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

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Abstract

Activated murine cytotoxic T cells express the NKG2D natural cytotoxicity receptor. This receptor recognizes 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 we 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.

Keywords

TMEV; cytotoxic T celll; enterovirus; Rae1; H60; Mult1; neurovirulence

Members of the picornavirus family of small, non-enveloped, 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-2^b MHC class I haplotype that CTLs specific for the $VP2_{121–130}$ TMEV peptide are responsible for viral clearance during the first two weeks of CNS infection (Myoung*et al*, 2007). However, the role of additional host factors such as co-stimulatory molecules has not been thoroughly explored.

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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). While NKG2D is a direct mediator of cytotoxicity for NKCs, it serves as a co-stimulatory 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 co-stimulation 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 PFU i.c.; 4–6 week old C57Bl/6J mice; all experiments adhered to Mayo IACUC guidelines). We found that at 3 days postinfection (dpi) 6 ± 0.4% of $CD45^{hi}$ BILs were CD8-positive, of which 20 ± 0.6% were also positive for the NKG2D costimulatory receptor. By 7 dpi the BILs were comprised of 24 ± 2% CTLs, with 76 ± 2% of these cells positive for NKG2D (n=4–6 mice per timepoint; mean ± 95% CI). Thus, between 3 and 7 dpi the phenotype of immune cells infiltrating the CNS shifted toward a population that was enriched in $NKG2D^+CD8^+CD45^{hi}$ CTLs (Figure 1A–1F). The presence of NKG2D-positive CTLs within the infected hippocampus (Wada and Fujinami, 1993) was confirmed by simultaneously immunostaining cryosections from mice at 7 dpi with anti-CD8 (53-6.7, 1:100) and anti-NKG2D (CX5, 1:50) (Figure 2A–2E) (Howe *et al*, 2004).

In order to determine the viral specificity of the NKG2D-positive CTLs at 7 dpi we co-stained BILs with anti-CD8, anti-NKG2D, and the TMEV $VP2_{121–130}$-specific tetramer (Beckman Coulter Immunomics, San Diego, CA; 1:100) (Howe *et al*, 2007). Flow cytometry revealed that 74 ± 2% of $CD45^{hi}CD8^+$ cells were VP2-specific (Figure 1G), while none of these cells were stained by an irrelevant D^b/E7 tetramer (data not shown) (Mendez-Fernandez *et al*, 2005). Moreover, 81 ± 2% of the VP2-specific CTLs were also NKG2D-positive (Figure 1H), while 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 upregulation of NKG2D ligands following infection in a variety of peripheral tissues (Eagle and Trowsdale, 2007). However, the upregulation of these ligands by picornavirus infection has not been previously explored. We found that Rae1, Mult1, and H60 were all upregulated within the hippocampus during acute TMEV infection as determined by RTPCR analysis of RNA isolated from the excised hippocampus (see Table 1 for conditions). Of the three ligands we analyzed, Rae1 showed the most robust upregulation, with a peak induction of 10 ± 1 fold over the uninfected hippocampus at 7 dpi ($F(4,9)=4.845$, $P=0.017$ by one-way ANOVA; $q(9,5)=5.841$, $P=0.012$ with the SNK pairwise comparison between 0 and 7 dpi; mean ± 95% CI) (Figure 3A). H60 and Mult1 were also upregulated 2 ± 0.1 fold and 2 ± 0.2 fold over uninfected, respectively, at 7 dpi (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 186107; 1:25; R&D Systems) and anti-H60 (clone 205326; 1:100;

R&D Systems) antibodies as well as a pan-specific NKG2D ligand antibody (goat anti-Rae1; 1:100; Santa Cruz Biotechnology) and the pan-specific NKG2D-Fc chimeric molecule (R&D Systems 139-NK; 1:10). Likewise, we attempted to confirm the expression of the NKG2D ligands at the protein level within the hippocampus by western blot analysis of hippocampal lysates. Of the numerous antibodies tested (same as immunostaining, above), only the anti-H60 antibody (clone 205326; 1:100; R&D Systems) successfully identified a prominent band that was present within the hippocampus only after infection and which was also present in lysate from the YAC-1 NK cell target line (Figure 3B). The apparent molecular weight of this prominent band (ca. 50 kDa) matches that described by Hasan and colleagues (Hasan *et al*, 2005). The relative difference in mobility between the putative H60 band in the hippocampal lysate and the YAC-1 cell lysate may reflect differential expression of one of the three recently identified isoforms of H60 that share high sequence homology but differ in molecular weight (Takada *et al*, 2008). We conclude that NKG2D ligands such as H60 are upregulated at both the transcriptional and translational level within the hippocampus following acute TMEV infection, but we are unable to identify the cells that express these ligands with the currently available immunologic tools.

Finally, we asked whether interruption of NKG2D-mediated recognition of NKG2D ligands was sufficient to alter viral load within the hippocampus. We used the CX5 function blocking antibody following a therapeutic regime adapted from Lanier and colleagues (100 ug/mouse i.p. on −1, +1, and +4 dpi; kindly provided by Dr. Lanier, UCSF) (Ogasawara *et al*, 2003). Control mice received an equivalent dose of polyclonal rat IgG (Kim *et al*, 2007). Plaque assay (Pavelko *et al*, 1998) of freshly excised brain homogenates at 7 dpi revealed that CX5 treatment led to 9 ± 2 times more infectious virus as compared to control IgG-treated mice ($U(5,5)=15$, $P=0.008$, Mann-Whitney rank sum test; mean ± 95% CI) (Figure 3C). Based on these observations, we propose that VP2-specific CTLs expressing NKG2D recognize NKG2D ligands such as Rae1 and H60 expressed on infected cells within the brain and thereby engage an enhanced cytotoxic response (Markiewicz *et al*, 2005) that contributes to the clearance of picornaviruses from the CNS.

In addition to expression on activated CTLs, NKG2D is also a primary cytotoxicity receptor for NKCs 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 $CD45^{hi}$ cells are NK1.1-positive at 7 dpi; 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 mutliple cells 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 picornaviruses 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; Lodoen *et al*, 2004; Vilarinho *et al*, 2007) and add to the potential repertoire of therapeutic strategies for reducing and controlling CNS picornaviral infections (Buenz and Howe, 2006; Buenz *et al*, 2006).

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Abbreviations

CNS	central nervous system
TMEV	Theiler's murine encephalomyelitis virus
CTL	cytotoxic T lymphocyte
NKG2D	natural-killer group 2, member D
NKC	natural killer cell
Rae1	retinoic acid early transcript 1
H60	minor histocompatibility protein 60
Mult1	murine UL16-binding-protein-like transcript 1
MHC	major histocompatibility complex
BILs	brain-infiltrating leukocytes
dpi	days postinfection

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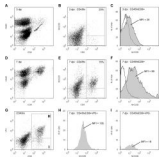

Figure 1.

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 VP2121–130 H-$2D^b$-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).

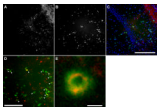

Figure 2.
Hippocampal sections were stained with anti-NKG2D (A) and anti-CD8 (B). Colocalization (C–E) confirmed the presence of $CD8^+$ (green) and NKG2D-positive (red) cells in the hippocampus at 7 dpi. DAPI is shown in blue in (C). Arrowheads in (D) indicate cells that are double-positive for CD8 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.

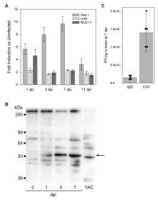

Figure 3.

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. qRTPCR 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 YAC-1 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.

Table 1

RT-PCR conditions for NKG2D ligands.

Gene	Forward Primer	Reverse Primer	UPL Probe
Rae-1γ	ATACACCAACGGGCTGGAT	CTTCGCTTCATACCAGAGAGG	cccagcag
H-60	ACAGCATAGCATCTACTTTTATCCAC	TCCATGGCACTGCTGTTATC	cctggaga
Mult-1	AGCTCATGTTGCACTGGAAA	TCATCAAGGTACTGAAAGATCCTG	tctggagc
GAPDH	AGCTTGTCATCAACGGGAAG	TTTGATGTTAGTGGGGTCTCG	catcacca

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 sec, 55°C anneal for 30 sec, and 72°C elongate for 5 sec. Primers are given as 5'-to-3'.