NK1.1+ Cells and Murine Cytomegalovirus Infection: What Happens In Situ?


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NK1.1<sup>+</sup> Cells and Murine Cytomegalovirus Infection: What Happens In Situ?<sup>1</sup>

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NK cells mediate early host defense against viral infection. In murine CMV (MCMV) infection NK cells play a critical role in controlling viral replication in target organs, such as spleen and liver. Until now it has not been possible to directly examine the role of NK cells in MCMV-induced inflammation in situ due to the inability to stain specifically for NK cells in infected tissues. In this study, we describe a method of in vivo fixation, resulting in the first identification of NK cells in situ using NK1.1 as the marker. Using this method, we characterize the NK1.1<sup>+</sup> cellular component of the inflammatory response to wild-type MCMV in the spleen, liver, and lung of genetically susceptible and resistant mice following i.p. infection. This study provides the first in situ description of the cellular response mediated specifically by NK cells following MCMV infection. *The Journal of Immunology, 2001, 166: 1796–1802.

Cytomegaloviruses are ubiquitous, host-specific pathogens capable of establishing life-long infections in immunocompetent hosts. Acute infection with murine CMV (MCMV)<sup>3</sup> elicits an immune response that is incapable of fully resolving the infection (1) and as a result the virus persists in the host, usually in a state of latency (2).

Several studies have highlighted the importance of NK cells as early mediators of host defense against viral infections (reviewed in Ref. 3), including acute infection with MCMV (4). Once activated, NK cells kill infected targets via effector mechanisms that vary at different physiological sites. In the spleen NK cells predominantly regulate MCMV replication via perforin-dependent cytolysis, whereas in the liver their effector function is mediated mainly via the production of IFN-γ (5). A subset of NK1.1<sup>+</sup> cells has been shown to limit the replication of MCMV in the spleen and, to a lesser extent, in the liver via operation of the Cmv1 locus (6). Cmv1 is an autosomal dominant locus first identified in mice of the MCMV-resistant C57BL/6J background, but not on the MCMV-susceptible BALB/c background, hence leading to the designation of these strains as Cmv1<sup>r</sup> and Cmv1<sup>s</sup>, respectively (7). Mice congenic to the BALB/c background have been produced to have C57BL/6J alleles for varying regions of the NK gene complex on mouse chromosome 6 (8). This complex encompasses the Cmv1 locus and the Ly55c gene that encodes NK1.1. These strains include the BALB.B6-Cmv1<sup>r</sup> strain, which has the NK1.1<sup>+</sup>, Cmv1<sup>r</sup> phenotype, and the BALB.B6-Ct6 strain, which is NK1.1<sup>+</sup>, Cmv1<sup>s</sup>. These strains are particularly useful to study NK cell function during virus infection because of the NK1.1<sup>+</sup> phenotype on both susceptible and resistant backgrounds.

In contrast to cells such as monocytes and neutrophils, it has been difficult to study the role of NK cells in situ due to the inability to use a specific marker for immunohistologic identification. The NK1.1 Ag is the most widely used murine pan NK marker. PK136, a mAb against NK1.1, was first described by Koo and Peppard in 1984 (9), but numerous attempts to detect NK1.1 in situ with this reagent have been unsuccessful. Several studies have identified NK cells on the basis of asialo-GM1, IFN-γ, or perforin expression (10–12); however, these markers are not specific for NK cells and can be detected on several other cell types, including macrophages and activated T cells. In situ histological analysis of NK cells therefore has been difficult, as it relies on subtractive analysis.

In this report, we describe the first demonstration of anti-NK1.1 staining in situ to identify NK cells using a modified in vivo perfusion/fixation method. Using this method, we have compared the acute NK1.1<sup>+</sup> cellular response to wild-type MCMV infection in the visceral organs of genetically susceptible intra-NK complex recombinant BALB.B6-Ct6 mice with that in resistant C57BL/6J and BALB.B6-Cmv1<sup>r</sup> mice (all NK1.1<sup>+</sup>). Expression of viral Ags and the consequences of infection on other cellular subsets have also been analyzed in this study. In susceptible mice the results demonstrate that MCMV infection in the marginal zones of splenic white pulp results in local changes in various cellular constituents, including macrophages and NK cells. In the liver distinct foci of infection were comprised of large numbers of macrophages and NK1.1<sup>+</sup> cells surrounding infected cytomegalic cells. In resistant mice, MCMV infection was predominantly in the red pulp of the spleen and was associated with increased NK1.1<sup>+</sup> cell and macrophage accumulation at sites of viral infection.

Materials and Methods

**Animals**

Inbred, specific pathogen-free C57BL/6J and BALB/c were obtained from the Animal Resources Center (Perth, Australia). The congenic mouse
strains BALB.B6-CT6 and BALB.B6-Cmv1, described previously (8), were bred in-house. All animal experimentation was performed with the approval of the animal ethics and experimentation committee of the University of Western Australia and according to the guidelines of the National Health and Medical Research Council of Australia.

**Cells**

Primary mouse embryo fibroblasts were grown in MEM (Life Technologies, Grand Island, NY) supplemented with 10% newborn calf serum (Life Technologies). Single-cell suspensions of mouse splenocytes were prepared in RPMI 1640 (Life Technologies) supplemented with 10% FCS (Tissue Culture Grade; CSL, Melbourne, Australia), l-glutamine (10 mM), 2-mercaptoethanol (20 µM), and antibiotics (100 µg/ml penicillin, CSL; 40 µg/ml gentamicin, Pharmacia & Upjohn, Sydney, Australia). To isolate splenocytes, spleens were dissociated in Dounce homogenizers (Kontes, Vineyard, NJ), and the dispersed cells were washed once in RPMI 1640 complete medium. Erythrocytes were lysed in NH4Cl, and the nucleated cells were washed an additional two times in complete medium to remove residual lysis buffer and undisrupted stromal elements. Splenocytes were suspended in mouse osmolarity-buffered saline (MOBS; 330 mosmol)/5% FCS for analysis by flow cytometry.

**Antibodies**

PE-conjugated anti-NK1.1 (PK136), CD11b (M1/70), CD4 (GK1.5), CD8 (53-6.72), and CD19 (ID3); FITC-conjugated anti-TCRβ (H57-596); biotin-conjugated anti-NK1.1 (PK136), biotin-conjugated anti-CD11c (HL3), and streptavidin-PE were purchased from PharMingen (San Diego, CA). Anti-asialo-GM1 was purchased from Wako Pure Chemicals (Osaka, Japan). The anti-MCMV mAb 3B2 has been described previously (13).

**Treatment of mice**

Groups of mice used for immunofluorescence analysis of tissue sections (two per group), FACS analysis (three per group), or plaque assays (three per group) were infected i.p. with 10^6 PFU of salmonella gland-propagated stocks of the virulent MCMV strain K181-Perth. Control animals were mock infected with MOBS/5% FCS. Mice were sacrificed at 1, 2, 3, 4, and 6 days after infection (p.i.), and tissue was collected as described below. Viral titers are expressed as the means from three infected animals.

**Removal of tissues and fixation protocols**

For in vitro fixation of tissues, mice were euthanized by cervical dislocation, and tissues were covered in Cryo-embed OCT (Tissue-Tek, Torrance, CA), snap frozen in liquid nitrogen, and stored at −80°C. For fixation of liver and spleen in situ, animals were anesthetized with Penthrane and perfused with 20 ml of the appropriate fixative via the left ventricle. Following perfusion/exsanguination, the mice were left at room temperature for 5 min to ensure appropriate penetration of tissues by the fixative. Following systemic perfusion, lungs were fixed by intratracheal injection of 10 ml of fixative. To ensure that lung architecture was maintained, perfusion was performed at a rate of 2 ml/min. Tissues were then removed and stored at −80°C.

**Table I. Fixation of tissue from unperfused animals**

<table>
<thead>
<tr>
<th>Fixative</th>
<th>Time (min)</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone</td>
<td>5</td>
<td>4°C</td>
</tr>
<tr>
<td>4% Parafomaldehyde</td>
<td>5</td>
<td>RT</td>
</tr>
<tr>
<td>PLP</td>
<td>5</td>
<td>24–26°C</td>
</tr>
<tr>
<td>Carnoy’s Fixative</td>
<td>15</td>
<td>RT</td>
</tr>
</tbody>
</table>

* RT, Room temperature.

**Preparation of tissue sections and immunofluorescence**

Sections (6 µm) were cut on a cryostat and mounted onto Silane (ICN Pharmaceuticals, Aurora, OH)-coated slides. Untreated tissues were fixed as described in Table I before rehydration in MOBS for 5 min. Fixed tissues were rehydrated, and after this step both types of tissue were treated identically. Paraformaldehyde-association autofluorescence was quenched by incubation in 50 mM NH4Cl for 10 min at 4°C before blocking with 5% normal goat serum (NGS) in MOBS containing 3% BSA for 30 min at room temperature. Primary Abs diluted in MOBS with 3% BSA were then added for 1 h at 4°C followed by the appropriate secondary reagent for 30 min at room temperature. Secondary Ab reagents were diluted in MOBS with 5% NGS and streptavidin-conjugated fluorochromes were diluted in 5% skin milk powder. Washing between each step was performed three times with MOBS. Sections were mounted in 50% glycerol/MOBS and visualized by either standard epifluorescence or on a confocal microscope (MRC 1000/1024 UV laser scanning confocal microscope; Bio-Rad, Hemel, U.K.). A focal series of seven horizontal planes of section spaced by 0.5 µm was monitored sequentially for FITC and PE using the 488- and 543-nm laser lines of an argon or a green helium-neon laser, respectively, a double-dichroic mirror for the excitation beam, an FITC band-pass 522/535-nm laser line, and a 580/32-nm filter for PE. The FITC/PE eight-bit-encoded 768- × 512-pixel images from the same plane of section were superimposed and visualized with a red/green pseudo color scale on a true color display monitor before printing using a Codonics NP-1600 printer (Codonics, Middleburg Heights, OH).

**Flow cytometry**

Flow cytometric analysis of splenocytes was performed using the mAbs described above. Cells were resuspended in 1 × 10^6/ml and nonspecific reactivity was blocked in MOBS/5% FCS/5% NGS for 30 min on ice. Labeling was performed for 60 min with the appropriate primary mAb, then with the second-step reagent for 30 min. All incubations were performed at 4°C, with three washes in MOBS/5% FCS between steps. Propidium iodide (2 µg/ml) was incorporated into the final wash to exclude dead cells. The labeled cells were then analyzed on a FACSscan (Becton Dickinson, San Jose, CA), and files of 10,000 events were collected and analyzed using CellQuest software (Becton Dickinson).

**In vivo growth of MCMV**

Mice were inoculated with the virulent MCMV strain K181-Perth as described above. At the designated times postinoculation, the animals were sacrificed, and spleens and livers were removed. All organs were individually weighed, homogenized in cold MEM/25% newborn calf serum, and centrifuged at 1,800 × g for 10 min at 4°C. Supernatant was stored at −80°C, and viral titers were subsequently quantified on mouse embryo fibroblasts using a standard plaque assay (15).

**Results**

**Identification of NK1.1+ cells in situ**

In situ identification of NK cells using the NK1.1 marker recognized by mAb PK136 has been elusive to date. To determine whether the inability to detect NK1.1 may be due to inappropriate fixation of this Ag, seven different fixation protocols were applied to cryostat sections of mouse spleen in an attempt to establish a reliable technique that allows in situ identification of cells expressing the NK1.1 Ag (Table II). Visualization of the NK1.1 marker was only possible after perfusion fixation before snap-freezing and cryostat sectioning. PLP fixation showed superior results to fixation with 4% paraformaldehyde (Table II), while fixation ex vivo resulted in no visible staining with PK136. Detection of other Ags, such as CD11b, was not dependent on the fixation protocol (Table II). Tissue architecture and Ag preservation were maintained optimally when fixation was performed for a minimum of 5 min postexsanguination.

To confirm the specificity of the PK136 Ab in situ, tissues from C57BL/6j (NK1.1+), BALB.B6-CT6 (NK1.1+), BALB.B6-Cmv1 (NK 1.1+), and BALB/c (NK1.1-) mice were tested. The resulting solution was added to three parts of lysine phosphate buffer supplemented with 2.5 mg/L sodium periodate and 600 µL/L glutaraldehyde. All solutions were stored at room temperature and used within 12 h of preparation.
expression of the NK1.1 Ag in these mouse strains is shown in Table III along with the strain susceptibility to MCMV infection and the Cmv1 phenotype (8). In all cases, fluorescent signal was only detected in NK1.1⁺ mouse strains. These data were complemented by dual labeling with anti-asialo-GM1 or TCRβ and confocal analysis (Fig. 1). Fig. 1A shows asialo-GM1⁺ cells that are NK1.1−, but no NK1.1⁺ cells that are asialo-GM1−. The ratio of asialo-GM1⁻/NK1.1⁺ to asialo-GM1⁺/NK1.1− was ~5:1. Fig. 1B shows that NK1.1⁺ cells can be distinguished from TCRβ⁺ and NK1.1⁻/TCRβ⁺ (NKT) cells. The distribution and localization of CD11b⁺ and NK1.1⁺ cells identified following MCMV infection are described below.

**Characterization of NK1.1⁺ and CD11b⁺ cellular responses to MCMV in situ**

Having devised a method for the specific detection of NK1.1⁺ cells in situ, immunohistologic studies were undertaken to determine the roles of these and other cell types to the response that follows MCMV infection.

**In the spleen.** Titers of MCMV in the spleens of susceptible mice were detectable by plaque assay on day 2 p.i. and peaked on day 4 (BALB/c day 2, 2.4 × 10⁵ PFU; day 4, 2.1 × 10⁶; day 6, 4.5 × 10⁶; BALB.B6-CT6, see Table IV) with MCMV Ag first detected by immunofluorescence in the splenic margins 2 days p.i. In susceptible BALB/c and BALB.B6-CT6 mice, a positive fluorescent signal to an MCMV intracellular Ag detected by mAb 3B2 was observed in the splenic margins by day 2 postinfection and became prominent in these areas 3–4 days p.i. (Fig. 2A). By day 6, the infection appeared to have been resolved, with low levels of staining for MCMV Ag evident only in the red pulp (Fig. 3A). The temporal appearance and intensity of staining of MCMV Ag were consistent with the titers of NK1.1⁺ detected in the spleen by plaque assay (Table IV).

In situ detection of NK1.1⁺ cells in uninfected, susceptible BALB.B6-CT6 mice showed a sparse distribution of cells, mainly within the red pulp regions. Concomitant with the early detection of MCMV Ag in situ, detection of NK1.1⁺ cells with mAb PK136 showed localization of NK1.1⁺ cells to the marginal zones of the white pulp on day 2 p.i. (Fig. 3B). Thereafter, NK1.1⁺ cells were only identified in the red pulp, where by day 6 p.i. the foci of NK1.1⁺ cells had markedly increased (Fig. 3B). Similar to NK1.1⁺ cells, CD11b⁺ macrophages were found in the red pulp by 18 h p.i., but were more detectable in the marginal zones of the white pulp 1 day p.i. (Figs. 2B and 3C). Accumulation of these cells in discrete foci was noted in both the red and white pulp regions on days 3 and 4 p.i., with an increase in cell numbers in these foci by day 6 (Table IV and Fig. 3C). Alterations to the number or distribution of CD4⁺ and CD19⁺ cells was unremarkable during this study. The number of CD8⁺ cells, especially CD11b⁺/CD8⁺ double-positive cells, increased on day 6 postinfection, particularly in susceptible strains (data not shown).

In contrast to the findings in susceptible mice, MCMV Ag was detected at very low levels in the marginal zones of splenic follicles of resistant C57BL/6J and BALB.B6-Cmv1⁺ mice (Fig. 3D). This correlated with the significantly lower titers of MCMV recovered from the spleens of resistant mice at the times and virus dose used in study (Table IV). Some MCMV-infected cells were also detected in the red pulp. At 6 days p.i. MCMV Ags were no longer detected by in situ analysis (Fig. 3D). The low levels of virus detected correlated with a significant increase in NK1.1⁺ cell numbers from day 2 onward (Table IV and Fig. 3E). In situ analysis showed accumulation of NK1.1⁺ cells into foci of the red pulp and in the margins of the white pulp as early as day 2 p.i. In resistant strains, NK1.1⁺ cells were present in significantly greater numbers from days 2 to 4 p.i. compared with the susceptible BALB.B6-CT6 mice, consistent with the number of NK1.1⁺ splenocytes detected by FACS analysis. Clusters of CD11b⁺ macrophages were observed at 2 days p.i. and reached significantly higher levels in the spleen than those observed for susceptible mice at days 2–4 p.i. (Table IV and Fig. 3F).

**In the liver.** Previous studies have looked at the presence of NK cells in the livers of MCMV-infected animals. However, the analysis was somewhat complicated by the use of asialo-GM1 as the marker for NK cells given the cross-reactivity with other cell types (10–12, 16, 17). In addition, the analysis was restricted to the MCMV-resistant C57BL/6J strain. Here, we specifically evaluate the role of NK1.1⁺ cells in MCMV-induced liver inflammation and compare the composition of the inflammatory infiltrate in NK1.1⁻/resistant and susceptible mouse strains. Viral titers in the liver of susceptible (BALB/c; BALB.B6-CT6) mice were highest at 2 (BALB/c, 4.6 × 10⁵ PFU; BALB.B6-CT6, 2.0 × 10⁶ PFU) and 4 (BALB/c, 6.3 × 10⁵ PFU; BALB.B6-CT6, 4.5 × 10⁶ PFU) days p.i. before decreasing by day 6 (BALB/c, 4 × 10⁵ PFU; BALB.B6-CT6, 1.8 × 10⁵ PFU). Although equivalent titers were observed in resistant mice (C57BL/6J and BALB.B6-Cmv1⁺) at days 2 and 4 p.i. (~2 × 10⁵ and 3 × 10⁵ PFU), infectious virus was undetectable by day 6 p.i. In susceptible and resistant strains of mice, MCMV Ag was first identified at 2 days p.i. in numerous

### Table II. Results of different fixation protocols on the ability to detect specific cellular Ags

<table>
<thead>
<tr>
<th>Fixation Method</th>
<th>Fixative</th>
<th>Spleen</th>
<th>Liver</th>
<th>Lung</th>
<th>NK1.1</th>
<th>CD11b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ex vivo tissue section on slide</td>
<td>Acetone</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Carnoy’s fixative</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>No fixative</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>4% Paraformaldehyde</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>PLP</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>In vivo whole mouse perfused</td>
<td>4% Paraformaldehyde</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>PLP</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*Ag visualization by fluorescence represented by +, where ++ > +.

### Table III. NK1.1 and Cmv1 phenotype in the mouse strains used in this study

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>Cmv1 Phenotype</th>
<th>NK1.1 Ag</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c</td>
<td>Susceptible (Cmv1⁺)</td>
<td>Negative</td>
</tr>
<tr>
<td>C57BL/6J</td>
<td>Resistant (Cmv1⁻)</td>
<td>Positive</td>
</tr>
<tr>
<td>BALB.B6-CT6</td>
<td>Susceptible (Cmv1⁺)</td>
<td>Positive</td>
</tr>
<tr>
<td>BALB.B6-Cmv1⁺</td>
<td>Resistant (Cmv1⁻)</td>
<td>Positive</td>
</tr>
</tbody>
</table>

* Refer to Ref. 12 for development of the congenic strains.
foci throughout the liver parenchyma, with infected cells showing classic signs of cytomegaly. The foci of infection were frequently associated with NK cells at this time (Fig. 4). By 4 days p.i. these cells were surrounded by foci of inflammation consisting of large numbers of macrophages (CD11b⁺) and NK (NK1.1⁺) cells. Moderate numbers of CD4⁺ T cells were observed in tissues from Cmv+ strains.

In the lungs. The role of different cellular constituents in the inflammatory response to MCMV was also examined in the lungs to determine whether the same cellular subsets mediate inflammatory responses at different physiological sites. Firstly, we characterized the extent of MCMV infection at this site. Viral titers were first observed in the lungs of susceptible mice at 4 days p.i. (<10^2 PFU), but reached titers of ~10^3 PFU at 6 days p.i. Immunofluorescence analysis with MCMV mAb showed low levels of viral Ags in the lungs of susceptible mice at 4 days p.i. This finding was associated with preferential accumulation of macrophages (CD11b⁺) and dendritic cells (CD11c⁺) at the sites of infection. However, the clusters of inflammatory cells associated with infection were small compared with those seen in spleen and liver. By 6 days p.i. a low level of virus infection was still detectable, and MCMV-positive cells were found in association with an infiltration of NK (NK1.1⁺) cells (Fig. 5). No evidence of infection with MCMV was identified in the lungs of resistant mice at the virus dose used in this study.

Discussion

Previous data have characterized the number and the cytotoxicity of NK1.1⁺ cells ex vivo following viral infection. However, very little is known about the nature of the NK1.1⁺ cellular response in vivo. The successful in situ detection of NK1.1⁺ cells in this study was performed using the PK136 mAb following perfusion fixation with PLP. PLP fixation may facilitate recognition of NK1.1 via oxidation of carbohydrate moieties by periodate followed by lysine-mediated cross-linking of the newly formed aldehyde groups. This fixative is likely to preserve the antigenic structure of NK1.1 in a configuration closest to its native form. Recognition of NK1.1⁺ cells following PLP incubation was absolutely dependent upon in vivo fixation, with tissue architecture and Ag preservation best obtained following the method described. This method of in situ detection of NK1.1⁺ cells allows analysis of NK cell interactions with other cells of the immune system in situ and hence will be of great value, since it will enable the specific analysis of NK cells and their roles in diseases including cancer, allergy, and viral infection.

Double labeling with PK136 and anti-asialo-GM1 (Fig. 1A), shows that all NK1.1 cells are asialo-GM1 positive, but the reverse does not hold true. This is expected, since NK1.1 is thought to be specific for NK cells, whereas asialo-GM1 has been shown to be expressed on NK cells, activated T cells, and macrophages (10–12, 16, 17). We have also shown that it is possible to separate NK cells from NKT cells on the basis of TCRβ expression (Fig. 1B).

In addition to describing the first specific identification of NK cells in situ we report on the inflammatory response that follows infection with MCMV and compare it in spleen, liver, and lungs of susceptible and resistant mouse strains. The extent of virus Ag detected by immunofluorescence correlated with titers of infectious virus recovered during acute infection. In the spleen sequential section analysis indicated that MCMV infection was occurring...
primarily in cells that were CD11b\(^+\) (Fig. 2), consistent with previous studies that have detected MCMV infection in mononuclear cells of peripheral tissues (18–20). Given the rapid accumulation of CD11b\(^+\) cells to the spleen, liver, and lungs, this population may represent critical players in the early dissemination of MCMV to peripheral tissues and/or the establishment of latency. In the spleen, monocytes are known to selectively migrate into the marginal zones (21) in a pattern consistent with that observed in this study. An increase in the number of CD11b\(^+\)/CD8\(^+\) cells was noted at day 6 p.i. McFarland and colleagues have previously shown that a subset of freshly isolated T cells taken after lymphocytic choriomeningitis virus infection is also CD11b\(^+\) (22). In that study these cells were recognized as the active effector and virus-specific memory CTLs. Our study focuses on the acute phase of MCMV infection; hence, the observed CD11b\(^+\)/CD8\(^+\) cells probably represent the active stage of CTL proliferation.

In the spleen, both FACS and in situ analysis demonstrated an earlier increase in NK1.1\(^-\) cells in splenocytes from resistant, compared with susceptible, mice. Nevertheless, both susceptible and resistant mice showed high levels of NK1.1\(^-\) cells 6 days p.i. (Table IV). At this time postinfection, the increase in NK1.1\(^-\) cells is partly due to NKT cells. The role of NKT cells in the response of susceptible and resistant strains of mice to infection with both wild-type and mutant MCMV (lacking specific open reading frames) has been analyzed and will be reported separately (E. H. Densley et al., manuscript in preparation).

In the liver of Cmv1\(s\) and Cmv1\(r\) mice, MCMV was first detected in situ at 2 days p.i. Peak titers of virus were equivalent in these strains at day 4 p.i, which was expected, since Cmv1 confers NK cell-mediated resistance to MCMV infection predominantly in the spleen and to a lesser extent in the liver (7). In both strains of mice, foci of infection were associated with prominent infiltration.

**Table IV. Analysis of viral replication and changes in proportions of splenocytes expressing NK1.1\(^-\) and CD11b\(^+\) following MCMV infection of NK1.1\(^-\)-resistant C57BL/6J and susceptible BALB.B6-CT6 mouse strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Day p.i.</th>
<th>FACS Analysis(^a)</th>
<th>Viral Titer(^b) (PFU/spleen)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>NK1.1</td>
<td>CD11b(^+)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(6.4 \pm 0.5)</td>
<td>(29.4 \pm 2.3)</td>
</tr>
<tr>
<td>BALB.B6-CT6</td>
<td>MOBS</td>
<td></td>
<td>(1.3 \times 10^9 \pm 0.6^c)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>(6.7 \pm 0.4)</td>
<td>(37.2 \pm 1.7^d)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>(5.6 \pm 0.7)</td>
<td>(37.5 \pm 3.5^e)</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>(15.6 \pm 1.2^f)</td>
<td>(44.2 \pm 9.3^e)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(1.8 \times 10^3 \pm 0.3^c)</td>
</tr>
<tr>
<td>C57BL/6J</td>
<td>MOBS</td>
<td>(7.4 \pm 1.1)</td>
<td>(38.6 \pm 3.2)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>(10.2 \pm 0.8^d)</td>
<td>(43.0 \pm 1.7)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>(9.0 \pm 1.4)</td>
<td>(54.3 \pm 1.4^e)</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>(12.0 \pm 1.0^f)</td>
<td>(53.3 \pm 4.8^d)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(0.4 \times 10^2 \pm 0.2^c)</td>
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\(^a\) FACS values are expressed as mean percentage of 10,000 live cells from three animals ± SEM.

\(^b\) Viral titers are shown as mean PFU/spleen of three mice ± SEM.

\(^c\) NA. Not applicable.

\(^d\) Significantly different from MOBS controls.

\(^e\) Significantly different between strains at the same sampling points.

**FIGURE 3.** Diagramatical representation of the NK1.1\(^-\) and CD11b\(^+\) cellular response to MCMV infection of the spleen (as detected using the 3B2 Ab). Large white circles represent the margins of the white pulp with the periarteriolar lymphoid sheath located inside. Sections through the spleen represent the time point of sampling from 6 h to 6 days p.i. MCMV Ag (a and d), NK1.1\(^-\) cells (b and e), and CD11b\(^+\) cells (c and f). Susceptible mice are represented on the left (a–c), while resistant mice are on the right (d–f).
of NK cells and macrophages by 4 days p.i. (Fig. 4). Dual labeling of infected livers at days 4–6 p.i. indicated that most CD8+ cells were also CD11b+ and CD11c+, probably representing activated CTLs. Although CD4+ T cells were observed in the livers of susceptible mice, these cells were absent in livers from infected resistant mice, consistent with previous data demonstrating that T cell responses are not required for the control of virus replication in the livers of resistant C57BL/6J mice (23). The absence of T cells in the livers of resistant mice may be a consequence of the early control of MCMV by NK cells in the spleen.

Lastly, we studied the inflammatory response to MCMV infection in the lung. Compared with those in spleen and liver, MCMV titers in the lung are lower and are detected later following i.p. infection. Nevertheless, accumulating CD11b+ macrophages, CD11c+ dendritic cells, and NK1.1+ cells were observed surrounding infected cells. Despite the appearance of NK cells, it is unclear whether these cells play a role in restricting early MCMV replication in the lung. Indeed, NK cells are important in preventing the dissemination of MCMV into the lung, but do not appear to play a crucial role with respect to viral clearance at this site (24).

The accumulation of macrophages observed in the lung postinfection with MCMV may be important for the establishment of viral latency at this site. Indeed, MCMV has been identified in macrophages of the lung alveolus 6 mo p.i. (2), and it has been postulated that this may result from viral invasion of alveolar macrophages during the acute stages of infection.

It is now clear that both the host genotype and the presence of viral immune modulators contribute to MCMV pathogenesis and virulence. A better understanding of the NK cellular response evoked by MCMV will improve our understanding of how specific cell types modulate the course of viral dissemination and persistence. Furthermore, identification of NK and other cell types involved in combating wild-type infection will be critical in determining how MCMV evades or subverts the immune response through comparison of cellular responses elicited by MCMV mutants deleted of potential immune modulators. With the in situ detection of NK1.1 reported here, it is now possible to better understand the role of NK cells in viral infection, cancer, and other disease states.

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References


