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Pulmonary Eosinophils and Their Role in Immunopathologic Responses to Formalin-Inactivated Pneumonia Virus of Mice

Caroline M. Percopo,* Zhijun Qiu,* Simon Phipps,† Paul S. Foster,† Joseph B. Domachowske,‡ and Helene F. Rosenberg²*²

Enhanced disease is the term used to describe the aberrant Th2-skewed responses to naturally acquired human respiratory syncytial virus (hRSV) infection observed in individuals vaccinated with formalin-inactivated viral Ags. Here we explore this paradigm with pneumonia virus of mice (PVM), a pathogen that faithfully reproduces features of severe hRSV infection in a rodent host. We demonstrate that PVM infection in mice vaccinated with formalin-inactivated Ags from PVM-infected cells (PVM Ags) yields Th2-skewed hypersensitivity, analogous to that observed in response to hRSV. Specifically, we detect elevated levels of IL-4, IL-5, IL-13, and eosinophils in bronchoalveolar lavage fluid of PVM-infected mice that were vaccinated with PVM Ags, but not among mice vaccinated with formalin-inactivated Ags from uninfected cells (control Ags). Interestingly, infection in PVM Ag-vaccinated mice was associated with a ~10-fold reduction in lung virus titer and protection against weight loss when compared with infected mice vaccinated with control Ags, despite the absence of serum-neutralizing Abs. Given recent findings documenting a role for eosinophils in promoting clearance of hRSV in vivo, we explored the role of eosinophils in altering the pathogenesis of disease with eosinophil-deficient mice. We found that eosinophil deficiency had no impact on virus titer in PVM Ag-vaccinated mice, nor on weight loss or levels of CCL11 (eotaxin-1), IFN-γ, IL-5, or IL-13 in bronchoalveolar lavage fluid. However, levels of both IL-4 and CCL3 (macrophage inflammatory protein-1α) in bronchoalveolar lavage fluid were markedly diminished in PVM Ag-vaccinated, PVM-infected eosinophil-deficient mice when compared with wild-type controls. The Journal of Immunology, 2009, 183:604–612.

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The development of safe and effective vaccines remains a standard and a benchmark for the control of respiratory virus infection. For infections with the ubiquitous human pathogen, respiratory syncytial virus (hRSV), this goal has not yet been reached. Along with efforts directed toward human clinical trials (1, 2), there is also significant interest in exploring alternative models for preclinical experimental research. The cotton rat and mouse models of hRSV challenge have been used extensively to study seroconversion and other immune responses to this virus, but these models face limitations with respect to issues of infectivity and virus replication in vivo (3, 4). The bovine pathogen, bRSV, shares remarkable sequence similarity with hRSV, although RSV disease in cattle and humans are distinguished by markedly different clinical findings and tissue pathology (5, 6). Live-attenuated vaccines against bRSV have been available for more than 20 years, although these formulations do not provide full protection against naturally acquired disease (7).

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We and others have characterized pneumonia virus of mice (PVM) infection, as disease pathogenesis in inbred strains of mice reproduces features of the more severe forms of hRSV (reviewed in Refs. 8, 9). Results of several recent studies suggest that PVM may also serve as a useful model for developing vaccine strategies, particularly those focused on acquisition of mucosal immunity to replicating virus pathogens. Specifically, Ellis and colleagues (10) demonstrated that mucosal inoculation with tissue culture-attenuated PVM resulted in protection against lethal challenge with the fully virulent strain via an IFN-γ-independent mechanism. Two groups have recently developed recombinant PVM using reverse genetics methodology (11, 12), which may prove to be useful for experimental vaccine-related studies.

However, among the factors that are credited with having delayed progress toward an effective hRSV vaccine is the feature known as “enhanced disease”, a hypersensitivity phenomenon initially observed in children inoculated with a formalin-inactivated vaccine who then responded aberrantly to a subsequent natural infection (reviewed in Refs. 13, 14). Rather than developing protective immunity, children inoculated with the formalin-inactivated vaccine developed pulmonary infiltrates containing mononuclear cells and eosinophils, both features characteristic of a skewed Th2 immune response. This response has been studied extensively and has been modeled in BALB/c mice inoculated with formalin-inactivated RSV components; the characteristic lung findings have been associated with overexpression of Th2 cytokines as well as with immune complex formation (15–18). Most recently, Moghaddam and colleagues (19) have provided biochemical evidence correlating this response with the presence of formalin-induced aldehydes on virus proteins, and Delgado and colleagues (20) have associated this response with lack of Ab affinity maturation and poor TLR signaling.

A Th2-skewed response may be a specific characteristic of formalin-inactivated paramyxovirus Ag vaccines, as hypersensitivity...
responses have been observed in similar preparations of bRSV (21, 22), measles virus (23), and human metapneumovirus (24). Given the interest in using PVM as a model for vaccination strategy, it will be crucial to establish its similarities to other infection and vaccine models. As such, we herein evaluate the responses of mice to formalin-fixed PVM Ags, prepared in manner analogous to the “lot 100” vaccine described in the initial clinical trials (25). Additionally, we explored the responses to formalin-fixed PVM Ags in eosinophil-deficient ΔδbGATA and TgPHIL mouse models (26, 27). Interestingly, despite substantial focus on eosinophil recruitment, it is not at all clear whether eosinophils actually promote systemic disease in response to formalin-inactivated vaccine challenge (28, 29). Given recent findings of Phillips and colleagues (30), who documented the role of eosinophils in augmenting virion clearance in the hRSV challenge model, we have focused on determining whether eosinophils serve to reduce virus titer and thereby promote host defense against PVM infection in response to this challenge.

**Materials and Methods**

**Mouse strains**

Wild-type BALB/c and C57BL/6 mice were obtained from Taconic. Eosinophil-deficient ΔδbGATA and TgPHIL mice models (26, 27). Interestingly, despite substantial focus on eosinophil recruitment, it is not at all clear whether eosinophils actually promote systemic disease in response to formalin-inactivated vaccine challenge (28, 29). Given recent findings of Phillips and colleagues (30), who documented the role of eosinophils in augmenting virion clearance in the hRSV challenge model, we have focused on determining whether eosinophils serve to reduce virus titer and thereby promote host defense against PVM infection in response to this challenge.

**Virus stocks and vaccine preparation**

The fully pathogenic PVM strain J3666 was originally obtained from Dr. A. Easton (University of Warwick, Coventry, U.K.) from virus stocks originating at the Rockefeller University. PVM strain J3666 has been maintained via continuous passage in vivo. Tissue culture-attenuated PVM strain 15 was obtained from the American Type Culture Collection (ATCC). Sequence similarities and differences between these two strains and the second variant strain 15 are as described (31). PVM strain 15 was used to prepare formalin-fixed vaccine Ag in a manner similar to that described for the RSV “lot 100” vaccine (25, 32, 33). In previous studies, mice inoculated with PVM strain 15 that recovered from acute infection served as control to PVM (SMART M-12; Biotech Trading Partners) and were completely protected from subsequent challenge with pathogenic PVM strain J3666, indicating conservation of crucial protective epitopes. To prepare vaccine Ags (PVM Ags), cells of the mouse monocye-macrophage RAW 264.7 cell line (ATCC), which permits moderate rates of replication of PVM at the permissive temperature of 32°C (34) (supplemental Fig. 1), 3 were grown to 50% confluence in IMDM with 10% heat-inactivated FCS, 2 mM glutamine, and penicillin/streptomycin and inoculated with PVM strain 15 at a multiplicity of infection of ~0.1. At 96 h after inoculation, the RAW 264.7 cells were scraped into the growth medium and sonicated to release cell-associated virions. Supernatants were clarified by high-speed centrifugation, washed twice with cold PBS, and resuspended in PBS at 100 μg/ml (as determined by Bradford assay) before addition of alum (Pierce Biotechnology). Control vaccine Ags (Ctrl Ags) were prepared precisely as above from parallel cultures of uninfected RAW 264.7 cells.

**Vaccination and infectious challenge**

Sera from age- and gender-matched mice were evaluated for seronegativity to PVM (SMART M-12) before initiating the experimental protocols (Fig. 1A). Ctrl Ags or PVM Ags were inoculated by sterile s.c. injection (100 μl/mouse) on days 0 and 14. On day 21, vaccinated mice were weighed and bled for sera to be used for detection of neutralizing Abs to PVM, and were then challenged with 30 PFU (34) pathogenic PVM strain J3666 in an 80-μl volume, with an identical inoculum of heat-inactivated virus or diluent control. Mice were weighed on days 25 and 26 (days 4 and 5 after challenge) and euthanized by cervical translocation under mild anesthesia on day 26, which was day 5 after infectious challenge. On day 5, differential physiologic responses (weight loss) are most pronounced (see Tables I and II). PVM-infected, but otherwise unmanipulated, mice (35, 36) and PVM-infected mice vaccinated with Ctrl Ags undergo rapid-onset mortality shortly thereafter.

**Evaluation of serum for neutralizing Abs**

Blood was taken from mice before vaccination on day 0 (before vaccination) and at day 21 (after the vaccinations on days 0 and 14, before infectious challenge) and serum was separated by standard methods. To determine whether neutralizing Abs developed in response to PVM Ags vaccination, paired pre- and postimmune sera from individual mice were diluted 1/10 in PBS (40 μl of serum and 360 μl of PBS). Virus (50 μl of undiluted PVM strain J3666) was added and samples were rotated end over end for 2 h at 4°C. One hundred twenty microtiter of the virus plus serum was added to RAW 264.7 cells growing in culture at 50% confluence in a 6-well plate (triplicate samples). Cultures were harvested 4 days after inoculation, and Triton X-100 lysates and RNA were prepared. Virus replication was detected by probing Western blots containing cell lysates with a 1/300 dilution of rabbit polyclonal anti-PVM N protein Ab, followed by a 1/1000 dilution of alkaline-phosphatase conjugated goat anti-rabbit IgG and standard developing reagents (Bio-Rad). Parallel blots were probed with a 1/300 dilution of chicken anti-actin following by a 1/1000 dilution of alkaline phosphatase-conjugated rabbit anti-chicken IgY (United States Biochemical) and developing reagents. Virus replication was also detected in a more quantitative fashion by quantitative RT-PCR using the dual standard curve method as described below. Negative control was sera from a normal, unmanipulated mouse; positive control was convalescent sera from C57BL/6 mice challenged with a sublethal inoculum of PVM strain 15 (10).

**Histopathology and immunolocalization of eosinophils**

For evaluation of histopathology, lung tissue was inflated slightly by transtracheal instillation of 0.2–0.3 ml cold phosphate-buffered 10% formalin. Lungs were removed and fixed in cold phosphate-buffered formalin before paraffin embedding. H&E-stained fixed tissue sections were prepared by Histoserv. Eosinophils were identified in formalin-fixed lung tissue sections via serial dilution of polyclonal rabbit anti-mouse eosinophil major basic protein antisera (generous gift of Dr. J. Lee and Dr. N. Lee, Mayo Clinic, Scottsdale, AZ) followed by goat anti-rabbit peroxidase staining (performed by Histoserv).

**Virus titer in lung tissue**

RNA was isolated from lung tissue that was isolated from mice and immersed in RNAlater (Ambion) and stored as per manufacturer’s instructions. RNA was isolated using the RNAzol Bee reagent (Tel-Test) (10) and virus titer was determined by quantitative RT-PCR with dual standard curve. This assay has been used to measure absolute copies of virus genome (PVMNp/10^6 GAPDH) generated per unit time with appropriate replication kinetics both in vivo, in mouse lung tissue, and in vitro in the mouse macrophage RAW 264.7 cell line (supplemental Fig. 1). In specific detail, RNA isolated as described was treated with DNase I to remove genomic contaminants. Reverse transcription to cDNA was performed using the first-strand cDNA synthesis kit (Roche; catalog no. 1,483,188), using random primers and including a no reverse transcriptase control. The quantitative PCR reactions were run in triplicate, with the ABI 2′ TaqMan reagent (primer-probe mixes and cDNA or plasmid standard in a 25-μl final volume (Applied Biosystems). Thermal cycling parameters for the ABI 7500 absolute quantitation program (Applied Biosystems) include 50°C for 2 min (UNG incubation), 95°C for 10 min (AmpliTaq Gold activation), and 40 amplification cycles alternating 95°C for 15 s and 60°C for 1 min. Primer-probe mixes include GAPDH-Vic (Applied Biosystems catalog no. 4308313) and PVMNp-Fam (custom design, primer 1, 5′-GCC GTC ATG AAC AUA GTA TGT-3′; primer 2, 5′-GCC TGA TGT GCC AGT GCT T-3′; probe 5′-FAM-CGC TGA TAA TGG CCT GCA GCA-GARMA). GAPDH standard curve includes serial dilutions of a mouse GAPDH nucleotide sequence (Ambion; DECA probe template, no. 7330) to 10^9, 10^8, 10^7, 10^6, and 10^5 molecules per reaction. PVMNp standard curve includes serial dilutions of the full-length PVM N open reading frame. (GenBank, http://www.ncbi.nlm.nih.gov/sites/entrez accession no. AY573815) in pBacPAK8 to 10^9, 10^8, 10^7, and 10^6 molecules per reaction. Experimental triplicate data points are interpolated to linear standard curves over the concentration range indicated.

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4 The online version of this article contains supplemental material.

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**Abbreviations used**

AGT GCT T-3

5 GCC GTC ATG AAC AUA GTA TGT-3′;

5′-FAM-CGC TGA TAA TGG CCT GCA GCA-GARMA

A gene product that is a ligand for the CX3C chemokine receptor CCR3.

GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

H&E: hematoxylin and eosin.

IMDM: Iscove’s modified Dulbecco’s medium.

PVMSH: PVM major basic protein.

PVMSH-Fam: custom-designed probe for PVMSH.

PVMSH-106 GAPDH: custom-designed primer for PVMSH.

PVMSH/106 GAPDH: custom-designed primer for PVMSH.

GTP: guanosine triphosphate.

H&E: hematoxylin and eosin.

IMDM: Iscove’s modified Dulbecco’s medium.

PVMSH: PVM major basic protein.

PVMSH-Fam: custom-designed primer for PVMSH.

PVMSH/106 GAPDH: custom-designed primer for PVMSH.

GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

H&E: hematoxylin and eosin.

IMDM: Iscove’s modified Dulbecco’s medium.

PVMSH: PVM major basic protein.

PVMSH-Fam: custom-designed primer for PVMSH.

PVMSH/106 GAPDH: custom-designed primer for PVMSH.

GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

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**Results**

**Microscopic and biochemical pathology associated with PVM infection after inoculation with formalin-inactivated Ags**

The experimental time line is shown in Fig. 1A. Lung tissue sections from mice vaccinated on days 0 and 14 with formalin-inactivated Ags prepared from uninfected cells (Ctrl Ags) and from PVM-infected cells (PVM Ags) before challenge on day 21 with actively replicating PVM are shown in Fig. 1, B–E. The histopathology observed in response to vaccination with Ctrl Ags followed by PVM infection is similar to that observed in PVM infection in naive, otherwise unmanipulated mice (supplemental Fig. 2); at day 26 we observed profound inflammation throughout the lung parenchyma (Fig. 1B), with neutrophils predominating at this time point in both lung tissue and in BALF (35) (Fig. 1C). In contrast, the inflammatory infiltrates in lung tissue from mice vaccinated with PVM Ags exhibit a more diffuse, patchy pattern (Fig. 2) with denser inflammatory infiltrates in the peribronchiolar and perivascular regions (Fig. 1E). Profound eosinophilia was observed in the BALF (Fig. 1F) and within the lung parenchyma (Fig. 2).

The detailed pattern of leukocyte recruitment is shown in Fig. 3A. Leukocyte recruitment in PVM-infected mice vaccinated with Ctrl Ags is similar to what we have observed previously in PVM infected, but otherwise naive mice (35). Specifically, by day 5 of infection (day 26 of the protocol, see Fig. 1A), eosinophils are present (2.7 ± 1.5%), but neutrophils predominate (93 ± 1.8%). In contrast, 66 ± 4.0% of the leukocytes in BALF from mice inoculated with PVM Ags, followed by PVM infection and evaluation as described. Lung tissue is stained with H&E; BALF cytospins are stained with modified Giemsa.

**Statistical analysis**

Each datum point was determined from duplicate or triplicate trials of samples obtained from 4–10 mice as indicated in the text. Each independent experiment was repeated two or three times. Statistically significant differences were determined via Student’s t test or Wilcoxon rank sum test, with p values obtained as indicated.

**Weight loss associated with PVM infection after vaccination with formalin-fixed Ags**

We measured weights of all mice on days 21, 25, and 26 of the experimental protocol (see Fig. 1A), which are days 0, 4, and 5 after inoculation of 30 PFU of replication-competent PVM, 30 PFU equivalents of heat-inactivated PVM, or diluent alone. As
which includes eosinophil recruitment to the lung tissue in response to PVM Ags elicits the characteristic hypersensitivity response, (Fig. 4A). IL-5 (pg/ml) in BALF of mice vaccinated and PVM infected as described in Materials and Methods. No neutralizing activity was detected among paired individual antisera at low dilution (1/10), which were evaluated qualitatively by Western blotting (Fig. 5A). Similarly, no neutralizing activity was observed when sera were tested, again at low (1/10) dilution, and virus genome equivalents were evaluated by quantitative RT-PCR (Fig. 5B).

Evaluating the role of eosinophils in promoting protective responses

Eosinophils have been shown to reduce the infectivity of the hRSV by target cells in vitro (41, 42). Most recently, Phipps and colleagues (30) have shown that hRSV clearance in a mouse model is augmented in the presence of pulmonary eosinophilia. As the antiviral response elicited by PVM Ags is not dependent on the presence of serum-neutralizing Abs, we proceeded to examine the role of the eosinophils in promoting these protective responses.

The ΔdblGATA mice have undergone a 21-nucleotide deletion of a palindromic GATA-binding enhancer site in the hematopoietic promoter of the gene encoding GATA-1. This deletion results in a unique deficiency of the eosinophil lineage with sparing of all other hematopoietic lineages (26). TgPHIL mice express a diphtheria toxin A transgene under the control of the eosinophil-specific eosinophil-peroxidase promoter, thereby resulting in lineage-specific cytosucide (27). Eosinophils can be identified in the BALF of PVM Ag-vaccinated, PVM-infected C57BL/6 wild type mice, but, as anticipated, no eosinophils are detected in identically treated eosinophil-deficient TgPHIL or ΔdblGATA mice (Fig. 6A).

As originally shown in Fig. 4A, virus titers among PVM-infected mice that had been inoculated with PVM Ags are diminished when compared with those inoculated with Ctrl Ags, although the difference is not dramatic. Interestingly, we find that eosinophil deficiency has no impact on virus titer in this experimental setting. Virus titer in lung tissue of PVM Ag-vaccinated,

Table I. Weights of wild-type mice vaccinated on days 0 and 14 with Ctrl Ags or PVM Ags followed by challenge on day 21 with actively replicating PVM, heat-inactivated PVM, or diluent alone

<table>
<thead>
<tr>
<th>Vaccination</th>
<th>Challenge</th>
<th>n</th>
<th>Day 0 (g) ± SD</th>
<th>Day 4 (g) ± SD</th>
<th>Day 5 (g) ± SD</th>
<th>Weight Loss (%) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctrl Ags</td>
<td>Diluent</td>
<td>5</td>
<td>21.9 ± 1.5</td>
<td>21.2 ± 1.1</td>
<td>21.4 ± 1.1</td>
<td>3.9 ± 0.4</td>
</tr>
<tr>
<td>Ctrl Ags</td>
<td>Inact. PVM</td>
<td>9</td>
<td>21.1 ± 1.4</td>
<td>ND</td>
<td>21.1 ± 1.3</td>
<td>ND</td>
</tr>
<tr>
<td>Ctrl Ags</td>
<td>PVM</td>
<td>10</td>
<td>21.9 ± 1.3</td>
<td>20.8 ± 1.3*</td>
<td>19.2 ± 1.3**</td>
<td>12 ± 3</td>
</tr>
<tr>
<td>PVM Ags</td>
<td>Diluent</td>
<td>5</td>
<td>21.0 ± 0.67</td>
<td>21.8 ± 0.28</td>
<td>21.0 ± 0.69</td>
<td>ND</td>
</tr>
<tr>
<td>PVM Ags</td>
<td>Inact. PVM</td>
<td>9</td>
<td>21.3 ± 0.96</td>
<td>ND</td>
<td>20.9 ± 0.83</td>
<td>ND</td>
</tr>
<tr>
<td>PVM Ags</td>
<td>PVM</td>
<td>9</td>
<td>22.3 ± 0.89</td>
<td>22.1 ± 1.07</td>
<td>21.0 ± 1.04*</td>
<td>6 ± 4†</td>
</tr>
</tbody>
</table>

*As shown, n indicates number of mice per group; weight loss for each mouse was calculated as [(weight at day 0 − weight at day 5) × 100]/weight at day 0; †, p < 0.002 vs respective weights at day 0; †, p < 0.001 vs respective weights at day 0; †, p < 0.001 vs weight loss observed in response to inoculation with Ctrl Ags followed by PVM challenge.
PVM-infected eosinophil-deficient mice (both ΔdblGATA and Tg-PHIL) are indistinguishable from those determined for their respective wild types; diminished virus titers are detected among all mice vaccinated with PVM Ags, compared with those vaccinated with Ctrl Ags, regardless of the presence or absence of eosinophils (Fig. 6, B and C).

As originally shown in Table I, we observe weight loss in response to PVM infection in both Ctrl Ags and PVM Ag-vaccinated mice, with a small but statistically significant degree of protection observed when comparing those inoculated with PVM Ags to those vaccinated with Ctrl Ags (p < 0.03; Table II). Similar findings were observed among the Ctrl Ags and PVM Ag-vaccinated and PVM-infected ΔdblGATA mice; eosinophil ablation had no impact on the degree of protection observed.

**Th2 cytokine responses in vaccinated and PVM-challenged wild-type and eosinophil-ablated mice**

A prominent Th2 response, including cytokines IL-4, IL-5 and IL-13, was detected in BALF of all mice that were inoculated with PVM Ags before PVM infection. Eosinophil ablation had no impact on detection of IL-5 or IL-13 (Fig. 7, A and B). In contrast, IL-4 detection in the eosinophil-deficient ΔdblGATA mice was markedly diminished; IL-4 levels in PVM Ag-vaccinated, PVM-infected ΔdblGATA mice were not significantly higher than those detected in response to Ctrl Ags vaccination (Fig. 7C). This finding is similar (although interestingly, not quite identical) to that reported by Jacobsen and colleagues (45) in their study of eosinophil-mediated recruitment of Th2 cells in response to allergen sensitization and challenge in vivo.

**Biochemical inflammatory responses in vaccinated and PVM-challenged wild-type and eosinophil-ablated mice**

Finally, we compared the biochemical inflammatory responses among the Ag-vaccinated, PVM-infected wild-type and eosinophil-deficient ΔdblGATA mice. Both wild-type and ΔdblGATA mice respond to PVM infection by producing a variety of proinflammatory mediators. Among the mediators of interest, CCL11 (eotaxin-1) is produced by respiratory epithelial cells in response to virus infection and is a unique mediator of eosinophil chemotraction (46); CCL3 (MIP-1α) recruits both neutrophils and eosinophils to the airways, and, as noted earlier, is a prominent biomarker of hRSV disease severity (39, 40); and IFN-γ, a cytokine with pleiotropic immunomodulatory properties (47), was recently shown to exert hierarchical control over the activities of CCL3 in...
CCL3 is completely unexplored. the role of eosinophils in promoting virus-induced expression of primarily by respiratory epithelial cells in response to PVM infection; those responding to PVM Ags vaccination. CCL3 is produced pri-
dblGATA mice, both the Ctrl Ag-vaccinated mice as well as 

CCL3 detected in BALF of PVM-infected eosinophil-deficient 
BALF. In contrast, we observed a marked reduction in the level of 

expression was markedly diminished in lung tissue determined by quantitative RT-PCR; 

n indicates number of mice per group; weight loss was calculated as described in Table 1; *, p < 0.05; **, p < 0.01 vs respective weights at day 0; †, p < 0.03 vs weight loss observed in response to inoculation with Ctrl Ags followed by PVM challenge.

Discussion

In this work we characterize the pathophysiologic responses of mice vaccinated with formalin-inactivated PVM Ags (PVM Ags) and demonstrate that they are similar to those reported for formalin-inactivated hRSV in the human vaccine trial and in exploratory rodent models. Specifically, we detect eosinophil-enriched inflammatory infiltrates in lung tissue and BALF associated with augmented levels of BALF Th2 cytokines. Interestingly, hyper-
sensitivity responses have been observed in response to formalin-inactivated Ags derived from a variety of paramyxovirus pathogens, including measles virus, bRSV, and, most recently, human metapneumovirus (21–24). The precise molecular mechanisms underly-
ing these responses remain uncertain. Several groups originally reproduced the hRSV response in mice by overexpression of G (attachment) protein alone in the absence of formalin (49–51). However, recent analyses suggested that, although there were some similarities, specifically pulmonary eosinophilia in association with Th2 cytokines, the responses to G protein overexpression occurred via distinct cellular mechanisms (reviewed in Refs. 13, 52). Among these, G protein-dependent eosinophilia required Vβ14+ T cells, while responses to formalin-inactivated hRSV Ags did not (53); likewise, formalin-inactivated hRSV Ags devoid of G protein were fully capable of eliciting hypersensitivity responses in BALB/c mice (54). From another perspective, the recent evidence suggesting that formalin-induced hRSV protein oxidation plays a role in promoting aberrant responses (19) is quite intriguing, and it will be interesting to determine, given that formalin inactivation results in more or less indiscriminate protein oxidation, why only a specific formalin-oxidized paramyxovirus protein (or proteins) induces Th2 skewing in vivo. In fact, it will be interesting to de-
terminate whether Th2 skewing is in fact limited to formalin-oxi-
dized paramyxovirus proteins only. The PVM model may be use-
ful in experiments designed to address these issues.

Regarding specificity, Piedra and colleagues (55) and Boelen and colleagues (56) both reported independently that formalin-inactivated nonvirus Ags elicited immunopathology in vaccinated cotton rats and BALB/c mice, respectively, an issue that is of con-
cern regarding validity of this model. We did not observe hyper-
sensitivity responses to formalin-inactivated Ags from uninfected 
cells in our experiments, but this may be attributed to the exper-
imental design more than specificity of the response. Among the 
differences between the models, the PVM strain J3666 used for 
infection is passaged in vivo and is thus devoid of tissue culture 
Ags that could readily cross-react with those used for preparing 
PVM for inoculation; in vivo passage of the infectious pathogen is 
ly, and it replicates to very high virus titer, sufficient for isolation 
of the infectious pathogen in mouse lung tissue in vivo (3, 
4) (supplemental Fig. 1). Thus, the only Ags ultimately present 
in the setting of PVM infection in vivo (48). Here, we find that BALF 
levels of CCL11, IFN-γ, and CCL3 were markedly diminished in 
PVM-infected mice that had been vaccinated with PVM Ags com-
pared with those vaccinated with Ctrl Ags (Fig. 8). Eosinophil 
deficiency had no impact on levels of CCL11 or IFN-γ detected in 
BALF. In contrast, we observed a marked reduction in the level of 
CCL3 detected in BALF of PVM-infected eosinophil-deficient 
ΔdblGATA mice, both the Ctrl Ag-vaccinated mice as well as 
those responding to PVM Ags vaccination. CCL3 is produced pri-
marily by respiratory epithelial cells in response to PVM infection; 
the role of eosinophils in promoting virus-induced expression of 
CCL3 is completely unexplored.

FIGURE 6. Comparison of wild-type and eosinophil-deficient mice: cellular inflammation and virus titer. A, BAL fluid from PVM Ag-vaccin-
nated, PVM-infected eosinophil-deficient ΔdblGATA and TgPHIL and 
wild-type mice; arrows indicate neutrophils (n) and eosinophils (e); cyto-

spin preparations were stained with modified Giemsa. B and C, Virus titer 
(PVMinf/10⁶ GAPDH) in lung tissue determined by quantitative RT-PCR; 

n = 4–10 mice/group; *, p < 0.05.

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Table II. Weights of wild type (BALB/c) and eosinophil-ablated (ΔdblGATA) mice vaccinated on days 0 and 14 with Ctrl Ags or 
PVM Ags followed by PVM infection on day 21*

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Vaccination</th>
<th>Challenge</th>
<th>n</th>
<th>Day 0</th>
<th>Day 4</th>
<th>Day 5</th>
<th>Weight loss (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c</td>
<td>Ctrl Ags</td>
<td>PVM</td>
<td>21</td>
<td>22.7</td>
<td>22.0</td>
<td>20.8</td>
<td>8.9 ± 4.6</td>
</tr>
<tr>
<td>BALB/c</td>
<td>PVM Ags</td>
<td>PVM</td>
<td>19</td>
<td>23.3</td>
<td>23.0</td>
<td>22.2</td>
<td>5.3 ± 4.7†</td>
</tr>
<tr>
<td>ΔdblGATA</td>
<td>Ctrl Ags</td>
<td>PVM</td>
<td>11</td>
<td>24.3</td>
<td>23.8</td>
<td>22.5</td>
<td>7.4 ± 2.6</td>
</tr>
<tr>
<td>ΔdblGATA</td>
<td>PVM Ags</td>
<td>PVM</td>
<td>11</td>
<td>25.8</td>
<td>25.7</td>
<td>24.9</td>
<td>3.8 ± 3.4†</td>
</tr>
</tbody>
</table>

* As shown, n indicates number of mice per group; weight loss was calculated as described in Table 1; *, p < 0.05; **, p < 0.01 vs respective weights at day 0; †, p < 0.03 vs weight loss observed in response to inoculation with Ctrl Ags followed by PVM challenge.
proteins, other than the fact that, in a natural setting, only replication of the respiratory virus in situ ultimately results in an Ag challenge of sufficient magnitude to initiate a hypersensitivity response to the formalin-inactivated vaccine components.

In addition to establishing that “enhanced disease” or hypersensitivity response is a characteristic of PVM Ags, we proceeded to use this model to explore the potential of pulmonary eosinophils elicited in this fashion to promote pathology or, as suggested in several recent publications (30, 41, 57, 58), to promote antiviral host defense. Of note, despite a remarkable amount of effort devoted to understanding the mechanisms via which eosinophils are recruited to the lung in response to formalin-inactivated virus Ags, it is not at all clear that eosinophils are in fact contributing to the physiologic dysfunction, specifically the respiratory dysfunction and systemic disease (28, 29), and recent results relating eosinophil recruitment, airway dysfunction, and systemic disease in response to hRSV G protein vaccination, a phenomenon recently shown to occur via mechanisms that are distinct from those contributing to formalin inactivation, suggest that eosinophils may be more or less “innocent bystanders” in the former process (59). In our earlier studies, we showed that isolated human eosinophils could reduce the infectivity of hRSV for target cells via the actions of their secretory mediators (41, 42); more recently, Phipps and colleagues (30) showed augmented hRSV clearance in IL-5 transgenic hyper-eosinophilic mice and induction of antiviral proteins in mouse eosinophils upon interaction with virions in vivo. As a first intriguing finding, we found that mice vaccinated with PVM Ags were protected against virus challenge; despite the absence of neutralizing Abs, we observed reduced total genome equivalents and diminished weight loss. These results suggest that the presence of eosinophils in the lung tissue and airways are associated with protection against virus infection. We repeated the vaccination and challenge protocol in eosinophil-deficient mice, and although this yielded several important biochemical alterations (to be discussed below), we found that eosinophil deficiency resulted in no change in virus titer and no change in the overall clinical picture.

Although we are still without explanation as to what is promoting protection against virus challenge in response to formalin-inactivated PVM Ags, among the larger questions vis-à-vis this work is why the responses of eosinophils differ here from what was observed in the hRSV challenge model (30), and why prominent virus clearance observed in the latter setting was not evident here. Among several possibilities, the most apparent relates to the nature of the pathogens and their relationship to the mouse model as a whole and with mouse eosinophils specifically. Overall, PVM is more virulent in mice than is hRSV, as PVM not only replicates in mouse lung epithelial tissue (38), but we have recently shown that PVM infects and replicates directly within mouse eosinophils (Ref. 60; K. D. Dyer, C. M. Percopo, E. R. Fischer, S. J. Gabryszewski, and H. F. Rosenberg, submitted for publication). We have not yet examined how PVM infection might alter the antiviral responses of eosinophils, but it is possible that infection might disable these cells, reduce their capacity to respond productively, and to clear virus infection, all elements that may serve to enhance the virulence of PVM in vivo.

As noted above, eosinophil ablation is associated with specific biochemical alterations in this model. While Th2 cytokine responses are detected in both wild-type and ΔdblGATA mice inoculated with PVM Ags, production of IL-4 is diminished specifically among the ΔdblGATA mice. This finding is consistent with those of Jacobsen and colleagues (45), who reported diminished recruitment of CD4+ T cells and IL-13 in BALF in eosinophil-ablated TgPHIL mice subjected to OVA sensitization and challenge, in a study which led to the conclusion that pulmonary eosinophils are crucial for the recruitment of Th2 lymphocytes in this traditional model of allergic lung disease. Here, IL-5 and IL-13 production persists among the ΔdblGATA mice, possibly derived from activated mast cells and basophils and NK and NKT cells, respectively (61–64), although the role of these cells in response to formalin-inactivated Ags has not been formally explored. Interestingly, diminished Th2 cytokine production in the absence of eosinophils is not a universal finding; we detected elevated levels of IL-5 in both the ΔdblGATA and TgPHIL eosinophil-deficient mouse strains during the Th2 phase of acute infection with the helminthic parasite Schistosoma mansoni (44).

In summary, we have clearly documented that a Th2-skewed hypersensitivity response, otherwise known as “enhanced disease”, is elicited by PVM infection following inoculation with formalin-inactivated PVM Ags, analogous to what is observed in response to formalin-inactivated hRSV, and in response to other formalin-inactivated paramyxovirus vaccines. While formalin-inactivated hRSV vaccine formulations are certainly not presently under consideration for human use, the developers of novel anti-RSV vaccine strategies continue to make a significant effort to demonstrate the absence of hypersensitivity reactions due to the serious nature of this problem (65–67). Given the recent interest in
PVM as a model for exploring mechanisms of disease and vaccine strategies, we have established an important point documenting similar immunopathologic responses to both human and mouse pathogens. Furthermore, we have also explored the role of eosinophils in this setting and found that they did not promote PVM clearance, despite clear evidence for eosinophil-mediated clearance of rRSV. The nature of the interactions between PVM and eosinophils remains a subject for future investigation.

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Disclosures

The authors have no financial conflicts of interest.

References


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Supplemental Figure Legends

Supplemental Figure 1. Kinetics of PVM replication in vivo and in vitro. (A) In vivo, wild type mice (n = 4 - 6 per time point) were sacrificed on days 0, 2, 3, and 5 after intranasal inoculation with 100 pfu (10^7 PVMSH) strain J3666 and lung tissue RNA was isolated for quantitative RT-PCR detection of PVM via the dual standard curve protocol as described in Methods. Day 0; mice were sacrificed immediately after intranasal inoculation; *p < 0.01 vs. day 2 and vs. day 3 as shown. (B) In vitro, cells of the RAW 264.7 cell line were plated at 5 x 10^5 per well in 6 well plates in IMDM medium as described in the Methods. 3 x 10^7 PVMSH (J3666) were added, incubated for 4 hours, non-adherent virus was removed by multiple washes, and fresh medium was added. At days 3, 5, and 7 after challenge, triplicate wells were scraped, and RNA prepared from individual wells for quantitative RT-PCR detection of PVM as described in the Methods; *p < 0.005 vs. day 3 as shown.

Supplemental Figure 2. (A) Normal mouse lung tissue (B) Lung tissue from an unvaccinated mouse infected with PVM, with focal peribronchiolar inflammatory infiltrates and diffuse involvement of the lung parenchyma.