 2. Severity of genital disease in wild-type B6 and RNase $L^{-/-}$ mice infected with HSV-2

Mice were infected with 2×10^6 pfu/mouse i.vag. with HSV-2 strain 333. A) Genital disease scores for 33 wild-type B6 and 43 RNase $L^{-/-}$ mice, and B) change in weight for 22 wild-type B6 and 28 RNase $L^{-/-}$ mice were recorded daily. Values represent the arithmetic mean ± standard error of the mean. *p value <0.001; **p value ≤0.0001.

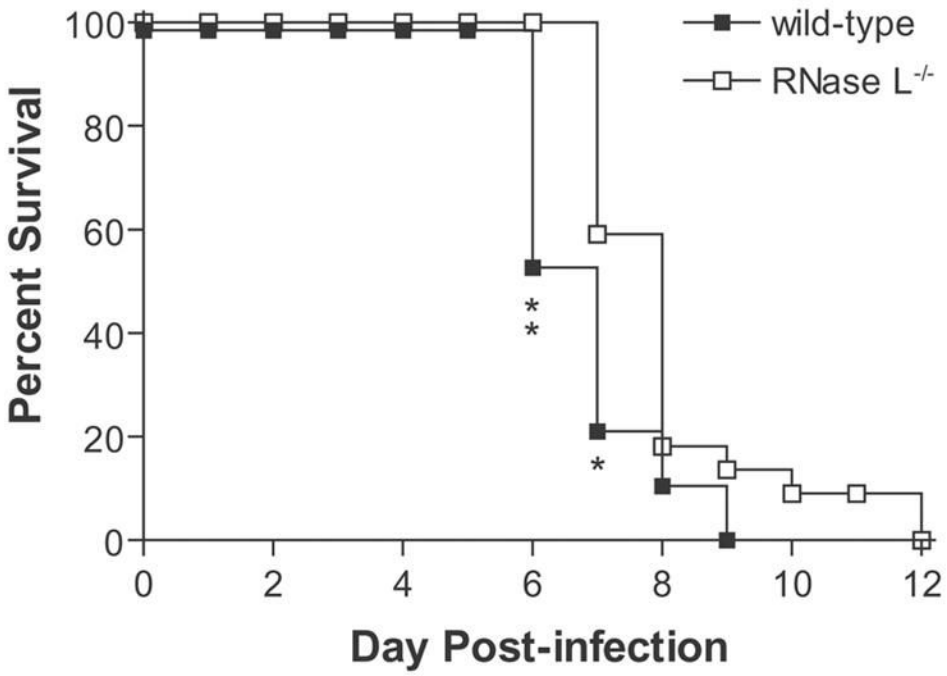

Figure 3. Survival time of mice after infection with a lethal dose of HSV-2

Wild-type B6 and RNase $L^{-/-}$ mice were infected i.vag. with 2×10^6 pfu/mouse with HSV-2 strain 333 and survival was recorded daily. n=19 for wild-type and n=22 for RNase $L^{-/-}$ mice. **p value=0.0003 on day 6; *p value= 0.0252 on day 7 post-infection.

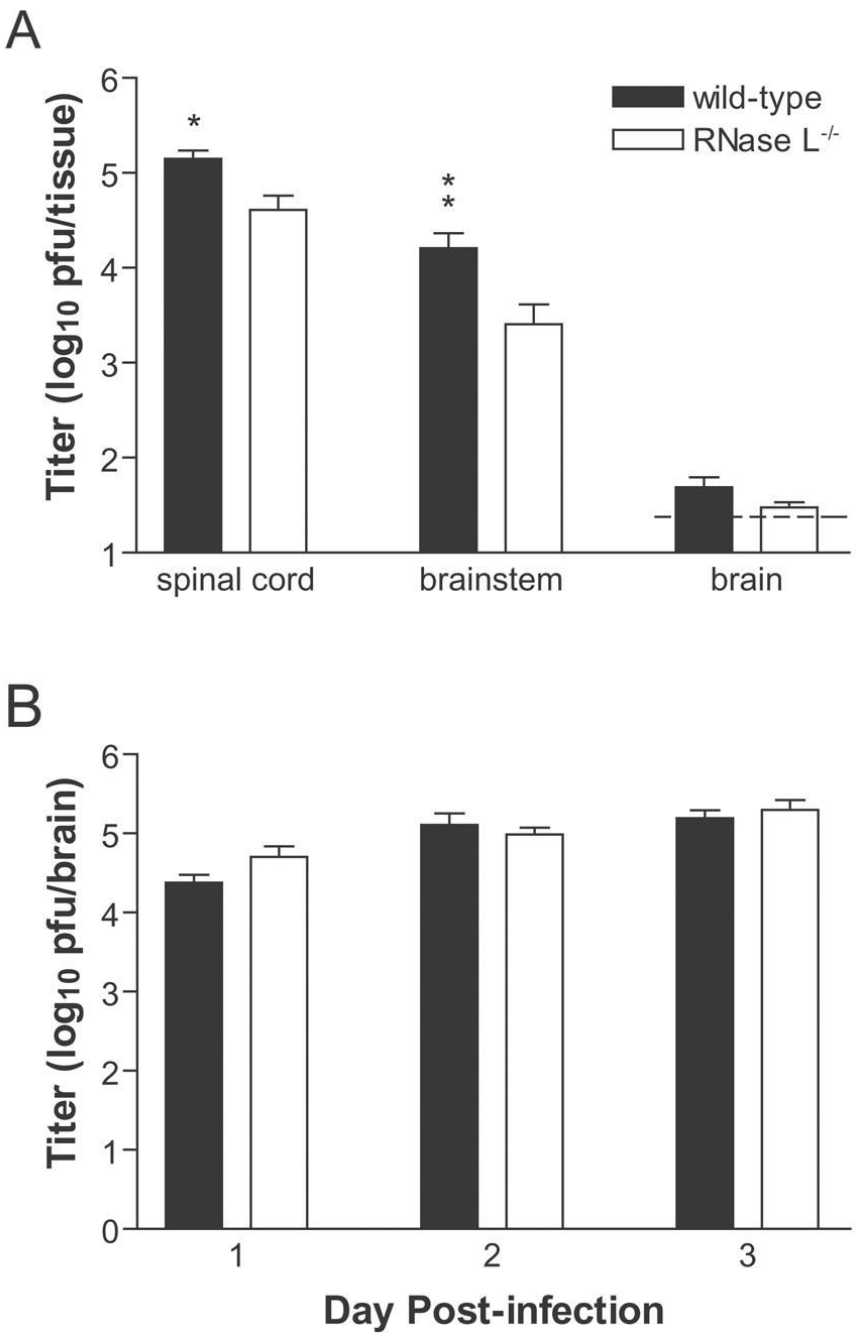

Figure 4. Replication of HSV-2 in the nervous tissues of wild-type B6 and RNase L$^{-/-}$ mice

A) Groups of mice surviving to day 6 post-infection with HSV-2 were sacrificed, and brain, brainstem, and spinal cord tissues were dissected. Viral titers in disrupted tissues were determined by plaque assay. The dashed line indicates the limit of detection. n=13 for wild-type B6 mice and n=22 for RNase L$^{-/-}$ mice. *p value=0.0152; **p value=0.0116. B) Groups of mice were injected intracranially with 5×10^3 PFU of HSV-2 and sacrificed 1, 2 or 3 days post-infection. Brain tissue was removed and viral titers in disrupted tissues were determined by plaque assay. Values represent the geometric mean ± SEM of 4 to 8 mice per group.

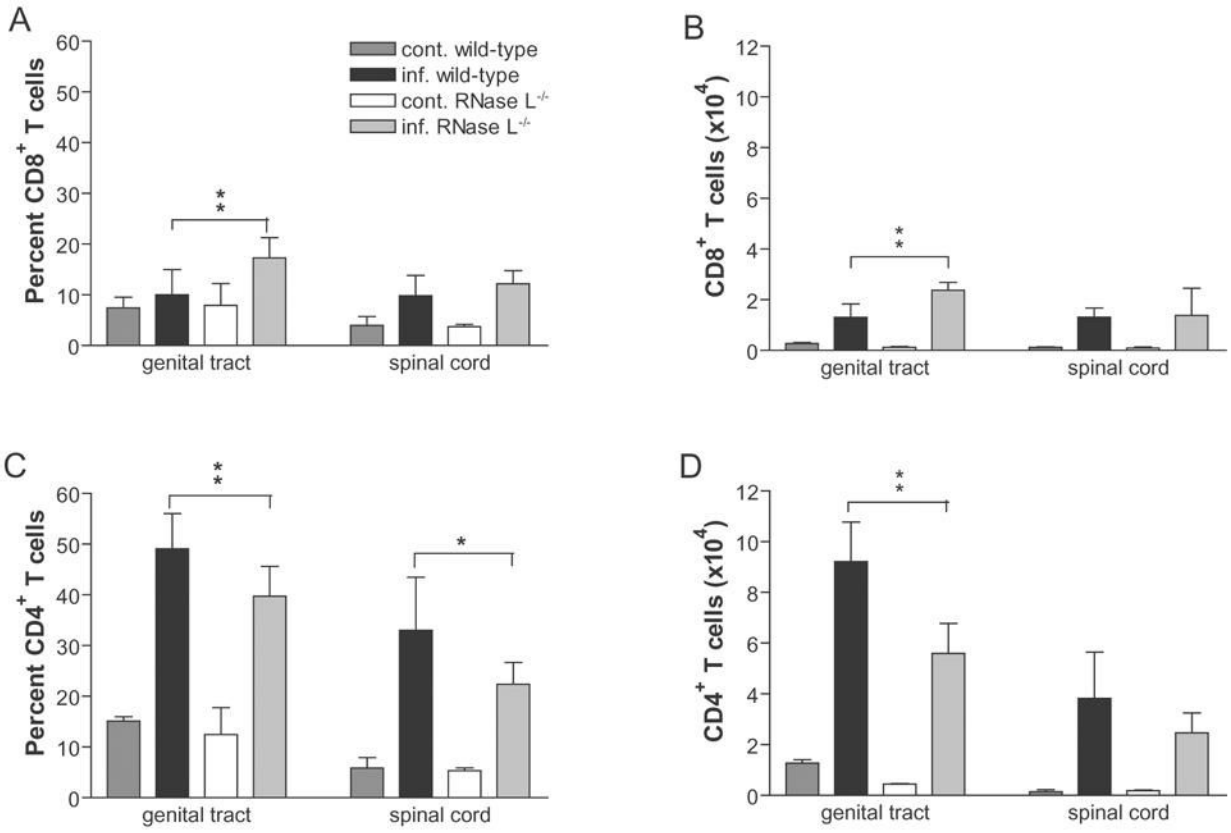

Figure 5. T lymphocyte infiltration into the genital tract and spinal cord

Groups of RNase $L^{-/-}$ or wild-type mice were uninfected or infected with HSV-2. After 6 days, genital tracts and lumbosacral spinal cords were dissected. Mononuclear cells enzymatically released from tissues of individual mice were stained with anti-CD45-APC, anti-CD8-PE, anti-CD4-FITC, and anti-CD69-PerCP and analyzed by flow cytometry. Regions were defined by $CD45^+$ cells of size and granularity typically associated with lymphocytes. Data from two individual experiments were pooled and represent A and B) the mean percentage ± SD of $CD8^+$ and $CD4^+$ T cells, respectively, recovered from 6 individual mice, and C and D) the mean number of $CD8^+$ and $CD4^+$ T cells, respectively, recovered. **p value=0.0301–0.0379; *p value=0.0438.