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Identification of a CD4 T cell epitope in the pneumonia virus of mice glycoprotein and characterization of its role in protective immunity

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Abstract

Pneumonia virus of mice (PVM) causes bronchiolitis and pneumonia in mice. Infection is associated with high levels of viral replication in the lungs and results in the functional inactivation of pulmonary virus-specific CD8 T cells. Due to its similarity to severe human respiratory syncytial virus (RSV) infection, PVM infection in mice has been proposed as an alternative RSV model. Here, we have delineated the minimal requirements for protective T cell immunity in the PVM model. Immunization with a CD8 T cell epitope from the PVM phosphoprotein P, combined with the ovalbumin (OVA) CD4 T cell epitope, did not confer protective immunity against lethal PVM challenge, suggesting a possible role of cognate CD4 T cell immunity. To determine the role of PVM-specific CD4 T cell responses, we mapped a PVM CD4 T cell epitope in the glycoprotein G, using a panel of overlapping peptides. Although immunization with this epitope provided some protection, solid protective immunity was only observed after immunization with a combination of the PVM-specific CD4 and CD8 T cell epitopes. Analysis of post-challenge T cell responses in immunized mice indicated that G-specific pulmonary CD4 T cells displayed a mixed Th1/Th2 phenotype, which was characterized by the presence of both IL-5 and IFN-γ secreting cells, in the absence of overt pathology.

Keywords: Pneumonia virus of mice; CD4 T cells; Protective immunity; Th2 responses; Intereleukin-5

Introduction

Pneumonia virus of mice (PVM) belongs to the *Pneumovirinae* subfamily of the paramyxoviruses and is a natural pathogen for mice. PVM is highly related to the human and bovine respiratory syncytial viruses (RSV) and to the human metapneumovirus, and has been proposed as a tractable model for severe RSV infection (Rosenberg et al., 2005). PVM causes bronchiolitis and pneumonia in mice, both of which resemble human RSV infection. Contrary to RSV in mice, PVM infection results in very high levels of virus production in the lungs (Cook et al., 1998). As such, PVM infection in mice could provide a useful alternative model for RSV vaccine development. An attractive feature of this model is that it can be used to delineate the requirements of Pneumovirus protective immunity in the context of a natural virus-host relationship. However, only limited data exist at present on the nature of the antiviral T cell responses and the further development of PVM as an alternative model for human RSV requires a better understanding of these responses and their targets.

We have recently shown that PVM infection in BALB/c mice results in a strong CD8 T cell response that is directed against epitopes in the phosphoprotein ($P_{261–269}$), matrix protein ($M_{43–51}$) and fusion protein ($F_{304–312}$) (Claassen et al., 2005). PVM-specific CD8 T cells in the lungs of PVM-infected mice display a partially inactivated phenotype: only 10–20% of P_{261}-specific CD8 T cells are capable of producing IFN-γ or

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TNF-α upon antigenic stimulation (Claassen et al., 2005). This is similar to the partially inactivated phenotype of RSV-specific CD8 T cells in the lungs of RSV-infected mice (Chang and Braciale, 2002). However, data on the antiviral CD4 T cell responses are still lacking. A further study of the PVM-specific CD4 T cell response is important for the following three reasons. First, identification of a CD4 T cell epitope is needed in order to determine the requirements for PVM protective immunity. Second, CD4 T cell responses most likely play a key role in the Th-2 biased T cell responses observed in mice, cattle and in humans after RSV infection (Openshaw et al., 2001; Varga and Braciale, 2002), and translation of these results to the PVM model requires the identification of epitopes. In fact, PVM infection has the capacity to prime mice for respiratory allergy (Barends et al., 2004) and is associated with eosinophilia (Domachowske et al., 2000), indicating a potential Th-2 bias of the virus-induced CD4 T cell response. Third, it is possible that CD4 T cell responses play a role in the functional inactivation of antiviral CD8 T cells. Support for this possibility is provided by the fact that CD8 T cell functionality can be rescued by IL-2 in RSV-infected mice (Chang et al., 2004).

To investigate these issues, it was essential to first identify PVM CD4 T cell epitopes. For the purpose of epitope mapping, we focused on the G protein because the major immunopathogenic CD4 T cell epitope in RSV-infected mice is located in the RSV G protein (Varga et al., 2000). Herein, we report the mapping of an epitope at the C-terminus of the protein. We show that immunization with this CD4 T cell epitope provided partial protection against lethal PVM challenge, but that the protective efficacy could be enhanced by including the P_{261} CD8 T cell epitope in a peptide vaccine. PVM-specific CD4 T cells appear to have a mixed Th-1/Th-2 phenotype, similar to RSV G-specific CD4 T cells (De Graaff et al., 2004; de Waal et al., 2004; Varga et al., 2001; Varga et al., 2000). *In vitro* stimulation of pulmonary lymphocytes from infected mice with the PVM G peptide resulted in the secretion of both IFN-γ and IL-5.

Results

Identification of a CD4 T cell epitope in the PVM G protein

In order to study the role of CD4 T cell responses in the generation of protective immunity, we set out to identify PVM-specific CD4 T cell epitopes. Since the major RSV CD4 T cell epitope was identified in the G protein (Varga et al., 2000), we focused our epitope mapping effort on its PVM counterpart. Thus, we obtained a set of 78 overlapping 15-mer peptides spanning the entire PVM G protein (396 amino acids) and used these peptides to stimulate splenic lymphocytes from PVM-infected mice (50 pfu, day 8 post infection). IFN-γ ELISPOT responses were considered positive if they exceeded the average value plus two standard deviations. Based on an average number of spots of 5.2 and a standard deviation of 6.6, resulting in a cutoff of 18 spots (indicated in Fig. 1a), we identified positive responses with peptides 77 and 78 (42 and 30 spots/10^6 splenocytes, respectively), as well as against peptide 70 (26 spots) (Fig. 1a). Peptide 70 had 18 spots, which is at the cutoff value. The highest responder, peptide 77, encompasses residues 381–395 (PYWCPMLQLFPRRSN), at the C-terminus of the G protein. The preceding peptide 76 (residues 376–390) did not induce any response. To confirm these results and to determine whether the $G_{381–395}$ peptide indeed harbored a CD4 T cell epitope, we harvested pulmonary lymphocytes at days 8 and 28 post infection (p.i.) and stimulated these cells with peptide 77 ($G_{381–395}$). Responses were visualized by intracellular IFN-γ staining. Unfortunately, we found that the background IFN-γ response in unstimulated cells at day 8 p.i. was high (data not shown), thereby reducing the sensitivity of the assay. Since analysis at day 28 resulted in considerably less background signal, we focused our experiments on this timepoint. At day 28 p.i., we found that 0.06–0.08% of total pulmonary CD4 T cells responded to peptide $G_{381–390}$ (Fig. 1b). No CD8 T cells responded to this peptide (data not shown). Thus, these data show that peptide 77 encompasses a CD4 T cell epitope. The $G_{381–395}$-specific response represented only a minor proportion of the total numbers of activated ($CD11a^{hi}$ $CD62L^{low}$ $CD43^{hi}$) (Cauley et al., 2002; Hogan et al., 2001) CD4 T cells in the lungs at days 28 post infection (42% $CD62L^{low}$ CD4 T cells, and 25% $CD11a^{high}$ $CD43^{high}$ CD4 T cells, versus 19% and 3.5% in naive mice, respectively) (Fig. 1c).

Role of PVM-specific CD4 T cell memory in protective immunity

Having identified a PVM-specific CD4 T cell epitope, we evaluated its importance in protective immunity. BALB/c mice were immunized with combinations of the newly found G_{381} peptide and the previously identified P_{261} CD8 T cell epitope, all emulsified in IFA. Four groups of mice were used. Group 1 was immunized with only the CD4 T cell epitope, group 2 was immunized with a combination of the CD4 and CD8 T cell epitopes, group 3 was immunized with a combination of the CD8 T cell epitope and the CD4 T cell epitope from ovalbumin ($OVA_{323–339}$) and group 4 was mock-immunized with PBS. The $OVA_{323–339}$ CD4 T cell epitope was included in the IFA emulsion to provide T cell help (van der Most et al., 1996), which is essential to prevent the generation of 'helpless' CD8 T cells after challenge (Janssen et al., 2003). Mice were challenged with PVM 4 weeks later. As the primary read-outs for protective immunity, we used survival time. As shown in Fig. 2, immunization with the CD4 T cell epitope provided partial protection, 9/16 mice survived. This result was significantly better than the PBS control group, in which 3/20 mice survived ($P=0.022$). Combining the G_{381} CD4 T cell epitope with the P_{261} CD8 T cell epitope provided the best protection: this combination protected 17/20 mice from lethal infection ($P<0.0001$, compared to PBS group). In all cases, we found that protective immunity prevented the severe symptoms that were associated with lethal infection, such as obstructed breathing and nasal mucus formation (data not shown), indicating a much less severe pathology. Although protection was partial, it is clear that the efficacy of the combination

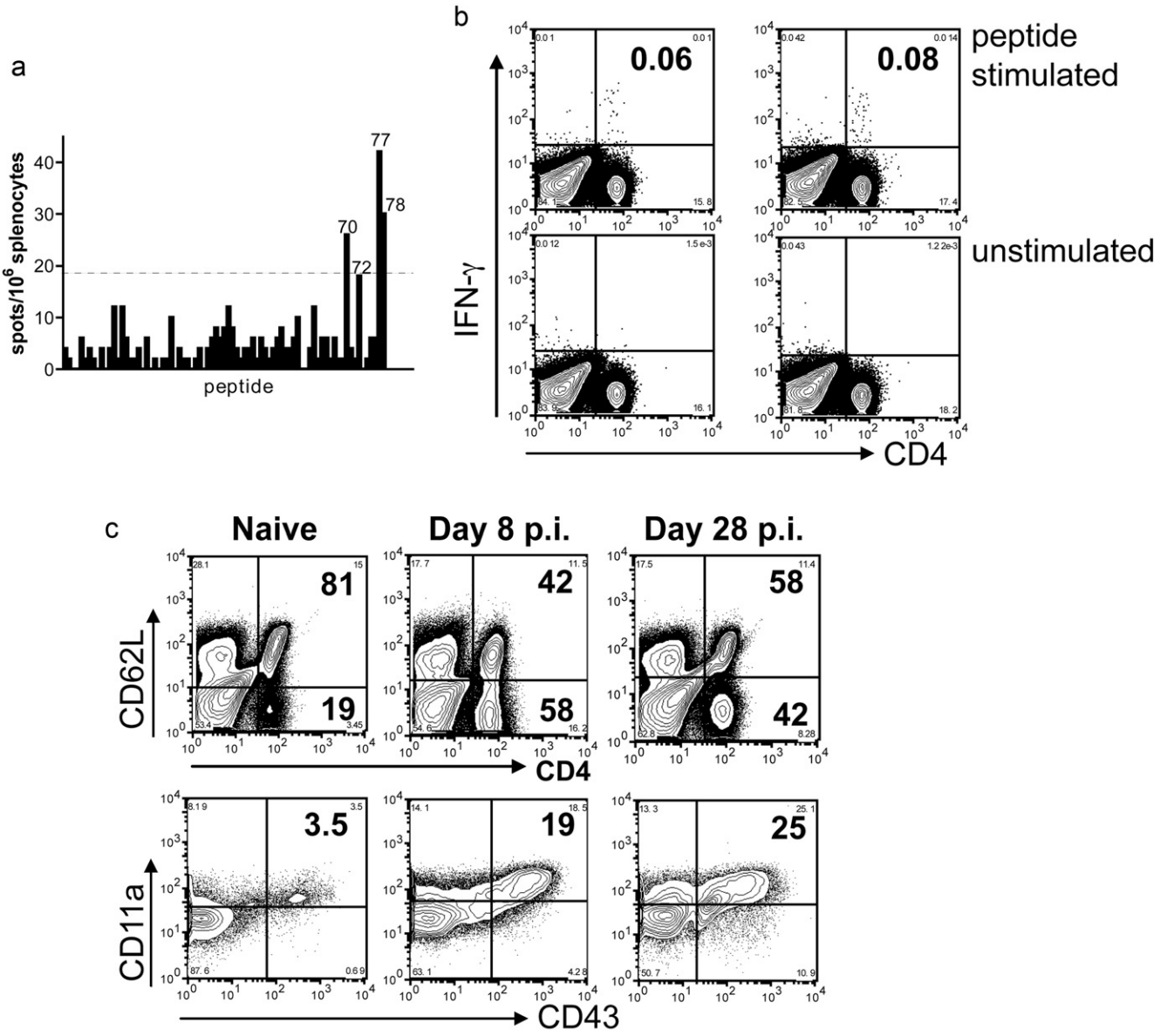

Fig. 1. Identification of a PVM-specific CD4 T cell epitope. (a) IFN-γ ELISPOT analysis of splenocytes obtained at day 8 after PVM infection (50 pfu). Cells were stimulated with a set of overlapping 15-mer peptides spanning the entire G protein. Stimulations were done with individual peptides. (b) Pulmonary lymphocytes were harvested as described in the Materials and methods at day 28 after PVM infection (500 pfu, respectively) and stimulated with peptide 77 (upper panels) or were stimulated with a negative control peptide (lower panels). Responses were visualized by intracellular IFN-γ staining. Representative data are shown. (c) Pulmonary lymphocytes were harvested from PVM-infected mice (day 8 and day 28 p.i.) or from naive mice and stained with antibodies against CD4, CD43, CD62L and CD11a. Data shown were gated on forward and side scatter (upper panel) or on forward/side scatter and on $CD4^+$ cells (lower panel).

peptide vaccine ($G_{381}+P_{261}$) was superior to the CD8 T cell epitope alone ($P=0.0026$). A trend towards significance in the two-tailed t test ($P=0.054$) was observed for the difference between the $G_{381}+P_{261}$ group and the G_{381} group.

Functional characterization of PVM-induced CD4 T cell responses

To determine whether PVM infection would also result in the generation of a Th-2 biased memory response, and to study the effect of immunization on this potential response, we analyzed the cytokine profile of PVM-specific CD4 T cell responses in surviving mice at day 28 post-challenge by IFN-γ and IL-5 ELISPOT assays. It is obvious that this analysis results in a survivor bias. However, our specific question dealt with the nature of the CD4 T cell response rather than its relation with protective immunity. As a control, P_{261}-specific responses were included.

As shown in Fig. 3, we observed that the levels of P_{261}- or G_{381}-specific T cells in surviving mice correlated with the presence of the epitopes in the vaccines, consistent with a vaccine-primed prime-boost effect. Thus, the highest P_{261} and G_{381} responses were measured in mice that had been vaccinated with the respective peptides. This was observed in the lung as

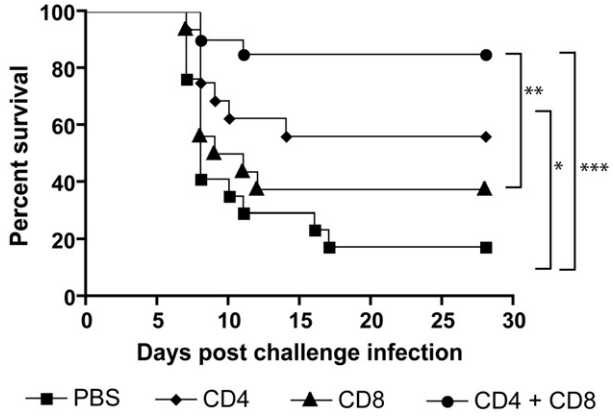

Fig. 2. Protective immunity. Kaplan–Meier survival plot, indicating the efficacy of P_{261} ('CD8'), G_{381} ('CD4') and $P_{261}+G_{381}$ ('CD4+CD8') T cell immunization. Mice were immunized with peptides emulsified in IFA ($n=16$ for $P_{261}+OVA_{323}$, $n=12$ for G_{381} only, and $n=16$ for $P_{261}+G_{381}$), or with PBS in IFA (mock, $n=16$), and challenged 4 weeks later. Percentage survival after PVM challenge (500 pfu) of peptide- or mock-immunized mice is shown (*$P=0.022$; **$P=0.0026$; ***$P<0.0001$).

well as in the spleen. G-specific IFN-γ^+ as well as IL-5^+ responses was detected in the lungs of infected mice by ELISPOT. G-specific IL-5 responses were readily identified in all mice in which IFN-γ producing G_{381}-specific T cells were detected (Fig. 3). Consistent with the G_{381}-specific IFN-γ ELISPOT data, the strongest IL-5 responses were measured in PVM survivors that had been immunized with $G_{381–395}$ only or with the combination of G_{381} and P_{261}.

While analyzing T cell responses in PVM challenge survivors, we observed that surviving mice that had been immunized with the combination of the $P_{261}+OVA_{323}$ peptides harbored IFN-γ-producing OVA-specific T cells not only in their spleens (data not shown) but also in their lungs (Fig. 4). This suggests that OVA-specific memory cells were recruited into the lungs during the antiviral response. Surprisingly, we found that pulmonary OVA-specific T cells also produced IL-5 after PVM challenge (Fig. 4). Thus, pulmonary OVA-specific T cells, apparently recruited into the lungs during PVM infection, appeared to display the same mixed Th1/Th2 phenotype as the antigen-driven G_{381}-specific response (Fig. 3).

CD4 T cell memory does not prevent functional inactivation CD8 T cells

Finally, we used the CD4 G epitope to address the question whether vaccine-induced PVM-specific memory CD4 T cells could rescue the functionality of post-challenge pulmonary CD8 T cells. To test this, P_{261}-specific CD8 T cells at 28 days post-challenge were quantitated by P_{261} pentamer staining (Claassen et al., 2005) and their functionality was assessed by intracellular IFN-γ staining after peptide stimulation. Consistent with our previous study, we found that only 10–20% of P_{261}-specific CD8 T cells produced IFN-γ after peptide stimulation (Claassen et al., 2005) and that IFN-γ^+ responses did not exceed 1.4% of all pulmonary CD8 T cells (Fig. 5). As shown in Fig. 5, the presence of a PVM-specific recall CD4 T cell response did not change this ratio between pentamer-positive and IFN-γ producing cells (Fig. 5). Irrespective of the vaccine composition used, i.e., CD8 or CD4 T cell epitopes only, CD4/CD8 combined or mock-immunized, we observed 4–10-fold higher frequencies of pentamer-staining cells than IFN-γ^+ cells (Fig. 5). These data suggest that the better protective efficacy of the $P_{261}+G_{381}$ peptide vaccine cannot be explained by the rescue of functional pulmonary CD8 T cells after PVM challenge. However, frequencies of P_{261}-specific CD8 T cells were higher in CD4+CD8 immunized mice (Fig. 5).

Discussion

In the present study, we have mapped a CD4 T cell epitope in the PVM glycoprotein G, spanning residues 381–395 (PYWCPMLQLFPRRSN). The $G_{381–395}$ peptide exclusively stimulated CD4 T cells and not CD8 T cells. However, we cannot yet exclude that the $G_{381–395}$ peptide might contain an overlapping B cell epitope. The sequence of peptide $G_{381–395}$ has no significant homology with any other protein. Identification of this epitope allowed us to define the requirements for protective immunity against lethal PVM challenge, and characterize the Th-1/Th-2 phenotype of the PVM CD4 T cell response.

Protective immunity

The data presented herein indicate that a minimal immunization protocol with two T cell epitopes, such that both CD4 and CD8 T cell responses are primed, provides protection in the stringent PVM challenge model. Protective immunity against PVM challenge was also described recently by Ellis et al., using mucosal vaccination with attenuated virus. Protective immunity was associated with the presence of neutralizing antibodies in the serum after vaccination (Ellis et al., 2007). Our data revealed that immunization with the CD4 T cell epitope only provided partial protection, whereas immunization with solely the CD8 epitope failed to protect mice. Thus, protective immunity most likely requires both CD4 and CD8 memory T cells. However, we cannot yet exclude that either peptide could induce antibody responses, which could contribute to immunity. Perhaps the most intriguing possibility would be when the $G_{381–395}$ peptide would harbor overlapping CD4 T cell and B cell epitopes. This is currently under investigation.

A conspicuous feature of our experiments was that immunization with the P_{261} CD8 T cell epitope, when combined with non-cognate CD4 help, failed to induce protective immunity. It could be argued that the efficacy of P_{261} peptide vaccination could be improved by using multiple immunizations. However, the point of this study is that the protective efficacy of a relatively weak vaccination method (single IFA-peptide vaccination) is enormously improved when a PVM CD4 T cell epitope is included. In fact, a recent study, using a fusion protein of the P_{261} peptide with the detoxified ricin B

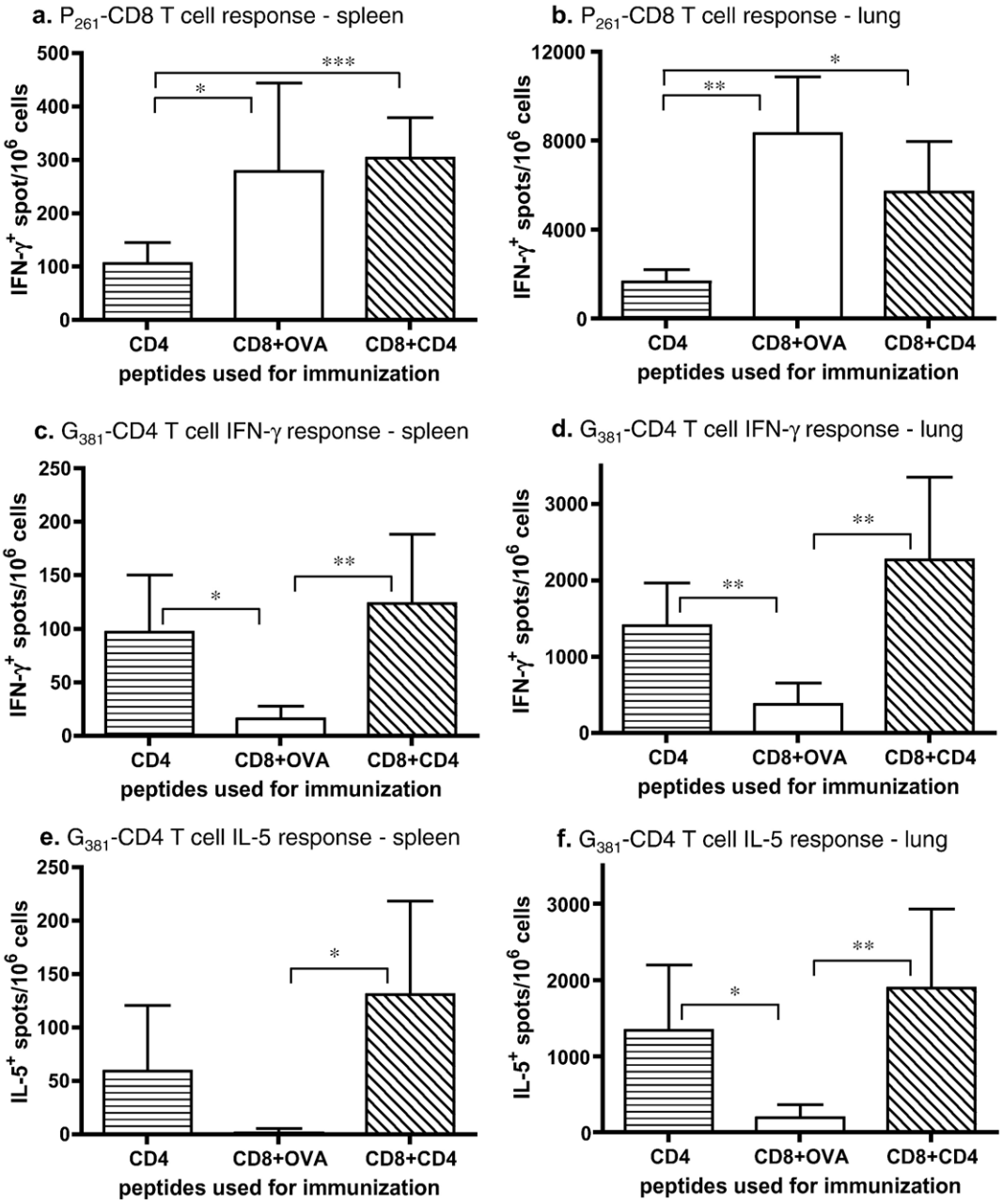

Fig. 3. T cell responses in immunized PVM survivors at day 28 post-challenge infection, as measured by IFN-γ or IL-5 ELISPOT. The immunization protocols are indicated at the bottom of each graph. Lymphocytes were stimulated with peptide P_{261} (a, b), G_{381} (c–f) or were left unstimulated. Background was subtracted to yield the number of specific spots. (a) P_{261}-specific IFN-γ responses in the spleen (*P=0.0236; ***P<0.0001). (b) P_{261}-specific IFN-γ responses in the lungs (**P=0.0033; *P=0.0139). (c) G_{381}-specific IFN-γ responses in the spleen (*P=0.016; **P=0.0095). (d) G_{381}-specific CD4 IFN-γ responses in the lungs (**P=0.007). (e) G_{381}-specific IL-5 responses in the spleen (*P=0.016). (f) G_{381}-specific IL-5 responses in the lungs (*P=0.0291; **P=0.010).

chain showed only partial protection that was not improved by boosting (Grimaldi et al., 2007). Thus, we hypothesize that protective PVM immunity is a matter of quality, not quantity.

Why is cognate CD4 T cell memory so important for protective immunity? Conceivably, the CD4 T cell recall response at the time of PVM infection could accelerate development of CD8 T cell responses or boost post-challenge B cell responses, leading to a more rapid production of neutralizing antibodies. As mentioned before, we cannot yet exclude that the $G_{381-395}$ peptide itself induced an antiviral B cell response. Alternatively, $CD4^+$ effector T cells could play a role independent of antibodies or CD8 T cells, possibly via IFN-γ (Hogan et al., 2001; Zhong et al., 2001).

Mixed Th-1/Th-2 phenotype of the G-specific response

The G_{381}-epitope induces production of IFN-γ, as well as the Th2-cytokine IL-5. This mixed Th1/Th2 cytokine response pattern resembles the RSV G-specific CD4 T cell responses observed in humans (De Graaff et al., 2004; de Waal et al., 2004) and mice (Varga et al., 2000). Increased frequencies of IL-5 producing G_{381}-specific CD4 T cells were observed in

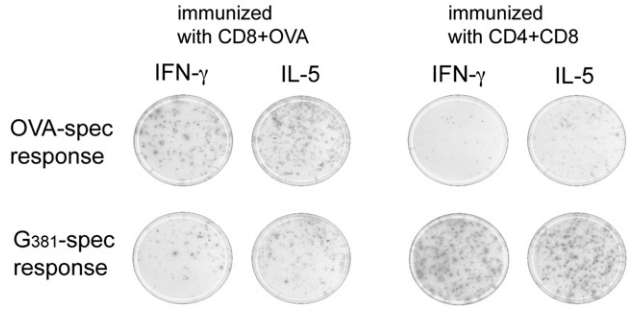

Fig. 4. Pulmonary OVA_{323}-specific mixed Th-1/2 responses, after immunization with CD8+OVA (left panel) or CD4+CD8 (right panel). IFN-γ ELISPOT responses were measured at day 28 post infection in immunized mice, as indicated. Lymphocytes were stimulated with either the G_{381} peptide or the OVA peptide, as indicated, or were left unstimulated. Note that OVA-specific Th-1 and Th-2 cells are only detected in CD8+OVA immunized and PVM-challenged mice.

protected mice that had been immunized with the G_{381} peptide or with the protective G_{381}/P_{261} combination. Thus, pulmonary IL-5 production can be involved in protective immune responses. Indeed, IL-5 plays an crucial role in the production of IgA antibodies in mucosal tissues (Moon et al., 2004). Similar to RSV in mice (Varga et al., 2001) and BRSV in cattle (Antonis et al., 2003, 2006), Th-1 and Th-2 responses co-exist during PVM infection. These observations are consistent with observations that pulmonary Th-2 responses in fact depend on Th-1 responses (Randolph et al., 1999). A salient detail in our findings is that OVA-specific CD4 T cells were also recruited into the lungs. This recruitment of non-specific CD4 T cells is consistent with previously reported data (Stephens et al., 2002; Chapman et al., 2005). These cells displayed a mixed Th1/Th2 phenotype. Interestingly, Stephens and coworkers described that non-specific recruitment of Th-2 biased cells into the lungs is also driven by a Th-1 inflammatory response (Stephens et al., 2002). This could provide an explanation for the presence of IL-5-secreting OVA-specific cells that persist in the lungs after PVM infection. The presence of such Th1/Th2 memory CD4 T cells in the lungs after PVM infection could have profound implications for our understanding of post-virus bronchial hyperreactivity phenomena.

Pulmonary PVM-specific CD8 T cells are not rescued from inactivation by recall CD4 T cell responses

The inclusion of the CD4 epitope in the vaccine did not rescue PVM-specific pulmonary CD8 T cells from functional inactivation. Thus, the eventual inactivation of PVM-specific CD8 T cells does not impede protective immunity, consistent with a recent study showing that attenuated PVM would confer protective immunity in IFN-γ receptor deficient mice (Ellis et al., 2007), indicating that IFN-γ itself may not be a critical effector molecule. Similarly, functionally inactivated M2-specific CD8 T cells were observed in vaccinia virus-M2-immunized and RSV-infected mice (Chang and Braciale, 2002). The key question now is how virus-specific pulmonary CD8 T cells are inactivated, while involved in a protective response. We assume that our epitope vaccines do not induce neutralizing antibodies, which would imply that the virus will undergo some initial replication and will trigger T cell activation (Ostler et al., 2001) in immunized animals. Most likely, this early viral replication suffices to inactivate antiviral CD8 T cells. As

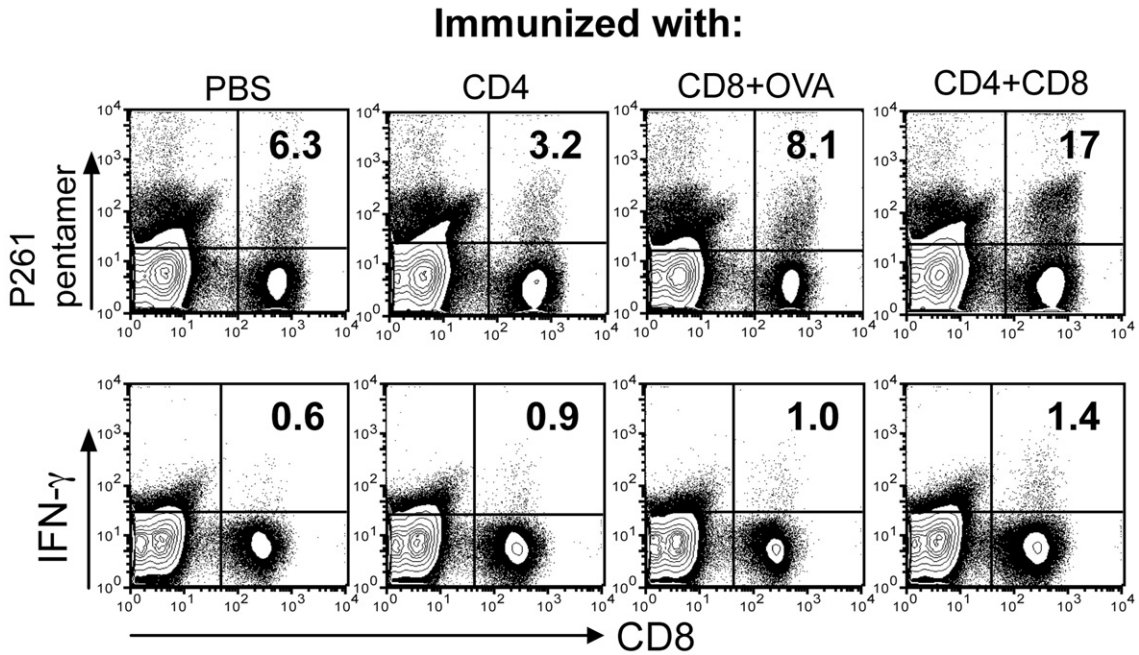

Fig. 5. Vaccine-induced G_{381}-specific CD4 memory T cells do not prevent P_{261}-specific CD8 T cell inactivation in the lungs. P_{261}-specific responses were measured by MHC class I pentamer staining (upper row) and by intracellular IFN-γ staining following peptide stimulation (lower row). Mice had been immunized as shown. Representative data of 3–4 mice are shown. No responses were measured in unstimulated cells.

recently shown, RSV replication may lead to induction of inhibitory B7 family members on lung epithelium (Stanciu et al., 2006). This, in turn, could induce functional inactivation of CD8 T cells through PD-1, as shown recently (Barber et al., 2006).

The magnitude of the pulmonary CD4 T cells response

Responses against the G_{381} epitope were relatively small. In all cases, we found that frequencies of G_{381}-specific CD4 T cells were much lower than frequencies of activated ($CD11a^{hi}$ $CD43^{hi}$ $CD62L^{low}$) (Cauley et al., 2002; Hogan et al., 2001) CD4 T cells. There are several possible explanations for this. First, the PVM proteome may harbor additional, and possibly more dominant, CD4 T cell epitopes. Second, it is possible that not all pulmonary G_{381}-specific CD4 T cells produce IFN-γ. An exciting possibility is that CD4 T cells are functionally inactivated, similar to the CD8 T cells. In fact, human alveolar macrophages have been reported to inactivate antigen-specific CD4 T cells (Blumenthal et al., 2001). Also, RSV-infected dendritic cells have a profound negative effect on CD4 T cell proliferation and cytokine production (de Graaff et al., 2005), which appears to be mediated through the combined action of IFN-α and IFN-λ(Chi et al., 2006). It is tempting to speculate that PVM also infects dendritic cells, with similar negative effects on the antiviral CD4 T cell response. Third, bystander CD4 T cells may be recruited into the inflamed lungs. It has been shown for Sendai virus that this indeed happens during the acute antiviral response (Chapman et al., 2005; Stephens et al., 2002), and our observation that OVA-specific CD4 T cells are recruited into the lungs is consistent with this. However, arguing against a major role of bystander activation is the fact that the phenotype of the pulmonary CD4 T cells after PVM infection ($CD11a^{high}$ $CD62L^{low}$ $CD43^{high}$) closely resembles the phenotype of MHC class II tetramer-positive Sendai virus-specific CD4 T cells in the lungs of Sendai virus-infected mice (Cauley et al., 2002). This suggests that the $CD11a^{high}$ $CD62L^{low}$ $CD43^{high}$ phenotype truly identifies antigen-specific CD4 T cells. However, we cannot yet exclude any of these possibilities and it is possible that all three contribute to the discrepancy between G_{381}-specific and activated CD4 T cell frequencies.

Materials and methods

Mice, virus and infections

Four- to six-week-old female BALB/c ByJIco mice were obtained from Charles River Nederland (Maastricht, The Netherlands). Mouse-passaged stocks of PVM strain J3666 (Cook et al., 1998) ($\sim 1 \times 10^5$ PFU/ml) were provided by Dr. Andrew Easton and grown as described (Claassen et al., 2005). Viral stocks were defrosted and diluted in PBS immediately prior to intranasal inoculation (50 μl; 50–500 pfu for PVM) under light ether anesthesia, as described previously (Claassen et al., 2005). The mouse study protocol was approved by the Animal Ethics Committee of the Veterinary Faculty of the University of Utrecht.

Peptides

Overlapping 15-mer peptides spanning the entire PVM G protein (Thorpe and Easton, 2005) were synthesized, resulting in a total of 78 peptides. All peptides were synthesized by the Peptide and Protein Facility (Department of Immunology, Utrecht University).

Peptide immunization

Peptides $G_{381–395}$ (PYWCPMLQLFPRRSN), $P_{261–269}$ (CYLTDRARI) (Claassen et al., 2005) and $OVA_{323–339}$ (KISQAVHAAHAEINEAG) (all at 50 nmol/mouse) were emulsified in Incomplete Freunds Adjuvant (IFA) in the appropriate combinations. The negative control formulation consisted of an emulsion of PBS and IFA. Four week-old BALB/c mice were immunized by subcutaneous injection (50 μl) at the base of the tail. After 28 days, the mice were challenged with 500 PFU of PVM strain J3666. Challenged mice were observed for a maximum of 28 days and then sacrificed for T cell analysis.

Sampling and tissue preparation

Mice were sacrificed by intraperitoneal (i.p.) injection of sodium pentobarbital. Before recovery, the lungs were perfused with PBS containing 50 units/ml heparine. Lungs and spleen were recovered under aseptic conditions and transferred to 2 ml of prechilled RPMI 1640 medium (Invitrogen Life Technologies). Lungs were mechanically minced and incubated in PBS containing collagenase (2.4 mg/ml; Roche Applied Science, Roche) and DNAase (1 mg/ml; Roche Applied Science), for 20 min at 37 °C. Single-cell suspensions were prepared from the pre-treated lungs and spleens by passage through cell strainers. Spleen cells were depleted of erythrocytes by treatment with a buffered 0.83% ammoniumchloride solution.

In vitro stimulations

For intracellular cytokine staining, lung and spleen lymphocytes (1×10^6) were stimulated in 96-well flat-bottom plates in 200 μl RPMI (Invitrogen Life Technologies), containing 10% FCS, 1% L-glutamine, 5 μg/ml peptide and 10 μg/ml Brefeldin A (Sigma-Aldrich) for 6 h at 37 °C, 5% CO_2. The same, single peptide concentration was used for all experiments. No peptide dose–response analyses were done.

ELISPOT assay

The IFN-γ ELISPOT and IL-5 ELISPOT assays were performed using the mouse IFN-γ ELISPOT pair (BD Biosciences, San Jose, USA) or IL-5 ELISPOT pair (BD Biosciences, San Jose, USA) in combination with MultiScreen-IP filter plates (Millipore, Billerica, MA), according to the instructions of the manufacturer. Cells where stimulated in 200 μl RPMI 1640 (Invitrogen Life Technologies), containing

10% FCS, 1% L-glutamine and 5 μg/ml peptide for 24 h at 37 °C, 5% CO_2. ELISPOT plates were analyzed using an AELVIS ELISPOT reader with ELI.Analyse software (A.EL. VIS GmbH, Hannover, Germany).

Staining and flow cytometry

The following antibodies were used: anti-CD3 PerCP (145–2C11), anti-CD4 PE and PerCP (L3T4), anti-CD8 APC and FITC (Ly-2), anti-CD11a FITC (2D7), anti-CD62 L PE (MEL-14), anti-IFN-γ FITC (XMG1.2), anti-TNF-α PE (MP6-XT22) and anti-CD43-FITC (1B11). All antibodies were purchased from BD Biosciences (San Jose, USA). PE-labeled $P_{261–270}$ H-$2K^d$ pentamer constructs were purchased from ProImmune (Oxford, UK). Intracellular stainings were done using the Cyto-Fix/CytoPerm solution and Perm/Wash buffer purchased from BD Biosciences (San Jose, CA) according to the instructions of the manufacturer. Cells were acquired on a FACScalibur flow cytometer (BD Biosciences, San Jose, CA) using CellQuest software. Data analysis was done with CellQuest (BD Biosciences, San Jose, CA) and FlowJo (TreeStar, Ashland, OR) software.

Statistics

T cell responses as measured by ELISPOT were compared using the *t* test, using two-tailed *P* values. Statistical analysis of Kaplan–Meier survival plots was done with the log-rank test. Prism Graphpad software (GraphPad Software, San Diego, CA) was used for statistical analysis.

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