Predominant clonal accumulation of CD8+ T cells with moderate-avidity in the CNS of Theiler’s virus-infected C57BL/6 mice

Hyun Seok Kang1 and Byung S. Kim1

1Department of Microbiology-Immunology, Northwestern University Medical School, Chicago, Illinois 60611

Running Title: Anti-viral T cell repertoire in the CNS

Keywords: Avidity, CDR3, CNS, T cell, Vβ repertoire, TMEV

All correspondence should be made to Dr. Byung S. Kim, Department of Microbiology-Immunology, Northwestern University Medical School, 303 E. Chicago Ave, IL 60611. Tel: 312-503-8693; Fax: 312-503-1399; e-mail: bskim@northwestern.edu
Abbreviations

aa, amino acid; APL, altered peptide ligand; CDR3, complimentary determining region 3; MS, multiple sclerosis; CNS, central nervous system; CNS-ILs, CNS-infiltrating lymphocytes; dpi, days post-infection; PFU, plaque forming unit; RT-PCR, reverse transcriptase - polymerase chain reaction; TCR, T cell receptor; TMEV, Theiler's murine encephalomyelitis virus; TMEV-IDD, TMEV-induced demyelinating disease
ABSTRACT

Induction of antigen-specific CD8+ T cells bearing high-avidity TCR is thought to be an important factor in anti-viral and anti-tumor immune responses. However, the relationship between TCR diversity and functional avidity of epitope-specific CD8+ T cells accumulating in the CNS during viral infection is unknown. Hence, analysis of T cell diversity at the clonal level is important to understand the fate and function of virus-specific CD8+ T cells. In this study, we examined the Vβ diversity and avidity of CD8+ T cells specific to the predominant epitope (VP2121-130) of Theiler’s murine encephalomyelitis virus (TMEV). We found that Vβ6+CD8+ T cells, associated with the epitope-specificity, predominantly expanded in the CNS during viral infection. Further investigations of the antigen-specific Vβ6+CD8+ T cells with CDR3 spectratyping and sequencing indicated that distinct T cell clonotypes are preferentially increased in the CNS as compared to the periphery. Among the epitope-specific Vβ6+CD8+ T cells, MGX-Jβ1.1 motif bearing cells, which could be found in a high precursor frequency in naïve mice, were expanded in the CNS and tightly associated with IFN-γ production. These T cells displayed a moderate-avidity to the cognate epitope rather than the high-avidity normally observed with memory/effector T cells. Therefore, our findings provide new insights into the CD8+ T cell repertoire during immune responses toward viral infection in the CNS.
INTRODUCTION

Theiler’s murine encephalomyelitis virus (TMEV) is a member of the Cardiovirus genus within the Picornaviridae family (43). This virus is a common enteric pathogen among wild mice but rarely causes neurological disease (57). However, when infected intracerebrally in susceptible mice (e.g., SJL/J (SJL) strain), it reproducibly induces a chronic immune-mediated demyelinating disease that has been studied as an infectious model for human multiple sclerosis (MS) (10, 30). In contrast, infection in resistant mice like the C57BL/6 (B6) strain results in strong anti-viral immune responses that clear the virus effectively and prevent disease development (24, 31). Therefore, immune responses in B6 mice have been often compared to those in susceptible SJL mice to understand the nature of protective vs. pathogenic immunity in these mice.

It has been shown that the MHC H-2D locus is a critical genetic factor for resistance to TMEV-induced demyelinating disease (9, 49). For example, the expression of H-2D\(^b\) transgene leads to susceptible FVB mice resistant, by inducing strong H-2D\(^b\)-restricted VP\(2_{121-130}\)-specific CD8\(^+\) T cell responses (36). This acquired resistance is abolished when VP\(2_{121-130}\)-specific T cells are tolerized by introducing VP2 transgene (45). These results strongly suggest that CD8\(^+\) T cells generated in the presence of H-2D\(^b\) are critical for viral clearance from the CNS. Since the cardinal difference between resistant B6 and susceptible SJL strains is the quantity, not the quality, of virus-specific CD8\(^+\) T cells (23, 32), strong CD8\(^+\) T cell responses are probably required to prevent viral persistence and the consequent development of demyelinating disease. Greater than 3-fold more virus-specific CD8\(^+\) T cells were found in the CNS of resistant B6 mice compared to those of susceptible SJL mice at the acute phase of infection. Thus, the level of virus-specific CD8\(^+\) T cells at an early phase of immune response may be a critical factor for
Many recent investigations indicate that oligoclonal CD8+ T cells accumulate in the CNS of MS patients (4, 38, 51). In addition, CD8+ T cells may also induce the development of EAE (54). Therefore, clonal expansion of certain CD8+ T cells may be associated with the pathogenesis of demyelinating diseases. However, B6 mice, that are resistant to TMEV-induced demyelinating disease, induce strong CD8+ T cell responses to a single predominant epitope (VP2121-130), i.e. ≥70% of CNS infiltrating CD8+ T cells (41, 42). These CD8+ T cells result in effective viral clearance, yet remain at a low level in the CNS more than 120 days post-infection without detectable pathology (42). This inconsistency led us to investigate the shape and quality of the TCR repertoire accumulating in the CNS of B6 mice.

The CD8+ T cell responses induced after viral infection have previously been investigated with other animal viruses including influenza virus, lymphocytic choriomeningitis virus (LCMV), mouse hepatitis virus (MHV), and Borna disease virus (11, 14, 35, 47, 58). Among these models, detailed T cell Vβ repertoire in the CNS was described only in MHV model (46). CD8+ T cell responses against TMEV in B6 mice are primarily against a single predominant epitope (22, 36, 41). However, virtually no study on the TCR Vβ repertoires of virus-specific CD8+ T cells has been reported. Furthermore, it is not yet known whether a particular TCR Vβ repertoire is associated with avidity and/or function of CD8+ T cells in the CNS. Since protective versus pathogenic CD8+ T cells may correlate with their Vβ repertoire and T cell function, these studies may help to elucidate the underlying mechanisms of protection vs. pathogenesis of CD8+ T cells in the CNS.

In this study, we have addressed several important questions in CD8+ T cell repertoire in the CNS. First, what is the pattern of Vβ usage in TMEV-infected B6 mice? Second, are there
differences in the antigen-specific CD8$^+$ T cell clonotypes between the CNS and periphery? Third, are the T cell clonotypes maintained in the CNS during the viral infection? Fourth, what is the functional avidity of CNS accumulating T cells during the virus infection? Last, what possible factors are associated with repertoire selection and expansion in the CNS? Our results show that V$\beta$6$^+$CD8$^+$ T cells preferentially expand in the CNS during the viral infection. Further analyses of the CDR3 region of antigen-specific V$\beta$6$^+$CD8$^+$ T cells by spectratyping and sequencing indicate that distinct T cell clonotypes are expanded in the CNS as compared to those in the periphery. T cells expressing a particular V$\beta$6-CDR3-J$\beta$1.1 sequence are preferentially retained in the CNS during the course of viral infection. Interestingly, these T cells are capable of producing IFN-$\gamma$ upon stimulation and display moderate-avidity to the cognate epitope. We believe that our findings will provide important information regarding the CD8$^+$ T cell repertoire during viral infection and these results may help to provide better understanding in anti-viral CD8$^+$ T cell immunity in the CNS.
MATERIALS AND METHODS

Mice and TMEV infection. C57BL/6, SJL/J and B6.S (Jackson Laboratories, Bar Harbor, ME) and 129S2/SP mice (Charles River, Fredric, VA) were housed in the Center for Comparative Medicine, Northwestern University, Chicago, IL. Under the guidelines of the Animal Care and Use Committee, 6-8 week old female mice were inoculated by intracerebral injection with 3 X 10^6 PFU of BeAn strain of TMEV in 30 µl of Dulbecco’s modified Eagle’s medium.

Peptides. All synthetic peptides purified by high-performance liquid chromatography to >95% purity were obtained from Genemed Synthesis, San Francisco, CA. These include TMEV VP2_{121-130} (FHAGSLLVFM) (6, 13) and VP2_{M130L} (FHAGSLLVFL) peptides (41).

Tetramer generation. H-2D^b tetramers were generated as previously described (3). Briefly, H-2D^b and human β2-microglobulin genes were subcloned into pET28 bacterial expression vector. BL21/DE3 was transformed and protein expression was induced with IPTG for 4 hrs. Inclusion bodies were purified and refolded in the presence of peptides. Soluble H-2D^b/peptide was biotinylated with BirA at room temperature. Excess biotin was removed by ultrafiltration and then tetramerized with Streptavidin-PE conjugate (Invitrogen, Carlsbad, CA). H-2D^b-LCMV-GP33 tetramer was purchased from Beckman Coulter (Fullerton, CA).

Isolation of CNS-infiltrating cells. Mice were anesthetized with isoflurane and perfused with 30 ml of cold HBSS. Brains and spinal cords were removed, minced with steel mesh and treated with collagenase IV and DNase I (Sigma) for 45 min at 37°C. Mononuclear cells were isolated after 100 % Percoll continuous gradient centrifugation at 27,000 g for 30 min as previously described (16).
**Flow cytometry.** Freshly isolated CNS-infiltrating mononuclear cells were cultured in 96-well round bottom plates in the presence of relevant peptide or PBS and Golgi-Stop™ for intracellular cytokine staining as previously described (50). Allophycocyanin-conjugated anti-CD8 (clone Ly2) or anti-CD4 (clone L3T4) antibody with PE-labeled rat monoclonal anti-IFN-γ (XMG1.2) antibody was used for intracellular cytokine staining. Cells were analyzed on a Becton Dickinson FACS Calibur or LSRII cytometer. Live cells were gated based on light scatter properties.

**Wildtype and mutant recombinant viruses.** The generation of mutant viruses was previously described (41). Briefly, viral RNA was transcribed *in vitro* from full-length wildtype (pSBW) or mutant (pSBW-VP2_{M130L}) viral plasmid using T7 RNA polymerase in the presence of RNasin (Promega, Madison, WI). Viral RNA was transfected into BHK cells using Lipofectamine 2000® (Invitrogen, Carlsbad, CA). Supernatant was collected 5-7 days post-transfection and used to infect fresh BHK cells to obtain high titer (~2 x 10^9 PFU/ml) viral stock.

**Isolation of IFN-γ secreting cells.** IFN-γ secreting cells were isolated using an enrichment kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer’s instruction (33). Briefly, CNS and spleen cells from virus-infected mice were stimulated *in vitro* for 4-6 hrs in the presence of 2 μM of VP2_{121-130}. Cells were then labeled with an affinity matrix for secreted IFN-γ (catch reagent). After 45 min of incubation at 37 °C, IFN-γ bound to the catch reagent was stained with a PE-conjugated IFN-γ-specific antibody (detection reagent). IFN-γ⁺ T cells were isolated by magnetic cell sorting using anti-PE microbeads.

**cDNA synthesis and PCR amplifications.** Total RNA was purified using TRIZOL™ reagent (Invitrogen, Carlsbad, CA) and then cDNA was generated from the RNA using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA) and oligo(dT)_18. Relative concentrations of cDNA were
estimated on the basis of β-actin amplification levels following PCR for 25-30 cycles. Vβ chain usage was assessed by PCR using a sense primer specific to different Vβ genes and a common antisense Cβ (Table 1).

**CDR3 length spectratyping.** CDR3 length spectratyping was performed as previously described with minor modifications (25, 44). Primary amplification of cDNAs from isolated antigen-specific T cells was performed for 35 cycles using Vβ-Cβ primer pairs at 58 °C for annealing in the presence of PCR enhancer solution (GIBCO, Invitrogen Corporation, Grand Island, NY). The primary PCR product (1 µl) was subjected to a secondary PCR with P32-end-labeled Vβ chain primer and 12 different Jβ primers (Table 1). The secondary PCR for Vβ-Jβ combinations was carried out for 25 cycles at 60 °C for annealing in the presence of 30 mM (NH4)2SO4 to avoid cross-amplification. The radioactive PCR products were separated on a 5% acrylamide sequencing gel. CDR3 lengths were then determined after exposure to Kodak X-OMAT LS film (Rochester, NY) or using phosphoimager (Bio-Rad personal FX).

**Functional avidity test.** CNS-infiltrating mononuclear cells from mice at 8 and/or 16 dpi were restimulated in vitro with ten-fold serial dilutions of peptides starting from 1 µM. Levels of CD8+ T cell responses were determined based on the IFN-γ production by intracellular cytokine staining. Avidity thresholds were set as previously described (53): < 10⁻³ µM, high avidity; 10⁻¹ – 10⁻³ µM, moderate avidity; > 10⁻¹ µM, low avidity.

**Sequencing of CDR3 bands.** To analyze individual CDR3 spectratype bands, DNA was eluted from each gel band and re-amplified with appropriate primer pairs for 30 cycles. These PCR products were cloned into pGEM®-T easy vector (Promega, Madison, WI) and sequenced (Macrogen Co. Korea or Northwestern genomic core facility). CDR3 size was defined as the
number of residues between the aligned C (CASS) in the Vβ gene element and the FGXG in the Jβ region minus 4 (25).

**Real-time PCR for Jβ and CDR3 usage.** Primary Vβ6 PCR products from CNS mononuclear cells and splenocytes from infected mice or naïve splenocytes were used to determine their Jβ chain and CDR3 usage. Briefly, 1:500-1000 diluted primary PCR products were re-amplified by real-time PCR in CYBR® green PCR mix (Bio-Rad). Amplification of CAS conserved region to Cβ region or only Cβ region was used as internal control. For Jβ chain analysis, a Vβ6 primer and twelve different Jβ chain primers were used. For CDR3 analysis, degenerate primers for CDR3 motifs corresponding to the same amino acid sequences were used (Table 1).

**Data presentation and statistical analysis.** Data are presented as mean ±SD of either 2-3 independent experiments or one representative result with triplicates from at least three independent experiments. Significance of differences was determined by Student’s t test. p values of < 0.05 were considered as statistically significant.
RESULTS

**V\(\beta\)6-bearing viral antigen-specific CD8\(^+\) T cells are predominant in virus-infected mice.** The majority of CD8\(^+\) T cells in the CNS of TMEV-infected B6 mice are reactive to the predominant VP\(2_{121-130}\) epitope. However, the clonal nature of CD8\(^+\) T cells remains unknown. To analyze the epitope-specific CD8\(^+\) T cells, the proportion of the epitope-specific cells in the CNS and periphery were analyzed at 8 days after TMEV infection using flow cytometry after staining with H-2D\(b\)-VP\(2_{121-130}\) (D\(b\)-VP2) tetramer (Fig. 1A) or intracellular IFN-\(\gamma\) (Fig. 1B). Our D\(b\)-VP2-tetramer staining was specific for VP2-specific CD8\(^+\) T cells: A negative control D\(b\)-LCMV-GP33-41-tetramer did not react with CD8\(^+\) T cells from TMEV-infected mice (Fig 1A) nor did the D\(b\)-VP2-tetramer react with CNS cells from MOG-induced EAE mice (not shown). As shown previously (17, 60), the proportion of virus-specific CD8\(^+\) T cells was 7-10 fold higher in the CNS than in the spleen. Greater than 80% of CNS-infiltrating CD8\(^+\) T cells and greater than 10% of splenic CD8\(^+\) T cells from infected mice reacted to D\(b\)-VP2 tetramers, while approximately one half of the D\(b\)-VP2 tetramer-positive cells produced IFN-\(\gamma\). Therefore, the great majority of CD8\(^+\) T cells observed in the CNS after TMEV infection appear to be virus-specific.

We further assessed the relative proportions of different V\(\beta\)-bearing VP\(2_{121-130}\)-specific CD8\(^+\) T cells in the CNS and spleens of virus infected mice at 8 days post-infection using flow cytometry after staining for V\(\beta\)s, CD8, and D\(b\)-VP2. CNS-infiltrating CD8\(^+\) T cells from three individual mice showed predominant V\(\beta\)6 expression ranging from 23.4 to 41%. Splenic VP2-specific CD8\(^+\) T cells showed a similar predominance of V\(\beta\)6 usage (29 - 49.3%). Interestingly, the percentage of V\(\beta\)6-bearing VP2-specific T cells (36.7% in average) was similar to V\(\beta\)8.1/2-
bearing cells (32.7% in average) in the spleen. However, a significantly reduced level (~2 fold) of Vβ8.1/2-bearing cells (18.9% in average) was found in the CNS in contrast to Vβ6-bearing cells (36.5% in average). These results strongly suggest that VP2-specific CD8⁺ T cells are differentially accumulated in the CNS in a Vβ-associate manner. However, proportions of dominant Vβ6⁺ cells within virus-specific CD8⁺ T cells remain similar in the CNS and periphery of TMEV-infected B6 mice.

**CDR3 repertoires of epitope-specific CD8⁺ T cells in the CNS and periphery are distinct.** Since Vβ6⁺CD8⁺ antigen-specific T cells are predominantly found in the CNS, Vβ6⁺ T cells may be preferentially maintained in the CNS, perhaps in conjunction with local antigen presenting cells (APCs). To test this possibility, the clonal nature of Vβ6⁺CD8⁺ T cells was assessed by Vβ6-CDR3-Jβ spectratyping of isolated tetramer positive cells and IFN-γ producing cells at 8 days post-infection (Fig. 2). As shown in Fig. 2A, the Vβ6 repertoires from tetramer binding cells in the CNS were distinct from those in the spleen. For example, among Vβ6-Jβ1.3, Vβ6-Jβ1.4 and Vβ6-Jβ1.5, the CDR3 size spectra of CNS cells were different from those of splenic cells although Vβ6-Jβ1.1 and -Jβ1.2 were similar. Since similar CDR3 spectra may not reflect identical clonotypes, selective samples with the same length CDR3 regions of epitope-specific CD8⁺ T cells from the CNS and the spleen were further sequenced (Fig. 2A-a & b). For example, seven amino acid (aa) sized bands in the Vβ6-CDR3-Jβ1.1 spectra from the CNS and spleen were composed of different amino acids. In contrast, the Vβ6-CDR3-Jβ1.1 bands from IFN-γ-producing CD8⁺ T cells (Fig. 2B-c & d) appear to selectively utilize Vβ6-MGX-TEVF both in the CNS and spleen. Nevertheless, it appears that the CDR3 repertoire of tetramer-positive CD8⁺ T cells is more heterogeneous than that of IFN-γ-producing cells.
Vβ6-Jβ size determinations using PCR (Fig. 2) may misrepresent the T cell repertoires due to the differential amplification by various Jβ primers. To examine this possibility, primary Vβ6-Cβ PCR products from the above experiments were directly sequenced after cloning into plasmids (Table 2). It is interesting to note that the frequency (38%) of CD8⁺ T cells with Vβ6-MGX-TEVF is significantly higher in the CNS compared to that (13%) in the spleen. In addition, the great majority of T cells reactive to this epitope both in the CNS and spleen appear to utilize Vβ6-XGX-TEVF. Again, the level of VP2-specific tetramer-reactive CD8⁺ T cells with XGX motif was higher in the CNS (81.4%) compared to that in the spleen (60.8%), and this trend was even higher with VP2-specific IFN-γ producing cells (91.1 % in the CNS vs. 76.9% in the spleen). Furthermore, tetramer-positive CD8⁺ T cells from the CNS (74.5 %) of TMEV-infected B6 mice preferentially utilized Jβ1.1 in conjunction with Vβ6, compared to those in the spleen (45.1%). However, only limited clonotypes were shared between CD8⁺ T cells in the CNS and spleen (e.g., CDR3 motifs paired with Jβ1.1 as indicated with asterisks). In contrast, IFN-γ-producing CD8⁺ T cells displayed similarly predominant Vβ6-Jβ1.1 (42%) and Jβ2.6 (42%) usages (Table 2). Interestingly, CDR3 clonotypes (indicated with **) containing MGX paired with Jβ1.1 (CNS, 38% vs. spleen, 27%:) and MGEQY paired with Jβ2.6 (CNS, 25% vs. spleen, 19%) were dominant both in the CNS and spleen. Therefore, the CDR3 region of virus-specific CD8⁺ T cells producing IFN-γ appears to be associated with the MGX CDR3 sequence. Since the usage of these CDR3 sequences by tetramer positive cells is less frequent, IFN-γ-producing CD8⁺ T cells with this motif may represent subpopulations within the tetramer positive cells (Table 2, marked with boxes).
Vβ6-MGX-Jβ1.1 CD8+ T cell clones remain predominant in the CNS during late infection. To examine the potential expansion and/or contraction of TCR repertoire during viral infection, CDR3-Jβ regions of CNS-infiltrating T cells were assessed by spectratyping at 4, 8, 16 and 24 days post-infection (Fig. 3). Since the number and percentage of specific cells in the CNS and spleens are too low at the early stage of virus-infection, isolation of tetramer positive cells was not feasible. Moreover, Vβ6-positive population is the predominant VP2-specific CD8+ T cells (Fig. 1) and Vβ6+CD4+ T cells are only a minor population in the CNS of virus-infected mice (not shown). Therefore, we determined the emerging Vβ6-CDR3 patterns among total Vβ6+ T cell populations in the CNS and spleens during viral infection to represent alterations in the magnitude of TCR CDR3 regions. As early as 4 days post-infection, preferential Jβ1.1 usage by Vβ6-bearing T cells in the CNS was evident when determined by real-time PCR (Fig. 3A). The relative frequencies of other Jβ usages (3 mice each group) were somewhat different from the sequencing results in Table 2 from 20 mice. In order to examine whether or not the inconsistency of other Jβ usages reflects the variation of individual mice, we further examined the Jβ usages in 4 individual mice. While the predominant Jβ1.1 usage was consistent (4/4 mice), the usages of other Jβs were not (Fig. 3B). Therefore, preferential Jβ usage other than Jβ1.1 appears to be somewhat variable depending on individual mice or experiments. The size distribution of Vβ6-CDR3-Jβ1.1 was further analyzed by spectratyping (Fig. 3C). The CDR3 sizes of splenic T cells from naïve mice showed a distribution with the strongest peak at 9 aa (Fig. 3C first row). Interestingly, the predominance of 7 aa CDR3 TCR spectrum, including Vβ6-MGX-Jβ1.1, became apparent in the CNS at 4 days post-infection and continued through the viral infection (Fig. 3C). However, this clonotype peaked in the spleen at 8 days post-infection and waned at 16 days post-infection to levels indistinguishable from that of normal
splenocytes, probably reflecting the low proportion of virus-specific CD8+ T cells, when the viral load was significantly decreased in the CNS (Fig. 3D).

Relative levels of CDR3 regions utilized by IFN-γ-producing VP2121-130-specific cells during viral infection were determined by real-time PCR using degenerate primers encoding deduced Vβ6-CDR3-Cβ amino acid residues containing MGN, MGF, MGE, RV and ITPT (Fig. 4A). The frequency of MGN and MGE CDR3 sequences representing dominant IFN-γ-producing virus-specific CD8+ T cells was significantly higher in the CNS at 4 days post-infection and continued to increase: 6000, 50000, and 8000 fold over the level of infrequently used ITPT sequence at 8, 16, and 24 days, respectively. The dominance of MGN and MGE was consistent in a separate experiment (not shown). These data suggest that VP2121-130-specific CD8+ T cells with CDR3 region sequences of MGN and MGE dominantly accumulated in the CNS. It is interesting to note that the frequency of MGN and MGE sequences is significantly higher (p < 0.01 and p < 0.001 respectively) in naïve splenic T cells than MGF, RV and ITPT sequences (Fig. 4B). These results suggest that the dominant utilization of CDR3 regions with MGN and MGE motifs by virus-specific IFN-γ-producing CD8+ T cells represents the selection from T cells with high precursor frequency in naïve B6 mice.

To further assess the Jβ distribution within MGN and MGE Vβ6-CDR3-Cβ motifs, the corresponding primary PCR products were analyzed with individual Jβ primers (Fig. 4B bottom a & c). Both MGN and MGE motifs were primarily used in conjunction with Jβ1.1 at 8 and 16 days post-infection (Fig. 4B b & d), consistent with the sequencing results (Table 2). However, the use of other Jβs varied depending on individual mice (Fig. 3B) or experiments (not shown). These results indicate that Vβ6-MGX-Jβ1.1 is a stable dominant public repertoire and CD8+ T
cell clones bearing this CDR3 motif are preferentially accumulated in the CNS during viral infection. However, Vβ6-MGX-Jβ2.1/Jβ2.6 may be either semi-public or transient repertoires and thus CD8⁺ T cells bearing these CDR3s vary among different individual mice.

**Epitope-specific CD8⁺ T cells with moderate avidity are maintained during viral infection.** High-affinity/avidity TCR is an important factor for both anti-viral and anti-tumor CD8⁺ T cell functions (1, 2). However, the relationship of functional avidity to TCR diversity of CD8⁺ T cells accumulated in the CNS during viral infection is unknown. We have previously showed that native epitope (WT, VP2₁₂₁-₁₃₀) and altered peptide ligand (APL, M130L: substituted M with L at the position 130 of VP2₁₂₁-₁₃₀) induce similar IFN-γ production at a high concentration (41). However, several fold higher concentrations of M130L peptide are required to induce similar levels of IFN-γ compared to that of WT peptide at low concentrations, suggesting that this APL exhibits a relatively lower functional avidity (unpublished observation and Fig. 5A). To further explore the relationship between functional avidity and TCR diversity, we determined the ability to produce IFN-γ in response to varying concentrations of WT or M130L peptides at two different time points after viral infection. The functional avidity of CD8⁺ T cells against WT peptide appears to be very high (2 x 10⁻⁶ µM), but heterogeneous among the T cells at 8 days post-infection (Fig. 5A). However, the average avidity (8 x 10⁻³ µM) of the T cells was reduced to moderate level (p<0.001) at 16 days post-infection. In contrast, the avidity of CD8⁺ T cells against M130L APL seems to be moderate both at 8 and 16 days (4 x 10⁻³ and 8 x 10⁻³ µM, respectively, p>0.05). The proportions of both WT and M130L-tetramer reactive CD8⁺ T cells increased at 16 days, while the overall numbers of WT and M130L epitope-reactive cells declined. Interestingly, the number of WT epitope-reactive cells decreased more
rapidly than M130L reactive cells (Fig. 5B). These results suggest that virus-specific CD8\(^+\) T cells with moderate functional avidity are preferentially retained in the CNS during the viral infection. Because T cells with a high-affinity TCR are known to undergo activation-induced apoptosis (37), T cells with a moderate avidity may have a survival advantage under continuous viral antigenic stimulations in the CNS.

To exclude the possibility that either a low number or deficiency of APCs in the CNS of virus-infected mice may have affected the measurements of functional avidity of T cells, we further determined the functional CD8\(^+\) T cell avidity with or without additional splenic DCs from naïve B6 mice. If the number and/or function of APCs from the CNS of virus-infected mice were compromised, the deficiencies would be overcome by the presence of excess numbers of APCs. However, the levels of IFN-\(\gamma\) production were similar in the presence or absence of added DCs across different concentrations of the cognate peptide (data not shown). The lack of significant deficiencies in the CNS APCs from virus-infected resistant B6 mice is consistent with our previous observation (20). Therefore, it is very unlikely that the differences in functional avidity presented above in Fig. 5A reflect deficiencies in APC function or numbers.

Since V\(\beta\)6\(^+\)CD8\(^+\) T cells reactive to M130L APL display moderate avidity are preferentially retained in the CNS (Fig. 5A&B), we further compared the binding intensities of WT and M130L-loaded tetramers to V\(\beta\)6 and/or V\(\beta\)8.1/2-bearing CD8\(^+\) T cells from mice at 8 days post-infection (Fig. 5C). Interestingly, similar numbers of V\(\beta\)6\(^+\)CD8\(^+\) T cells displayed high and low-intensity binding to WT-tetramer, whereas greater than 80% of V\(\beta\)6\(^+\)CD8\(^+\) T cells showed low-intensity binding to M130L-tetramer (Fig. 5C left). These differences in the tetramer reactivity of V\(\beta\)6\(^+\)CD8\(^+\) T cells are also apparent by their respective mean fluorescence intensity of tetramer binding. However, such differential reactivity was not observed with subdominant
Vβ8.1/2+CD8+ T cells reactive to these tetramers (Fig. 5C right). The overall tetramer-binding intensity of Vβ8.1/2+CD8+ T cells was much higher than that of Vβ6-bearing cells. The relationship between tetramer-binding intensity and functional avidity is unclear since these are not tightly associated with each other (12, 52). Nevertheless, these tetramer-binding results are consistent with the above functional assessment of avidity via IFN-γ production using epitope peptides (Fig. 5A), supporting that VP2-specific CD8+ T cells from WT virus-infected mice display intermediate avidity towards APLs.

We further compared Vβs utilized by CD8+ T cells reactive to WT and M130L-loaded tetramers at 8 and 16 days post-infection using RT-PCR (Fig. 5D). Vβ6 dominance was observed in CD8+ T cells reactive to M130L at both 8 and 16 days post-infection similar to WT epitope-reactive CD8+ T cells. The nucleotide sequences of CDR3-Jβ1.1 spectratyping bands equivalent to 7 aa residues of WT and M130L reactive cells were further analyzed to examine the potential association of moderate avidity TCR and MGX motif (Fig. 5E). As expected, the majority of sequences showed the MGX motif both in WT and M130L-reactive CD8+ T cells (7/11 and 10/11, respectively) at 16 days post-infection, when T cells with moderate avidity are predominant in the CNS. Taken together, these results strongly suggest that virus-specific CD8+ T cells with moderate avidity display a unique CDR3 motif and are capable of producing IFN-γ, and preferentially retained in the CNS for a prolonged time period.

Efficient induction of moderate avidity Vβ6-MGX-Jβ1.1 requires WT epitope peptide in vivo. It is known that the affinity of viral-peptide/MHC to TCRs could affect the magnitude of antigen-specific CD8+ T cell responses (28, 59). Since the percentage of M130L-reactive cells increased in the CNS during viral infection (Fig 5B), we examine the possibility
that M130L-reactive cells expand more efficiently \textit{in vivo} following infection with M130L-containing virus. At 8 days post-infection, we compared the magnitude of CD8$^+$ T cells and V$\beta$ repertoires by using WT and M130L-loaded tetramers (Fig. 6A). Similar proportions of V$\beta$6$^+$ (35-42%) and V$\beta$8.1/2 CD8$^+$ T cells (10-20%) from both WT and M130L virus-infected mice recognized WT and M130L tetramers. These data strongly suggest that virus expressing WT ligand most efficiently induces CD8$^+$ T cells reactive not only to WT but also to M130L epitopes.

Despite the induction of a predominant V$\beta$6$^+$ VP2-reactive CD8$^+$ T cell response by the mutant viruses, it is conceivable that these APL-bearing viruses may preferentially induce V$\beta$6$^+$CD8$^+$ T cells with different CDR3 motifs. To examine this possibility, relative levels of MGX CDR3 in mice infected with WT and M130L viruses were assessed at 8 days post-infection using real-time PCR (Fig. 6B). To our surprise, M130L virus infection failed to induce a vigorous response of CNS-infiltrating T cells with MGN and MGE CDR3 (<10-fold) when compared to the WT virus. However, T cells induced by M130L virus maintained a similar hierarchy of V$\beta$6 TCR with the CDR3 motifs. These results strongly suggest that moderate avidity T cells bearing the MGX motif (Fig. 1, Table 2, Fig. 4 and Fig. 5) expand more efficiently in the CNS in response to WT virus expressing unmodified ligand. Thus, the failure of APL-bearing virus to induce vigorous V$\beta$6$^+$ VP2-reactive CD8$^+$ T cells may be attributable to the inefficient induction of T cells bearing the predominant MGX motif.

\textbf{Expansion of CD8$^+$ T cells with MGX CDR3 correlates with high precursor frequency and MHC haplotype.} We speculated that the V$\beta$6 dominance of VP2-specific CD8$^+$ T cells reflects the high precursor frequencies of V$\beta$6-bearing VP2-specific T cells in naïve
mice. To examine this possibility, we further determined the precursor frequency of Vβ6+CD8+ T cells with the MGX CDR3 sequences in naïve mice to understand the potential mechanisms of high frequency of VP2-reactive CD8+ T cells bearing this motif (Fig. 7A). Relative frequencies of the dominant Vβ6-associated MGX motif (MGN, MGF and MGE) and two rare motifs (RV and ITPT) in splenic T cells from naïve B6 (H-2b), SJL (H-2s) and B6.S (H-2s under the B6 background) mice were assessed using real-time PCR (Fig. 7A). Surprisingly, levels of MGX expression were similarly high in all these naïve mice, in contrast to low to undetectable levels of rare motifs. These results suggest that the precursor frequency of T cells with this motif is intrinsically high in mice regardless of their MHC haplotypes. This observation is consistent with the very recent report indicating that T cell repertoire diversity and response magnitude are associated with the precursor frequency of naïve antigen-specific T cells (39).

Since the level of MGX expression is similarly high in mice with different MHC haplotypes, TMEV infection may cause non-specific expansion of T cells with MGX motif due to the presence of an unknown viral superantigen rather than VP2-epitope. In order to discern whether or not the expansion of Vβ6 MGX repertoire is epitope-specific MHC-dependent process, B6 and SJL mice were infected with TMEV and their CNS-infiltrating T cells were analyzed at 8 days post-infection (Fig. 7B). We reasoned that SJL mice expressing a limited Vβ repertoire (one-half number of B6 Vβs) would better utilize Vβ6, if Vβ6 MGX dominance were not specific for VP2/H-2Db stimulation. Despite the high precursor frequency of the MGX motif both in naïve B6 and SJL mice, T cells with the MGX motif (particularly, Vβ6-MGN and Vβ6-MGE) were expanded only in B6 mice bearing H-2b after virus infection (Fig. 7B). Interestingly, the expansion pattern was very similar between H-2b-bearing B6 and 129 mice (Fig. 7C). In particular, the preferential Jβ1.1 usage was a mirror image between these mice although the
overall magnitude was higher in 129 mice. These results indicate that the expansion of CD8+ T cells with the Vβ6-MGX CDR3 region and Jβ1.1 restriction is both viral epitope and H-2b haplotype dependent.

DISCUSSION

In this study, we have analyzed the clonal nature of virus-specific CD8+ T cells accumulating in the CNS of resistant B6 mice during infection with neurotropic Theiler’s virus. Our results indicate that Vβ6+CD8+ T cells are the predominant virus-specific population in the CNS. Similar preferential expansions of Vβ-restricted CD8+ T cells were reported following infection with many other viruses (8, 11, 29, 46, 48). Interestingly, the skewed Vβs are different depending on the viruses, suggesting that the preferential expansion of CD8+ T cells reflects epitope-dependent responses. Alternatively, skewed expansion of Vβ6+ T cells after infection with TMEV may reflect the involvement of a superantigen (5, 19). However, this is unlikely since the preferential expansion of CD8+ T cells is dependent on the presence of a particular CD8+ T cell epitope. For example, B6-P1 (virus capsid antigens) transgenic mice, which are tolerant to the CD8+ T cell epitope (42), displayed negligible levels of CD8+ T cells including Vβ6+ populations in the CNS after TMEV infection (unpublished data). Furthermore, the precursor frequency of Vβ6-MGX CDR3 regions that are specific for the epitope is high in naïve mice regardless of MHC haplotype, but these motif bearing cells expanded only in response to the epitope in conjunction with H-2b (Fig. 7B). Therefore, the preferential use of Vβ6 TCR may reflect the combination of high precursor frequency of T cells with the Vβ-associated CDR3 sequences and utilization of the CDR3 region by the epitope-specific CD8+ T cells. Interestingly,
however, preferential Vβ6 usage was largely maintained after infection with APL-bearing virus, suggesting that such skewing is due to the collective property of epitope rather than a particular residue within the epitope (Fig. 6).

We have observed that CD8+ T cells accumulated in the CNS of virus infected resistant B6 mice displayed a skewed Vβ6 usage with distinct CDR3 sequences compared to CD8+ T cells in the spleen (Fig. 1 & 2). Our results differ from those found in previous studies of influenza virus or LCMV infection indicating that T cell Vβ repertoires are similar in systemic and local sites (11, 29). However, CD8+ T cells induced in monkeys following SIV infection utilize heterogeneous Vβs with some degree of predominance in the brain (34). Similarly, infection with neurotropic MHV virus induced epitope-specific CD8+ T cells with a preferential Vβ in the CNS (46). Thus, virus-specific CD8+ T cells with restricted Vβs may represent unique property of the CNS compartment. Alternatively, differences in the precursor frequencies of CD8+ T cells specific for different viruses may results in different patterns of Vβ skewing. Furthermore, it is conceivable that increases in the proportion of VP2-specific CD8+ T cells in the CNS (Fig. 1) may reflect infiltration and perhaps local amplification of virus-specific CD8+ T cells at the site of viral reservoir for neurotropic TMEV.

CD8+ T cells producing IFN-γ have commonly been utilized to enumerate the number of antigen-specific CD8+ T cells, because the numbers of IFN-γ-producing cells and tetramer-positive populations are similar as shown in mice infected with influenza virus and LCMV (15, 40). However, recent studies with HIV infection have demonstrated that there are distinct CD8+ T cell populations producing IFN-γ and/or granzyme B within the tetramer-positive CD8+ T cell population (18, 27). No attempts have previously been made to elucidate whether these
functionally distinct populations represent different T cell clonotypes or reflect different differentiation stages of the same T cell population. Our current study strongly suggests that IFN-γ-producing epitope-specific CD8+ T cell subpopulation displays distinct TCR repertoires; hence these cells express a unique Vβ6-CDR3 motif within the VP2\textsubscript{121-130} tetramer-positive CD8+ T cells (Fig. 2 & Table 2). To the best of our knowledge, this is the first example demonstrating that there are preferred TCR clonotypes of CD8+ T cells producing IFN-γ. Although the underlying mechanism of restricted TCR clonotypes associated with IFN-γ production is unknown, CD8+ T cells with certain TCR-dependent functional avidity may be able to preferentially trigger IFN-γ production or induce the differentiation of particular type of CD8+ T cells, similar to that of CD4+ T cell responses (7, 55, 56).

The avidity of TMEV epitope-specific CD8+ T cells in the CNS during the peak immune responses appears to be relatively high, whereas the avidity during subacute phase following the peak responses is intermediate (Fig. 5). Since the expansion of CD8+ T cells with a moderate avidity towards the ligand correlates with an increase in T cell numbers with a particular TCR CDR3 motif during subacute phase of TMEV infection (Fig. 4), a subpopulation of T cells with restricted TCRs may be preferentially accumulated/retained in the CNS during late infection. Alternatively, prolonged antigenic stimulation during subacute phase may lead to functional exhaustion of CD8+ T cells by upregulating expression of inhibitory receptors. However, stimulation of T cells isolated from the CNS during late infection with cognate epitope peptide, anti-CD3/CD28 antibodies or PMA/inomycine (by passing TCR signal cascade) resulted in similar levels of IFN-γ-producing CD8+ T cells. Furthermore, differences in the expression of neither PD-1 nor TIM3, which are known to be involved in CD8+ T cell desensitization, were detected during the course of viral infection (data not shown). Therefore, CD8+ T cells with high
and moderate avidity may be initially selected from the mixed TCR pool but T cells with moderate avidity are preferentially retained during the later phase in the presence of CD4⁺ T cell help (26).

It is interesting to note that the dominance of CD8⁺ T cells with intermediate avidity in our study highly contrasts with the previous observation that the functional avidity of CD8⁺ T cells specific for LCMV is drastically increased in the periphery during the course of viral infection (52). In addition, it has recently shown that high affinity CD8⁺ T cells more likely expand and survive as memory cells rather than very low affinity cells, although very low affinity T cells specific for microbes also manage similar initial activation and proliferation (59). However, it is conceivable that CD8⁺ T cells with very high avidity with efficient cytotoxic function may undergo apoptosis and be removed from the CNS in order to void potential pathogenic outcome. Furthermore, a recent report suggests that lower TCR avidity resulting in shorter contact time is more beneficial in attacking multiple target cells compared to CD8⁺ T cells with higher avidity (21). Therefore, T cells with moderate avidity may be able to efficiently control persistent virus without pathogenic function.

Our results indicate that intermediate avidity CD8⁺ T cells specific for TMEV preferentially expand in the CNS of virus-infected mice (Fig. 5). However, it is uncertain at this time whether intermediate avidity cells can be efficiently converted into memory CD8⁺ T cells in the CNS. Therefore, it would be interesting to compare the TCR repertoire of memory CD8⁺ T cells in the CNS and periphery to correlate them with the avidity differences in future studies. In addition, susceptible mice fail to induce strong initial CD8⁺ T cell responses to efficiently clear viral loads from the CNS in contrast to resistant mice. Further studies on differences of CD8⁺ T cells between resistant B6 and susceptible SJL mice may also help to understand the nature of
anti-viral CD8+ T cell responses in the CNS, the site of chronic viral infection. These studies may ultimately provide the means to intervene persistent viral infections and the pathogenesis of virally induced CNS inflammatory diseases.
Acknowledgements

We would like to thank Ben Haley, Stacy Ryu, and Heeyoung Yang for their help in preparing this manuscript. This work was supported by grants from the National Institutes of Health (RO1 NS28752 and RO1 NS33008) and the National Multiple Sclerosis Society (RG 4001-A6).

Disclosures

The authors have no financial conflict of interest.
REFERENCES


41. Myoung, J., W. Hou, B. Kang, M. A. Lyman, J. A. Kang, and B. S. Kim. 2007. The immunodominant CD8+ T cell epitope region of Theiler's virus in resistant C57BL/6 mice is critical for anti-viral immune responses, viral persistence, and binding to the host cells. Virology 360:159-71.


FIGURE LEGENDS

FIG. 1. Assessment of Vβ usages by antigen-specific CNS-infiltrating and peripheral T cells. Detection of Db-VP2_{121-130} vs. Db-LCMV-GP33 (negative control) tetramer positive CD8⁺ T cells (A) and IFN-γ-producing CD8⁺ T cells (B) after stimulation with PBS or VP2_{121-130} peptide in the CNS and spleen. (C) Proportion of CD8⁺ T cells bearing different Vβs and reactive to VP2-tetramer in the CNS and spleen. Plots represent gated CD8⁺ T cells. Antibodies to Vβs were selected based on RT-PCR analyses. Three mice were individually analyzed. Data are a representative of three independent experiments. All experiments were done at 8 days post TMEV infection.

FIG. 2. Disparity of virus-specific CD8⁺ T cell repertoire between the CNS and periphery. (A) Vβ6-CDR3-Jβ size spectra of isolated tetramer positive cells from 12 mice. To assess the homogeneity of the identically sized CDR3s, bands (a & b), indicated with arrows, were eluted, re-amplified by PCR, and cloned for sequencing. (B) Vβ6-CDR3-Jβ size spectra of isolated IFN-γ producers. Bands (c & d), indicated with arrows, were processed as in (A). The spectratype figures are representative results of two separate experiments. Amino acid sequences were deduced from nucleotide sequences. C: CNS; S: Spleen. TEVF sequences in CDR3β loop are part of Jβ1.1 chain.

FIG. 3. Expansion of virus-specific CD8⁺ T cells with selective TCR repertoire. (A) Increase of Vβ6-Jβ1.1 mRNAs during the course of viral infection. cDNAs were prepared from splenocytes
from naïve mice and CNS-mononuclear cells from infected mice (3 mice per group) at 4, 8, 16 and 24 days post-infection. Relative Jβ chain usage was quantitatively measured from primary Vβ6 PCR products using real-time PCR. (B) The dominant use of Jβs other than Jβ1.1 by VP2\textsubscript{121-130}-specific Vβ6\textsuperscript{+} cells varies among individual mice. CNS infiltrating cells were obtained at 8 days post-infection from 4 individual mice and primary Vβ6-Cβ PCR product was similarly used to measure relative levels of Jβ chains by real-time PCR. (C) Vβ6-Jβ1.1 CDR3 size distribution in the CNS and spleen during the course of viral infection. Spectratyping was carried out using \textsuperscript{32}P-labeled Vβ6 primer as described in Materials and Methods. (D) Viral loads during the immune responses. Relative viral loads were determined by real-time PCR using cDNAs prepared from CNS cells at 4, 8, 16, and 24 days post-infection.

**FIG. 4.** Tracing clonotypic expansion of specific CDR3 repertoire in the CNS during viral infection (3 mice per group). (A) Diagrammatic presentation of tracing strategy of CDR3 clonotypes. (B) Expression of clonotype-specific CDR3s during viral infection. Relative abundance of CDR3 sequences was measured by real-time PCR using degenerate primers for MGN, MGF, MGE, RV and ITPT CDR3 aa sequences. The CDR3 clonotypes of naïve mice represent splenic T cells from uninfected mice because there is no significant T cell infiltration in the CNS of uninfected mice. Jβ restriction for the CDR3 sequences was further assessed by real-time PCR using indicated Jβ primers. Arrows indicate sources of the PCR templates. * and ** indicate $p < 0.01$ and $p < 0.001$ respectively compared with other CDR3 sequences and/or Jβ chains. Additional experimental results using mice infected with TMEV (3 mice per group) at 8 and 16 days post-infection indicate that the predominant use of Vβ6-MGX-Jβ1.1 is consistent between experiments. In contrast, the use of other CDR3 sequences were somewhat variable.
**FIG. 5.** Accumulation of CD8⁺ T cells with a moderate avidity in the CNS during viral infection.

(A) Functional avidity of CNS-ILs was measured by intracellular IFN-γ staining; CNS-ILs obtained at 8 and 16 days of post-infection were stimulated by ten-fold serial dilutions of wide type (WT) VP2₁₂₁₋₁₃₀ or mutant M130L peptides starting from 1 μM concentration. Functional avidity was based on the concentration of peptides required to attain 50% of the maximal IFN-γ production by CD8⁺ T cells. The maximum % of IFN-γ producing cells is set to 100%. * and ** are p < 0.001 and p > 0.01 respectively. (B) Proportions (top) and numbers (bottom) of tetramer positive (left) and IFN-γ producing (right) cells against WT and M130L peptides during TMEV infection. (A and B) are representative of three individual experiments. Pooled CNS cells from 15 mice were used for these analyses. (C) Comparison of WT and M130L tetramer-binding intensities of Vβ6 and Vβ8.1/2-bearing CD8⁺ T cells. Gated on CD8⁺ and tetramer-positive cells from the CNS of mice at 8 days post-infection were analyzed for their mean fluorescence intensity (MFI). (D) Assessment of Vβs utilized by WT (VP2₁₂₁₋₁₃₀) and M130L-reactive CD8⁺ T cells by RT-PCR. cDNAs were prepared from the CNS cells with each tetramer at 8 and 16 days post-infection. (E) Sequence analyses of 7 aa CDR3 regions within Vβ6-Jβ1.1. Vβ6-Jβ1.1 PCR products from cells isolated using WT and M130L-tetramers at 16 days post-infection were eluted from spectratyping gel, re-amplified by PCR, and then cloned followed by sequencing.

**FIG. 6.** Efficient expansion of Vβ6-MGX bearing moderate avidity cells in response to WT virus compared with APL-bearing viruses. (A) Percentage of Vβ6 and Vβ8.1/2-bearing cells among WT and M130L tetramer-reactive CD8⁺ T cells. CNS-CD8⁺ T cells from 3 mice infected with WT or M130L virus were stained with WT (VP2₁₂₁₋₁₃₀) or M130L peptide-loaded tetramers.
in combinations with anti-CD8 and anti-Vβ6 or Vβ8.1/2 antibodies. Tetramer and CD8 positive
gates were used to calculate the percentage of Vβ-bearing cells. (B) Measurements of Vβ6-
CDR3 mRNAs in the CNS after infection with WT and epitope-variant viruses using real-time
PCR. After normalization, based on their Cβ mRNA levels, the lowest value was set as one fold
expression.

**FIG. 7.** CD8⁺ T cell expansion level correlates with naïve T cell frequency and MHC haplotype.
(A) Relative frequency of Vβ6-MGX CDR3 expressed by splenic T cells from naïve mice. The
frequency of CDR3 regions was determined by real-time PCR using degenerate primers. (B)
Expansion levels of Vβ6-MGX CDR3 in the CNS of B6 (H-2ᵇ) and SJL (H-2ˢ) mice (3
mice/group) at 8 days post TMEV infection. (C) Vβ6-Jβ usage by T cells in the CNS of B6 and
129 (H-2ᵇ) mice (3 mice/group) at 8 days post-infection.
TABLE 1. Primer sequences used in Vβ RT-PCR, spectratyping and CDR3 real-time PCR

<table>
<thead>
<tr>
<th>Primer name (Forward)</th>
<th>Sequence</th>
<th>Primer name (Reverse)</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vβ1</td>
<td>CCCAGACAGCTCCAAAGCTACTTT</td>
<td>ACB4 (distal Cβ)</td>
<td>GACAGGTGTGGTGCCCTCTGCGCA</td>
</tr>
<tr>
<td>Vβ2</td>
<td>CCACAGGGGTGCTACGTACGGAGC</td>
<td>MCB1-1 (proximal Cβ)</td>
<td>ACCAAGGACCTCTGGGAGT</td>
</tr>
<tr>
<td>Vβ3</td>
<td>CCTCCTAACCTACCTGCAGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vβ4</td>
<td>TCAAAAGAAAACCATTTAGAC</td>
<td>JB1.1</td>
<td>ACTGTCGGTCTGTTCTCTTTCC</td>
</tr>
<tr>
<td>Vβ5</td>
<td>TCTGGGGTGTCCGACGTCTCCA</td>
<td>JB1.2</td>
<td>AAAAGCCTGGCTCCCTGAGCCGAAG</td>
</tr>
<tr>
<td>Vβ5.1</td>
<td>CATTATGGATAAATGGAGAGAGAT</td>
<td>JB1.3</td>
<td>CTCTCTTCTCCAAAATAGAGC</td>
</tr>
<tr>
<td>Vβ5.2</td>
<td>AAGGTGGAAGGAGAAAGGATGTC</td>
<td>JB1.4</td>
<td>GACAGGTGTGGTTCCATAGCCG</td>
</tr>
<tr>
<td>Vβ6</td>
<td>GAGAAGAAGTCATCTTTTCTTCT</td>
<td>JB1.5</td>
<td>GAGTCCCCCTCTCCAAAAGCCG</td>
</tr>
<tr>
<td>Vβ7</td>
<td>AAGAAGGCGGGGAGCTTTCC</td>
<td>JB1.6</td>
<td>TCACAGTGAGCCGGGTTGCTTCG</td>
</tr>
<tr>
<td>Vβ8</td>
<td>GGAGGCTCGACGTACCCAAAG</td>
<td>JB2.1</td>
<td>GTGAGTCGTTGTTCTTGTCGCCAG</td>
</tr>
<tr>
<td>Vβ8.1/2</td>
<td>AGCCAAAGAACTTCTGCTCATT</td>
<td>JB2.2</td>
<td>CAGCAGCTGTGAGCCTTTTGC</td>
</tr>
<tr>
<td>Vβ9</td>
<td>CCTAACAAATTCATTCTTCTGA</td>
<td>JB2.3</td>
<td>GTTCCCGAGCCAAAATACAGC</td>
</tr>
<tr>
<td>Vβ10</td>
<td>TCAGATAAAGCTCATTTGGAAT</td>
<td>JB2.4</td>
<td>GTGCCGCGACCAAAATACAG</td>
</tr>
<tr>
<td>Vβ11</td>
<td>CCCCAATCAATCGACACTCAAC</td>
<td>JB2.5</td>
<td>GTGCCGCGACCAAAATACAGG</td>
</tr>
<tr>
<td>Vβ12</td>
<td>CTATGGAAAGATGGTGGGGCT</td>
<td>JB2.6</td>
<td>CTAAAAAGCTGGAGCGCTGTGC</td>
</tr>
<tr>
<td>Vβ13</td>
<td>CCTAAAAGGAACAATCTCCCTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vβ14</td>
<td>TCAGGTGTGGCCACCTACGCACT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vβ15</td>
<td>CCCAATTATCTATTTTCCTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vβ16</td>
<td>CCAGATGTGTCATATTTTCAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vβ17</td>
<td>AGTGTCCCGAACTCAGAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vβ18</td>
<td>CAGCCCGGCAACCAATCTAAATTCTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vβ19</td>
<td>CTGCTGACAAACATGACCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vβ20</td>
<td>TCTGAGCGGCTGGAATC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CASS-MGN</td>
<td>CTCTGTCGACAGCTGTTATGGINANAY*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CASS-MGF</td>
<td>CTCTGTCGACAGCTGTTATGGINNTY*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CASS-MGE</td>
<td>CTCTGTCGACAGCTGTTATGGINNAR*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CASS-RV</td>
<td>CTCTGTCGACAGCTGTTATGGINNRT*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CASS-ITPT</td>
<td>CTCTGTCGACAGCTGTTATGGINNACN*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAS (IR)**</td>
<td>GAGCGGTTTTTTCTGAGCCAGC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Degenerate CDR3 regions are underlined; ** IR represents internal reference
## Table 2. Analysis of V\(\beta\)6 CDR3 region from tetramer binders and IFN-\(\gamma\) producers

### Tetramer Binders

<table>
<thead>
<tr>
<th>Freq.</th>
<th>V(\beta)6</th>
<th>CDR3-J(\beta)</th>
<th>FGXG</th>
<th>J(\beta)</th>
<th>SIZE</th>
</tr>
</thead>
<tbody>
<tr>
<td>4/59</td>
<td>CASS</td>
<td>MGTEVF</td>
<td>FGKG</td>
<td>J(\beta)1.1</td>
<td>7</td>
</tr>
<tr>
<td>3/59</td>
<td>CASS</td>
<td>MGTEVF</td>
<td>FGKG</td>
<td>J(\beta)1.1</td>
<td>7</td>
</tr>
<tr>
<td>2/59</td>
<td>CASS</td>
<td>MGTEVF</td>
<td>FGKG</td>
<td>J(\beta)1.1</td>
<td>7</td>
</tr>
<tr>
<td>* 2/59</td>
<td>CASS</td>
<td>MNGTEVF</td>
<td>FGKG</td>
<td>J(\beta)1.1</td>
<td>7</td>
</tr>
<tr>
<td>1/59</td>
<td>CASS</td>
<td>MGEQY</td>
<td>FGPG</td>
<td>J(\beta)2.6</td>
<td>5</td>
</tr>
</tbody>
</table>

* 8/59 CASS RGFTEVF FGKG J\(\beta\)1.1 7  
* 2/59 CASS LGFTEVF FGKG J\(\beta\)1.1 7  
* 1/59 CASS AGNTVEF FGKG J\(\beta\)1.1 7  
* 2/59 CASS GGFTEVF FGKG J\(\beta\)1.1 7  
* 1/59 CASS SGYTEVF FGKG J\(\beta\)1.1 7  
* 1/59 CASS LGSTEVF FGKG J\(\beta\)1.1 7  
* 1/59 CASS PGNTVEF FGKG J\(\beta\)1.1 7  
* 1/59 CASS QGNTVEF FGKG J\(\beta\)1.1 7  
* 1/59 CASS RGSTEVF FGKG J\(\beta\)1.1 7  
* 1/59 CASS WGNTEVF FGKG J\(\beta\)1.1 7  
* 1/59 CASS PGGDYEQF FGPG J\(\beta\)2.1 9  
* 1/59 CASS GQQLY FGEG J\(\beta\)2.2 5  
1/59 CASS QGQVF FGKG J\(\beta\)1.1 5  
1/59 CASS TGPEVF FGKG J\(\beta\)1.1 6  
1/59 CASS IGGSTINTEVF FGKG J\(\beta\)1.1 10  
1/59 CASS TQVNGAPL FGEG J\(\beta\)1.3 8  
* 2/59 CASS MSGYEF FGKG J\(\beta\)2.6 5  
1/59 CASS GGSATLY FGSG J\(\beta\)2.8 6  
1/59 CASS IGGAPDTQY FGPG J\(\beta\)2.5 9  
1/59 CASS SGEQY FGPG J\(\beta\)2.6 6  
1/59 CASS IGGGYEQY FGPG J\(\beta\)2.6 9  
* 3/59 CASS RVEVP FGKG J\(\beta\)1.1 5  
* 2/59 CASS SYEQY FGPG J\(\beta\)2.6 5  
1/59 CASS SAEFV FGKG J\(\beta\)1.1 5  
1/59 CASS TERRGVF FGKG J\(\beta\)1.1 7  
1/59 CASS NQAEQY FGFG J\(\beta\)2.3 7  
1/59 CASS ITGGAPONTLY FGSG J\(\beta\)2.4 11  
1/59 CASS SYEQY FGPG J\(\beta\)2.6 5  
1/59 CASS GYEQY FGPG J\(\beta\)2.6 5

### IFN-\(\gamma\) producers

<table>
<thead>
<tr>
<th>Freq.</th>
<th>V(\beta)6</th>
<th>CDR3-J(\beta)</th>
<th>FGXG</th>
<th>J(\beta)</th>
<th>SIZE</th>
</tr>
</thead>
<tbody>
<tr>
<td>**8/24 CASS</td>
<td>MGTEVF</td>
<td>FGKG</td>
<td>J(\beta)1.1</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>**2/24 CASS</td>
<td>MGTEVF</td>
<td>FGKG</td>
<td>J(\beta)1.1</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>1/24 CASS</td>
<td>MGSSAXLY</td>
<td>FGSG</td>
<td>2.3</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>**8/24 CASS</td>
<td>MGEQY</td>
<td>FGPG</td>
<td>2.6</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>

**1/24 CASS GGYTEVF FGKG J\(\beta\)1.1 7  
1/24 CASS PQGQVQMTLY FGEG J\(\beta\)1.3 10  
1/24 CASS WGGAEQF FGPG J\(\beta\)2.1 7  
1/24 CASS SGGNAYEQF FGPH J\(\beta\)2.1 9  
2/24 CATT QYEQY FGPG J\(\beta\)2.6 5  
1/24 CASS DGYEQY FGPG J\(\beta\)2.6 6  
1/24 CASS ITGGGAYEQF FGPG J\(\beta\)2.6 11  
1/24 CASS DGGYEQY FGPG J\(\beta\)2.6 5

- All CDR3 amino acid sequences are deduced from DNA sequencing analysis from T-vector cloned V\(\beta\)6 PCR products.
- CDR3 sized was counted between CASS and FGXG conserved motifs.
- Horizontal boxes are MGX motif containing clones.
- J\(\beta\)1.1 and J\(\beta\)2.6 are indicated with small boxes.
- * and ** marks represent tetramer positive and IFN-\(\gamma\) producing cells with the same CDR3 motif respectively.
A. Tetramer isolated Vβ6

<table>
<thead>
<tr>
<th>Vβ6</th>
<th>CDR3</th>
<th>Jβ1.1</th>
<th>Freq.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CASS</td>
<td>MGN</td>
<td>TEVFFGKG</td>
<td>2/34</td>
</tr>
<tr>
<td>CASS</td>
<td>MGG</td>
<td>TEVFFGKG</td>
<td>4/34</td>
</tr>
<tr>
<td>CASS</td>
<td>MGY</td>
<td>TEVFFGKG</td>
<td>3/34</td>
</tr>
<tr>
<td>CASS</td>
<td>MGF</td>
<td>TEVFFGKG</td>
<td>2/34</td>
</tr>
<tr>
<td>CASS</td>
<td>MGH</td>
<td>TEVFFGKG</td>
<td>1/34</td>
</tr>
<tr>
<td>CASS</td>
<td>MGQ</td>
<td>TEVFFGKG</td>
<td>1/34</td>
</tr>
</tbody>
</table>

B. IFN-γ Producer isolated Vβ6

<table>
<thead>
<tr>
<th>Vβ6</th>
<th>CDR3</th>
<th>Jβ1.1</th>
<th>Freq.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CASS</td>
<td>MGN</td>
<td>TEVFFGKG</td>
<td>2/4</td>
</tr>
<tr>
<td>CASS</td>
<td>MGF</td>
<td>TEVFFGKG</td>
<td>1/4</td>
</tr>
<tr>
<td>CASS</td>
<td>MGQ</td>
<td>TEVFFGKG</td>
<td>1/4</td>
</tr>
</tbody>
</table>
A

SPLEEN

Naive

4 dpi

8 dpi

16 dpi

24 dpi

Fold Expression

1 2 3 4 5 6
Jp1 Jp2

B

Mouse #1

Mouse #2

Mouse #3

Mouse #4

Fold Expression

1 2 3 4 5 6
Jp1 Jp2

C

SPLEEN

N/A

CNS

Naive

D

Viral RNA

Days post infection

4 8 16 24

9 7 7 5

CDR3 SIZE