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Activation and Inactivation of Antiviral CD8 T Cell Responses during Murine Pneumovirus Infection

Erwin A. W. Claassen,1* Patrick A. A. van der Kant,* Zuzana S. Rychnavska,* Grada M. van Bleek,† Andrew J. Easton,‡ and Robbert G. van der Most3*

Pneumonia virus of mice (PVM) is a natural pathogen of mice and has been proposed as a tractable model for the replication of a pneumovirus in its natural host, which mimics human infection with human respiratory syncytial virus (RSV). PVM infection in mice is highly productive in terms of virus production compared with the situation seen with RSV in mice. Because RSV suppresses CD8 T cell effector function in the lungs of infected mice, we have investigated the nature of PVM-induced CD8 T cell responses to study pneumovirus-induced T cell responses in a natural virus-host setting. PVM infection was associated with a massive influx of activated CD8 T cells into the lungs. After identification of three PVM-specific CD8 T cell epitopes, pulmonary CD8 T cell responses were enumerated. The combined frequency of cytokine-secreting CD8 T cells specific for the three epitopes was much smaller than the total number of activated CD8 T cells. Furthermore, quantitation of the CD8 T cell response against one of these epitopes (residues 261–270 from the phosphoprotein) by MHC class I pentamer staining and by in vitro stimulation followed by intracellular IFN-γ and TNF-α staining indicated that the majority of pulmonary CD8 specific for the P261 epitope were deficient in cytokine production. This deficient phenotype was retained up to 96 days postinfection, similar to the situation in the lungs of human RSV-infected mice. The data suggest that PVM suppresses T cell effector functions in the lungs. The Journal of Immunology, 2005, 175: 6597–6604.

Human respiratory syncytial virus (RSV)4 and human metapneumovirus are the leading causes of lower respiratory tract infections in infants and children (1, 2). A striking feature of RSV infection is the common occurrence of reinfections throughout life (3, 4), which cannot be fully explained by antigenic variation of the virus. Although the glycoprotein (G) shows considerable antigenic variation (giving rise to the two major antigenic groups A and B), neutralizing Abs against the fusion protein (F) are much more cross-reactive between the groups (5). Frequent reinfections are also observed in calves infected with the related bovine RSV (6). This indicates that natural infection does not confer adequate, long-lived immunity.

The immunology of RSV infection has been most extensively studied in the murine (BALB/c) infection model. Data from this model have implicated CD8 T cells as key mediators of protective immunity (7–10). Immunization with CD8 T cell epitopes (10), or recombinant viruses expressing such epitopes (11), induces protective immunity. However, RSV-specific CD8 T cells in the lungs have been shown to undergo functional inactivation during infection, and this anergic phenotype is retained long after the virus is cleared, suggesting that RSV-specific CD8+ effector-memory in the lungs is partly dysfunctional (12, 13). Ostler and Ehl (7) have argued that this immunosuppression does not impair protective immunity because RSV-primed mice were protected against recombinant vaccinia viruses expressing RSV proteins. However, protection against (intranasal) vaccinia virus challenge does not depend on lung-resident effector-memory cells (14), and the implications of these results for immunity to natural RSV infections are not at all clear.

One possible disadvantage of the murine RSV infection model is the species-specificity of pneumoviruses. Although the RSV BALB/c infection model has yielded excellent results, it depends, in part, on the assumption that the human-adapted RSV proteins interact with murine host factors. It is now clear that at least five RSV proteins, the nonstructural proteins 1 and 2 (NS1 and NS2), the SH protein, and the F and G glycoproteins, interact with the immune system (15–20). Some of these effects, such as the F protein-mediated inhibition of lymphocyte proliferation (19), appear to be species specific. One way to circumvent this potential problem is to study natural virus-host combinations, in parallel to the murine RSV model. Bovine RSV infection in cattle closely mimics human infection (21) but is limited by a lack of reagents and high costs. Pneumonia virus of mice (PVM) would be a good candidate for an authentic pneumovirus model in mice. PVM is a natural mouse pathogen and induces a severe granulocytic bronchiolitis that is associated with extensive viral replication (22–25). PVM infection in mice resembles severe human RSV infection more closely than murine RSV infection does in terms of viral load and pathogenesis (22, 25). Because both the murine RSV and PVM models have advantages and disadvantages, a deeper insight in PVM T cell immunology would extend and build on the work done with RSV.
In the present study, we have used the PVM infection model to investigate antiviral CD8 T cell responses, including possible CD8 T cell inactivation, in a natural pneumovirus-host interaction. We have identified the first CD8 T cell epitopes to be described for this virus and have used these to examine antiviral responses by MHC class I multimer staining and functional assays. The data indicate that murine pneumovirus infection is associated with a massive influx of activated CD8 T cells into the lungs. However, pulmonary (but not splenic) CD8 T cell responses specific for a codominant epitope in the phosphoprotein (P) displayed a defect in their capacity to produce the proinflammatory cytokines IFN-γ and TNF-α. The inactivation was more extreme than that observed in RSV-infected mice (12, 13) and suggests that a productive pneumovirus infection may severely impair the functionality of CD8+ effector-memory in the lungs. The consequences of these findings for pneumovirus immunity will be discussed.

Materials and Methods

**Mice, virus, and infections**

Five- to 6-wk-old female BALB/c ByJ/co mice were obtained from Charles River Nederland. Mouse-passaged stocks of PVM strain J3666 (~1 × 10^7 PFU/ml) and a stock of RSV A2 (~5 × 10^5 PFU/ml) were used for infection experiments. Influenza virus A/PRI/8/34 was generously provided by Dr. A. Nowak (University of Western Australia, Nedlands, Australia). Viral stocks were defrosted and diluted in PBS immediately prior intranasal inoculation (50 μl) (50–400 PFU for PVM, 2.5 × 10^5 PFU for RSV and 0.5 hemagglutination units (~5 × 10^5 hemagglutination units) for influenza PR8) under light ether or isofluorane anesthesia. The mouse study protocol was approved by the Animal Ethics Committee of the Veterinary Faculty of the University of Utrecht.

**Peptides**

The PVM proteome (26), with the exception of the L protein, was screened for predicted epitopes for the H-2D^d, H-2K^d, and H-2L^d alleles, using the peptide prediction algorithms of SYFPEITHI (www.syfpeithi.de) (27) and Bioinformatics and Molecular Analysis Section (BIMAS) web sites (http://bimas.dcnr.nih.gov/molbio/hla_bind) (28). Only the predicted epitopes with the highest scores in both databases were chosen. All peptides were synthesized by the Peptide and Protein Facility (Department of Immunology, Utrecht University). A summary of epitopes per viral protein is provided in Table I. A full list of peptides is available online.

**Peptide immunization**

Peptides P_{261-270} (50 nmol/mouse) and OVA_{323-339} (KISQAVHAA HAEINEAG) (50 nmol/mouse) were emulsified in IFA. The control formulation consisted of an emulsion of PBS and IFA. Four-week-old HAINEAG) (50 nmol/mouse) were emulsified in IFA. The control for-

**Sampling and tissue preparation**

Mice were sacrificed by i.p. injection of sodium pentobarbital. Before recovery, the lungs were perfused with PBS containing 50 U/ml heparin.

Table I. Distribution of MHC I motif binding peptides in the PVM proteome

<table>
<thead>
<tr>
<th>Protein</th>
<th>D^a</th>
<th>K^d</th>
<th>L^d</th>
<th>Epitopes</th>
</tr>
</thead>
<tbody>
<tr>
<td>NS1</td>
<td>2</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NS2</td>
<td>3</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>4</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>4</td>
<td>1</td>
<td></td>
<td>P_{261-270} CYLTDRARl (K^d)</td>
</tr>
<tr>
<td>M</td>
<td>3</td>
<td>8</td>
<td></td>
<td>M_{43-52} LPKNSVMDL (L^d)</td>
</tr>
<tr>
<td>M2</td>
<td>3</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SH</td>
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<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>6</td>
<td></td>
<td></td>
<td>F_{304-315} WYCHNAGSL (K^d)</td>
</tr>
<tr>
<td>G</td>
<td>1</td>
<td>5</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

*Numbers of motif-fitting peptides per viral protein and their distribution over the restriction elements are indicated. Peptide sequences of the three epitopes are shown. Anchor residues are shown in bold.

In vitro stimulations

For intracellular cytokine staining, the lung and spleen cells (1 × 10^6) were stimulated in 96-well flat-bottom plates in 200 μl of RPMI 1640 medium (Invitrogen Life Technologies), containing 10% FCS, 1% l-glutamine, 5 μg/ml peptide, and 10 μg/ml brefeldin A (Sigma-Aldrich) for 4 h at 37°C, 5% CO_2.

ELISPOT assay

The IFN-γ ELISPOT was performed using the mouse IFN-γ ELISPOT pair (BD Biosciences) and MultiScreen-IP filter plates (Millipore), according to the instructions of the manufacturer. Cells where stimulated in 200 μl of RPMI 1640 medium (Invitrogen Life Technologies), containing 10% FCS, 1% l-glutamine, and 5 μg/ml peptide for 24 h at 37°C, 5% CO_2.

**Staining and flow cytometry**

The following Abs were used: anti-CD3 PerCP (145-2C11), anti-CD8 al-llophycocyanin and FITC (Ly-2), anti-CD11a FITC (2D7), anti-CD62L PE (MEL-14), anti-IFN-γ FITC (XMG1.2), and anti-TNF-α PE (MP6-XT22) were purchased from BD Pharmingen and anti-NKG2A FITC (28D5) was purchased from eBioscience. PE-labeled P_{261-270} H-2K^d pentamer and tetramers were purchased from ProImmune. The pentamer construct was used for all experiments shown in this article. The P_{261} tetramer was solely used as a control for specific CD8 T cell staining and to confirm the inactivated phenotype. The allophycocyanin-labeled RSV M2 H-2K^d tetramer was provided by the MHC Tetramer Core Facility of the National Institute of Allergy and Infectious Disease. The PE-labeled influenza virus HA H-2K^d tetramer (H_{A9-23,44-48} IYSTVASSL) was provided by Dr. A. Currie (University of Western Australia). Intracellular stainings were done using the CytoFix/CytoPerm solution and Perm/Wash buffer purchased from BD Pharmingen according to the instructions of the manufacturer. Cells were acquired on a FACScalibur flow cytometer (BD Biosciences) using CellQuest software. Data analysis was done with CellQuest (BD Pharmingen) and FlowJo (Tree Star) software.

Results

PVM infection in mice induces activation and expansion of CD8 T cell in the lungs

Infection of BALB/c mice with relatively low doses of PVM strain J3666 results in lethal pneumonia (22, 23, 29). Intranasal inoculation of 200 or 400 PFU of the stock of PVM J3666 used here resulted in lethal infection, with mice succumbing to the infection at day 8–10 postinfection (p.i.). A nonlethal dose of 50 PFU was associated with pneumonia and weight loss (data not shown).

To assess the extent of CD8 T cell activation in the lungs of mice undergoing a nonlethal PVM infection, cells were stained for the expression of NKG2A, CD11a (LFA-1), and L-selectin of effector CD8 T cells in the lungs (31). In addition, CD11a can be used as a surrogate marker for virus-specific CD8 T cells while activation of effector CD8 T cells in the lungs (31). The expression of NKG2A, CD11a (LFA-1), and L-selectin was associated with pneumonia and weight loss (data not shown).

Infection of BALB/c mice with relatively low doses of PVM strain J3666 results in lethal pneumonia (22, 23, 29). Intranasal inoculation of 200 or 400 PFU of the stock of PVM J3666 used here resulted in lethal infection, with mice succumbing to the infection at day 8–10 postinfection (p.i.). A nonlethal dose of 50 PFU was associated with pneumonia and weight loss (data not shown).

To assess the extent of CD8 T cell activation in the lungs of mice undergoing a nonlethal PVM infection, cells were stained for the expression of NKG2A, CD11a (LFA-1), and L-selectin (CD62L). As demonstrated recently by Gold et al. (30), NKG2a is a useful surrogate marker for virus-specific CD8 T cells while expression of CD11a has been shown to play a role in the retention of effector CD8 T cells in the lungs (31). In addition, CD11a can also be used as a surrogate marker for virus-specific CD8 T cells (32). CD62L down-regulation identifies effector and effector-memory CD8 T cells (14). Staining of pulmonary CD8 T cells at day 8 p.i. revealed that 40% had up-regulated NKG2a expression, whereas only 2% of CD8 T cells in the lungs of uninfected mice expressed NKG2A (Fig. 1a). Further analysis showed that ~40% of the CD8 T cells in the lungs of infected mice displayed CD11a^{high} and CD62L^{low} phenotype (Fig. 1b), consistent with an activated phenotype. PVM infection also resulted in a mild lymphopenia with spleen cell counts 2.5-fold reduced in comparison to
normal levels. Thus, acute PVM infection in BALB/c mice is associated with a large influx of activated CD8 T cells in the lungs and a simultaneous decrease in the total number of splenocytes.

**Detection of virus-specific T cell responses in the spleen and in the lungs of infected mice**

Although the presence of large numbers of activated CD8 T cells in the lungs of PVM-infected mice suggested that the virus induced strong antiviral T cell responses, analysis of the expression patterns of activation markers does not establish specificity. Because no MHC class I-restricted PVM epitopes have been described so far, it was necessary to identify the antigenic targets of the response. To do this, most of the PVM proteome (9 of 10 viral proteins, excluding the L protein) was scanned for the presence of potential CD8 T cell epitopes using the algorithms predicting MHC class I binding for the H-2D\(^d\), H-2K\(^d\), and H-2L\(^d\) alleles (27, 28, 33). As shown in Table I, analysis of the 9 viral proteins yielded a total of 56 motif-fitting peptides. Splenocytes and lung lymphocytes, harvested from infected mice at day 8 p.i., were stimulated with synthetic peptides representing all of the predicted epitopes, and responses were quantified using IFN-\(\gamma\) ELISPOT and intracellular cytokine staining. Splenocytes from infected mice responded positively to three of the predicted MHC class I binding peptides, which are derived from the P (peptide P\(_{261}\)), matrix protein (M; peptide M\(_{43}\)), and the F protein (peptide F\(_{304}\)) (Fig. 2a and Table I). The epitopes in the P and F proteins contained the canonical H-2K\(^d\) binding motif, whereas the M protein epitope contained the H-2L\(^d\) binding motif (Table I). The frequencies of PVM-specific CD8\(^+\) T cells in the spleen were low, with 14, 42, and 69 specific spots per 5 \(\times\) 10\(^5\) splenocytes for the F\(_{304}\), M\(_{43}\), and P\(_{261}\) epitopes, respectively (Fig. 2a). This is equivalent to frequencies of 0.02–0.1% of splenic CD8 T cells. These responses were at the limit of detection for the intracellular cytokine staining assay (data not shown). Stronger CD8 T cell responses were observed in the lungs of infected animals. Responses against all three epitopes were detected at frequencies of 1.5–2% of lung CD8 T cells at day 8 p.i. (Fig. 2b). No clear immunodominance hierarchy among the three epitopes was detected with the epidopes appearing to be codominant.

**P\(_{261}\)-specific CD8 T cells in the lungs are functionally inactivated**

The relatively low frequency of PVM-specific pulmonary CD8 T cells, as compared with the specific responses in RSV-infected mice (7, 12), appears to be inconsistent with the high numbers of activated cells (NKG2A\(_{\text{high}}\), CD11a\(_{\text{high}}\), and CD62L\(_{\text{low}}\); Fig. 1) in
the lungs. This apparent inconsistency may have been due to activation of cells to an epitope not identified in the search of PVM protein sequences because not all epitopes contain the canonical motifs (13, 34) or could indicate that the frequency of IFN-γ-producing, PVM-specific CD8 T cells is lower than the total number of virus-specific cells. A discrepancy between IFN-γ-secreting and MHC I tetramer+ CD8 T cells has been described previously for RSV-specific CD8 T cells in mice (12, 13). To investigate this further, a MHC I pentamer construct for the P261 epitope was used to accurately quantify the response in the lung, and the number of pentamer+ CD8 T cells was compared with the frequency of IFN-γ-secreting cells after P261 peptide stimulation. The acute response was analyzed at day 8 p.i. As shown in Fig. 3a, we found that 2.1% of CD8 T cells produced IFN-γ after stimulation, compared with 0.6% in unstimulated cells. Similar data were obtained by measuring the production of TNF-α by intracellular cytokine staining (data not shown). Strikingly, the P261 pentamer stained 11% of total pulmonary CD8 T cells in infected mice (Fig. 3b). Thus, the functional responses (IFN-γ+) comprised ~20% of the total P261-specific CD8 T cell response. Comparison of the results from P261-pentamer staining with those from CD11a staining indicates that the total P261-specific response accounts for 28% of the total number of activated (CD11a<sup>high</sup>) CD8 T cells in the lungs (Fig. 3c). We found that pentamer+ cells were CD11a<sup>high</sup>, CD62L<sup>low</sup> and CD43<sup>high</sup> (Fig. 3c and data not shown). As a specificity control for P261-pentamer CD8 T cell staining, we used the pentamer to stain lung samples from RSV-infected mice. This did not result in positive staining, demonstrating that the pentamer does not stain inflamed lung tissue per se but is truly PVM specific (Fig. 3d). We then performed several control experiments to verify the discrepancy between MHC I pentamer staining and intracellular IFN-γ staining. First, we repeated staining experiments in PVM-infected mice with a P261 tetramer, allowing us to compare data obtained with two independent MHC multimer constructs. P261 tetramer staining confirmed the results obtained with the pentamer, i.e., we observed a discrepancy between numbers of IFN-γ-producing and MHC multimer-binding cells (data not shown). Second, the staining procedure itself was controlled using RSV-infected mice and influenza virus-infected mice because the ratios of IFN-γ-secreting and tetramer-staining cells have been well established in these models (12, 13). Using the dominant MHC class I epitope in the RSV M2 protein, we found that 22% of pulmonary CD8 T cells were positive with the M2-tetramer at day 8 post-RSV infection, whereas only 15% of CD8 T cells secreted IFN-γ in response to the M2 peptide (Fig. 4a), reproducing the published results on tetramer:IFN-γ ratios in murine RSV infection. Furthermore, we observed a 1:1 ratio for the influenza virus A/PR/8/34-specific response at day 8 p.i.: 12% of pulmonary CD8 T cells stained positive with a tetramer specific for the immunodominant epitope in the HA protein (HA<sub>533–541</sub>, IYSVASSL) (35) and responded to HA<sub>533–541</sub>-peptide stimulation by IFN-γ production (Fig. 4b).

In conclusion, the data suggest that the main reason for the discrepancy between the total number of PVM-specific CD8<sup>+</sup> T cells and the quantity of virus-specific, cytokine-secreting CD8 T cells is functional inactivation, similar to that seen with RSV infection in mice.

Chang et al. (12) reported that the proportion of functionally inactivated M2-specific CD8 T cells in the lungs of RSV-infected mice increases with time. Therefore, we assessed the change in the ratio of total to functional PVM-specific CD8 cells during the transition of the effector response toward memory. P261 pentamer and intracellular IFN-γ staining experiments performed at day 28 p.i. revealed a ratio of 10:1 between pentamer+ and IFN-γ+ CD8 T cells, compared with the ratio of ~5:1 at day 8 p.i., indicating that the anergic phenotype was retained in effector-memory CD8 T cells (Fig. 5a and b). At day 28 p.i., the P261-specific response (4.4% of total; Fig. 5b) constituted ~14% of all CD11a<sup>high</sup>-activated cells (32% of total; Fig. 5c).

FIGURE 3. Discrepancy between functional P261-specific CD8 T cells and total numbers of P261-specific CD8 T cells. a, Functional P261-specific responses in the lungs at day 8 p.i. Frequencies of IFN-γ+ cells as a percentage of total CD8 T cell numbers are shown in the upper right quadrants. b, Total numbers of P261-specific cells in the lungs at day 8 p.i. Frequencies of P261-pentamer+ cells as a percentage of total CD8 T cell numbers are shown in the upper right quadrants. c, Pentamer+ CD8 T cells are CD11a<sup>high</sup>. Cells were gated on CD8<sup>+</sup> T cells using side scatter vs CD8<sup>+</sup> (FL4) gates. Frequencies of pentamer+ and pentamer− CD11a<sup>high</sup> as percentage of the total number of CD8 T cells are indicated in the upper and lower right quadrants, respectively. d, PVM-specificity of the P261-pentamer. Pulmonary lymphocytes from a RSV-infected mouse (day 8 p.i.) were stained with the P261-pentamer.
Deficient cytokine production during secondary responses

A remarkable feature of functional CD8 T cell inactivation in the RSV murine model is that it occurs even during secondary infection, i.e., after immunization with vaccinia virus recombinants expressing the RSV M2 or F proteins (12, 13). To investigate whether this was also the case for the PVM infection model, mice were immunized with the P261 peptide emulsified in IFA together with the OVA323 CD4 T cell epitope. Immunized mice were challenged 4 wk later, and pulmonary CD8 T cell responses were analyzed at day 28 postchallenge. The challenge infection was associated with less severe clinical symptoms (data not shown), suggesting that PVM-specific CD8 memory T cells had been induced by the immunization and that these cells provided partial protection (E. Claassen and R. van der Most, unpublished data). As before, the number of P261-pentamer staining cells exceeded the number of IFN-γ- or TNF-α-secreting cells (by ~6-fold; Fig. 6 and data not shown). The secondary P261-specific response (5.5% of total CD8 T cells; Fig. 6a) comprised ~30% of the total number of activated CD8 T cells (18% of total CD8 T cells; Fig. 6b), indicating that primed P261-specific CD8 T cells had been recruited to the lungs.

Maintenance of antiviral CD8 T cell memory

As discussed above, analysis of the CD8 T cells in the lungs of infected mice showed that up to 90% of pulmonary effector-memory (CD62Llow) CD8 T cells were functionally inactive at day 28 p.i. The longevity of PVM-specific CD8 T cell memory was assessed by analyzing responses in the spleen and in the lungs at 96 days p.i. Surprisingly, at this time, functional T cell memory levels in the spleen exceeded those in the lungs (Fig. 7a). P261-specific CD8 T cell responses in the spleen were 10-fold stronger at day 96 (1.2% of total CD8 T cells) than at day 8 (0.1%; Fig. 7a). Levels of P261-specific CD8 T cells in the lungs remained constant (compared with day 28 p.i.) at 0.3–0.5% of CD8 T cells. As before, the numbers of P261-pentamer staining cells exceeded the number of cytokine-producing cells in the lungs (Fig. 7b). These P261-specific CD8 T cells expressed the IL-7R (CD127) at slightly higher levels than the nonstaining CD8 T cell population in the lungs (mean fluorescence intensity = 31 for pentamer+ cells vs 18 for nonstaining cells, n = 3) (Fig. 7c), suggesting that these dysfunctional memory cells were maintained by IL-7 (36).

Discussion

The murine pneumovirus PVM causes a severe granulocytic and eosinophilic bronchiolitis, with very high viral titers in the lungs...
FIGURE 6. PVM-specific responses at day 28 postchallenge in P<sub>261</sub>-immunized mice. a. Functional P<sub>261</sub>-specific responses in the lungs at day 28 p.i. The frequencies of IFN-γ<sup>+</sup> cells as a percentage of total CD8 T cell numbers are shown in the upper right quadrant (right panel). Total numbers of P<sub>261</sub>-specific cells in the lungs at day 28 p.i. Frequencies of P<sub>261</sub>-pentamer<sup>+</sup> cells as a percentage of total CD8 T cell numbers are shown in the upper right quadrant (left panel). b. Total numbers of activated CD8 T cells in the lungs. The frequency of CD11a<sup>hi</sup> CD8 T cells as a percentage of total CD8 T cell number is shown in the upper right quadrant.

FIGURE 7. Longevity of memory. a. Splenocytes (upper panels) and lung lymphocytes (lower panels) were harvested at day 96 after PVM infection and stimulated with P<sub>261</sub> peptide (right panels) or were left unstimulated (left panels). Frequencies of IFN-γ<sup>+</sup> cells as a percentage of total CD8 T cell numbers are shown in the upper right quadrant. b. P<sub>261</sub>-pentamer<sup>+</sup> CD8 T cells in the lungs at day 96 after PVM infection. The frequencies of P<sub>261</sub>-pentamer<sup>+</sup> cells as a percentage of total CD8 T cell numbers are shown in the upper right quadrant. c. P<sub>261</sub>-pentamer<sup>+</sup> CD8 T cells in the lungs express the IL-7R (CD127).

(23, 24, 37). The infection is associated with an inflammatory infiltrate in which up to 12% of the cells are eosinophils at early times to be replaced by an infiltrate consisting entirely of neutrophils (24). The PVM model has been proposed as a tractable model for the replication of a pneumovirus in its natural host, which mimics the process of severe infection of humans with RSV (22).

Cellular immune responses play a key role in the control of many, if not all, viral infections (38) and are particularly critical in the pathogenesis of RSV infection in mice (7, 11). Although some aspects of the immune response to PVM infection in mice have been described, no information about specific T cell responses have yet been reported (22, 39, 40). Having shown that there is an expansion of activated T cells in the lungs of infected mice, we investigated the virus specificity of response. To date, no PVM-specific T cells have been described. Motif-based epitope mapping for the H-2D<sup>b</sup>, H-2K<sup>d</sup>, and H-2L<sup>d</sup> alleles identified the first reported T cell epitopes for PVM, which is derived from the P, M, and F proteins.

A surprising observation was that the level of functional (i.e., cytokine producing) PVM-specific CD8 T cells in the lungs of infected mice at day 8 p.i. was only 1.5–2% of the total number of CD8 T cells. The levels of functional virus-specific T cells in the spleen were even lower. Although it was possible that this was due to T cell activation by an unidentified, yet more dominant, epitope, a similar phenomenon has been reported for RSV infection in mice, which was shown to be due to functional inactivation of the virus-specific cells (12, 13). Analysis of the PVM-specific CD8 T cells showed that they were impaired in their capacity to produce cytokines upon antigenic stimulation. This conclusion was supported by the facts that 1) the cells were unable to produce either IFN-γ or TNF-α, which were assayed separately, 2) the results were reproduced with a MHC class I tetramer (instead of the original pentamer), and 3) the IFN-γ/tetramer ratios measured in RSV-infected control mice were within the range of published data, showing that the staining procedures were appropriate and consistent (7, 12, 13). Deficient cytokine production was evident during the effector response, at 8 days post infection. The ratio between cytokine-producing and pentamer-binding CD8 T cells further decreased during the transition toward memory, with <10% of P<sub>261</sub>-specific cells producing IFN-γ and TNF-α at 96 days p.i. Similar to the situation reported for RSV-infected mice, the secondary CD8 T cell responses, detected after immunization with the P<sub>261</sub> peptide followed 4 wk later with a virus challenge, were also subject to functional inactivation. The responses in PVM-infected and RSV-infected mice differ in the extent of inactivation. In the RSV model, 40–60% of virus-specific CD8 T cells were inactivated (12, 13), whereas with PVM the levels of functional cells dropped below 10% of the total number of specific cells. This loss of function of antiviral CD8 memory T cells cannot be explained by a concurrent loss of CD4 T cell responses: functionally inactivated P<sub>261</sub>-specific CD8 T cells are still detected at 230 days p.i., in the presence of functional PVM-specific CD4 memory T cells (E. A. W. Claassen et al., manuscript in preparation).

Suppression of antiviral responses could be caused either directly, by viral gene products (e.g., NS1, NS2, F or G proteins), or
indirectly, by immunosuppressive factors released from infected cells (12). In either case, immunosuppression would be a function of viral loads, and this may explain the difference between RSV and PVM. In the case of PVM, an inoculum of 50 PFU results in peak viral loads of $10^6-10^8$ PFU/g lung, in contrast to the situation in RSV-infected mice (29). In addition, PVM is an authentic murine virus, and its proteins could therefore be better adapted to counteract murine CD8 T cells. The NS1/NS2 protein-mediated antagonism of IFN-α/β responses (15, 18, 19) could be particularly important in this respect since it has been shown that type I IFNs are critical for the induction of CD8 T cell responses (41). Although the RSV NS1/NS2 proteins are functional in mice (42), it is possible that the effects of PVM NS1/NS2 are stronger in the lungs of PVM-infected mice as a result of higher viral loads. Irrespective of the mechanism, the net result is that functional antiviral memory responses in the lung following pneumovirus infection are poorly sustained. There, the data presented here support the suggestion that functional inactivation of pulmonary effector memory cells could also be responsible for the susceptibility to reinfection as has been described for human and bovine RSV infections (12).

The dynamics of T cell memory in the spleen in PVM-infected mice was different to that seen in the lung. During an acute infection, PVM-specific responses in the spleen were very weak and could only be detected by the more sensitive IFN-γ ELISPOT assay. Surprisingly, splenic T cell memory responses at day 96 p.i. were greater than the responses seen at day 8. It is possible that migration of effector-memory cells from the lungs to the spleen, thereby differentiating into central memory cells, could explain this increase (14). If this were the case, it would suggest that memory T cell inactivation can be reversed. Indeed, it has been shown that such a reversal can be accomplished by in vivo exposure to IL-2 (43). An alternative explanation for the increased frequencies of splenic PVM-specific memory is that these memory cells proliferate. Homeostatic T cell proliferation is essential for the maintenance of T cell memory (44) and is amplified under lymphopenic conditions (45). PVM-infection is accompanied by mild lymphopenia, and it is possible that the splenic PVM-specific memory T cell pool benefits from the ensuing phase of homeostatic proliferation. It seems likely that both factors could play a role: anergic effector-memory cells slowly differentiate toward central-memory cells and migrate to the spleen (14). In this process, these cells would regain effector functions and proliferate by exposure to homeostatic, common γ-chain (CD132) binding, cytokines, which may be present at elevated levels as a result of PVM-induced lymphopenia. Irrespective of the mechanism, the different behavior of splenic and pulmonary memory responses provides further, indirect, support for a role of viral replication in functional inactivation because only the pulmonary T cells are directly exposed to viral products.

In conclusion, we have identified three PVM T cell epitopes that are recognized during a natural infection. Analysis of the T cell response indicates that PVM-specific CD8 T cells are deficient in IFN-γ and TNF-α production during primary and secondary infection and remain in this dysfunctional state for prolonged periods of time. This is consistent with the results obtained in the RSV model and supports the suggestion that pneumovirus replication, either directly or indirectly, is associated with functional inactivation of CD8 T cells. The data also support the hypothesis that virus-induced immune suppression could explain the repeated infections observed in both human and bovine RSV infections. Thus, it will be important to determine the susceptibility of mice to recurrent PVM infections.

During the preparation of this article, Gray et al. (46) reported that respiratory infection of mice with the paramyxovirus SV5 also results in the generation of functionally inactivated CD8 T cells. PVM and SV5 belong to different subfamilies of the Paramyxoviridae family, which suggests that this phenomenon of immune escape by CD8 T cell inactivation may be a general characteristic of paramyxovirus infections. This could provide important clues toward the identification of a common mechanism.

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