Lytic Cycle T Cell Epitopes Are Expressed in Two Distinct Phases During MHV-68 Infection

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Lymphatic Cycle T Cell Epitopes Are Expressed in Two Distinct Phases During MHV-68 Infection

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Murine herpesvirus-68 (MHV-68) is a type 2 γ herpesvirus that productively infects alveolar epithelial cells during the acute infection and establishes long-term latency in B cells and lung epithelial cells. In C57BL/6 mice, T cells specific for lytic cycle MHV-68 epitope p56/Db dominate the acute phase of the infection, whereas T cells specific for another lytic cycle epitope, p79/Kb, dominate later phases of infection. To further understand this response, we analyzed the kinetics of Ag presentation in vivo using a panel of lacZ-inducible T cell hybridomas specific for several lytic cycle epitopes, including p56/Db and p79/Kb. Two distinct peaks of Ag presentation were observed. The first peak was at day 6 in the mediastinal lymph nodes and correlated with the initial pulmonary lytic infection. The second peak was at day 18 in both the mediastinal lymph nodes and spleen and correlated with the peak of latent infection. Interestingly, the p56 epitope was detected only in the mediastinal lymph nodes at day 6 after infection whereas the p79 epitope was predominantly presented in the spleen at day 18, suggesting that differential epitope presentation drives the switch in T cell responses to this virus. Phenotypic analysis of APCs at day 18 postinfection revealed that dendritic cells, macrophages, and B cells all presented lytic cycle epitopes. Taken together, the data suggest that there is a resurgence of lytic cycle Ags in the spleen, which explains the kinetics and specificity of the T cell response to MHV-68. The Journal of Immunology, 1999, 163: 868–874.

The γ herpesviruses (γHV) are characterized by their ability to establish lifetime latent infection in host lymphocytes (1). For example, EBV and Kaposi’s sarcoma-associated herpes virus (KSHV/HHV-8) induce persistent infection in humans and are associated with malignancies such as nasopharyngeal carcinoma, B cell lymphomas, and Kaposi’s sarcoma (1, 2). Although γHV have evolved multiple strategies to avoid elimination by the host, they are nevertheless under the close control of the host immunity throughout the infection. In particular, it is believed that the CD8+ T cell-mediated immune response plays a critical role in controlling the acute and latent phases of infection. The T cell response to γHV infection has been extensively studied, particularly in the case of EBV infection in humans (3, 4). However, very little is known about the induction of T cell responses in terms of the sites and kinetics of Ag presentation. This issue has been difficult to address because of the lack of a suitable animal model.

Recently, murine herpesvirus-68 (MHV-68), a natural pathogen of small rodents, has been classified as a type 2 γHV with similar genomic and pathobiological features to those of EBV and Kaposi’s sarcoma-associated herpes virus (5–7). When introduced intranasally into laboratory mice, MHV-68 establishes a transient productive infection in alveolar epithelium, resulting in acute interstitial pneumonia (8). Concurrent with the acute infection, the virus establishes a latent infection in B cells and lung epithelial cells, which persists for the lifetime of the animal (9, 10). Another key feature of the viral infection is the development of an infectious mononucleosis (IM)-like syndrome following viral clearance from lung. It is characterized by splenomegaly and activated CD8+ T cells in the peripheral blood, similar to that associated with EBV infection (5, 11, 12). Interestingly, a large subset of the activated CD8+ T cells express the Vβ4 TCR irrespective of the haplotype of the infected animal (12).

CD8+ T cells have been shown to play a key role in clearing lytic virus from the lungs during the acute infection and have also been implicated in the control of latently infected B cells (13, 14). Recent studies have identified several MHV-68 lytic cycle CD8+ T cell epitopes (15, 16) (L. Liu, E. J. Usherwood, M. A. Blackman, and D. L. Woodland, manuscript in preparation). These include the dominant p56/Db epitope (from ORF6, an immediate early gene encoding an ssDNA-binding protein), the p79/Kb epitope (from ORF61, an early gene encoding the large ribonucleotide reductase subunit), and the subdominant gB604–612/Kb epitope (from ORF8, a late gene encoding gp110). Despite the fact that both p56/Db and p79/Kb are dominant epitopes derived from early gene products, the CTL activity against these two peptides showed distinct kinetic patterns (16). The p56-specific response was initially prominent in the acute infection but declined rapidly after the virus was cleared, analogous to the CTL response in a nonpersistent virus infection. In contrast, the CTL response against p79 reached its peak in the early IM phase (about 18 days after infection) and was maintained at a relatively high level. Whether this sustained CTL activity represents the continuous Ag presentation to the specific T cells or the prolonged survival of the activated T cells is not clear.

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In the current study, we took advantage of the MHV-68 model system to investigate the induction of T cell response to a γHV infection. In particular, we addressed the question of whether there was sustained presentation of lytic cycle epitopes that would explain the specificity and kinetics of the T cell response to MHV-68. The data demonstrate that there are two waves of lytic Ag presentation that correlate with the initial acute infection in the lung and the subsequent peak of latent infection in splenic B cells.

Materials and Methods

Mice

Female C57BL/6 mice (H-2b) were purchased from Jackson ImmunoResearch Laboratories (Bar Harbor, ME). H-2LΔ-deficient B6C2D mice (17) (under license from GenPharm International, Mountain View, CA) were bred at St. Jude Children’s Research Hospital. Mice were housed under specific pathogen-free conditions until MHV-68 infection and in BL3 containment after infection.

Cell lines and culture conditions

The T cell hybridomas and L cell lines were grown in complete tumor medium (CTM) containing 10% FCS at 37°C with 10% CO2 (18). L cells transfected with the KΔ, DΔ or L-AΔ MHC genes have been described previously (19, 20). NIH 3T3 cells were grown in DMEM (BioWhittaker, Walkersville, MD) containing 10% FCS. All adherent cell lines were removed with 0.25% trypsin/EDTA (Life Technologies, Grand Island, NY) before use in the assays. The BWZ.36 fusion partner was a gift from Dr. N. Shastri (University of California, Berkeley, CA) (21).

Virus stocks and virus infections

The original stock of MHV-68 (clone G2.4) was obtained from Prof. A. A. Nash (Edinburgh, U.K.) as a cell-free lysate derived from infected baby hamster kidney cells. This was then propagated in owl monkey kidney fibroblasts (ATCC 15656CRL; American Type Culture Collection, Manassas, VA) and titrated on NIH 3T3 cells (14). Mice were anesthetized with Avertin (2,2,2,tribromoethanol) and infected intranasally with 400 PFU of MHV-68 (in 40 μl PBS) at 8–12 wk of age. Mediatinal lymph node (MLN), spleen, and bronchoalveolar lavage (BAL) were then isolated at various times after infection. The inflammatory cells in BAL were first absorbed on plastic petri dishes (Falcon, Lincoln Park, NJ) for 60 min at 4°C. Red blood cells were lysed with Gey’s solution for all the inflammatory cells in MLN and spleen. Erythrocytes were lysed with Gey’s solution for all inflammatory cells in BAL and MLN. The inflammatory cells in BAL were harvested from B6C2D (MHC class II-deficient) mice, and the clones giving the highest percentage of positive cells were selected for further study.

Synthetic peptides

The p79 (TSINFVKI) and p56 (AGPHNDMEI) peptides were kind gifts from Dr. P. C. Doherty (St. Jude Children’s Research Hospital, Memphis, TN). Stock solutions of peptides (1 mg/ml) were prepared in PBS.

Ag presentation assays

The ex vivo Ag presentation assay has been described previously (26). Briefly, MLNs and spleens were removed from three to seven mice at various times postinfection and pooled before preparing single cell suspensions. T cells were depleted by incubation with an anti-Thy1 mAb, AT83 (28), and a mixture of rabbit and guinea pig complement (Cedarlane, Westbury, N.Y.). The cells were then fixed with 0.125% paraformaldehyde, a dye that we have previously shown to block latent virus reactivation in vitro (data not shown), and 2-fold serial dilutions of the cells were prepared in 96-well flat-bottom plates, starting at 106 cells per well. Hybridoma cells (104 per well) were then added to each well, and the plates were then incubated for 20 h. Responding hybridomas were identified using an X-Gal assay. The numbers of blue cells per well were counted using an inverted tissue culture microscope, and estimates of APC frequencies were determined from the highest dilution giving a detectable response above background. Background responses were determined by culturing the hybridomas with serial dilutions of naive spleen cells and were always under 20 spots per well. In all of the ex vivo Ag presentation assays, duplicate titrations of unfixed APCs were also included to assess Ag presentation due to reactivating latent virus. To assess Ag expression in vivo, 2 × 104 MHV-68-specific hybridomas (in 100 μl PBS) were injected i.v. into naive or d18 MHV-68-infected C57BL/6 mice. After 18 h, spleens from individual mice were taken and single cell suspensions were prepared. The cells were distributed into six-well plates (Costar, Cambridge, MA) at 2 × 105 cells per well, and an X-Gal assay was performed immediately.

To assess T cell hybridoma specificity, 103 hybridomas were cultured with 104 MHV-68-infected or uninfected L-KΔ, L-DΔ, or L-AΔ cells in flat-bottom microtiter plates in the presence or absence of peptides (pB6A64-66, pB6A62, pB6A56, pB6A57, pA34). Peptides were added to the cultures at a concentration of 10 μg/ml as appropriate. In some experiments the L cells were prepped with 50 μg/ml peptide, washed, and then added to serially diluted fresh spleen cells before the Ag presentation assay.

X-Gal assay

The response of lacZ+ hybridomas was assessed in a standard X-Gal assay (26). Cells were washed once with PBS and then fixed with 100 μl/well cold 2% formaldehyde/0.2% glutaraldehyde for 5 min. The cells were washed again with PBS and then overlaid with PBS containing 1 mg/ml X-Gal, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 2 mM MgCl2. After 6–18 h incubation, the numbers of blue cells per well were counted using an inverted tissue culture microscope.

Abs and flow cytometry

Spleen cells were stained for three-color cell sorting with combinations of the following Abs: anti-CD45R-C5 conjugate (B220; cat No. 01128B), anti-CD11c-FITC conjugate (90704D), and anti-CD11b-PE conjugate (01715B) (26). Samples were then sorted on a FACSStar+ flow cytometer (Becton Dickinson) into CD45R+ (B cells), CD11b+ /CD11c+ /CD45R− (dendritic cell-enriched), and CD11b+ /CD11c+ /CD45R+ (macrophage-enriched) populations. The purity after sorting was 92% or greater. All Abs were purchased from PharMingen (San Diego, CA).

Infective center and limiting dilution assays

The frequency of virus-associated cells was estimated by an infective center assay as previously described (14). Briefly, single cell suspensions were T cell depleted (in the case of spleen cells), plated on monolayers of NIH 3T3 cells, and then overlaid with carboxymethyl cellulose medium. Following 6 days of culture, the carboxymethyl cellulose overlay was removed, and virus plaques were quantitated by staining infected NIH 3T3 cell monolayers (1.5 × 106 per well) in flat-bottom 96-well tissue culture plates. Twenty-four wells were plated for each dilution. The numbers of wells exhibiting cytopathic effect were counted after 3-week culture. To

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analyze the presence of lytic virus, the samples were freeze/thawed before the assay.

Results

Generation of lacZ-inducible T cell hybridomas specific for lytic MHV-68 Ags

To study Ag expression in the course of an MHV-68 infection, we first generated panels of MHC class I-restricted lacZ-inducible T cell hybridomas specific for lytic cycle MHV-68 epitopes. MHC class II-deficient B6C2D mice were infected with MHV-68, and, 9 days later, cells from the MLN and BAL were harvested for fusion. Approximately 150 hybridomas were generated in three independent fusions. The hybridomas were initially screened for their reactivity to C57BL/6 spleen cells infected with MHV-68 in vitro, and 10 hybridomas were shown to be MHV-68 specific (data not shown). All 10 hybridomas expressed high levels of CD8. These hybridomas were then subcloned, and clones giving the strongest response to anti-TCR Ab were chosen for further studies. The specificity of these hybridoma clones was then assessed using vaccinia recombinants encoding MHV-68 lytic cycle proteins gB and gp150 and the previously defined MHV-68-derived peptides (p11, p56, p77, p79, and gB604–612) (15). As shown in Table I, five MHC class I-restricted T cell hybridomas were identified with specificity for the p56/Dd, p79/Kb, and gB604–612/Kb epitopes. The other five MHV-68-reactive hybridomas did not recognize any of the known peptide epitopes or the gp150 protein when screened with vac-gp150 (the data for one of these hybridomas, 4801.3, is shown in Table I). For the rest of the studies, we selected a panel of four hybridoma cell lines that collectively recognized all of the Ags identified in Table I: 4951.5 (Kb/gB604–612), 4943.4 (Kb/p79), 49100.2 (Dd/p56), and 4801.3 (Kb/unknown Ag).

Kinetics of lytic Ag expression during the course of MHV-68 infection

LacZ-expressing hybridomas have previously been shown to be sensitive reagents for analyzing Ag presentation ex vivo (26). Since we wished to use the MHV-68-specific lacZ+ hybridomas for similar studies, we first assessed the ability of the hybridomas to detect low frequencies of APCs under optimal conditions. Thus, L cells were pulsed with the appropriate peptides, and titrated numbers of cells were tested for their ability to stimulate the hybridomas. As shown in Fig. 1, two of the hybridomas specific for p79/Kb and p56/Dd (4943.4 and 49100.2, respectively), were very sensitive and able to detect as few as three peptide-pulsed L cells. Hybridoma 4951.1 (specific for gB604–612/Kb) was much less sensitive, requiring over 1000 APCs to generate a detectable response. Note that the sensitivity of hybridoma 4803.1 could not be determined, because the peptide Ag has not been identified. We also tested the sensitivity of the hybridomas to low numbers of peptide-pulsed L cells in the presence of varying numbers of spleen cells to determine whether large numbers of spleen cells affected the detection of APCs. The ability of the hybridomas to detect small numbers of APCs was not affected by an excess of up to 1 × 10^6 spleen cells.

Table I. Specificity analysis of the MHC class I-restricted hybridomas

<table>
<thead>
<tr>
<th>Hybrid</th>
<th>Source</th>
<th>L-Kb Cell</th>
<th>L-Dd Cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>4722.2</td>
<td>MLN</td>
<td>51</td>
<td>&gt;300</td>
</tr>
<tr>
<td>4951.1</td>
<td>MLN</td>
<td>136</td>
<td>&gt;300</td>
</tr>
<tr>
<td>4943.4</td>
<td>MLN</td>
<td>110</td>
<td>&gt;300</td>
</tr>
<tr>
<td>49102.2</td>
<td>MLN</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>49104.1</td>
<td>MLN</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>4801.3</td>
<td>BAL</td>
<td>&gt;300</td>
<td>—</td>
</tr>
</tbody>
</table>

* LacZ-inducible T cell hybridomas were generated using T cells from BAL or MLN of B6C2D mice at day 9 post-MHV-68 infection.
* The number of lacZ+ cells per well.
* —, Indicates that there was no response by the hybridoma.

FIGURE 1. LacZ hybridomas recognize low frequencies of APCs. L cells were pulsed with 50 μg/ml p79 (A), p56 (B), or gB604–612 (C) at 37°C for 4 h. After three washes, titrated numbers of peptide-pulsed or unpulsed L cells were plated in 96-well flat-bottom plates. Hybridomas (1 × 10^3) were added into each well, and an X-Gal assay was performed after 18 h incubation. The data are presented as the numbers of lacZ+ cells per well and are representative of three independent experiments.
10^6/well spleen cells, which is the maximal number of cells in the ex vivo presentation assay (data not shown).

We next used the hybridomas to study the kinetics of Ag presentation in MHV-68-infected C57BL/6 mice. MLNs and spleens were taken between days 4 and 70 postinfection, and APC frequencies were assessed using the panel of \( \text{lacZ} \) hybridomas. A potential concern with this experimental system was that latent virus might reactivate upon in vitro culture of latently infected cells, resulting in an overestimation of Ag presentation. To avoid this problem, we fixed the APCs with paraformaldehyde within 3 h of harvesting (early gene products for other \( g \)HVs do not appear before 4 h during reactivation of virus in vitro) (29). The frequency of APCs that presented Ag to the hybridoma was calculated from the reciprocal of the number of cells in the last well that elicited a detectable hybridoma response. As shown in Fig. 2, the hybridomas generally detected two distinct peaks of Ag presentation during the course of an MHV-68 infection of C57BL/6 mice. The first peak was detected exclusively in the MLN by all four hybridomas at around day 6–7 postinfection. This time point corresponds to the peak of virus titers in the lung during the acute phase of the infection (8, 14). The frequency of APC detected by each of the hybridomas at this time was fairly consistent, ranging from 1 in 3 \( \times 10^5 \) to 1 in 10^6 MLN cells. These are minimal estimates of APC frequencies. A second peak of lytic Ag presentation was detected by three of the four hybridomas in both the MLN and spleen around day 18, long after the acute infection has resolved and lytic virus cleared from the lungs. This time point is just subsequent to the peak prevalence of latently infected B cells in spleen (8, 9, 14). It should be noted that increased frequencies of APCs were detected during the second peak of Ag presentation when unfixed cells were used in the assay. This suggests that there was significant secondary infection, or viral reactivation, in vitro and underscores the importance of fixing the APC for these studies. It is interesting that the p56/Db-specific hybridoma (49100.2) did not detect Ag in the spleen or MLN at day 18, even though it readily detected Ag in the MLN at day 6. The poor or low presentation of the p56 epitope is consistent with the observation that T cells specific for this epitope are dominant in the acute phase of infection but are subdominant in the later mononucleosis stage. In contrast, the p79 epitope was presented strongly in the latent phase of infection in the spleen, consistent with the prolonged T cell response to this epitope (16). The lytic Ags were not detected by any of the hybridomas 1 mo after infection and did not reappear at later time points (up to day 70, data not shown).

The observation that there was a second peak of lytic Ag presentation after lytic virus had been cleared from the lung was surprising. To rule out the possibility of virus reactivation and viral gene expression in vitro before fixation, the hybridoma cell lines were injected directly into uninfected or day 18 MHV-68-infected C57BL/6 mice. Twenty hours later, single cell suspension was prepared from the spleens and added into 6-well plates at 2 \( \times 10^7 \)/well. X-Gal assay was performed, and the numbers of \( \text{lacZ}^+ \) cells per well were counted.

**FIGURE 2.** Kinetics of lytic Ag expression during the course of MHV-68 infection. Cells obtained from MLNs (A) or spleens (B) of C57BL/6 mice at different time points postinfection were titrated into 96-well plates after T cell depletion. The cells were either unfixed or fixed with 0.1% paraformaldehyde before the assay. T cell hybridomas (1 \( \times 10^3 \)) were then added into each well, and the plates were incubated for 18 h. An X-Gal assay was performed and the numbers of \( \text{lacZ}^+ \) cells per well were counted. The frequencies of APCs carrying the lytic Ags were estimated by the reciprocal of the APC numbers in the last well that a hybridoma response was observed. Cells were pooled from at least three mice for each time point, and most time points were assessed by two to five replicate experiments.

**FIGURE 3.** In vivo lytic Ag expression in spleen at day 18 post-MHV-68 infection. Hybridoma cells (2 \( \times 10^5 \)) were i.v. injected into uninfected or day 18 MHV-68-infected C57BL/6 mice. Twenty hours later, single cell suspension was prepared from the spleens and added into 6-well plates at 2 \( \times 10^7 \)/well. X-Gal assay was performed, and the numbers of \( \text{lacZ}^+ \) cells per well were counted.
because p56/D expression had not been detected in the spleen at day 18 (Fig. 2).

There are three possible explanations for the presence of lytic Ag in the MLN and spleen at day 18. First, it is possible that Ag-loaded APC persist for some time after the clearance of lytic virus, as has been described for Sendai virus infection of mice (26). However, it is unlikely that these cells would persist for over a week and would not produce a second peak of Ag presentation. Second, if it is possible that lytic virus is not completely cleared and persists in the spleen, it would be difficult to clear from the spleen, after the pulmonary infection has been cleared. This seems unlikely given the strong immune response to this virus and the fact that previous studies have not detected lytic virus at this time (8, 14). The third possibility is that there is reactivation of latent virus in vivo at this time point, which results in the presentation of Ag, but may, or may not, result in the release of lytic virus. To further investigate these possibilities, we looked for lytic virus in the spleen. As expected, an infective center assay detected lytically infected spleen cells between days 14–40 postinfection, with the highest frequency at day 14 (about 1 in 250 T-depleted spleen cells) (Fig. 4A). However, a highly sensitive limiting dilution assay additionally detected a low level of lytic virus in the spleen at day 14 postinfection (<1 infectious particle per 100,000 T-depleted spleen cells) (Fig. 4B). Thus, these data confirm that lytic virus is present in the spleen at late time points and explains the presentation of lytic Ag at this time. At this point, we cannot distinguish between persistent lytic virus and reactivation of latent virus. However, we favor the latter possibility, given the correlation between the kinetics of latency and lytic Ag presentation.

**Phenotypic analysis of APCs presenting lytic Ags**

Given that there was lytic cycle Ag presentation in the spleen at day 18, it was of interest to determine which cell population(s) was presenting Ag. Thus, B cells (CD45R+), dendritic cells (CD45R−/CD11b−/CD11c−) and macrophages (CD45R+/CD11b+/CD11c+) were enriched by flow cytometry from the spleens of C57BL/6 mice 18 days after infection and tested for their ability to stimulate the hybridomas. As shown in Table II, dendritic cell-, macrophage-, and B cell-enriched populations all presented Ag to the class I-restricted hybridomas, with the predominant presenting populations being dendritic cells and macrophages. The enrichment of APCs was less than expected, when compared with unsorted populations (Fig. 2). Thus, it is possible that additional spleen cell populations not tested here were presenting lytic Ag to the hybridomas. However, we also noted that there was substantial loss of total B cell numbers during the sorting procedure. This was the case only for B cells isolated from MHV-68-infected mice, raising the possibility that we were specifically losing latently infected B cells. We have not been successful in improving the recovery of these cells. Nonetheless, the data clearly show that there is substantial Ag presentation in B cell-, dendritic cell-, and macrophage-enriched populations. To determine whether these sorted populations were associated with virus, an infective center assay was performed (Fig. 5). Consistent with the Ag expression experiments, both the dendritic cell- and macrophage-enriched populations were associated with virus, with titers higher than detected in the B cells. Taken together, these data suggest that these cells were either directly infected by the virus, or that they acquired virus particles from B cells in which the virus had reactivated.

**Discussion**

Understanding the role of the immune system in the control of γHV infection and latency is essential for the development of effective vaccines and therapeutics. However, relatively little is known about the induction of CD8+ T cell responses to γHV...
infections. In the current manuscript, we have analyzed the presentation of lytic phase Ags with a view to understanding the kinetics of the CD8+ T cell response to MHV-68. The data show that Ag presentation in the initial acute phase of the infection is very similar to that seen with nonpersistent respiratory viruses, such as Sendai virus (26). Thus, APCs are predominantly present in the regional nodes and their numbers essentially correlate with the amount of lytic virus in the lungs, which peaks at day 6. However, unlike the situation with nonpersistent respiratory viruses, there is also a second wave of Ag presentation that occurs in both the lymph nodes and the spleen. This activity peaks at day 18 postinfection and correlates with the presence of low levels of lytic virus. These data demonstrate that there is effective Ag presentation during the latent phase of infection and explain the kinetics of the CD8+ T cells response to this virus.

Cytotoxic CD8+ T cells in the lung play a key role in controlling viral replication during the primary infection, and lytic virus is essentially cleared from the lung between days 7 and 10 (8, 14). The observation that T cell Ag presentation is limited to the MLN during this initial phase of infection is also consistent with data from other respiratory viruses, such as Sendai virus (26). However, the second wave of MHV-68 Ag presentation in the MLN and spleen is clearly distinct from the situation with other respiratory viruses and suggests a resurgence of lytic cycle virus. The peak of this second wave of Ag presentation occurs in the third week after infection and correlates with numerous events related with the establishment of latency. First, there is a major increase in the numbers of latently infected cells that peaks between days 14 and 18 before dropping to a stable low level of latency at around 3 wk postinfection (9, 14). Second, there is a large increase in activated CD8+ T cells in the spleen and blood starting around day 14 and then persisting for many months (12). This syndrome is highly reminiscent of the IM syndrome associated with EBV infection in humans. Third, there is also an expansion of Vb47/CD8+ T cells from day 14 after infection that persists for the life of the animal (12). This response does not seem to be classically MHC restricted, and the Ag that drives this response is thought to be expressed between days 13 and 19.8 The data presented here now add the observations that there is an increase in the presentation of lytic Ags and that there is a low level of lytic virus present at this time.

The increase in lytic Ag presentation and the presence of a low level of lytic cycle virus in the spleen at day 14 was surprising, since lytic cycle virus is reported to be cleared by this time (8, 14). It is currently unclear whether this represents virus that was not cleared during the acute infection or virus that has reactivated from a latent state. However, it seems unlikely that lytic cycle virus from the acute phase of the infection would increase at this time, given the strong immune response to the virus. Thus, we favor the hypothesis that the increase in Ag presentation is due to virus that has reactivated from latently infected B cells during the large expansion of latently infected cells. This hypothesis is consistent with the observation that the increase in Ag presentation correlates with the increase in infective centers. In addition, our data show that there is significant viral reactivation and a subsequent increase in Ag presentation that is revealed when spleen cells are not fixed before analysis (Fig. 2). A similar discrepancy between fixed and unfixed samples in the MLN was not observed at the acute phase of the infection (days 6 and 7), consistent with the idea that there is relatively little latency in the MLN at this time, and consequently, little reactivation of virus. Interestingly, we observed a small peak of Ag presentation in the spleen at day 7 when unfixed APC were used, suggesting that there is a peak in the number of latently infected cells in the spleen at this time. This is consistent with a report suggesting a small rise and fall in the numbers of infective centers in the spleen around day 5 after infection (8).

We had initially assumed that Ag would be presented predominantly by B cells and that it would be biased to epitopes derived from proteins expressed early in the replicative cycle, such as p56 and p79. The assumption was that the majority of B cells harboring reactivated virus would be cleared by the immune response before releasing infectious particles. However, the data indicate that there was strong presentation of the gB Ag on B cells, indicating that late viral genes were being expressed in these cells. In addition, lytic virus could be detected in the spleen, and there was substantial presentation of Ag by dendritic cells and macrophages, suggesting either secondary infection or secondary processing of viral particles. The fact that infective centers were detected in both dendritic cell and macrophage populations supports the general idea that these cells are associated with virus. In this regard, there is evidence for MHV-68 latency in non-B cell populations in B cell-deficient mice (30, 31). But this may not represent the situation in normal mice since the infection was i.p. established. Taken together, the data are consistent with the hypothesis that B cells harboring reactivating virus are being partially controlled by the immune system and that a low level of viral particles is being released. Indeed, other studies have shown that immunosuppressive regimens result in the reactivation of a lytic infection (14).

The observation that there is a second peak of Ag presentation explains the strong CD8+ T cell response to some lytic phase Ags for several weeks after the clearance of lytic virus from the lung (16). For example, we detected prolonged presentation of the p79K8 Ag in the spleen, which correlated with the kinetics of the T cell response to this Ag (16). However, we were only able to detect presentation of the p56/0 Ag in the MLN during the acute infection. Interestingly, the CD8+ T cell response to the p56 Ag peaked during the acute phase, around day 10, and was significantly reduced at later stages of the infection (16). It has been speculated that the change in T cell specificity during the course of infection reflects differences in Ag production and processing in different cell types (i.e., alveolar macrophages vs B cells). Our data are clearly consistent with this general idea.

The data presented here demonstrate the utility of using lacZ hybridomas for analyzing Ag presentation ex vivo. The advantage of this system is that it reliably detects low frequencies of APCs. In addition, the fact that T cells are used for Ag detection demonstrates that Ag is expressed in a relevant form. This is particularly important in the case of herpes viruses that can potentially interfere with Ag presentation and thus fail to present Ag even though the antigenic protein can be demonstrated to be present. Other approaches to analyze Ag presentation have also been described, such as the development of Abs specific for particular peptide MHC complexes (32, 33). These reagents are particularly useful for histochemical analysis of Ag presentation. But the hybridoma approach has the advantage that the reagents are very easy to generate and test and are highly specific, allowing one to assess multiple Ags, as was done here. We also show that the hybridomas can be introduced directly into the animal to confirm the presence of Ag in vivo. The key disadvantage of this and other techniques is that APCs expressing low levels of Ag will not be detected. Thus, the frequencies of APCs determined by this method are likely to be underestimates. Nonetheless, the data clearly reveal important information about the general kinetics of Ag presentation and can be used to unequivocally demonstrate the presence of Ag in certain organs or at certain time points.

Taken together, the data show that MHV-68 infection of C57BL/6 mice involves a complex interplay between the host and the virus. Viral Ags are present for some time after the initial infection of the lung has been cleared, and there is consequently a sustained period of Ag presentation. These data explain the kinetics of the CD8+ T cell response to MHV-68 infection and have implications for understanding the host response to γHV infections in general.

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