

Kinetics of antiviral CD8 T cell responses during primary and post-vaccination secondary bovine respiratory syncytial virus infection

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Abstract

We have measured antiviral CD8 T cells responses in bovine respiratory syncytial virus (bRSV) infected calves that had been immunized with either formalin-inactivated (FI) or live-attenuated (L) bRSV, with evidence of immunopathology following challenge of calves vaccinated with FI-bRSV. In all cases, bRSV infection induced potent pulmonary CD8 T cell responses. The kinetics of the post-challenge response in L-bRSV immunized animals was accelerated compared to the FI-bRSV and PBS groups, suggesting that only the L-bRSV vaccine, and not the FI-bRSV vaccine, had primed memory T cells. The differences between primary and post-vaccination secondary infection were very minor, in terms of the proliferation status of pulmonary CD8 T cells. Functional IFN- γ^+ CD8 responses were slightly higher in the FI-bRSV vaccinated animals. Furthermore, the existence of strong IFN- γ^+ CD8 responses in FI-bRSV vaccinated animals after challenge suggests (i) that these IFN- γ^+ responses in FI-bRSV immunized animals do not protect against immunopathology, and (ii) that Th-2 biased responses during bRSV challenge after vaccination with FI-bRSV have a limited impact on the CD8 responses in the bronchoalveolar lavage fluid. Thus, several response patterns (Th-1/Th-2) seem to co-exist during bRSV infection.

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1. Introduction

The human and bovine respiratory syncytial viruses (hRSV and bRSV) are major causes of lower respiratory tract infections. Both with respect to epidemiology and pathogenesis, bRSV infection in calves closely resembles hRSV infection in infants and bRSV therefore, provides a valuable animal model for hRSV [1]. Vaccine development has been hampered by the fact that immunization with formalin-

inactivated (FI) virus primes for a much more severe, and sometimes even lethal, form of respiratory disease [2–6]. This phenomenon was first observed in a human vaccine trial in the 1960s [4] and was later found to also occur in cattle immunized with formalin- or beta-propiolactone-inactivated bRSV [5,6]. Enhanced disease resulting from immunization with FI-virus has an immunopathological basis and has now been modeled in hRSV-infected mice [2,7–9] and monkeys [10] and in bRSV-infected cattle [1,11–13]. In mice, immunization with inactivated virus evokes a Th-2 biased CD4 T cell response, which is associated with eosinophilia and clinical symptoms upon challenge [2]. In cattle, enhanced clinical symptoms post-immunization were associated with pulmonary eosinophilia and increased IgE titers [1].

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RSV-specific CD8 T cell responses play a key role in the control of infection and in the maintenance of antiviral immunity [2,3,14]. This was most convincingly shown by T cell subset depletion experiments in cattle and mice [14,15]. Depletion of CD8 T cells in gnotobiotic calves resulted in more prolonged virus shedding and more severe pathologic lesions (i.e., more extensive lung consolidation), after bRSV infection. CD4 T cell depletion did not affect the kinetics of viral clearance, but did result in more serious symptoms. Consistent with a key role of the CD8 T cell response, McInnes et al. [16] showed that acute bRSV infection in cattle is associated with an influx of activated CD8 T cells into the lungs and the trachea, peaking at Day 10 post-infection. On the other hand, CD8⁺ cytotoxic T lymphocytes also have the capacity to contribute to lung pathogenesis, as shown in hRSV-infected mice [14,17,18]. Th-1 and Th-2 responses seem to co-exist in a delicate balance during RSV infection. Several studies in the mouse model have provided evidence for mutual down-regulation of CD8 and Th-2-biased CD4 T cell responses [9,19,20], whereas other studies have shown that antiviral CD8 T cell responses are essential in the development of pulmonary eosinophilia [21]. In addition, IL-5 and IFN- γ producing G-specific CD4 T cells co-exist in RSV-infected BALB/c mice [22,23]. Thus, the combined literature indicates that in murine RSV-induced immune responses, Th-1, and/or CD8, and Th-2-biased CD4 T cell responses could either co-exist or down-regulate each other.

The role of CD8⁺ CTLs in the control of infection and in enhancement of lung pathology [17,18], combined with the pathogenic potential of a Th-2-skewed CD4 T cell response [23–25] and the limited longevity of cellular immunity [26], provides a challenge for vaccine development [27]. Most pneumoviruses, including bRSV and hRSV are species-specific. Thus, these specific virus–host relationships provide an argument to study natural host–pathogen interactions, in parallel to the murine hRSV model. The bRSV challenge model in calves is promising because FI-bRSV associated enhanced pathogenesis can be experimentally reproduced in bRSV-infected animals [1,13]: after challenge, FI-bRSV immunized calves presented with severe symptoms, eosinophilia and high IgE titers, with specificity for the F protein (Antonis et al., unpublished observations).

In the present study, we asked how antiviral CD8 T cell responses develop during primary bRSV infection, and how vaccination with FI-bRSV or L-bRSV would affect this response, both in terms of kinetics and magnitude. We focused on CD8 T cell responses because their role in pulmonary eosinophilia has been ambiguous [28]. Our data indicate that post-challenge T cell responses in PBS- and in FI-bRSV immunized animals display similar kinetics, whereas those responses in L-bRSV immunized animals peaked earlier. Because accelerated recall T cell responses, indicative of the recruitment of memory cells, were observed only in L-bRSV animals, it appears that the FI-bRSV vaccine was not very efficient in priming long term T cell memory. However, IFN- γ ⁺ T cell responses were readily detectable in FI-bRSV-

immunized and challenged animals. This suggests that Th-1 CD8 responses per se do not protect against severe symptoms. In our model, IFN- γ ⁺ CD8 T cell responses appear to co-exist with Th-2 associated phenomena such as eosinophilia and elevated IgE levels.

2. Methods and materials

2.1. Viruses and animals

bRSV field strain *Odijk* [29] was used for viral challenge. Vaccination and challenge experiments have been described in detail previously [1]. Briefly, bRSV was cultured on embryonic bovine trachea (EBTr) cells. After clarification, supernatant was treated with formalin (37% formaldehyde solution, 1:4000 at 37 °C for 72 h), followed by ultracentrifugation. A vaccine dose consisted of 1 ml sedimented virus material, equivalent to 0.75 mg protein or 10^{6.7} TCID₅₀, mixed with 1 ml 2% Al(OH)₃. In the case of the live-attenuated bRSV vaccine, we used a dose of 10^{4.6} TCID₅₀ of bRSV strain *Lelystad* in 2 ml EMEM, supplemented with 10% FBS. SPF- and bRSV-naïve calves (six per group), which had been obtained by caesarean section [1], received two intramuscular vaccinations with FI-bRSV, L-bRSV (strain *Lelystad*) or PBS, and were challenged with bRSV *Odijk* [29] (at 10^{5.5} TCID₅₀/ml, 2 ml dose) approximately four months later. Intranasal virus inoculation was done using a nebulizer. Bronchoalveolar lavage fluids (BALF) were obtained as described [1]. Approximately 100 ml of BALF was obtained from each animal after instillation of 120 ml PBS. BALF samples were centrifuged (200 g, 10', 4 °C) and lavage cells were resuspended in PBS containing 25% FCS, 0.5% BSA and 0.01% NaN₃, counted and used for further analysis. Lymph node and spleen samples were obtained during postmortem analysis as described [1]. PBMC were isolated by Ficoll centrifugation of heparinized blood samples. bRSV *Lelystad* strain was grown in embryonic bovine trachea cells (EBTr), as described [1].

2.2. Vaccinia virus recombinants

To construct recombinant vaccinia viruses expressing bRSV genes, viral RNA was isolated from BALF from a bRSV *Odijk*-infected animal or from bRSV *Lelystad*-infected EBTr cells [1], using RNA isolation kits from Qiagen (Valencia, CA). RNA preparations were subjected to RT-PCR amplification, using *Pfu* polymerase (Promega) and primer sets designed to amplify the complete open reading frames for the N, P, M, M2 and G genes, as described previously [1]. cDNAs encompassing the N, P, M and M2 sequences were amplified from viral RNA derived from the *Odijk* strain, whereas the G-protein cDNA was generated from the *Lelystad* RNA. PCR-amplicons were subjected to sequence analysis to confirm the absence of unwanted mutations, and inserted into pCRII (Invitrogen). Inserts were then subcloned

into the vaccinia virus transfer vector pSC11. Recombinant vaccinia viruses (strain *Western Reserve*) were generated using standard methodology, as described [30]. The MVA-F recombinant was provided by Dr. Gerd Sutter (Institute of Molecular Virology, GSF-Research Center and Technical University Munich, Munich, Germany) as part of the EU IMPRESSUVAC project. Details on the construction of the MVA-F recombinant will be described elsewhere. Expression of the inserted bRSV genes was confirmed using (i) an immuno-peroxidase monolayer assay (IPMA) and (ii) by metabolic ^{35}S -Met labeling of vaccinia virus recombinant infected cells followed by radioimmunoprecipitation either with a polyclonal goat anti-hRSV (A + B) serum (Biodesign International) or with monoclonal antibodies directed against the bRSV N, P, G, and F proteins (Animal Sciences Group, Lelystad).

2.3. Antibodies and flow cytometry

Cells harvested from BALF were incubated with the appropriate antibodies for 30 min on ice. FITC- or R-PE-labeled antibodies against CD4 (clone CC8), CD8 (clone CC63), and IFN- γ (clone CC302, IgG1) were purchased from Serotec (Oxford, UK). Allophycocyanin (APC)-labeled anti-bovine CD8 antibody was prepared by labeling antibody, purified from the supernatants of hybridoma cultures (clone CC63, obtained from the ATCC), with the APC labeling kit from Prozyme (San Leandro, CA). R-PE-labeled Ki-67 antibody (clone B56, IgG1), and the isotype control (MOPC-21), were obtained from BD Biosciences (Mountain View, CA). The B56 antibody is cross-reactive with the bovine protein [31]. Surface stainings were done in FACS buffer (PBS supplemented with 1% BSA and 0.01% NaN_3). For intracellular Ki-67 staining, cells were fixed and permeabilized using the FACS lysing and FACS permeabilization reagents from BD Biosciences. After surface and/or intracellular staining, cells were fixed in 1% paraformaldehyde and acquired on a FACScalibur flow cytometer. Data were analyzed using CellQuest (BD Biosciences, Mountain View, CA) and FlowJo (Tree Star, San Carlos, CA) software.

2.4. Intracellular cytokine staining

Lymphocytes were stimulated with bRSV *Lelystad* (m.o.i.=0.1) (or medium from uninfected EBTr cells as the negative control), or with recombinant vaccinia or MVA viruses expressing bRSV genes, or control recombinants (wt MVA and T7 RNA polymerase expressing vaccinia virus) (m.o.i. = 1). After overnight incubation (37 °C, 16 h), brefeldin A was added and incubation was continued for another 6 h at 37 °C. As a positive control, cells were stimulated with SEB (Sigma) in the presence of brefeldin A. Cells were then fixed using FACS lysing solution, permeabilized using FACS permeabilization solution, stained with APC- and FITC-labeled antibodies against CD8 and IFN- γ , and acquired using a FACScalibur flow cytometer. The

MOPC-21 IgG1 monoclonal antibody was used as an isotype control.

2.5. Statistics

Statistical analysis was done with Prism and InStat3 software (Graphpad Software), using the Mann–Whitney test and the Kruskal–Wallis nonparametric test). Differences were considered significant when P was <0.05.

3. Results

3.1. Primary bRSV infection coincides with CD8 T cell proliferation and migration into the lungs

Acute bRSV infection is associated with an influx of activated CD8 T cell lymphocytes into the lung [16]. To further study the dynamics of this response and the proliferation status of the infiltrating T cells, we analyzed the lymphocyte populations isolated from bronchoalveolar lavage fluid. For this experiment, two calves were infected with bRSV strain *Odijk* [1,29]. This strain was selected because it induces a symptomatic infection in calves [29]. BALF samples were collected daily from Day 5 through 9 after infection, and cells were stained with monoclonal antibodies against CD4 and CD8 and, after permeabilization, with an antibody against the proliferation marker Ki-67. Lymphocytes were gated using forward and side scatter parameters (Fig. 1a). As shown in Fig. 1b, infiltrating CD4 and CD8 T cells were first detected at Day 7 post-infection (p.i.). Apparently, an influx of T cells starts between Days 6 and 7 p.i. At Day 8 post-infection, approximately 16% of all BALF cells consisted of CD4 and CD8 T cells (Fig. 1a and b). The data shown in Fig. 1 are representative for the two animals. These kinetic data are consistent with results described by McInnes et al. [16]. The majority (60–65%) of the infiltrating CD8 T cells were proliferating (Ki-67^{high}) at Day 7 p.i. (Fig. 1c). From Day 7 through 9, the numbers of CD8 T cells in the lungs increased, but expression of Ki-67 slightly decreased (40–45% at Day 9), suggesting that fewer cells were cycling. Since CD8 T cell proliferation is usually a function of TCR stimulation after antigen encounter in the local lymph nodes, it seems likely that the dividing CD8 T cells observed in the lungs are predominantly bRSV-specific.

However, non-specific recruitment of CD8 T cells also occurs during respiratory infections [32]. Thus, to assess the bRSV-specificity, we stimulated BALF cells (obtained at 9 days post-infection) with virus (m.o.i. = 0.1, virus harvested from EBTr cells) and visualized responding cells using intracellular IFN- γ staining and flow cytometry. The cells were incubated overnight with bRSV, after which the Golgi-inhibitor brefeldin A was added, followed by another 6 h incubation. As controls, cells were stimulated with the superantigen SEB or were incubated in the presence of medium from uninfected EBTr cells. Cells were then permeabilized

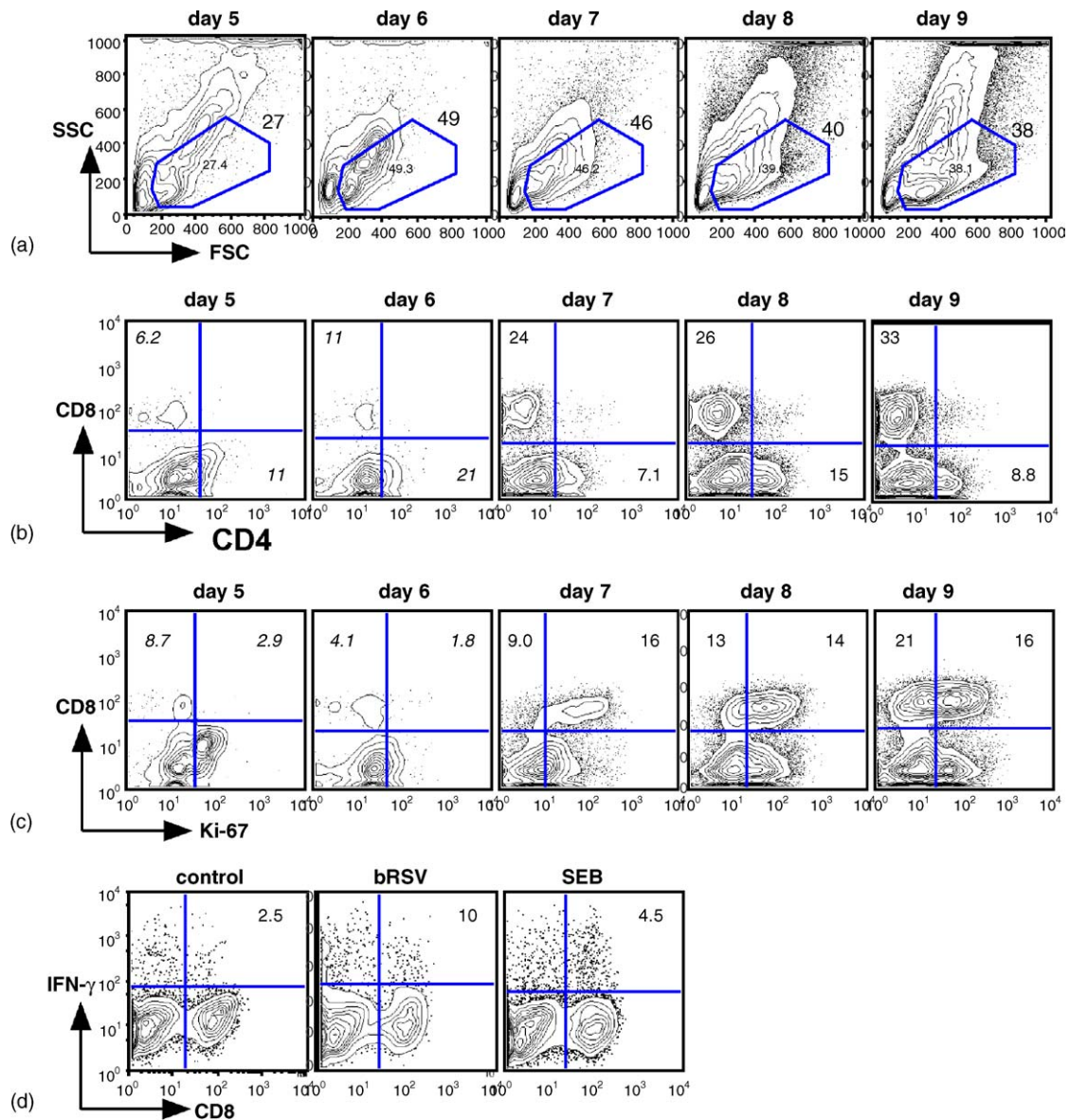


Fig. 1. Kinetics and specificity of pulmonary CD8 T cell responses during acute bRSV infection. Two animals were infected with bRSV *Odijk* and BALF were collected daily from Day 5 through 9 post-infection. All data shown are from one of the two animals and are representative (a) percentage of lymphocytes of BALF and gating strategy based on forward (FSC) and side scatter (SSC) parameters; (b) influx of predominantly CD8 T cells at Day 7 post-infection. Percentages of the relevant cell populations are indicated in the quadrants; (c) infiltrating CD8 T cells at Days 7–9 are proliferating (Ki-67^{high}). Percentages of Ki-67⁺ CD8 T cell populations are indicated in the upper right quadrant. Percentages in italics (Days 5 and 6) indicate that a very small of cells were analysed; (d) specificity of infiltrating lymphocytes. Day 9 BALF were stimulated with bRSV (m.o.i. = 1), SEB or control medium from uninfected EBTr cells, and analyzed by intracellular cytokine staining. Percentages IFN- γ ⁺ CD8⁺ cells of total CD8⁺ cell numbers are shown. Note that the SEB-stimulated sample appears to have more IFN- γ ⁺ cells than the bRSV-stimulated sample; this is, however, solely the result of different total cell numbers in the two plots.

and stained with monoclonal antibodies against CD8 and IFN- γ . We found that up to 10–15% of all pulmonary CD8 T cells produced IFN- γ upon bRSV stimulation (Fig. 1d), suggesting that at least a significant fraction of the infiltrating T cell population is bRSV-specific. It is possible that this underestimates the fraction of the response that is bRSV-specific, since stimulation with whole (live) virus may not result in optimal antigen presentation. The control SEB-stimulation yielded a 4–7% response of IFN- γ ⁺ CD8 T cells. Thus, quantitative analysis of T cell proliferation through Ki-67

staining provides us with a rough estimate of the dynamics and magnitude of the antiviral responses. Such an estimate is particularly useful in situations in which few T cell epitopes have been identified, as is the case for bRSV.

3.2. CD8 T cell proliferation in bRSV-infected calves: impact of immunization and immunopathogenesis

We have recently described a bRSV challenge model for Th-2-driven FI-vaccine-induced immunopathology [1]. We

now extended these studies by analyzing the CD8 T cell responses after bRSV challenge. Eighteen SPF calves, immunized intramuscularly with FI-bRSV, L-bRSV, or mock-immunized with PBS, and challenged four months later, were analyzed for T cell responses. The clinical and serological results of this study have been described previously [1]. The extent of viral replication was assessed by measuring RNA levels in the BALF by RT-PCR, as reported previously [1]. Briefly, viral RNA was detected in the BALF of PBS-immunized animals in 4/6 animals at Day 4 p.i. and in all 6/6 animals at Day 7 p.i. [1]. No viral RNA was detected in the BALF samples from the immunized animals at either Day 4 or 7 p.i., with the exception of a weak PCR signal in one L-bRSV immunized animal at Day 7 [1]. Thus, both vaccines led to reduced levels of viral RNA in the BALF, but only the L-bRSV vaccine provided partial protection against symptoms. Immunization with FI-bRSV primed animals for immunopathogenesis. Thus, this experimental setting allowed a comparison of primary bRSV infection with two modes of secondary infection, i.e., semi-protective (L-bRSV) and immunopathogenic (FI-bRSV).

BALF samples were collected at 3 days before challenge and at Days 1, 4, 7 and 9 p.i., and were first used to visualize the kinetics of CD4 and CD8 T cell influx into the lungs. During primary infection, i.e., in PBS-immunized animals, we found that the dynamics of T cell influx were consistent with the data shown in Fig. 1, infiltrating T lymphocytes were first detectable at Day 7 p.i. (Fig. 2), and were present at slightly increased levels at Day 9 after bRSV infection. Post-challenge CD8 T cell responses in FI-bRSV immunized animals were very similar in kinetics but smaller in magnitude (Fig. 2a). In contrast, we observed accelerated post-challenge responses in L-bRSV immunized animals, with the response peaking at Day 7 post-challenge instead of Day 9 (Fig. 2a). The CD8 T cell responses in L-bRSV immunized animals at Day 7 were significantly different from those in PBS- or FI-bRSV immunized animals ($P=0.041$ and 0.004 , respectively). The largest responses were observed in the PBS-immunized group (i.e., the animals undergoing primary infection) at Day 9. These responses differed significantly from the number of CD8 T cells in L-bRSV-immunized animals ($P=0.026$) and there was a trend towards significance when compared with FI-bRSV-immunized animals ($P=0.065$). A similar trend was seen for the numbers of CD4 T cells in the BALF (Fig. 2b). Responses in L-bRSV-immunized animals appeared to peak earlier, at Day 7, and this was significantly different from the response in FI-bRSV-immunized animals ($P=0.04$). The differences between numbers of CD4 T cells at Day 9 were not significant.

We then tested whether the CD8 T cell populations in the three different groups displayed the same proliferation characteristics, by staining for the Ki-67 antigen. Based on the results shown in Fig. 1, we analyzed Day 7 BALF samples for Ki-67 expression. Again, we found only minor differences between the three groups (Fig. 3), and these were not

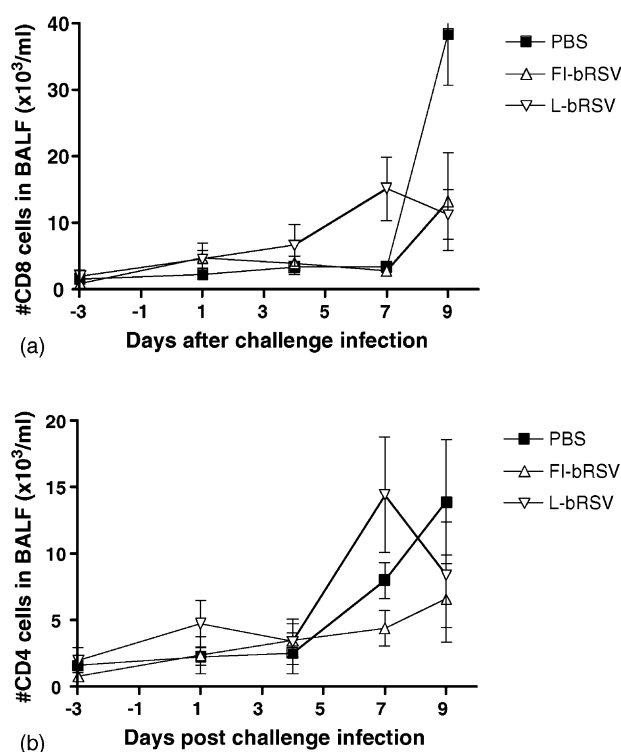


Fig. 2. Total numbers of CD8⁺ (a) and CD4⁺ (b) cells in the BALF, at 3 days before infection and Days 1, 4, 7 and 9 post-challenge infection. Cell numbers were calculated using the total numbers of cells recovered from BALF [1] and the frequencies of CD4⁺ and CD8⁺ cells determined by flow cytometry, using the ungated population of cells. Error bars indicate the standard error of the mean as determined using Graphpad Prism software. Significance was determined using the Mann–Whitney and Kruskal–Wallis tests (InStat3 software).

significant ($P>0.05$ for all comparisons). In all animals, we found that high numbers of the CD8 cells in the BALF stained positive for Ki-67⁺, indicating a high level of proliferation (Fig. 3).

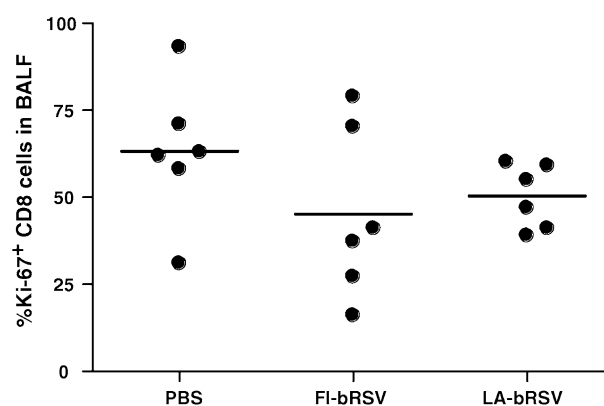


Fig. 3. Proliferation of CD8⁺ cells in the lungs at Day 7 post-infection. Percentages Ki-67⁺ cells of total CD8⁺ cells are indicated for all animals. Vaccination history and average percentages Ki-67⁺ cells for each group are indicated. Significance was determined using the Wilcoxon–Mann–Whitney test (InStat3 software). No significant differences were observed. PBS vs. FI-bRSV, $P=0.3$; PBS vs. L-bRSV, $P=0.13$; FI-bRSV vs. L-bRSV, $P=0.5$.

3.3. Antiviral peripheral and pulmonary CD8 T cell responses

Although staining for the Ki-67 antigen shows the extent of T cell proliferation, it does not reveal functionality or antigenic specificity. To assess the bRSV-specificity of the T lymphocytes infiltrating the lungs, we stimulated BALF cells with an MVA-recombinant expressing the viral F protein. As shown by Nixon and co-workers, infection of human or simian lymphocyte preparations with vaccinia virus recombinants results in effective stimulation of CD8 T cells, after which responses can be quantitated by IFN- γ ELISPOT or by intracellular cytokine staining [33,34]. Gaddum et al. recently validated this approach for bRSV-specific memory T cell analysis [35].

To assess the specificity of antiviral responses, we analyzed F-specific CD8 T cell responses before and after vaccination and after the challenge in the peripheral blood. Pre-challenge and post-challenge responses were also analyzed in BALF samples. Lymphocytes were isolated from heparinized blood before vaccination, on Day 14 post-vaccination, on Day 3 before challenge infection and on Days 1, 4, 7 and 9 post-infection. Lymphocytes were also isolated from BALF

samples on Day 3 before infection and on Days 1, 4, 7 and 9 post-infection. In all cases, lymphocyte samples were infected with the MVA-F recombinant, or, as a control, with wild type MVA virus. Uninfected lymphocytes were used as an additional specificity control. Cells were stained with monoclonal antibodies against CD8 and IFN- γ . We found no F-specific CD8 T cell responses in the periphery before challenge and at Days 1, 4 and 7 post-challenge (data not shown). However, F-specific responses were detected in PBMC in 5 out of 18 animals at Day 9 p.i., (Fig. 4a). Interestingly, three of these five animals had received the FI-bRSV vaccine (Fig. 4). This could suggest that somewhat stronger F-specific IFN- γ ⁺ responses existed in the six FI-bRSV immunized animals. However, the differences were not significant ($P > 0.05$ for all comparisons). Thus, in 10 out of 12 other animals, no or very weak IFN- γ -producing F-specific CD8 T cells were detected in PBMC samples, indicating that there was little systemic involvement in these animals. In the BALF samples, we found IFN- γ ⁺ responses in most of the animals at Day 7 p.i. (Fig. 5a), and no significant differences were observed between the groups ($P > 0.05$ for all comparisons). No responses were measured before challenge and at Days 1 and 4 p.i. (not shown). Note that these samples did not contain

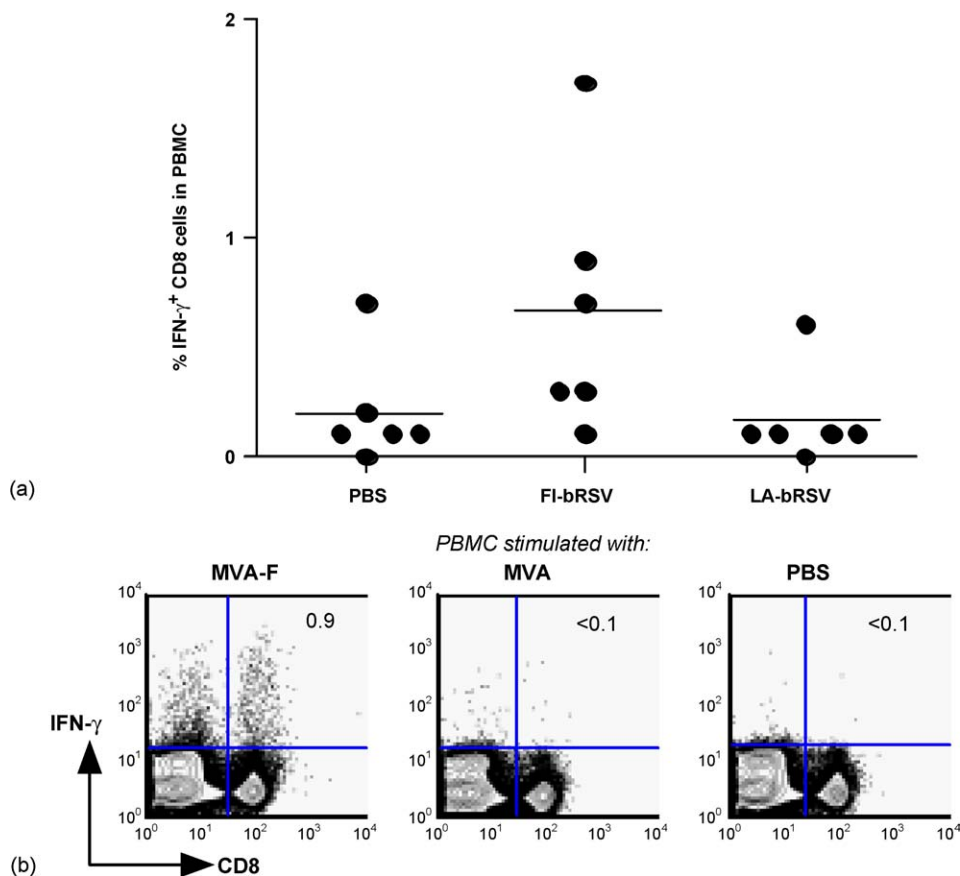


Fig. 4. (a) Peripheral IFN- γ ⁺ CD8 T cell responses during acute bRSV infection in all animals, after in vitro stimulation with MVA-F. Stimulation with wt MVA yielded responses <0.1%. Vaccination history and average percentages IFN- γ ⁺ cells for each group are shown; (b) PBMC from animal #48 (vaccinated with FI-bRSV, as indicated) were stimulated with MVA-F, MVA or PBS, and stained for IFN- γ expression. Frequencies are expressed as percentages IFN- γ ⁺ CD8⁺ cells of total CD8⁺ cells. Significance was determined using the Wilcoxon–Mann–Whitney test (InStat3 software).

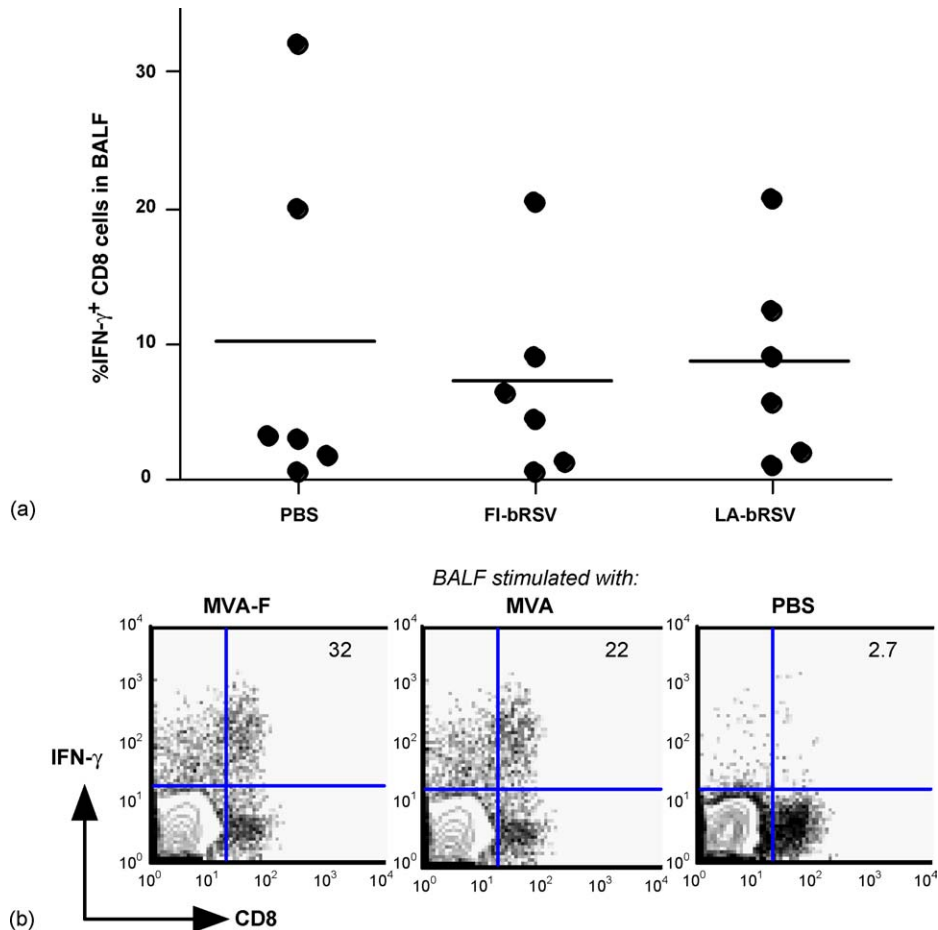


Fig. 5. (a) IFN- γ responses in Day 7 BALF from all animals, after in vitro stimulation with MVA-F. Stimulation with wt MVA yielded responses of ~60% of MVA-F induced responses. Vaccination history and average percentages IFN- γ ⁺ cells for each group are shown; (b) BALF from animal #43 (vaccinated with PBS) were stimulated with MVA-F, control MVA or PBS. Frequencies are expressed as percentages IFN- γ ⁺ CD8⁺ cells of total CD8⁺ cells. Significance was determined using the Wilcoxon–Mann–Whitney test (InStat3 software).

CD8 T cells (Fig. 2). Unfortunately, however, as illustrated in Fig. 5b, the responses in BALF samples measured at Day 7 p.i. were observed not only after stimulation with the MVA-F recombinant but also after stimulation with wild type, non-recombinant MVA virus (although the percentage of IFN- γ ⁺ CD8 cells was ~40% lower). No such background responses were measured in unstimulated cells (Fig. 5b), or in MVA-stimulated PMBC or lymph node cells (Figs. 4 and 6).

3.4. Antiviral CD8 T lymphocytes recognize multiple bRSV proteins

To further determine the antigenic specificity of the response, we measured antiviral responses in the draining lymph nodes using intracellular IFN- γ staining. In these experiments, we focused on the breadth of the CD8 T cell response and the potential differences in specificity between the groups, focusing on the difference between the mock-immunized and the FI-bRSV-immunized groups. To identify the relevant antigens, we constructed a set of vaccinia virus recombinants expressing the viral N, P, M, G and M2 proteins,

in addition to the MVA-F recombinant. Lymphocytes were isolated from the tracheobronchial and mediastinal lymph nodes at Day 10 after infection and were stimulated by infection with the recombinant viruses expressing the different bRSV proteins, including the MVA-F recombinant. After overnight incubation, cells were permeabilized and stained with monoclonal antibodies against CD8 and IFN- γ . Within the group of 14 bRSV-infected animals that we analyzed, we found responses against all proteins tested (Table 1). The results showed that whereas similar numbers of PBS immunized and FI-bRSV immunized calves recognized the P, M, G, M2 and F proteins, there was a bias towards N protein recognition in FI-bRSV immunized calves compared with PBS controls. Thus, five out of six FI-bRSV immunized calves recognized the N protein compared with only one out of six PBS immunized calves ($P = 0.065$). A representative animal from the FI-bRSV immunized group (#47) with strong bRSV-specific responses (including N) is shown in Fig. 6a. It is interesting that CD8 T cell responses against the G protein were observed in several animals (Table 1 and Fig. 6b). From these experiments we conclude that immunization did

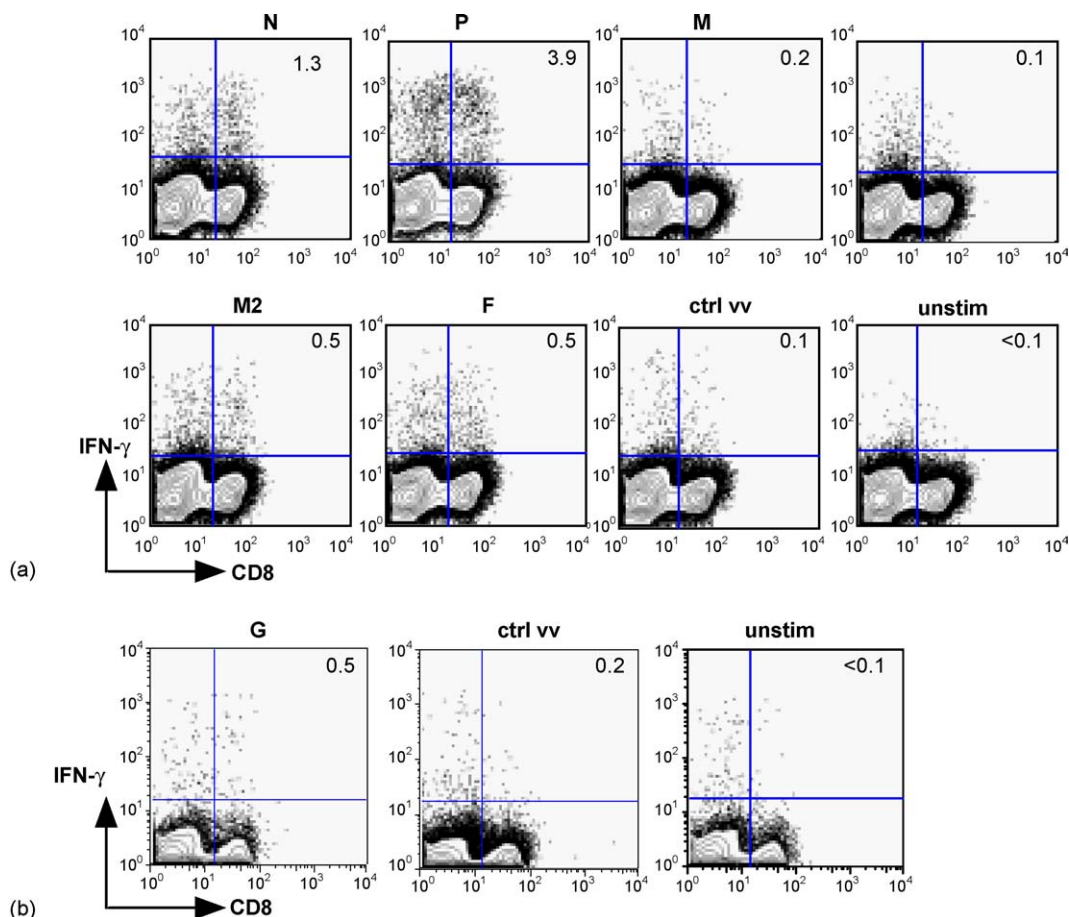


Fig. 6. Specificity and breadth of the response in animal #47 (vaccinated with FI-bRSV) (a) and illustration of G-specific responses in animal #42 (vaccinated with PBS) (b). Lymphocytes from the draining lymph nodes from animals #47 (tracheobronchial lymph nodes) and #42 (mediastinal lymph nodes) were stimulated with recombinant viruses and stained for IFN- γ expression. The bRSV or control genes expressed by the recombinant viruses are indicated. Frequencies are expressed as percentages IFN- γ ⁺ CD8⁺ cells of total CD8⁺ cells.

not result in a strong specificity bias towards individual viral proteins, with the possible exception of the N protein, and that most viral proteins are potential targets for CD8 T cells.

4. Discussion

In the present study, we have analyzed antiviral CD8 T cell responses in a symptomatic bRSV infection model [1]. In particular, we have compared T cell responses in infected calves undergoing primary infection and in calves undergoing secondary infection after FI-bRSV or L-bRSV immunization. Both immunizations result in reduced viral replication, but only the L-bRSV vaccine provides partial protection against symptoms [1]. FI-bRSV vaccination primes for enhanced pathogenesis that is associated with eosinophilia and increased IgE titers. Recent data indicate that these IgE antibodies are specific for the bRSV F protein (Antonis et al., unpublished observations). Antiviral T cell responses were quantitated by measuring (i) the kinetics of T cell influx and proliferation status in the lungs and (ii) the

specificity and frequencies of IFN- γ producing CD8 T cells in the lungs, draining lymph nodes and periphery. IFN- γ producing cells were visualized by stimulating lymphocytes using bRSV antigens produced by recombinant vaccinia or recombinant MVA vectors. Since it is possible that not all bRSV-specific CD8 T cells respond to recombinant vaccinia-produced antigens by IFN- γ production [36], we cannot exclude that our assay underestimates the true magnitude of the response. Furthermore, a particular technical problem that we encountered with our intracellular IFN- γ staining assay was that stimulation of BALF samples with the control MVA virus yielded a strong background response. These results are consistent with a recently published study by Sandbulte et al. [37] and as suggested by these authors, could reflect superantigenic properties of MVA, or stimulation of innate pathways. An alternative explanation is that the CD8 T cells in the lung have a low activation threshold, with the result that viral (i.e., MVA) replication could trigger IFN- γ production. It is obvious that this complicates the interpretation of these data: although it is clear that the BALF samples from infected animals contain considerable numbers of CD8 T cells that can be activated to produce

Table 1
%IFN- γ ⁺ CD8 cells in draining lymph nodes after stimulation with recombinant vaccinia viruses

Stimulation	PBS immunized animals						FI-bRSV immunized animals						L-bRSV	
	38	39	40	41	42	43	44	45	46	47	48	49	50	51
N	1.2 ¹	0.2	0.1	0.1	0.3	0.1	0.2	0.7	0.7	1.3	1.1	1.2	0.1	0.1
P	0.9	0.3	0.1	0.2	1.1	0.1	0.1	0.2	0.1	3.9	0.2	0.6	0.1	0.1
M	0.4	0.1	0.2	0.2	0.2	0.3	0.1	0.7	0.1	0.2	0.2	1.2	0.2	0.2
G	1.4	0.1	0.2	0.5	0.5	0.1	0.6	0.4	0.1	0.1	0.6	0.4	0.2	0.1
M2	0.8	0.7	0.1	0.7	0.9	0.1	0.3	0.3	0.2	0.5	0.6	0.9	0.4	0.2
F	0.5	1.2	0.2	0.1	0.9	0.1	0.7	0.4	0.2	0.5	0.4	0.4	0.5	0.3
ctrl vv	0.5	0.3	0.1	0.2	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
unstim	0.3	0.4	0.2	0.2	0.1	0.1	0.2	0.1	0.1	0.3	0.3	0.3	0.3	0.2

^a% IFN- γ ⁺ CD8 cells responding to vaccinia viruses expressing bRSV genes (N, P, M, G, M2, F). 'Ctrl vv' indicates stimulation with a control vaccinia virus expressing T7 polymerase. 'Unstim' indicates unstimulated lymphocytes. Shaded boxes indicate where responses above background levels have been measured. 'Above background' was operationally defined as average of all Ctrl vv values (0.16) + 2 × S.D. (2 × 0.12 = 0.24), leading to a cut-off of 0.4.

IFN- γ (and therefore, display a Th-1-like phenotype), their specificity cannot be determined in detail using this method.

Our study makes three points. First, we found potent virus-induced pulmonary CD8 T cell responses in all three experimental groups, although the magnitudes, expressed as total numbers of T cells in the BALF, displayed different kinetics. Accelerated responses, indicative of the recruitment of memory T cells, were observed in the L-bRSV-immunized group, but not in the FI-bRSV-immunized group, as compared to the PBS group. The largest responses, in terms of the numbers of T cells in the BALF, were seen in PBS-immunized calves. The levels of IFN- γ ⁺ T cells were similar in the three groups, although IFN- γ ⁺ CD8 T responses were slightly higher and more focused to the N protein in the FI-bRSV group. Because recalled memory T cell responses in the immunized animals should result in an accelerated recruitment of antiviral T cells as compared to naive animals, our data suggest that the L-bRSV vaccine has the capacity to prime bRSV-specific memory cells. In contrast, no such accelerated recruitment was observed for the FI-bRSV immunized group, leading to the conclusion that this vaccine did not prime very high levels of long-term T cell memory. It cannot be excluded that the FI-bRSV vaccine induced a weak N-specific memory response. The conclusion that FI-bRSV vaccination fails to prime CD8 T cell responses conclusion is supported by recent work from Woolums et al. [38]. In contrast, West et al. [39] reported that vaccination with modified-live bRSV resulted in somewhat accelerated antiviral CTL responses post bRSV challenge, suggesting that the modified-live vaccine did prime CD8 memory in calves, similar to our data. The greater magnitude of the T cell response (in terms of numbers of T cells in the BALF) in PBS-immunized animals compared to vaccinated animals could be explained by different levels of antigen: both vaccines were very efficient in

reducing viral replication [1]. Second, Th-1 (IFN- γ) and Th-2 (IgE) [1] associated responses co-exist in FI-bRSV immunized and bRSV-challenged animals. BRSV-specific T cells were readily identified by IFN- γ staining. In a separate study, we analyzed production of the Th-2 cytokine IL-4 by bRSV-induced T cells, but we did not find any IL-4 secreting T cells after in vitro stimulation with bRSV antigens (Antonis and van der Most, unpublished data). We are currently developing IFN- γ , IL-4 and IL-5 Q-PCR assays to better assess the Th-2 bias of the response. Combined, the data suggests that IFN- γ ⁺ pulmonary CD8 T cell responses do not necessarily protect against severe symptoms (which were only observed in the FI-bRSV group) [1]. A similar Th1/Th2 co-existence has been demonstrated in RSV-infected BALB/c mice, in which the pathogenic V β 14⁺ population of G-specific CD4 T cells is characterized by the simultaneous production of the both IFN- γ and IL-5 [23]. Third, the antigenic targets recognized by the antiviral CD8 T cells in the draining lymph nodes are diverse: the N, P, M, M2, F and G proteins are all recognized in different animals, indicating that immunization did not result in a strong specificity bias, with the possible exception of N, which is more often recognized in FI-bRSV immunized animals. Salient detail is the detection of CD8 T cell responses specific for the G protein in several animals, especially since a lack of G-specific CD8 T cell responses in RSV-infected BALB/c mice has been proposed as the key factor in enhanced pathogenesis [20]. Clearly this is not the case in bRSV-infected calves, since even FI-bRSV immunized animals with severe symptoms harbored anti-G CD8 T cell responses (see animals 44 and 48). Consistent with our data, showing a broad response, Gaddum et al. recently identified the N, F and M2 proteins as antigenic targets of memory CD8 T cell responses in bRSV-infected MHC-I homozygous animals [35].

Our previous study revealed that vaccination with either the FI-bRSV or L-bRSV vaccines resulted in reduced levels of viral RNA in the lungs [1]. Assuming that the FI-bRSV vaccine is indeed a poor inducer of long-lived CD8 memory responses, it appears that the main protective component of this vaccine is the level of antibodies. The efficacy of the L-bRSV vaccine, which reduces viral replication in the absence of immunopathology, could perhaps be explained by its capacity to induce bRSV-specific memory T cells. If this is indeed the case, then it seems plausible that RSV vaccination strategies can be optimized by further enhancing the capacity of such vaccines to induce CD8 T cell memory.

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