Murine \(\gamma\)-herpesvirus infection causes \(V_\beta^4\)-specific CDR3-restricted clonal expansions within CD8\(^+\) peripheral blood T lymphocytes

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Abstract

Infection of mice with the \(\gamma\)-herpesvirus MHV-68 results in lytic infection in the lung cleared by CD8\(^+\) cells and establishment of lifelong latency. An Epstein–Barr virus (EBV)-like infectious mononucleosis (IM) syndrome emerges \(\sim\)3 weeks after infection. In human IM, the majority of T cells in the peripheral blood are monoclonal or oligoclonal and are frequently specific for lytic or latent viral epitopes. However, a unique feature of MHV-68-induced IM is a prominent MHC haplotype-independent expansion of CD8\(^+\) T cells, the majority of which utilize \(V_\beta^4\) chains in their \(\alpha\beta\)TCR. The ligand driving the \(V_\beta^4\) expansion is unknown, but the \(\beta\) bias and MHC haplotype independence raised the possibility that these cells were responding to a virally encoded or a virally induced endogenous superantigen (sAg). The aim of this study was to determine whether this rapidly proliferating subset is composed of polyclonally or clonally expanded T cells. Complementarity-determining region (CDR)-3 size analysis of \(V_\beta^4\) CD8\(^+\) cells in infected mice demonstrated CDR3-restricted expansions in the \(V_\beta^4\) family as a whole. More refined analysis demonstrated major distortions in every \(J_\beta\) subfamily. V–D–J junctional region sequencing indicated that these CDR3 size-restricted expansions were composed of clonal or oligoclonal populations. The sequences were largely unique in individual mice, although evidence for ‘public’ or highly conserved T cell expansions was also seen between different mice. Taken together with previous studies showing an apparent MHC independence, the data suggest that a novel ligand, distinct from conventional sAg and peptide–MHC, drives proliferation of \(V_\beta^4\) CD8\(^+\) T cells.

Introduction

Murine \(\gamma\)-herpesvirus-68 (MHV-68), a \(\gamma\)2-herpesvirus, shares biological features and sequence homology with human herpesvirus 8 and Epstein–Barr virus (EBV) (1,2). Intranasally infected mice develop an acute respiratory illness that is rapidly resolved by CD8\(^+\) T cell-mediated clearance of replicating virus in the lung (3,4). Latent infection, which persists for the life of the animal, is subsequently established in B cells, epithelial cells and macrophages (5–9). Associated with the establishment of latency is an EBV-like infectious mononucleosis (IM) phase, characterized by splenomegaly and the presence of activated CD8\(^+\) T cells in the peripheral blood (6,10). A feature of MHV-68-associated IM that distinguishes it from EBV-associated IM is the pronounced, selective expansion of CD8\(^+\) T cells utilizing \(V_\beta^4\) chains in their \(\alpha\beta\)TCR
An extensive analysis of ~18 strains of MHV-68-infected amplified during the IM stage of MHV-68 infection using a PCR- and sequencing-based strategy (reviewed in 16). Our results clearly demonstrate the presence of multiple clonal and oligoclonal expansions within the Vβ4+CD8+ population. These expansions dramatically distort the Vβ4+ T cell repertoire and suggest an important role for the CDR3 region of the αβ TCR in the Vβ4+CD8+ expansion associated with the IM-like phase of MHV-68 infection.

Methods

Mice

Female C57BL/6 mice, 8–12 weeks of age, were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were housed under specific pathogen-free conditions until MHV-68 infection and in BL3 containment after infection. All animal procedures in these experiments were approved by the Institutional Animal Care and Use Committee at St Jude Children’s Research Hospital, Memphis, TN.

Virus stocks and plaque assay

The original stock of MHV-68 (clone G2.4) was obtained from Professor A. A. Nash, University of Edinburgh, Edinburgh, UK. Virus was grown in owl monkey kidney cells (ATCC, Rockville, MD; 1566 CRL), and titered by plaque assay on NIH 3T3 cells (ATCC; CRL 1566), as previously described (4).

Infection of mice and tissue sampling

Mice were anesthetized via i.p. injection of ~300 µl avertin (2% 2,2,2-tribromoethanol and 2% t-amyl-alcohol), then infected intranasally with 400 p.f.u. of MHV-68 in 30 µl HBSS. Mice to be sacrificed were given 400 µl avertin i.p. and ~100 U heparin sodium (1000 USP U/ml; Fujisawa, Healthcare Inc, Deerfield, IL). Blood (750–1000 µl per mouse) was collected from an incision in the left ventricle, and transferred to a 15 ml tube containing 10 ml of HBSS and heparin (0.6 USP U/ml; Sigma, St Louis, MO; cat. no. 210-6) and stored on ice until processing. Blood was collected from MHV-68-infected mice 1 month post-infection.

Vα repertoire analysis

The TCR Vα repertoire was assessed using mAb to Vα2 (B20.1), Vα3.2 (RR3-16), Vα8 (B21.14) or Vα11.1, 11.2 (RR8-1). Antibodies were either biotinylated and used in association with streptavidin–phycoerythrin (PE) (Biosource, Camarillo, CA) or PE conjugated. Anti-Vα antibodies were either generated from concentrated culture supernatant or purchased (PharMingen, San Diego, CA), and used in a three-color staining protocol with Vβ4+FITC (KT4) (PharMingen) and CD8–TriColor (CT-CD8a; Caltag, Burlingame, CA). Cells were stained in 96-well round-bottom plates, and 100,000 events were acquired on a FACScan and analyzed using CellQuest software (Becton Dickinson, San Jose, CA).

In vivo CD4 depletion of mice

GK1.5 anti-CD4 antibody was grown in a mAb production module (Technne, Princeton, NJ). Mice were injected i.p. with 0.5 ml of GK1.5 antibody for 7 days every second day. Depletion efficacy was monitored by staining peripheral blood...
lymphocytes (PBL) with CD4-FITC clone RM4-4, which is not blocked by GK1.5, V_{µ}4-PE (PharMingen) and CD8-TriColor (Caltag). Depletion of CD4 cells was ~95%. The percentage of CD8{sup+} T cells in the peripheral blood of CD4-depleted mice was 5.9 ± 0.5% for naive mice and 17.7 ± 4.5% for MHV-68-infected mice.

**Extraction of RNA and preparation of cDNA**

RNA was extracted from CD4-depleted PBL (>10{sup6} lymphocytes per mouse) using the Qiagen RNeasy kit and eluted in a volume of 40 µl (cat. no. 74104). Then 38 µl of RNA was denatured at 95°C for 5 min and the following sequentially added: 2 µl RNasin (Promega; cat. no. N2511), 16 µl 5× first-strand buffer (Gibco/BRL, Gaithersburg, MD), 8 µl dNTP (10 mM), 4 µl random primers (Gibco/BRL; cat. no. 48190-011), 8 µl 0.1 M DTT and 4 µl Superscript II RT (Gibco/BRL; no. 18064-014). The mixture was incubated at room temperature for 5 min, followed by 42°C for 1 h and finally at 95°C for 5 min, and stored at −20°C.

**PCR reactions and CDR3 size analysis**

PCR reactions were performed in 50 µl reaction volumes using a Perkin-Elmer 480 thermal cycler (Perkin-Elmer, Foster City, CA). First round PCR was performed by adding 1 µl of cDNA to the following mixture: 5 µl 10×PCR buffer (Perkin-Elmer), 3 µl MgCl{sub}2 (25 mM; Perkin-Elmer), 4 µl dNTP (10 mM each; Perkin-Elmer), 5 µl 5’ primer and 5 µl 3’ primer (10 µM each), 0.25 µl Taq polymerase (Perkin-Elmer), and 28 µl milliQ H{sub}2O. PCR reactions were carefully optimized and run to saturation. PCR conditions were as follows: 2 min at 95°C, followed by 35 cycles of 95°C for 1 min, 65°C for 1 min and 72°C for 50 s. A final extension at 72°C for 7 min was performed. PCR products were visualized following electrophoresis on 2% agarose gels (NuSieve 3:1 agarose; FMC BioProducts, Rockland, ME) with ethidium bromide staining. Unlabeled V_{µ}4, C{sub}β, and J{sub}β, and 3’ Fam-labeled C{sub}β and J{sub}β primers (30) were synthesized (Center for Biotechnology, St Jude Children’s Research Hospital). PCR run-off reactions were performed by adding 1 µl first-round PCR product to the following: 1 µl 10×PCR buffer, 0.6 µl MgCl{sub}2 (25 mM), 0.8 µl dNTP (10 mM each), 1 µl 3’ Fam-labeled primer (10 µM), 0.25 µl Taq polymerase and milliQ H{sub}2O added to 10 µl. Run-off conditions were as follows: 2 min at 95°C, followed by 5–9 cycles of 95°C for 1 min, 65°C for 1 min and 72°C for 50 s. A final extension at 72°C for 7 min was performed.

Fam-labeled run-off products were denatured at 95°C for 2 min. Then 2 µl of run-off product was mixed with 2 µl loading buffer and 1 µl size standards (Genescan 1000 ROX; Applied Biosystems, Brisbane, Australia) and separated on a 6% polyacrylamide gel using an Applied Biosystems 373A DNA sequencer. Data were analyzed using Genescan analysis 2.1 Software (Applied Biosystems) which records the size and fluorescence intensity of each peak. The area of each individual peak was expressed as a percentage of total peak area. Only profiles with fluorescence intensity >700 U were analyzed. Major expansions were those which were at least 50% greater than the highest corresponding value in naive mice. CDR3 sizes were calculated from codons 95–106, inclusive (11).

**Cloning of PCR products for V–D–J junctional region sequencing**

Excess primers and nucleotides were removed from V_{µ}4–C{sub}β PCR products using the Qiagen PCR purification kit (cat. no. 28104), according to the manufacturer’s instructions. An aliquot of 1 µl of this cleaned product was used as a template for subsequent V_{µ}4–J{sub}β reactions using the above PCR conditions. V_{µ}4–J{sub}β PCR product quality was checked by gel electrophoresis (as above) and 1.5 µl of the reaction product cloned using the TOPO TA cloning kit and pCR® II-TOPO vector (Invitrogen; cat. no. K4600-01). White insert-containing colonies were picked and grown for 16–18 h in 2.5 ml LB medium at 37°C on an orbital shaker. DNA purification was performed using the Qiagen Miniprep kit (cat. no. 27106). Samples of 10 µl of DNA were sequenced on an Applied Biosystems 377 sequencer (Center for Biotechnology, St Jude Children’s Research Hospital) using M13-21 and reverse primers, and TaqFS dye terminator chemistry. Sequences were analyzed using GCG software (Wisconsin Package).

**Results**

**Major CDR3-restricted distortions in the V_{µ}4{sub}+CD8{sup+} T cell population**

Our previous studies of MHV-68 infection have shown that expansion of V_{µ}4{sub}+CD8{sup+} T cells is seen in mice of diverse MHC haplotypes (10; Hardy et al., submitted for publication). At present it is unclear what drives this dramatic T cell expansion and whether it is composed of clonally or polyclonally restricted populations. To determine the clonal composition of the expanded cells, V_{µ}4 region repertoire diversity was characterized using a PCR- and sequencing-based protocol that determines CDR3 length and clonotype distribution (30). This technique provides a global overview of diversity within given V_{µ}4 families and also permits focused analysis of individual J{sub}β subfamilies. The presence of clonal or oligoclonal expansions is demonstrated by distortion of an otherwise Gaussian distribution of peak sizes (16).

In order to avoid analysis of the CD4{sup+} T cell CDR3 region, C57BL/6 mice were depleted of CD4 cells by in vivo injection of GK1.5 antibody. Previous studies from our laboratory demonstrated that V_{µ}4{sub}+CD8{sup+} T cells had reduced viability ex vivo (unpublished observations), and for this reason we avoided potentially deleterious manipulations such as in vitro depletion and flow cytometric sorting. Additionally, we have shown that depletion of CD4{sup+} cells during the first week post-infection prevents expansion of V_{µ}4{sub}+CD8{sup+} T cells (31). However, depletion subsequent to 14 days post-infection has no effect on the magnitude of V_{µ}4{sub} expansion (31). Mice in the current study were depleted from 22 days post-infection.

Initially, CDR3 size analysis of the entire V_{µ}4 family using Fam-labeled C{sub}β in the run-off reaction was performed on four naive mice and six MHV-68-infected mice. Peripheral blood cDNA from CD4-depleted naive mice yielded Gaussian CDR3 size distributions (Fig. 1 shows the data for two representative naive mice) indicative of a diverse or polyclonal repertoire, as expected. In contrast, there was clear evidence of repertoire distortion among V_{µ}4 chains in five out of six infected mice (Fig. 1 shows the data for two representative mice). Infected
V<sub>β</sub>4<sup>+</sup>CD8<sup>+</sup> T cell clonal expansions in MHV-68-infected mice

Fig. 1. V<sub>β</sub>4–C<sub>β</sub> CDR3 size distribution in CD8<sup>+</sup> T cells from naive and MHV-68-infected mice. cDNA extracted from CD4-depleted peripheral blood of naive mice and mice 1 month post-MHV-68 infection was subjected to PCR amplification using V<sub>β</sub>4<sup>+</sup> and C<sub>β</sub> primers, followed by a run-off reaction with Fam-labeled C<sub>β</sub> primer. Products were separated on a sequencing gel and size distribution analysis using appropriate software. Relative fluorescence intensity, plotted on the y-axis, was always >700. U. Expansions at CDR3 of 14 or 11 amino acids were detected in the infected mice as indicated (asterisk).

mouse #1 showed a prominent expansion of CDR3 14 amino acids in length (10% of the repertoire), whilst CDR3 of this size were not seen in either naive mouse. Infected mouse #2 showed a prominent expansion of CDR3 11 amino acids in length (29%), with the corresponding values for the naive mice being 14 and 17% respectively. Flow cytometric analysis of the two naive mice indicated that~6% of CD8<sup>+</sup> cells expressed V<sub>β</sub>4<sup>+</sup> αβ<sub>β</sub>TCR, whilst the corresponding values for MHV-68-infected mice (infected #1 and infected #2) were 31 and 42%, respectively.

Having established that the V<sub>β</sub>4–C<sub>β</sub> repertoire is non-uniformly distorted in MHV-68-infected mice, we determined the extent of J<sub>β</sub> gene involvement by CDR3 size analysis using 3′<sup>+</sup> primers to each of the 12 mouse J<sub>β</sub> genes. The fact that V<sub>β</sub>4 expansion has been consistently seen in all C57BL/6 mice examined to date (data not shown) justified focussing the detailed analysis on two individual naive and two individual infected animals. The CDR3 size distribution for most J<sub>β</sub> elements in naive mice was overall normally distributed (polyclonal) (Fig. 2). In contrast, the CDR3 size distribution in each of the infected mice was markedly skewed across most J<sub>β</sub> repertoire profiles (Fig. 2). Infected mouse #1 showed prominent expansions in each of the J<sub>β</sub> subfamilies, whilst infected mouse #2 showed prominent expansions in all but the J<sub>β</sub>2.5 subfamily. In every case an expansion was considered significant if the percentage of the repertoire at a particular CDR3 size was at least 50% greater than the corresponding value in either naive mouse—the expansions in infected mice were frequently ≥200% of the values in naive mice. It is noteworthy that the expansion in the J<sub>β</sub>2.3 subfamily at CDR3 of 14 amino acids in infected mouse #1, which represented 57% of the repertoire, was also detected in the V<sub>β</sub>4–C<sub>β</sub> profile (Fig. 1). In contrast, CDR3 of 14 amino acids were undetectable in the V<sub>β</sub>4–C<sub>β</sub> and V<sub>β</sub>4–J<sub>β</sub>2.3 analysis of either naive mouse. Overall, the MHV-68-induced V<sub>β</sub>4<sup>+</sup>CD8<sup>+</sup> expansion appears to be unusual in that it consists of multiple V<sub>β</sub>4–J<sub>β</sub> selective expansions that impact dramatically on the available diversity within the circulating repertoire.

V–D–J junctional region sequencing demonstrates clonal and oligoclonal expansions

CDR3 size-restricted expansions are strong evidence of selective clonal or oligoclonal expansions (16–18). To determine the distribution of individual clonotypes within the V<sub>β</sub>4<sup>+</sup>CD8<sup>+</sup> expansions in infected C57BL/6 mice, we performed V–D–J junctional region sequencing. PCR products from two J<sub>β</sub> subfamilies which showed expansions at identical CDR3 sizes in the two infected mice, J<sub>β</sub>1.6 and J<sub>β</sub>2.2, were sequenced to determine whether similar or identical TCR sequences had been selected. Sequences obtained from cloned V<sub>β</sub>4–J<sub>β</sub>1.6 PCR products in infected mouse #1 were predominantly 10 amino acids in length (87%), with one major clonal expansion (with a junctional amino acids sequence of QDAGN) which represented 70% of total sequences (Fig. 3). Similarly, the majority of V<sub>β</sub>4–J<sub>β</sub>1.6 sequences in infected mouse #2 were 10 amino acids in length (75%), being comprised of one dominant clone (QEWGL; 38%: Fig. 3).

Sequence data obtained from cloned V<sub>β</sub>4–J<sub>β</sub>2.2 PCR products in infected mouse #1 demonstrated that 96% of sequences had CDR3 sizes of 11 amino acids, with two clones detected which represented 57 and 25% of total sequences (QDWDA and QDGLGWD respectively; Fig. 4). Whilst the majority of V<sub>β</sub>4–J<sub>β</sub>2.2 expansions in infected mouse #2 were also of 11 amino acids (80%), as predicted by CDR3 size analysis, three obvious clonal expansions (QDWDA, QDWGA and QDWGAG) were detected which accounted for 28, 16 and 20% of total sequences respectively. The glutamine (Q) in these CDR3 is presumably derived from the V<sub>β</sub>4 germline sequence (32), although amino acids in this position can be non-germline encoded. Nevertheless, there appears to be selective pressure to maintain the DWXA motif derived in part from the D<sub>β</sub>2.1 germline sequence (33). An additional expansion at CDR3 of 12 amino acids (QEEGGGA; 16% of sequences) was also seen (Fig. 4). Thus, although both infected mice had V<sub>β</sub>4–J<sub>β</sub>1.6 expansions with a CDR3 of 10 amino acids, these were composed of clones with distinct sequences. Although several of the 11 amino acid V<sub>β</sub>4–J<sub>β</sub>2.2 expansions in the two infected mice were unique, one clone with identical nucleotide sequence (QDWDA; Fig. 4) was seen in both mice.

It is important to note that CDR3 size analysis and V–D–J sequencing gave similar profiles for CDR3 length distribution (Table 1). For example, in the V<sub>β</sub>4–J<sub>β</sub>2.2 analysis of infected mouse #2, CDR3 size analysis showed that CDR3 of 11 and 12 amino acids represented 62 and 26% of the repertoire respectively. This was consistent with sequencing data, which showed that CDR3 of 11 and 12 amino acids represented 80 and 16% of the repertoire respectively (Table 1). The slight difference in absolute values from either method may reflect the statistical error imposed by sequencing a limited number.
CDR3 size distribution of individual J\(\beta\) subfamilies in naive and MHV-68-infected mice. CDR3 size analysis was performed as described in Fig. 1, except that Fam-labeled J\(\beta\)1.1–6 (upper panel) and J\(\beta\)2.1–7 (lower panel) primers were used in the run-off reactions. Relative fluorescence intensity, plotted on the y-axis, was always >700 U. The CDR3 size distribution for each J\(\beta\) subfamily shows a largely normal distribution for the naive mice. In contrast, multiple expansions (indicated by an asterisk) were detected in most J\(\beta\) subfamilies in both infected mice.

Fig. 2. CDR3 size distribution of individual J\(\beta\) subfamilies in naive and MHV-68-infected mice. CDR3 size analysis was performed as described in Fig. 1, except that Fam-labeled J\(\beta\)1.1–6 (upper panel) and J\(\beta\)2.1–7 (lower panel) primers were used in the run-off reactions. Relative fluorescence intensity, plotted on the y-axis, was always >700 U. The CDR3 size distribution for each J\(\beta\) subfamily shows a largely normal distribution for the naive mice. In contrast, multiple expansions (indicated by an asterisk) were detected in most J\(\beta\) subfamilies in both infected mice.

Thus, the sequencing of J\(\beta\)1.6 and J\(\beta\)2.2 chains showed evidence for oligoclonal and clonal expansions, some of which were shared between the two individual mice sequenced. These data are consistent with earlier random sequencing of V\(\beta\)4-C\(\beta\) V–D–J junctional region sequences from four individual MHV-68-infected mice, which also showed evidence for unique and ‘public’ expansions (data not shown).

V–D–J junctional region sequences in naive mice
Although the CDR3 size analysis results from the naive mice showed largely Gaussian profiles for the individual J\(\beta\) subfamilies, V–D–J junctional region sequencing was performed in naive mouse #2 to determine whether individual J\(\beta\) subfamilies were composed of distinct populations of cells. V–D–J junctional region sequencing from the J\(\beta\)1.6 subfamily showed that there was a diverse distribution of CDR3 sizes (Fig. 5 and Table 1), although several repeat sequences of a CDR3 size of 12 amino acids (QDRVN) were found. V–D–J junctional region sequences from the J\(\beta\)2.2 subfamily showed
Fig. 3. \(V_{\beta 4}--J_{\beta 1.6}\) junctional region sequences in MHV-68-infected mice. cDNA extracted from CD4-depleted peripheral blood of two individual mice 1 month post-infection was subjected to PCR using \(V_{\beta 4}\) and \(C_{\beta}\) primers. A second PCR reaction was performed with \(V_{\beta 4}\) and \(J_{\beta 1.6}\) primers using 1.5 \(\mu\)l of the above PCR product as template. The PCR reactions were performed completely independently of those for CDR3 size analysis. The PCR products were cloned and DNA from insert-containing colonies sequenced. Two nucleotide sequences encode for the N–D–N sequences QDAGN and QEWGL in mouse #1 and #2 respectively. The differences in the nucleotide sequences of the relevant codons are in bold and underlined. The frequency of prominent expansions is in bold. CDR3 size was calculated from codons 95–106 inclusive.
Vβ4/H11001 CD8+ T cell clonal expansions in MHV-68-infected mice

**Fig. 4.** Vβ4-Jβ2.2 junctional region sequences in MHV-68-infected mice. The PCR reactions and sequencing were performed as described in the legend to Fig. 3. In infected mouse #1 two nucleotide sequences encode for the N-D-N sequence QDWGA. The differences in the nucleotide sequences of the relevant codons are in bold and underlined. The N-D-N sequences QDWDA (indicated by double asterisks) had identical nucleic acid sequences in both mice. The frequency of prominent expansions is in bold. CDR3 size was calculated from codons 95–106 inclusive.

10 unique sequences and a CDR3 size distribution which broadly mirrored that obtained using CDR3 size analysis (Fig. 6 and Table 1).

**Vα usage by Vβ4+CD8+ T cells**

To further characterize receptor diversity, we analyzed Vα usage by Vβ4+CD8+ cells (Table 2). The data show that Vα8 was consistently paired with Vβ4 at higher frequency in infected compared to naive mice, in both spleen (22.1 ± 3.4 versus 8.9 ± 1.1 % respectively) and blood (20.8 ± 6.6 versus 6.9 ± 1.1 % respectively). However, this was not unique to Vβ4 chains, as there was also an increase in Vα8 usage among all CD8+ T cells from infected mice. In one mouse expansion of Vα3.2/α chains was observed exclusively in the Vβ4 subset of CD8+ T cells in both spleen and blood, whilst this change was not seen in the other three
**V_{β}^{4}/CD8^{+} T cell clonal expansions in MHV-68-infected mice**

Table 1. Numbers of amino acids in the CDR3 regions of V_{β}^{4}/CD8^{+} T cells from naive and MHV-68-infected mice

<table>
<thead>
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<th>J_{β}</th>
<th>Method</th>
<th>Mouse</th>
<th>CDR3 size (amino acids)</th>
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<td></td>
<td></td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>J_{β1.6}</td>
<td>CDR3 size</td>
<td>naive #1</td>
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<tr>
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<td></td>
<td></td>
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<td>–</td>
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<td></td>
<td></td>
<td>infected #2</td>
<td>–</td>
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<td>infected #1</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td>infected #2</td>
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^{a}Determined by CDR3 size analysis or V-D-J junctional region sequencing.

^{b}Percent of repertoire at each CDR3 size.

^{c}Not tested.

**Fig. 5.** V_{β}4–J_{β1.6} junctional region sequences in a naive mouse. The PCR reactions and sequencing were performed as described in the legend to Fig. 3, using cDNA for naive mouse #2. Two nucleotide sequences encode for the N-D-N sequence NKH. The differences in the nucleotide sequences of the relevant codons are in bold and underlined. CDR3 size was calculated from codons 95–106 inclusive.

Table 2. N-Diversity values for V_{β}4 and J_{β1.6} germlines

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<th>V_{β}4 germline</th>
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<th>CDR3</th>
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<td>Q---</td>
<td>C---S---S---S---</td>
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</tbody>
</table>

There was little change in the frequency of V_{α}2 usage, and a small compensatory reduction in relative usage of V_{α}11 in both splenic and peripheral blood V_{β}4^{+}CD8^{+} T cells as a consequence of MHV-68 infection. These results indicate that there is diversity in the V_{α} repertoire of V_{β}4^{+}CD8^{+} T cells in MHV-68-infected mice.
Vβ4–Jβ2.2 junctional region sequences in a naive mouse. The PCR reactions and sequencing were performed as described in the legend to Fig. 3. CDR3 size was calculated from codons 95–106 inclusive.

Table 2. Vα utilization by Vβ4+CD8+ lymphocytes

<table>
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<th>Vα</th>
<th>Spleen</th>
<th>Blood</th>
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<tr>
<td></td>
<td>Naive</td>
<td>MHV-68</td>
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<tr>
<td>Vβ4+CD8+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vβ4</td>
<td>3.65 ± 1.0</td>
<td>2.7 ± 1.3</td>
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<tr>
<td>Vβ3.2</td>
<td>3.4 ± 0.7</td>
<td>15.0 ± 25.7</td>
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<tr>
<td>Vβ8</td>
<td>8.9 ± 1.1</td>
<td>22.1 ± 3.4</td>
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<tr>
<td>Vβ11</td>
<td>8.8 ± 2.6</td>
<td>1.5 ± 0.9</td>
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<tr>
<td>Vβ2</td>
<td>6.3 ± 1.6</td>
<td>4.5 ± 0.7</td>
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<tr>
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<td>3.5 ± 0.1</td>
<td>7.2 ± 9.2</td>
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<tr>
<td>Vβ8</td>
<td>8.2 ± 1</td>
<td>18.3 ± 3.9</td>
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<tr>
<td>Vβ11</td>
<td>2.0 ± 0.1</td>
<td>2 ± 0.4</td>
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<tr>
<td>CD8+</td>
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<tr>
<td>Vβ2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vβ3.2</td>
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<tr>
<td>Vβ8</td>
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<tr>
<td>Vβ11</td>
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*Mean ± SD from four mice per group.

Discussion

Infection of mice with MHV-68 causes a dramatic expansion of peripheral blood CD8+ T cells which, in some aspects, mirrors EBV-induced IM. However, unlike EBV, there is a strong bias among the peripheral blood T cells during the IM phase toward those that utilize Vβ4 chains in their αβTCR, resulting in an increased percentage of Vβ4+ T cells among the CD8+ T cells. The Vβ4+CD8+ T cell expansion occurs in virtually all mouse strains examined, although to varying extents, and the effect has been shown to be independent of MHC haplotype (10; Hardy et al., submitted for publication).

The nature of the stimulatory ligand remains elusive, but it does not appear to be dependent on MHC class I or II molecules nor on the non-classical MHC class I molecule CD1 (23). We investigated CDR3 size restriction and V-D-J junctional region sequence diversity of Vβ4+CD8+ T cells solely in C57BL/6 mice since this strain shows the most pronounced expansion and has been used extensively to study the biology of MHV-68 infection (4,23,34). Our results clearly indicate the presence of multiple clonal and oligoclonal expansions within the Vβ4+CD8+ T cell subset amplified during the IM phase of MHV-68 infection. T cells specific for lytic and latent epitopes have been
identified in EBV-infected patients (35–37) and large oligo-
clonal expansions are seen within the activated CD8+ set
during acute IM (26,27). Additionally, clonal expansions to a
latent EBV epitope were found in healthy EBV-seropositive
individuals (38). Despite these findings in EBV, it appears that
at least the Vγ4+ component of the activated CD8+ T cells in
the peripheral blood during the IM phase of MHV-68 infection
is not specific for lytic or latent epitopes. Several acute phase
MHV-68 viral epitopes in H-2b mice (28,29,39) and a latent
CTL epitope in H-2d mice have been identified (40). In contrast
to the situation for EBV (27), T cells specific for lytic epitopes
are relatively infrequent, generally 1–11% of the blood CD8+
set (28). In addition, with the emergence of Vγ4+CD8+ T cells in
the peripheral blood at ~20 days post-infection, the fre-
cency of CD8+ T cells specific for the lytic epitopes drops
(28). Analysis of a panel of Vγ4+CD8+ hybridomas generated
from MHV-68-infected mice during the IM phase and reactive
to latently infected spleen cells (23) failed to identify reactivity
to the lytic or latent epitopes (unpublished data).

A role for sAg in herpesviruses has previously been sug-
gested (24,25,41). In addition, a product of the herpesvirus
saimiri ORF14, that is homologous to the MMTV sAg, Mtv7,
encodes a protein that binds to MHC class II molecules and
stimulates T cells. However, this activity differs from a
conventional sAg because there is no Vβ bias to the polyclonal
T cell activation (42). This product has recently been shown
to play an essential role in T cell transformation by the virus,
and for high-level persistent infection (43).

We previously postulated that the striking Vγ4 bias of the
CD8+ T cell expansion may be due to the presence of a sAg
(10). Hallmarks of sAg responses are dependence on class
II MHC expression, lack of MHC restriction, stimulation of
specific Vβ-bearing T cells and lack of antigen-processing
requirement (19,20). The presence of oligoclonal expansions
described here are unusual for known sAg. Contact between
the TCR and bacterial or viral sAg is largely dependent upon
interactions with CDR1, CDR2 and the hypervariable region
4 (HV4) loop of the TCR β chain (19–22), distinct from the
CDR3 region which is predominantly responsible for the fine
antigen specificity of the T cell (13). Consistent with this,
CDR3 size analysis of sAg-reactive T cells has failed to
detect evidence for CDR3 involvement in two reported studies
(44,45). Nevertheless, the TCR α chain and CDR3β sequence
can stabilize TCR–sAg interactions which are suboptimal or
involve unusual sAg (46–49). This is illustrated by the finding
that T cell recognition of the sAg produced by Mycoplasma
arthritidis mitogen (MAM) is dependent upon two amino acids
located within the Vγ17 CDR3 region (50). Thus, although the
CDR3-size restricted oligoclonal populations of T cells and
the putative ‘public’ expansion (QDWDA) seen in Vγ4–Vγ2.2
sequences are characteristic of antigen-driven expansions, it
remains formally possible that the expansion is mediated by
an unusual sAg, similar to MAM. Although sAg have been
reported to modulate CD8+ T cell responses (51,52), the
possibility that a putative MHV-68-associated sAg reactivates
Vγ4+CD8+ T cells expanded during the lytic phase seems
remote as these cells are not reactive to known lytic epitopes
(28).

Numerous studies have demonstrated TCR Vγ-specific
expansions in response to virus infection in mice (53–56).
However, whilst all of the above Vγ4-specific expansions are
driven by known peptide–MHC combinations, they differ from
the Vγ4 expansion in MHV-68-infected mice which is MHC
independent (10,23). Lin and Welsh examined the Vγ8.1+ T
cells responding to lymphocytic choriomeningitis infection,
and demonstrated a surprisingly diverse repertoire which was
unique to each mouse examined (57). A diverse Vγ17+ T cell
repertoire is also seen during the response to influenza A
matrix peptide M158–66 in the human (58). Others have shown
highly restricted repertoires in response to a number of viral
and non-viral peptide–MHC antigens (17,18,55,59). Thus,
whilst CDR3β amino acids sequence restriction or diversity
does not in itself indicate that the immune response is driven
by peptide–MHC, the presence of dominant clonal expansions
is generally seen to support this conclusion.

The question of whether T cells from the acute repertoire are
selected into the memory repertoire remains controversial
(17,59). In the case of lymphocytic choriomeningitis infection
it appears that the memory repertoire evolves from a represen-
tative sampling of the acute T cell repertoire (56,57),
consistent with findings in other systems (17). However, there
is evidence that the T cell repertoire during recall responses,
at least in the case of Listeria monocytogenes infection,
may be more ‘focussed’ (60). It would be of interest to perform
longitudinal studies of individual mice to determine whether
the Vγ4+CD8+ T cell repertoire evolves or remains stable
over time.

It would be anticipated that naive mice should have a
Gaussian CDR3 size distribution. However, V–D–J junctional
region sequencing of the Jβ1,6 subfamily showed evidence
of a small clonal expansion, although the Vγ4–Jγ1,6 CDR3
size distribution for this mouse was not markedly skewed.
Biases in Jγ usage are seen in the developing and mature
T cell repertoire, resulting in under-utilization of certain Jβ
elements, including Jβ1,6 (47,61,62). This bias may make the
sample size of the Jβ1,6 pool statistically unrepresentative
compared to more abundantly expressed groups such as
Jβ2,2. Thus, even small clones of cells may be sufficient to
distort the repertoire, thereby giving rise to repeat sequences
in naive mice. In naive C57BL/6 mice, memory/activated
(CD62L−) CD8+ T cells are somewhat enriched in the blood
compared to the spleen (data not shown). This raises the
possibility that clonally expanded cells are relatively frequent
in the blood and may account for some skewing of the ‘naïve’
repertoire.

The oligoclonality of the expanded Vγ4+CD8+ T cells is
consistent with the idea that a small subset of MHV-68-
reactive Vγ4+ precursors exists in the naïve repertoire and
are preferentially stimulated to proliferate. This hypothesis is
supported by the finding that a low frequency of Vγ4+ T
cells respond to MHV-68-infected splenocytes in vitro and
is consistent with the finding that a low frequency of random
Vγ4+CD8+ T cells hybridomas (~3%) can be stimulated by
MHV-68-infected spleen cells (unpublished observations).
This possibility is concordant with the ~1 week lag be-
tween detection of the stimulatory activity in the spleen and observed
elevated levels of Vγ4+CD8+ T cells in the peripheral blood
(10,23; Hardy et al., submitted for publication). Nevertheless,
the stimulus driving proliferation is strong, since >30–50% of
CD8+ T cells are Vγ4+ 3–4 weeks post-infection. This idea is
reinforced by the finding that large expansions of Vβ4+CD8+ cells are found in TAP1 and β2-microglobulin knockout mice which have low numbers and a restricted repertoire of CD8+ T cells (23,28,63,64).

In summary, the Vβ4+CD8+ T cells that dominate the activated T cells in the peripheral blood during MHV-68-induced IM represent preferentially amplified CDR3 size-restricted oligoclonal populations, a finding typical of peptide–MHC-driven T cell proliferation, but also consistent with unusual sAg, such as MAM. Taken together with previous studies suggesting that the reactivity is apparently independent of MHC class I and class II molecules, as well as β2-microglobulin or TAP1 expression (23), the data suggest that the ligand driving Vβ4 expansion is unconventional and neither fits into the sAg or antigen-specific TCR recognition paradigms.

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Abbreviations
CDR complementary-determining region
CTL cytotoxic T lymphocyte
EBV Epstein–Barr virus
IM infectious mononucleosis
MAM Mycoplasma arthritidis mitogen
MHV-68 murine γ-herpesvirus-68
PBL peripheral blood lymphocyte
sAg superantigen

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