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Does Toll-like receptor 3 play a biological role in virus infections?

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Abstract

The Toll-like receptor (TLR) family functions to recognize conserved microbial and viral structures with the purpose of activating signal pathways to instigate immune responses against infections by these organisms. For example, in vitro studies reveal that the TLR3 ligand is a double-stranded RNA (dsRNA), a product of viral infections. From this observation, it has been proposed that TLR3 is likely an important first signal for virus infections. We approached this issue by investigating the role of TLR3 in four different infectious viral models (lymphocytic choriomeningitis virus (LCMV), vesicular stomatitis virus (VSV), murine cytomegalovirus (MCMV), and reovirus) and in TLR3 genetically deficient ($^{-/-}$) mice. Our results indicate that TLR3 is not universally required for the generation of effective antiviral responses because the absence of TLR3 does not alter either viral pathogenesis or impair host's generation of adaptive antiviral responses to these viruses.

Keywords: Toll-like receptor 3; Virus; Mice

Introduction

Ten distinct mammalian Toll-like receptors (TLRs) have been described, most of which have been shown to function as receptors for pathogen-associated molecular patterns (Akira and Hemmi, 2003; Takeda et al., 2003). These receptors aid the host to combat infection as microbial ligands upon binding to various TLRs on antigen-presenting cells (APCs) trigger NF-κB leading to the upregulation of cytokines and co-stimulatory molecules with the presumed purpose of initially enhancing innate immune attack against the pathogen from which that ligand is derived (Aderem and Ulevitch, 2000; Anderson, 2000; Hallman et al., 2001; Kopp and Medzhitov, 1999). It has been hypothesized that loss of function of particular TLRs causes the host to become more susceptible to infections with TLR-reactive pathogens. Evidence supporting this concept comes from observations where the loss of TLR2 has resulted in increased susceptibility to *Staphylococcus aureus* infections (Takeuchi et al., 2000), loss of TLR9 resulted in ablation of the immunostimulatory properties of bacterial DNA (Hemmi et al., 2000), and loss of TL4 resulted in increased susceptibility to respiratory syncytial virus (Kurt-Jones et al., 2000). These results led to the proposal that TLR3, a receptor demonstrated to be reactive to double-stranded (ds) RNA (Alexopoulou et al., 2001; Matsumoto et al., 2002), a frequent byproduct of virus replication, functions as a universal virus receptor for priming the innate immune system to viral infections.

Of the TLRs studied, only TLR3 has been identified to respond to dsRNA (Alexopoulou et al., 2001) by specifically recognizing poly (I:C) and purified Lang reovirus genomic dsRNA, both of which resulted in the induction of IFN-β, IL-12, IL-6, and TNFα from macrophages obtained from TLR3-sufficient mice. The ability of TLR3 to induce IFN-β has been attributed to at least two factors: its MyD88-independent activation of IRF3 and to its induction of the traditional dsRNA-dependent protein kinase (PKR) pathway (Doyle et al., 2002; Horng et al., 2001; Oshiumi et al., 2003). Recognition of dsRNA ligand has been demonstrated to

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occur extracellularly by TLR3-blocking antibodies that prevented TLR3-mediated responses to poly (I:C) in vitro (Matsumoto et al., 2002). In vivo studies using TLR3-deficient ($^{-/-}$) mice showed protection of such mice from shock induced by poly (I:C) treatment, suggesting this receptor contributed to the in vivo recognition of and responses to extracellular dsRNA (Alexopoulou et al., 2001). Furthermore, it was shown that the effector responses elicited by TLR3–poly (I:C) interaction can inhibit murine γ herpesvirus 68 replication in bone marrow-derived macrophage cultures (Doyle et al., 2002). Based on the above findings, it has been repeatedly assumed that TLR3 likely played an important role in host defense against virus infections (Akira and Hemmi, 2003; Alexopoulou et al., 2001; Doyle et al., 2002; Harte et al., 2003; Matsumoto et al., 2002; Renshaw et al., 2002). However, it has not yet been determined if the physiologic amounts of dsRNA naturally made during viral infections per se were sufficient to trigger through TLR3 a biologically meaningful response to alter the outcome of disease. We set out to experimentally determine if TLR3 is essential for antiviral immunity or if TLR3 represents an expendable level of redundancy built into the dsRNA recognition system. Here we report our work that directly tests this hypothesis by studying both host immune responses and control of infection by a dsRNA virus, reovirus; two ssRNA viruses that make dsRNA intermediates, lymphocytic choriomeningitis virus (LCMV) and vesicular stomatitis virus (VSV); and a DNA virus, murine cytomegalovirus (MCMV), in TLR3 gene-disrupted mice. Our results indicate that TLR3 does not play a critical role in the host antiviral adaptive immune response to reovirus, LCMV, VSV, or MCMV as susceptibility to infection and generation of CD4 and CD8 T cell immune responses to these viruses are equivalent in TLR3-deficient and -sufficient mice.

Results and discussion

Our initial experiments studied the primary adaptive immune responses to LCMV infection in TLR3$^{-/-}$ mice. LCMV is an arenavirus containing a genome consisting of two negative-stranded RNAs and like the other viruses investigated in this report is able to trigger Type-I IFNs. Control of acute LCMV infection is totally mediated by virus-specific CD8$^+$ T cells through a perforin-mediated pathway (Anderson et al., 1985; Kagi et al., 1995). To test if TLR3 plays a role in the immune response to LCMV, TLR3$^{-/-}$ mice and control C57Bl/6 (B6) × 129 mixed background mice bred at TSRI vivarium were inoculated intraperitoneally with 1×10^5 pfu LCMV ARM53b. IFNγ responses from LCMV-specific T cells were assessed 8 days postinfection via intracellular cytokine staining and flow cytometry following a 5-h culture of splenocytes with brefeldin A, rIL2, and CD8 and CD4 LCMV-specific H-2^b-specific peptide epitopes, GP61 (aa GP61–80) and GP33 (aa GP33–41), respectively (Hudrisier et al., 1997; Murali-Krishna et al., 1998; Whitmire et al., 1998). TLR3$^{-/-}$ mice showed no difference compared to controls in their ability to generate both a robust CD8$^+$ and CD4$^+$ IFNγ response to LCMV (Fig. 1A). Furthermore, LCMV-specific CD8$^+$ T cells from TLR3$^{-/-}$ mice also displayed normal lysis of GP33 peptide-coated H-2^b-MC57 targets cells (Fig. 1B).

Hosts usually encounter pathogens during natural infections at significantly lower concentrations than utilized in many laboratory infectious models. To investigate a role for TLR3 during a low-level infection, we conducted experiments in which both the dose and route of inoculation of LCMV were varied. A series of ip and foot pad inoculations with doses of LCMV ranging from as little as 1×10^2 to 5×10^5 pfu LCMV were carried out and CD8$^+$ T cell IFNγ responses from both the draining lymph nodes and spleen were analyzed (Fig. 1C). Intravenous injection of a very high dose of LCMV (2×10^6 pfu) was also analyzed (data not shown); however, irrespective of the route and dose of virus inoculation, the absence of TLR3 did not affect the generation of the primary cellular response to LCMV.

We next assayed the primary T cell responses to a RNA virus, VSV, and a DNA virus, MCMV, in TLR3$^{-/-}$ mice. VSV, a member of the Rhabdovirus family, contains a single-stranded negative-sense RNA genome, and control of VSV in mice is dependent on the generation of virus-specific antibodies and complement (Lefrancois, 1984; Steinhoff et al., 1995). Furthermore, VSV infection leads to induction of Type-I IFNs triggered, in part, by the prevalence of structured dsRNA-defective interfering particles found during VSV infection (Holland, 1987). We found that TLR3$^{-/-}$ mice handle VSV infection as well as B6 × 129 control mice. Mice infected with VSV generated both CD4$^+$ and CD8$^+$ VSV-specific T cell responses to peptides NP415 and NP52, respectively (Fig. 2A), and were able to clear the virus (data not shown). MCMV is a dsDNA herpes virus that, like LCMV, is a natural pathogen of mice. MCMV infection of susceptible strains of mice results in lifelong latent infection punctuated by spontaneous reactivation from the latent state. Protection of the host from lethal MCMV infection is complex requiring contributions from both the innate and adaptive immune response (Koszinowski et al., 1990). The effect of the loss of TLR3 on the T cell response to MCMV in TLR3$^{-/-}$ mice was analyzed day 9 postinfection with 1×10^4 pfu ip. T cell IFNγ responses were measured after 5-h culture in αCD3-coated wells because there are no currently defined H-2^b T cell peptide epitopes for MCMV. These analyses did not identify differences between control and TLR3$^{-/-}$ mice in the primary T cell response to MCMV (Fig. 2B). Furthermore, the spleens from these mice were free of virus, indicating that the absence of TLR3 did not increase the susceptibility to MCMV (data not shown).

Next we determined the role TLR3 played in functional T cell memory responses. Intravenous inoculation of naive or immunosuppressed mice with LCMV variant Cl 13 (2×10^6 pfu) leads to establishment of persistent infection

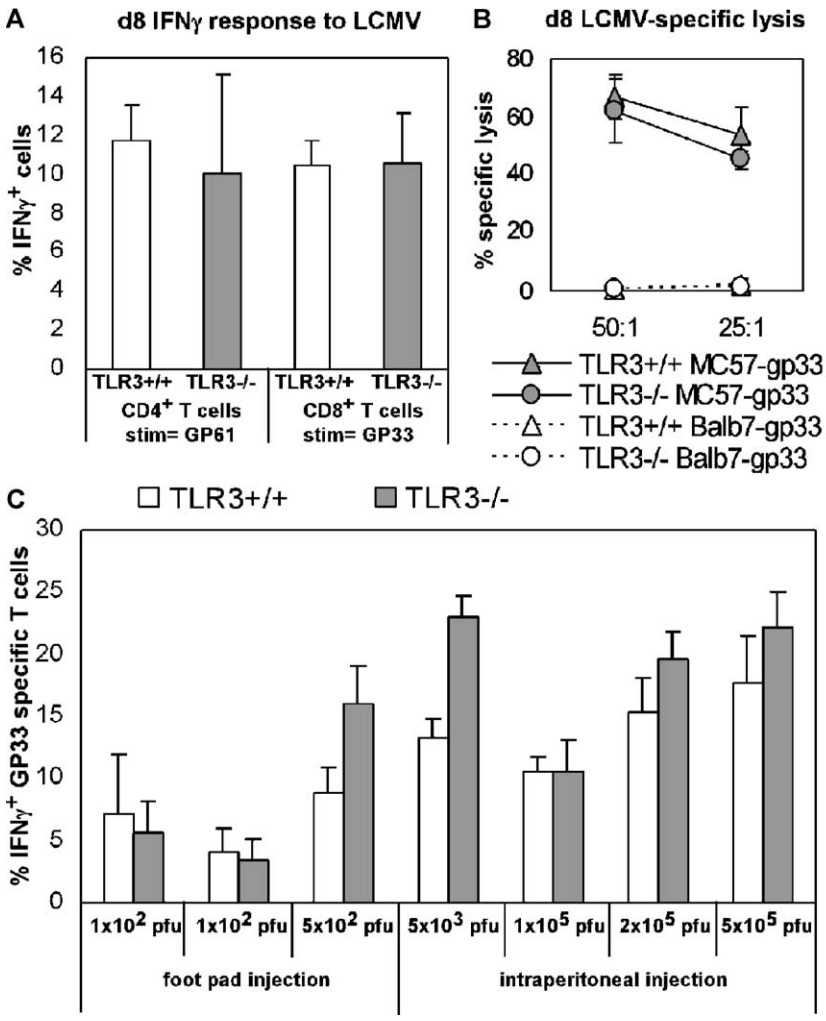

Fig. 1. Absence of TLR3 does not affect primary $CD4^+$ or $CD8^+$ T cell responses to LCMV. $TLR3^{-/-}$ mice and control mice (n = 3 mice per group) were infected with LCMV ARM53b (1×10^5 pfu ip), infection with $TLR3^{-/-}$ and control splenocytes was obtained 8 days later. (A) Cells were stimulated in vitro with either LCMV-specific $CD4^+$ or $CD8^+$ T cell peptide epitopes in the presence of brefeldin A. IFNγ responses were measured by intracellular cytokine staining in combination with $CD8^+$ and $CD4^+$ surface staining. Results are represented as percentage of IFNγ-positive cells within respective $CD4^+$ or $CD8^+$ populations. (B) Measurement of $CD8^+$ T cell lytic function was assessed via standard chromium release assay against GP33 peptide-coated syngeneic and allogeneic cells; MC57 and BALB17 cells respectively. (C) $TLR3^{-/-}$ mice respond efficiently to LCMV irrespective of route and dose of inoculation. $TLR3^{-/-}$ and control mice (n = 3–4 mice per group per condition) were inoculated with LCMV ARM 53b using either foot pad or ip injection and varying doses as indicted. IFNγ responses from GP-33-specific T cells were analyzed 8 days postinfection from draining lymph nodes in foot pad-injected mice and splenocytes from ip-injected mice. Results are represented as percent $IFN\gamma^+$ $CD8^+$ T cells of total $CD8^+$ T cell population. Data represented in A and B are derived from individual experiments while those for C are compiled from several experiments.

(Ahmed et al., 1984). In contrast, mice previously immunized with LCMV ARM53b develop T cell memory responses that are able to prevent establishment of persistent infection by subsequent LCVM Cl 13 challenge (Arbour et al., 2002). $TLR3^{-/-}$ mice were tested for the ability to generate a lasting functional memory response to LCMV infection. LCMV immune $TLR3^{-/-}$ and control mice (1×10^5 pfu LCMV ARM53b ip) were inoculated 3 months after primary challenge with 2×10^6 pfu LCMV Cl 13 via iv injection. The $CD4^+$ and $CD8^+$ T cell responses of these mice were analyzed 3 days after inoculation. All mice showed levels of IFN-γ^+ LCMV-specific T cells that were not statistically different, indicating normal induction of the T cell memory response (Fig. 2C).

The last series of studies employed a double-stranded RNA virus infection of neonatal $TLR3^{-/-}$ and control mice using Type 3 reovirus strain Dearing (T3D). Intracerebral inoculation of neonatal mice is the classic experimental model for evaluating virulence of neurotropic viruses such as reovirus. We therefore compared mortality, viral titer, antigen distribution, and neuropathology in newborn $TLR3^{-/-}$ and control $TLR3^{+/+}$ injected intracerebrally with T3D. There was no difference between T3D-infected $TLR3^{+/+}$ control mice and T3D-infected $TLR3^{-/-}$ mice in virus-induced CNS injury. The localization and the severity of viral CNS pathology were similar in both strains of mice, with the brunt of virus-induced injury present in the CA2–3 region of the hippocampus, cingulate gyrus, fronto-parietal cortex, and

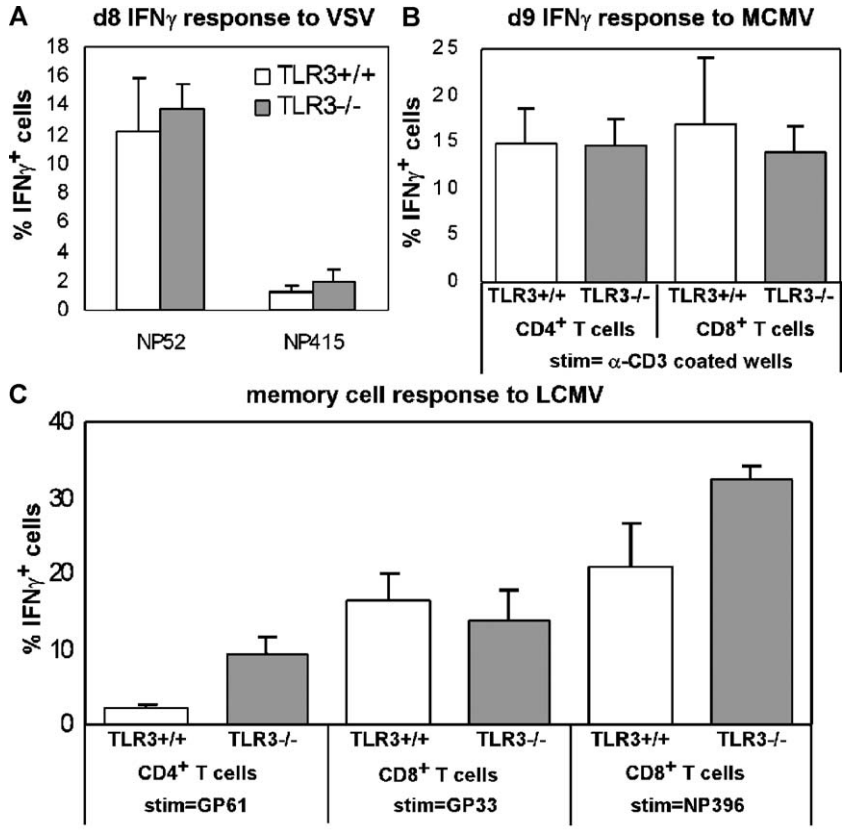

Fig. 2. (A–B) Primary T cell responses to VSV and MCMV are normal in TLR3$^{-/-}$ mice. Splenocytes were isolated from TLR3$^{-/-}$ and control mice 8 days postinfection with VSV (2×10^6 pfu iv) or 9 days postinfection with MCMV (1×10^4 pfu ip). Cells from VSV-infected mice were stimulated for 5 h with either CD8-specific peptide epitope NP52 or CD4-specific peptide epitope NP415, while cells from MCMV-infected mice were stimulated with α-CD3. Groups consisted of 3–4 mice. All data represent averages of results from individual mice derived from three experiments and are represented as percent IFNγ$^+$ T cells of respective CD8$^+$ or CD4$^+$ T cell populations. (C) TLR3$^{-/-}$ mice have functional T cell memory responses. TLR3$^{-/-}$ and control mice ($n = 2$ per group) were immunized with LCMV Arm 53b (1×10^5 pfu ip). These mice were challenged with LCMV Cl 13 (2×10^6 pfu iv) 3 months after immunization. Splenocytes were isolated 3 days post-secondary challenge, and responses to CD4 peptide epitope GP61 and CD8 peptide epitopes GP33 and NP396 were measured. Results are presented as percent IFNγ$^+$ cells of either CD4$^+$ or CD8$^+$ cell populations.

dorsal thalamus as previously reported (Figs. 3B–C) (Oberhaus et al., 1997; Raine and Fields, 1973; Richardson-Burns et al., 2002). CNS injury is evidenced by the presence of numerous pyknotic and apoptotic cells, some infiltrating inflammatory cells, disruption of cytoarchitecture, and loss of tissue integrity (see Fig. 3). The viral antigen co-localized with areas of CNS injury with anti-T3D immunohistochemical staining present in the hippocampus, cortex, and thalamus. Fig. 4 shows viral antigen staining in the dorsal thalamus and CA3 region of the hippocampus from T3D-infected TLR3$^{+/+}$ and TLR3$^{-/-}$ mice (Fig. 4). There was no difference in survival between T3D-infected TLR3$^{+/+}$ and control mice. Mortality was 40% by day 7 in TLR3$^{-/-}$ mice, with all six survivors appearing moribund. Mortality was 37.5% in control TLR3$^{+/+}$ mice, with all five survivors appearing moribund. Mean weight of mice on day of sacrifice (d7) was 3.53 g for TLR3$^{-/-}$ mice and 3.90 g for TLR3$^{+/+}$ control mice. Viral titer in the brain at time of sacrifice was the same in the two groups of mice (8.94 ± 0.06 $\log_{10}$ pfu/ml in TLR3$^{-/-}$ mice compared to 9.00 ± 0.13 in control TLR$^{+/+}$ mice).

In summary, our data indicate that the TLR3 signaling pathway does not appear to influence the generation of effective antiviral responses for a range of virus infections, thereby calling into question whether it represents a universal element in antiviral immunity. Although we did not observe enhanced susceptibility with our strain and dose of MCMV, others (Hoebe et al., 2003) using mice deficient in lps2, a downstream component of the TLR3 signaling pathway, noted increased susceptibility to MCMV at higher doses of virus. So it appears that there may be some conditions where TLR3 does enhance immunity, and that it may be restricted to the early control of an overwhelming viral infection requiring a fast and strong IFN type-I innate response to prevent host death. One question that arises from the current studies is whether TLR3 can in fact recognize any component of these viruses. Using an in vitro assay where the various TLR genes are transfected and expressed in HeLa and HEK cells, UV-inactivated LCMV Arm 53b and other arenaviruses were unable to activate cells expressing TLR3 while control poly I:C did, again indicating that TLR3 is unlikely to play a role in the

pathogenesis of arenaviruses. However, several of these arenaviruses did activate other TLR receptors (de Silva and Kunz, unpublished observations). It is also possible that TLR3 is not capable of recognizing the viral RNA intermediates of LCMV, VSV, and MCMV. This is not true for reovirus where Alexopoulou et al. (2001) identified TLR3 recognition of purified reovirus genomic dsRNA. Hence, it is possible that during these viral infections, TLR3 never comes in contact with unsequestered or exposed viral RNAs. Alternatively and a more likely scenario is that the concentrations of dsRNA made during natural infection are not sufficient to bind to and significantly influence TLR3 signaling when compared to the high concentrations used for in vitro studies.

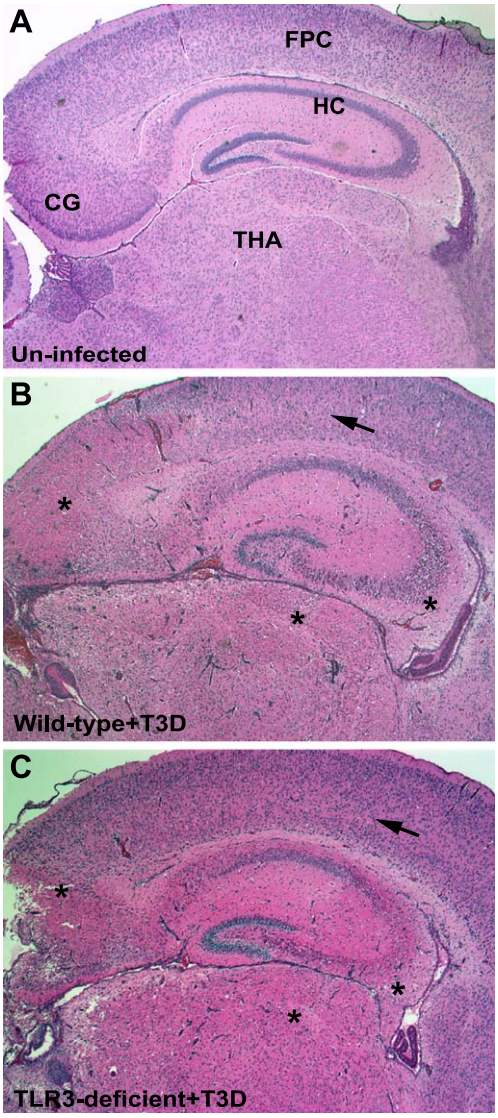

Fig. 3. There is no difference between TLR3-deficient mice and wild-type mice in T3 reovirus-induced injury or T3 reovirus antigen expression in the CNS. (A) Uninfected control mouse brain stained with hematoxylin and eosin (H&E) shows normal CNS cytoarchitecture and normal appearing nuclei of viable cells. (B) H&E-stained brain from T3D-infected TLR3$^{+/+}$ wild-type mouse shows characteristic T3 reovirus-associated injury throughout the brain evidenced by cell death, loss of normal cytoarchitecture, and loss of tissue integrity in the cingulate gyrus (CG), fronto-parietal cortex (FPC), hippocampus (HC), and thalamus (THA). (C) H&E-stained brain from T3D-infected TLR3-deficient (TLR3$^{-/-}$) mouse shows T3 reovirus-induced CNS injury comparable to that seen in T3D-infected TLR$^{+/+}$ wild-type mice. * = brain region with viral injury, cell loss, apoptotic cells, and loss of cytoarchitecture; arrow = brain region with cell loss.

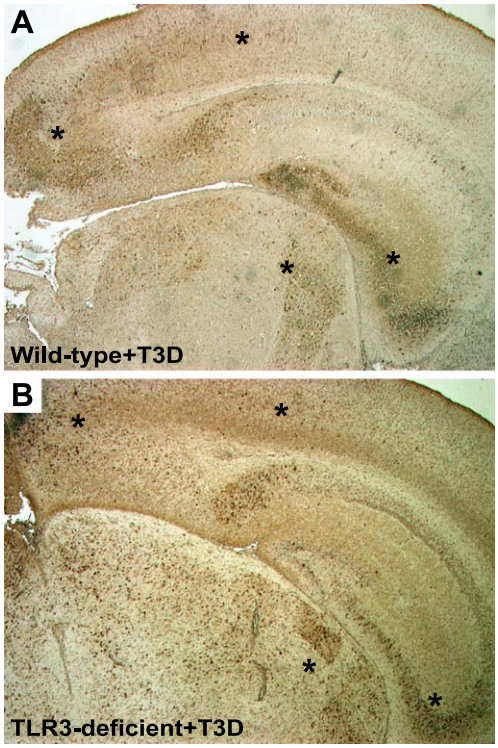

Fig. 4. There is no difference between TLR3-deficient mice and wild-type control mice in T3 reovirus-induced injury in the CNS. (A) Immunohistochemistry (IHC) for T3 reovirus antigen shows numerous T3D-positive cells (dark brown) in brains of T3D-infected TLR3$^{+/+}$ wild-type control mice. Antigen staining is evident in the same brain regions in which T3 reovirus injury is present (compare to Fig. 3). (B) IHC for T3 reovirus antigen in brain of T3D-infected TLR3$^{-/-}$ mouse shows similar antigen levels to that seen in the T3D-infected TLR3$^{+/+}$ wild-type control mouse brains.

Materials and methods

Mice

TLR3$^{-/-}$ mice on C57BL/6 × B129 mixed background and matched control mice were derived at Yale University and bred at The Scripps Research Institute. Mice were bred and maintained under specific pathogen-free conditions.

Virus strains

Stocks of both LCMV ARM 53b and variant LCMV Cl 13 were grown up via single passage in baby hamster kidney (BHK) cells from stocks that had been previously triple plaque purified in Vero cells (Ahmed et al., 1984; Dutko and Oldstone, 1983). VSV stocks were generated by collection of

supernatants from 100% CPE VSV-infected BHK-21 cells (Lyles et al., 1992). Stocks of the Smith strain of MCMV of virus derived from in vivo propagation in salivary glands of Balb/c mice were obtained from Dr. Raymond Welsh at the University of Massachusetts Medical School, Worcester, MA (Bukowski et al., 1984). Reovirus strain type 3 Dearing (T3D) was identified, passaged, and quantitated as previously reported (Tyler et al., 1985, 1989).

LCMV, VSV, and MCMV methods

LCMV ARM 53b was inoculated into mice intraperitoneally with either 5×10^3, 1×10^5, or 5×10^5 pfu where noted. Footpad injection experiments with LCMV ARM 53b were conducted with 1×10^2 or 5×10^2 pfu virus. Secondary challenges with LCMV Cl 13 were performed intravenously with a dose of virus previously demonstrated to result in persistent infection in naive mice (2×10^6 pfu). In experiments using VSV, the virus was injected intravenously at a dose of 2×10^6 pfu. Mice infected with MCMV were inoculated intraperitoneally with 1×10^4 pfu virus.

Reovirus methods

Two-day-old neonatal mice (weight: 1.5–2 g) were used in reovirus T3D studies. Eight control (C57BL/6 × 129) and 10 TLR3$^{-/-}$ mice on the same background were infected with 10^7 pfu of T3D intracerebrally in a 10-μl volume using a 29-gauge needle connected to a Hamilton microsyringe. Animals were sacrificed for tissue collection at day 7 postinfection, by which time all animals were moribund. Tissue was collected for histology, immunocytochemistry, and determination of viral titer by plaque assay. T3D infected and 5 were not virally infected (uninfected control). Nine TLR3$^{-/-}$ mice were T3D infected. Mice were inoculated with T3D (1×10^5 PFU) via intracerebral or intracranial (ic) injection with each mouse pup weighing about 1.7 g. Injections were made using a 29-gauge needle in a 10-μl volume. Animals were sacrificed by decapitation 7 days after infection.

Analysis of T cell responses

All data represent averages of results from individual mice with 3–5 per group. Splenocytes from LCMV- or VSV-infected mice were cultured for 5 h in RPMI 7% FCS with 50 U/ml rIL2 and 1 μg/ml MHC class-I or class-II restricted peptides. T cells from MCMV mice were cultured for 5 h in α-CD3-coated wells in RPMI 7% FCS + 50 U/ml rIL2. Brefeldin A (1 μg/ml) was added to all cultures at least 3 h before staining. Surface and intracellular staining procedures were performed as previously described using CD4-FITC, CD8-PE, and IFNγ-APC antibodies (Homann et al., 1998). Antibodies used in these experiments were obtained from Pharmingen. CD8$^+$ T cell cytolytic function was measured by standard 5 h ^{51}Cr release assay using MC57 and BALB17 mouse fibroblasts coated with LCMV peptide GP33–41 (1-h incubation with 1 μg/ml peptide followed by three washes).

MHC H-2^b restricted peptides

Peptides were obtained from PeptidoGenic Research. Peptides used were LCMV MHC Class-I: GP33–41, NP396–404; LCMV MHC Class-II: GP61–80; VSV MHC Class-I: NP52–59; VSV MHC Class-II GP 415–433.

Histology

For histopathologic and immunohistochemical staining, five or six whole mouse brains per treatment group were fixed by immersion in 10% buffered formalin for 24–30 h at room temperature (RT), then cut in half along the mid-coronal line for sectioning. Fixed tissues were transferred to 70% ethanol, paraffin embedded, and sectioned at 4-μm thickness. For each animal, two coronal sections were stained with hematoxylin and eosin (H&E) for studies of the extent of virus-induced pathology. Paraffin-embedded sections were baked at 57 °C for 5 min to enhance antigen retrieval, then de-paraffinized by immersion in mixed xylenes followed by rehydration in a series of descending ethanol concentrations followed by PBS.

Immunohistochemistry

Brain tissue sections were de-paraffinized by baking for 5 min at 57 °C, immersion in mixed xylenes, then rehydration in graded alcohols. For viral antigen staining, de-paraffinized tissues were permeabilized in PBS/0.1% Triton X (PBSX) for 1 h at room temperature (RT), then non-specific binding was blocked in 3% BSA/PBS for 1 h at RT. Tissue was incubated with primary antibody anti-T3D polyclonal (laboratory stock) diluted (1:200) in 3% BSA/PBSX for 1 h at 37 °C, then washed in PBSX. Secondary antibody anti-rabbit-Alexafluor594 (Molecular Probes, Eugene OR) diluted 1:100 in 1.5% BSA/PBSX was incubated with tissue for 1 h at RT in the dark. Tissues were washed in PBSX, exposed to Hoechst 33342 (Molecular Probes), diluted in PBS for 5 min at RT, washed in PBSX, then aqueous mounted with Vectashield (Vector Laboratories Inc, Burlingame, CA). All images were captured by digital bright-field or fluorescence microscopy at ×25–100 magnification using a Zeiss Axioplan 2 Digital Microscope with Cooke SensiCam 32 bit Camera.

Determination of viral titer

Reovirus T3 titer in brain tissue was determined by standard plaque assay techniques on confluent monolayers of L929 fibroblasts. Specimens were frozen (−70 °C) and thawed (RT) three times, briefly sonicated, and then diluted into gelatinized saline. Duplicate aliquots were serially di-

luted in 10-fold steps and samples from at least three consecutive dilutions were inoculated onto L929 cell monolayers. Monolayers were stained with neutral red dye at day 6 postinfection and plaques counted. Viral titer was determined by averaging the number of plaques seen at the most informative dilution and correcting for inoculum size and dilution factor. Results are presented as $\log_{10}$ plaque forming units (pfu)/ml ± SEM.

Acknowledgments

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