NKG2D contributes to efficient clearance of picornavirus from the acutely infected murine brain

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Activated murine cytotoxic T cells express the NKG2D natural cytotoxicity receptor. This receptor recognizes major histocompatibility complex (MHC) class I–like molecules expressed on the surface of infected cells and serves to augment T cell–mediated cytotoxicity. The role of NKG2D-mediated augmentation in the clearance of central nervous system viral infections has not been explored. Using the Theiler's murine encephalomyelitis virus model, the authors found that NKG2D-positive CD8⁺ cytotoxic T cells enter the brain, that NKG2D ligands are expressed in the brain during acute infection, and that interruption of NKG2D ligand recognition via treatment with a function-blocking antibody attenuates the efficacy of viral clearance from the central nervous system. *Journal of NeuroVirology* (2008) 14, 261–266.

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Members of the picornavirus family of small, nonenveloped, positive-stranded RNA viruses, which includes enterovirus 71, poliovirus, hepatitis A virus, the coxsackieviruses, and the rhinoviruses, are a frequent cause of infection worldwide. Despite the fact that members of this family infect more humans than any other group of viruses (Rotbart, 2002), host factors that control picornaviral replication are poorly understood. Even less is known about host factors that control picornaviral neurovirulence and clearance of picornaviruses from the central nervous system (CNS) (Buenz and Howe, 2006). We and others have used the Theiler's murine encephalomyelitis virus (TMEV) as a mouse model of picornavirus infection of the CNS (Buenz et al., 2006; Jin et al., 2007; Rubio et al., 2006; Tsunoda et al., 2006). Previous studies have extensively characterized the role of antiviral CD8⁺ cytotoxic T lymphocytes (CTLs) in the clearance of TMEV from the brain during acute infection (Mendez-Fernandez et al., 2003), and it is clear in mice of the H-2b major histocompatibility complex (MHC) class I haplotype that CTLs specific for the VP2₁₂₁−₁₃₀ TMEV peptide are responsible for viral clearance during the first 2 weeks of CNS infection (Myoung et al., 2007). However, the role of additional host factors such as costimulatory molecules has not been thoroughly explored.

NKG2D is a natural cytotoxicity receptor expressed on natural killer cells (NKCs) and on activated CTLs in mice (Eagle and Trowsdale, 2007). This receptor recognizes stress- and infection-regulated MHC class I–like molecules such as Rae1, Mult1, and H60 expressed on the surface of murine cells (Raulet, 2003). Although NKG2D is a direct mediator of cytotoxicity for NKCs, it serves as a costimulatory receptor for CTLs and augments cytotoxicity downstream from T-cell receptor recognition of peptides presented on MHC class I (Markiewicz et al., 2005). Because the mechanisms that direct CTL-mediated clearance of infected neural cells are unclear, we asked whether NKG2D-positive immune effectors are present in the CNS during acute TMEV infection, whether NKG2D ligands are expressed in the brain following infection,
and whether interruption of NKG2D-mediated costimulation of CTLs alters viral clearance from the CNS.

Using flow-cytometric analysis of a previously characterized preparation of brain-infiltrating leukocytes (BILs) (Howe et al., 2007), we measured the number of NKG2D-positive immune cells present in the CNS at 3 and 7 days after TMEV infection (2 × 10^5 plaque-forming units [PFU] intracerebrally [i.c.]; 4- to 6-week-old C57Bl/6 mice; all experiments adhered to Mayo Institutional Animal Care and Use Committee [IACUC] guidelines). We found that at 3 days postinfection (d.p.i.), 6% ± 0.4% of CD45^hi^ BILs were CD8-positive, of which 20% ± 0.6% were also NKG2D-positive.

![Figure 1](image-url)

**Figure 1.** NKG2D-positive antiviral CD8^+^ T cells infiltrate the brain during acute infection with TMEV. Brain-infiltrating leukocytes (BILs) were isolated from mice at 3 (A–C) and 7 (D–F) days postinfection (dpi) and analyzed by flow cytometry. The number of CD45^hi^CD8^+^ cells increased between 3 (A) and 7 (D) dpi. Likewise, the number of CD45^hi^ cells positive for both CD8 and NKG2D increased from 20% at 3 dpi (B) to 76% at 7 dpi (E). Moreover, the intensity of NKG2D surface labeling increased from a mean fluorescence intensity (MFI) of 35 at 3 dpi (shaded histogram in C) to 89 at 7 dpi (shaded histogram in F). The MFI for the isotype control was 4 at 3 dpi (open histogram in C) and 5 at 7 dpi (open histogram in F). BILs were further analyzed for antiviral specificity by costaining with an MHC class I tetramer loaded with the VP2_{121-130} H-2Db-specific viral peptide (G–I). Of the cells that were both CD8^+^ and tetramer-positive (box “H” in G), 81% were also NKG2D-positive, with an MFI of 136 (H). In contrast, only 12% of cells that were CD8^+^ but tetramer-negative (box “I” in G) were also NKG2D-positive, with an MFI of only 18 (I).
positive for the NKG2D costimulatory receptor. By 7 d.p.i., the BILs were comprised of 24% ± 2% CTLs, with 76% ± 2% of these cells positive for NKG2D (n = 4–6 mice per time point; mean ± 95% CI). Thus, between 3 and 7 d.p.i., the phenotype of immune cells infiltrating the CNS shifted toward a population that was enriched in NKG2D⁺CD8⁺CD45hi CTLs (Figure 1A to F). The presence of NKG2D-positive CTLs within the infected hippocampus (Wada and Fujinami, 1993) was confirmed by simultaneously immunostaining cryosections from mice at 7 d.p.i. with anti-CD8 (53-6.7; 1:100) and anti-NKG2D (CX5; 1:50) (Figure 2A to E) (Howe et al., 2004).

In order to determine the viral specificity of the NKG2D-positive CTLs at 7 d.p.i., we costained BILs with anti-CD8, anti-NKG2D, and the TMEV VP2121–130-specific tetramer (Beckman Coulter Immunomics, San Diego, CA; 1:100) (Howe et al., 2007). Flow cytometry revealed that 74% ± 2% of CD45hiCD8⁺ cells were VP2-specific (Figure 1G), whereas none of these cells were stained by an irrelevant D9/E7 tetramer (data not shown) (Mendez-Fernandez et al., 2005). Moreover, 81% ± 2% of the VP2-specific CTLs were also NKG2D-positive (Figure 1H), whereas only 12% ± 2% of the VP2 tetramer-negative CTLs were positive for NKG2D (Figure 1I) (n = 4 mice; mean ± 95% CI). We conclude that a robust population of NKG2D-positive VP2-specific CTLs are present within the brain at 7 days after infection with TMEV.

Others have reported the up-regulation of NKG2D ligands following infection in a variety of peripheral tissues (Eagle and Trowsdale, 2007). However, the up-regulation of these ligands by picornavirus infection has not been previously explored. We found that Rae1, Mult1, and H60 were all up-regulated within the hippocampus during acute TMEV infection, as determined by reverse transcriptase polymerase chain reaction (RT-PCR) analysis of RNA isolated from the excised hippocampus (see Table 1 for conditions). Of the three ligands we analyzed, Rae1 showed the most robust up-regulation, with a peak induction of 10 ± 1-fold over the uninfected hippocampus at 7 d.p.i. (F(4, 9) = 4.845, P = .017 by one-way analysis of variance [ANOVA]; q(9, 5)=5.841, P = .012 with the SNK pairwise comparison between 0 and 7 d.p.i.; mean ± 95% CI) (Figure 3A). H60 and Mult1 were also up-regulated 2 ± 0.1-fold and 2 ± 0.2-fold over uninfected, respectively, at 7 d.p.i. (n = 3 mice; mean ± 95% CI) (Figure 3A).

In order to identify the cellular locus of NKG2D ligand expression in the hippocampus, we attempted, unsuccessfully, to immunostain fresh frozen or paraffin-embedded sections of hippocampus after fixation with paraformaldehyde, methanol, or ethanol:acetone. We tested specific anti-Rae1 (clone

![Figure 2](https://example.com/figure2.png)

**Figure 2** Hippocampal sections were stained with anti-NKG2D (A) and anti-CD8 (B). Colocalization (C–E) confirmed the presence of CD8⁺ (green) and NKG2D-positive (red) cells in the hippocampus at 7 dpi. DAPI is shown in blue in (C). Arrowheads in (D) indicate cells that are double-positive for CD8 and NKG2D. A representative double-positive cell is shown in (E). Scale bar in C is 200 microns and refers to A–C; scale bar in D is 100 microns; scale bar in E is 5 microns.
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Table 1 RT-PCR conditions for NKG2D ligands

| Gene | Forward primer | Reverse primer | UPL probe |
|------|----------------|----------------|-----------|
| Rae-1γ | ATACACCAACGGGCTGGAT | CTTCCGGTCATACCAGAGG | cccagcag |
| H-60 | ACAGCATAGCATCTTTATCCAC | TCCATGCGACGGTATCTAC | cctgaga |
| Mult-1 | AGCTCATGTTGCACTGGAAA | TCATCAAGGTACGAAAGATCCTG | tcctggac |
| GAPDH | AGCTGTGCTATCAAGGGGAAG | TTTGATGTTAGTGGGGTCGG | ctaacca |

Note. Excised hippocampus was processed for RNA purification using the Qiagen RNeasy Lipid-Tissue kit. cDNA was prepared using the Roche 1st Strand cDNA Synthesis kit and analyzed using the Roche Universal Probe Library taqman probes identified above and the Roche TaqMan Master kit. Samples were amplified for 45 cycles using 95°C melt for 10 s, 55°C anneal for 30 s, and 72°C elongate for 5 s. Primers are given as 5′ to 3′.

Figure 3 NKG2D ligands are upregulated in the hippocampus during acute infection with TMEV and interference with NKG2D recognition of these ligands increases viral load in the CNS. qRT-PCR analysis of brain mRNA revealed a 10-fold upregulation of Rae1 at 7 dpi (A) as compared to the uninfected brain. Likewise, H60 and Mult1 were also upregulated throughout the acute phase of infection (A). Western blot analysis of hippocampal protein lysates showed the translational upregulation of H60, with peak expression at 7 dpi (B). The 50 kDa band (arrow) upregulated in hippocampus was also present in YAC1 cell lysates (B). Treatment with the CX5 anti-NKG2D function blocking antibody throughout the acute phase of infection resulted in a 10-fold increase in viral load within the brain at 7 dpi (C). Bar graph shows mean ± 95% confidence interval. Five mice were treated in each group and individual viral titers are shown as black circles in (C). All CX5-treated mice showed increased viral titer.
In addition to expression on activated CTLs, NKG2D is also a primary cytotoxicity receptor for NKC and NKC activity is certainly modulated by viral infection. We chose to specifically focus on the role of NKG2D on antiviral CTLs because the number of NKCs present within the brain during the peak of viral clearance is low compared to CTLs (ca. 10% of CD45hi cells are NK1.1-positive at 7 d.p.i.; data not shown). However, we certainly cannot rule out a role for NKC-mediated recognition of NKG2D ligands on infected neural cells as part of an early response required for viral clearance. Likewise, NKG2D is expressed by subpopulations of CD4 T cells (Saez-Borderias et al., 2006), γδ T cells (Dandekar et al., 2005), and macrophages (Baba et al., 2006), suggesting that multiple cell types may be inhibited by CX5 treatment. Regardless, our observations provide evidence that NKG2D-mediated recognition of virus-infected targets by CTLs participates in the clearance of picornviruses from the CNS. These findings extend previous studies regarding the immunovirology of NKG2D ligand expression, which have largely focused on viral evasion of immune surveillance (Lodoen et al., 2003, 2004; Vilarinho et al., 2007), and add to the potential repertoire of therapeutic strategies for reducing and controlling CNS picornavirus infections (Buenz and Howe, 2006; Buenz et al., 2006).

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