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J Immunol 2009; 183:604-612; doi: 10.4049/jimmunol.0802270
http://www.jimmunol.org/content/183/1/604

Supplementary Material
http://www.jimmunol.org/content/suppl/2009/06/18/183.1.604.DC1

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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*†

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.

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).

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 (13–18). 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 RSV (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 ΔdblGATA 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 Phipps 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 ΔdblGATA and TgPHIL mouse models were originally obtained from Dr. J. Lee and Dr. N. Lee. All animal studies were conducted under the review of National Institutes of Allergy and Infectious Diseases Animal Study Protocol LAD8E.

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 seroconverted to PVM (SMART-M12; 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 monocyte-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 centrifugation, and formalin was added (1/4000) to the clarified supernatants, thereby promoting host defense against PVM infection in response to this challenge.

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 microliters 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 Biological) 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 antiserum (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 imersed 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 (PVMAG1/106 GADPH) 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 reagents, 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 1 s and 60°C for 1 min. Primer-probe mixes include GADPH-Vic (Applied Biosystems catalog no. 4308313) and PVMAG1-Fam (custom design, primer 1, 5′-GCC GTG ATC AAC MCA GTG TGT-3′; primer 2, 5′-GCC TGA TGT GCC AGT GCT T-3′; probe 6FAM-CGC TGA TAA TGG CCT GCA GCA-TAMRA). GADPH standard curve includes serial dilutions of a mouse GADPH nucleotide sequence (Ambion; DECA probe template, no. 7330) to 103, 102, 101, and 100 molecules per reaction. PVMAG1 standard curve includes serial dilutions of the full-length PVM SH open reading frame. (GenBank, http://www.ncbi.nlm.nih.gov/sites/entrez) accession no. (AY573815) in PbacPAK8 to 109, 108, 107, and 106 molecules per reaction. Experimental triplicate data points are interpolated to linear standard curves over the concentration ranges indicated.

4 The online version of this article contains supplemental material.
Detection of leukocytes and proinflammatory mediators in bronchoalveolar lavage fluid (BALF) and lung tissue homogenates

BALF was collected from vaccinated and challenged mice by instillation and withdrawal of 0.8 ml of cold PBS with 0.5 mM BSA. Cytospin preparations were stained with modified Giemsa (Dade Behring) and the leukocyte differential was determined by visual inspection of 10 independent fields from six to eight mice per group (>200 cells/mouse). Concentrations of various proinflammatory mediators (eotaxin, IFN-γ, macrophage inflammatory protein 1-α, IL-4, IL-5, and IL-13) were determined by ELISA (R&D Systems) as per the manufacturer’s instructions.

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.

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. 1D) 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 PVM-infected mice that were vaccinated with PVM Ag were eosinophils. The BALF eosinophilia observed in the PVM Ag-vaccinated, PVM-infected mice was accompanied by elevated levels of the Th2 cytokine IL-5 (Fig. 3B), similar to what has been reported in mouse models of aberrant hypersensitivity to hRSV (16, 37). Minimal IL-5 (below detectable limits of the assay) was detected in response to PVM infection in mice inoculated with Ctrl Ags. Other Th2 cytokines (IL-4, IL-13) detected in response to infection in PVM Ags and Ctrl Ag-vaccinated mice are described below.

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
shown in Table I, we observed no statistically significant weight loss over the 5-day examination period (days 21–26) in response to diluent or in response to a single dose of heat-inactivated PVM regardless of the vaccination Ag (Ctrl Ags or PVM Ags). We did observe significant weight loss in response to actively replicating PVM in both Ctrl Ag- and PVM Ag-vaccinated mice. Interestingly, PVM-infected mice vaccinated with PVM Ags had lost only 6 ± 4% of their initial body weight at day 5, compared with infected mice vaccinated with Ctrl Ags, which had lost 12 ± 3% (p < 0.002) of their initial body weight at this time point.

**Virus titer and virus-induced cytokine responses**

In association with protection against weight loss, PVM-infected mice that were vaccinated with PVM Ags exhibit lower lung virus titers than did those that were vaccinated with Ctrl Ags (*, p < 0.05; Fig. 4A). Likewise, we detected CCL3 (MIP-1A), a chemokine produced locally by respiratory epithelial cells in response to virus infection and a biomarker of disease severity (38–40), in all mice inoculated with PVM-infected C57BL/6 wild type mice, but, as anticipated, no eosinophils are detected in identically treated eosinophil-deficient TgPHIL or ΔdblGATA mice (Fig. 6B). 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,

**Neutralizing serum Abs**

All mice used herein were documented seronegative for PVM before the start of the experiments (data not shown). Sera obtained before vaccination (before day 0) and paired sera obtained on day 21 (after Ag vaccination, but before infectious challenge) were evaluated for their ability to neutralize PVM in the tissue culture assay 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 for target cells in vitro (41, 42). Most recently, Philipp 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 (BALB/c background) and TgPHIL mice (C57BL/6 background) are devoid of eosinophils at baseline and remain so in response to profound Th2 stimulation (26, 43, 44). 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 diptheria toxin A transgene under the control of the eosinophil-specific eosinophil-peroxidase promoter, thereby resulting in lineage-specific cytosuicide (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 alonea

<table>
<thead>
<tr>
<th>Vaccination</th>
<th>Challenge</th>
<th>n</th>
<th>Day 0</th>
<th>Day 4</th>
<th>Day 5</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></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></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>
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</tr>
<tr>
<td>PVM Ags</td>
<td>Inact. PVM</td>
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<td>21.3 ± 0.96</td>
<td>ND</td>
<td>20.9 ± 0.83</td>
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</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>

a 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 < 0.04;**, p < 0.001 vs respective weights at day 0; †, p < 0.002 vs weight loss observed in response to inoculation with Ctrl Ags followed by PVM challenge.
PVM-infected eosinophil-deficient mice (both \( \Delta dblGATA \) 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 (\( p \leq 0.03 \); Table II). Similar findings were observed among the Ctrl Ags and PVM Ag-vaccinated and PVM-infected \( \Delta dblGATA \) 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 \( \Delta dblGATA \) mice was markedly diminished; IL-4 levels in PVM Ag-vaccinated, PVM-infected \( \Delta dblGATA \) 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 \( \Delta dblGATA \) mice. Both wild-type and \( \Delta dblGATA \) 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-1\( \alpha \)) recruits both neutrophils and eosinophils to the airways, and, as noted earlier, is a prominent biomarker of hRSV disease severity (39, 40); and IFN-\( \gamma \), a cytokine with pleiotropic immunomodulatory properties (47), was recently shown to exert hierarchical control over the activities of CCL3 in

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**FIGURE 4.** Virus titer and detection of virus-induced CCL3. A, Virus titer (\( \text{PVM}_{ag/10^6 \text{GAPDH}} \)) was determined by quantitative RT-PCR of cDNAs from lung tissue RNA from mice that were inoculated with Ctrl Ags (gray symbols) or PVM Ags (black symbols), followed by PVM infection (+PVM) or challenge with inactivated virions (+inact PVM) or diluent (dil) on day 21, and evaluation on day 26. Horizontal bar indicates mean value; \( n = 5-6 \) mice/group. B, CCL3 (MIP-1\( \alpha \), pg/ml) detected in BALF of mice that were inoculated and infected or challenged as described above; \( * \), \( p < 0.05 \).

**FIGURE 5.** Evaluation of sera for neutralizing Abs. PVM virions (\( 5 \times 10^3 \) PFU of strain J3666) were equilibrated with 1/10 dilutions of mouse sera in PBS and used to infect RAW 264.7 cell cultures (see Materials and Methods). A, Lysates from RAW cells infected with virions that had been subjected to equilibration with individual mouse sera were evaluated by Western blot probed with anti-PVM-N Ab. Negative control (neg) was serum from a normal, unmanipulated mouse; positive control (pos) was convalescent serum from a mouse that had recovered from acute PVM infection and had undergone seroconversion, as described (10). Parallel blots were probed with chicken anti-actin to demonstrate equivalent loading of all lanes. B, Absolute virus copy number was determined for each sample; controls were as described above. Individual points were assayed in triplicate; shown are average values \( \pm \text{SE} \) for five to six mice.
the role of eosinophils in promoting virus-induced expression of majorly by respiratory epithelial cells in response to PVM infection; those responding to PVM Ags vaccination. CCL3 is produced primarily in dblGATA mice, both the Ctrl Ag-vaccinated mice as well as CCL3 detected in BALF of PVM-infected eosinophil-deficient 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 determine whether Th2 skewing is in fact limited to formalin-oxidized paramyxovirus proteins only. The PVM model may be useful in experiments designed to address these issues.

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, hypersensitivity 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 underlying 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 regarding validity of this model. We did not observe hypersensitivity responses to formalin-inactivated Ags from uninfected cells in our experiments, but this may be attributed to the experimental 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 protein contamination; in vivo passage of the infectious pathogen is physiologically relevant to natural infection, which is not necessarily the case in all reported experimental trials. Furthermore, in contrast to the hRSV challenge model in mice and cotton rats, in which high-titer, highly concentrated viral inocula grown in tissue culture are cleared rapidly and generate limited physiologic responses, the PVM infectious inoculating dose is minimal and dilute, and it replicates to very high virus titer, sufficient for isolation and reinfection of a naive host in mouse lung tissue in vivo (3, 4) (supplemental Fig. 1). Thus, the only Ags ultimately present in high concentration come from replicated virus, similar to what one would expect to occur during a natural infection in a vaccinated host. As such, we do not truly understand the underlying specificity of this response. There actually may not be anything extraordinary or unique about formalin-inactivated paramyxovirus

### Table II. Weights of wild-type (BALB/c) and eosinophil-deficient (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 (%) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c</td>
<td>Ctrl Ags</td>
<td>PVM</td>
<td>21</td>
<td>22.7 ± 2.7</td>
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<td>BALB/c</td>
<td>PVM Ags</td>
<td>PVM</td>
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<td>23.3 ± 3.3</td>
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<tr>
<td>ΔdblGATA</td>
<td>Ctrl Ags</td>
<td>PVM</td>
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<td>24.3 ± 2.1</td>
<td>23.8 ± 2.5</td>
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<td>7.4 ± 2.6</td>
</tr>
<tr>
<td>ΔdblGATA</td>
<td>PVM Ags</td>
<td>PVM</td>
<td>11</td>
<td>25.8 ± 3.2</td>
<td>25.7 ± 3.1</td>
<td>24.9 ± 3.6</td>
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</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 hRSV. The nature of the interactions between PVM and eosinophils remains a subject for future investigation.

Acknowledgments

We thank Drs. James and Nancy Lee for the generous gift of the TgPHIL males for our breeding colony and anti-mouse MBP Ab. Drs. Craig Gerard and Alison Humble for providing the AdhB1GATA males for breeding. We also thank members of the Eosinophil Biology Section and Molecular Signal Transduction Section, Laboratory of Allergic Diseases, National Institute of Allergy and Infectious Diseases, for helpful discussions that contributed to this work.

Disclosures

The authors have no financial conflicts of interest.

References


