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# Host Genetic Determinants of Vaccine-Induced Eosinophilia During Respiratory Syncytial Virus Infection<sup>1</sup>

Tracy Hussell,<sup>2</sup> Andrew Georgiou, Tim E. Sparer, Stephen Matthews, Pietro Pala, and Peter J. M. Openshaw

In BALB/c mice, sensitization with the attachment protein (G) of respiratory syncytial virus (RSV) leads to CD4<sup>+</sup> T cell-mediated lung eosinophilia during subsequent challenge with RSV. To determine the host genetic influences on this model of lung eosinophilia, we tested 15 different inbred mouse strains. Eosinophilia developed in all H-2<sup>d</sup> (BALB/c, DBA/2n, and B10.D2), but not in H-2<sup>k</sup> (CBA/Ca, CBA/J, C3H, BALB.K, or B10.BR) mouse strains. Among H-2<sup>b</sup> mice, 129 and BALB.B developed eosinophilia, whereas C57BL/6 and C57BL/10 did not. Testing first generation crosses between sensitive and resistant strains showed that eosinophilia developed in all H-2<sup>dxk</sup> ( $n = 5$ ), irrespective of background genes, but not in H-2<sup>dxb</sup> ( $n = 2$ ) mice. In vivo depletion of CD8<sup>+</sup> T cells or IFN- $\gamma$  rendered C57BL/6, but not BALB.K mice, susceptible to eosinophilia. Analysis of B10 recombinant mice showed that the D<sup>d</sup> allele (in B10.A(5R) mice) prevented CD8<sup>+</sup> T cell accumulation in the lung, resulting in intense lung eosinophilia. However, the D<sup>b</sup> allele (in B10.A(2R) and B10.A(4R) mice) supported CD8<sup>+</sup> T cell expansion and prevented eosinophilia. Intracellular cytokine staining showed that lung eosinophilia correlated with reduced IFN- $\gamma$  and increased IL-10 expression in lung T cells. These results are compatible with the unifying model that Th2 cells mediate the disease but can be inhibited by CD8<sup>+</sup> T cells secreting IFN- $\gamma$ . Our findings have important implications for the development of protective, nonpathogenic vaccines for RSV disease. *The Journal of Immunology*, 1998, 161: 6215–6222.

**A**lthough more than forty years have elapsed since the discovery of respiratory syncytial virus (RSV),<sup>3</sup> the disease that it causes remains an important global problem. Bronchiolitis is the most common single cause of hospitalization during infancy in the western world, most cases of which are caused by RSV. Reinfections occur throughout life and may cause severe problems in the elderly and immunocompromised individuals. In the 1960s, vaccine trials using formalin inactivated virus proved disastrous, causing exacerbated lung disease and, in some cases, death among vaccine recipients. Although the World Health Organization, many governmental bodies, and industrial organizations have identified RSV as a major target for vaccine development, a safe and effective vaccine has yet to be developed for human use.

The BALB/c mouse model of RSV infection reproduces some important features of the human disease and has provided many insights into possible protective and pathogenic immune processes in man. Protective Ab responses are directed primarily against the fusion (F) and attachment (G) glycoproteins, both of which are expressed on the surface of the virion. Vaccination with F expressed by recombinant vaccinia virus (rVV) generates Ab, cytotoxic T cells, and CD4<sup>+</sup> Th1 cells after virus challenge (1–4),

whereas the glycoprotein G of RSV (rVV-G) leads to Ab production and CD4<sup>+</sup> Th2 cells, but no detectable cytotoxic T cells (5–9). The patterns of lung pathology after virus challenge are also distinctive, in that F-primed mice develop augmented lung disease characterized by lung hemorrhage and neutrophilia, whereas G-primed mice develop pulmonary eosinophilia (10, 11).

Pulmonary eosinophilia was also found in the lungs and peripheral blood of some children vaccinated with formalin inactivated RSV (see, for example, Ref. 12). To improve our understanding of vaccine augmentation, we and others have performed extensive studies of the immune determinants of RSV-induced lung eosinophilia in BALB/c mice sensitized to G. In drawing general conclusions about the relevance of this response to disease in diverse hosts, mapping genetic responses in inbred mice appears vital. It is well established that CD4<sup>+</sup> T cells are instrumental in causing eosinophilic disease augmentation, and that CD8<sup>+</sup> T cells making IFN- $\gamma$  may be crucial regulators of this response (5, 13). MHC gene effects are therefore likely to play a critical role in the genetic control of augmented RSV disease. Associations between MHC and susceptibility to infection have been reported (14–18) and indicate the difficulties encountered when trying to design vaccines for outbred human populations. Different inbred strains of mice have provided an insight into the influence of MHC haplotype on the outcome of infection in many infectious disease models. However, there has been no systematic study of the genetic influences on the induction of vaccine-augmented pathology during RSV infection.

In the present studies, we sought to determine the immunologic mechanisms and genetic influences on RSV-induced lung eosinophilia using 15 inbred and 7 first generation (F<sub>1</sub>) crossbred mice. Our results indicate that eosinophilic lung disease requires CD4<sup>+</sup> T cell recognition, and that, in mouse strains susceptible to eosinophilia, CD8<sup>+</sup> T cells secreting IFN- $\gamma$  play an important regulatory role. The results obtained using different inbred and recombinant mouse strains may explain the variability in the response to

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<sup>3</sup> Abbreviations used in this paper: RSV, respiratory syncytial virus; F, fusion glycoprotein; G, attachment protein; rVV, recombinant vaccinia virus;  $\beta$ -gal,  $\beta$ -galactosidase; BAL, bronchoalveolar lavage.

RSV infection in humans and give insights into mechanisms that must be considered in designing vaccines for use in outbred human populations.

## Materials and Methods

### Virus

Recombinant vaccinia expressing rVV-G and the A2 strain of RSV were grown in HEp-2 cells, snap frozen in liquid nitrogen, and assayed for infectivity. Vaccinia expressing  $\beta$ -galactosidase (rVV- $\beta$ -gal) or HEp-2 cells were used as respective controls. All virus and control Ag preparations were shown to be free of mycoplasma contamination by DNA hybridization (Gen-Probe, San Diego, CA).

### Mice

Eight- to 10-wk-old female mice were purchased from Harlan Olac (Bicester, U.K.) and kept in pathogen-free conditions. The following strains of mice were used: BALB/c, DBA/2n, B10.D2 (all H-2<sup>d</sup>); BALB.B, 129, C57BL/6, C57BL/10 (all H-2<sup>b</sup>); BALB.K, B10.BR, CBA/Ca, CBA/J, C3H/HeN (all H-2<sup>k</sup>); and B10.A (2R), (4R) and 5(R) (H-2 h2, h4, and i2, respectively). First generation crosses were derived from the following mouse strains: C3H  $\times$  DBA/2n, B10.BR  $\times$  B10.D2, BALB.K  $\times$  B10.D2, BALB.K  $\times$  BALB/c, B10.BR  $\times$  BALB/c (all H-2<sup>dkk</sup>), and C57BL/6  $\times$  BALB/c, C57BL/6  $\times$  B10.D2 (both H-2<sup>bd</sup>) (for a review of these mouse strains see Ref. 19). On day 0, groups of 4–5 mice were scarified on the rump with either  $3 \times 10^6$  plaque-forming units of rVV-G or rVV- $\beta$ -gal in a final volume of 10  $\mu$ l as previously described (20). On day 14, mice were challenged intranasally with 50  $\mu$ l of RSV stock (equivalent to  $10^6$  plaque-forming units/mouse) or HEp-2 control Ag. All animal experimentation was performed under the guidelines set out by the Home Office.

### Determination of vaccinia virus replication in different strains of mice

The skin from individual mice, sensitized with rVV-G, was removed at different time points after scarification and placed in serum-free medium (RPMI 1640). The skin and s.c. tissue were mechanically disrupted, and  $10^6$  cells were pelleted and sonicated for 1 min in a water bath sonicator. The supernatant was assayed for vaccinia virus using a standard HEp-2 cell plaque assay.

### Determination of respiratory syncytial virus titers in lung tissue

Lungs were removed from four G-primed mice 4 and 7 days after RSV challenge and homogenized. Clarified supernatant was added to a HEp-2 monolayer and virus titers were determined as described (21).

### Recovery of lung cells

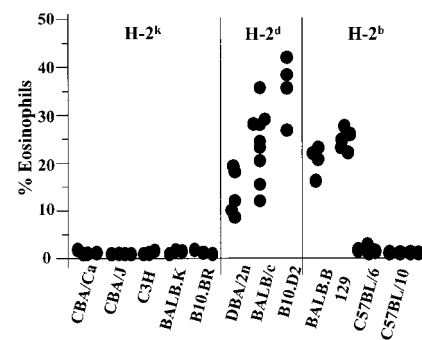
After 7 days, mice were injected i.p. with 3 mg/mouse pentobarbitone and exanguinated via the femoral vessels. Bronchoalveolar lavage (BAL) was performed as described previously (20). Two hundred microliters of lavage fluid from each mouse was stored in individual Eppendorf tubes for subsequent cytospin preparations. Samples from individual mice were examined by flow cytometry for expression of surface markers and intracellular cytokine expression. All samples were kept in ice cold RPMI 1640 containing 10% FCS, 2 mM/ml L-glutamine, penicillin (50 U/ml), and streptomycin (50  $\mu$ g/ml) (R10F). Mediastinal lymph nodes were removed from individual mice in each experimental group and placed in ice cold R10F. All samples were washed, counted, and resuspended at a concentration of  $10^6$  cells/ml.

### Cyocentrifuge preparations

Lavage cells from individual mice were cyocentrifuged onto glass slides at 700 rpm for 5 min. Slides were air-dried for 30 min and stained with Wright/Giemsa (Sigma Immunochemicals, Dorset, U.K.) according to the manufacturer's instructions. At least 300 cells were counted in three separate fields for each preparation. Eosinophils were identified by their distinct eosinophilic granules on light microscopy (allowing them to be distinguished from other granulocytes) and, additionally, by size and granularity on flow cytometry.

### Flow cytometric analysis of intracellular and surface Ags

Intracellular IFN- $\gamma$  (AN18-FITC), IL-4 (11B11-PE), IL-5 (TRFK5-PE), and IL-10 (JES5-16E3 PE) were assessed in CD4<sup>+</sup> (H129.19-QR) or CD8<sup>+</sup> (15-6.7-QR) T cells by flow cytometry as described previously (22). Unstimulated cells from individual mice were also stained with Abs



**FIGURE 1.** The effect of G protein vaccination in mice with different MHC haplotype. Mice were scarified with rVV-G and then challenged intranasally with RSV. The presence of eosinophils was assessed using Wright/Giemsa-stained cyocentrifuge preparations. The results show the percent of cells possessing the typical characteristics of eosinophils for individual mice.

to CD4 (QR), CD8 (FITC), and CD45RB (PE). Samples were analyzed on a Coulter (Luton, U.K.) EPICS Elite flow cytometer, collecting data on at least 30,000 gated lymphocytes from each sample.

### Determination of G-specific Ab by ELISA

G-specific Ab was assessed by ELISA as described previously (20). ELISA Ag was prepared by infecting HEp-2 cells with either rVV-G or rVV- $\beta$ -gal. When significant cytopathic effect was observed, the infected cells were harvested, centrifuged, resuspended in 500  $\mu$ l, and then subjected to 2 min of sonication in a sonicator (Ultrawave, Cardiff, U.K.). Aliquots (10  $\mu$ l) were stored at  $-20^{\circ}\text{C}$  until required. Briefly, microtiter plates were coated overnight with 100  $\mu$ l of either rVV-G or control rVV- $\beta$ -gal Ag. After blocking with 2% normal rabbit serum for 2 h, dilutions of test sample were added for another hour at room temperature. Bound Ab was detected using peroxidase-conjugated rabbit anti-mouse Ig and the *o*-phenylenediamine substrate. Color changes were quantified in an ELISA plate reader at 490 nm. This method to detect G-specific Ab was standardized using G-specific mAbs (kindly provided by Dr. Jose Melero, Madrid, Spain). The amount of G-specific Ab was determined by subtracting the optical density obtained by incubating serum on rVV- $\beta$ -gal-coated plates from the same sample incubated on rVV-G coated plates.

### Depletion of T cell subsets

Mice were treated with an i.p. injection of 200  $\mu$ g/mouse/day of depleting Abs to CD4 (YTS191.1 + YTA3.1) or CD8 (YTS169.4 + YTS156.7) Abs from day 8 to 18 after scarification with rVV-G. On day 14, mice were challenged intranasally with RSV, and tissues were sampled on day 21. Depletion of IFN- $\gamma$  was performed by i.p. injection of rat anti-mouse IFN- $\gamma$  (XMG 1.2) or control Ab, starting 2 days before RSV challenge and daily for 5 days, repeated on days 4, 7, and 10 of infection.

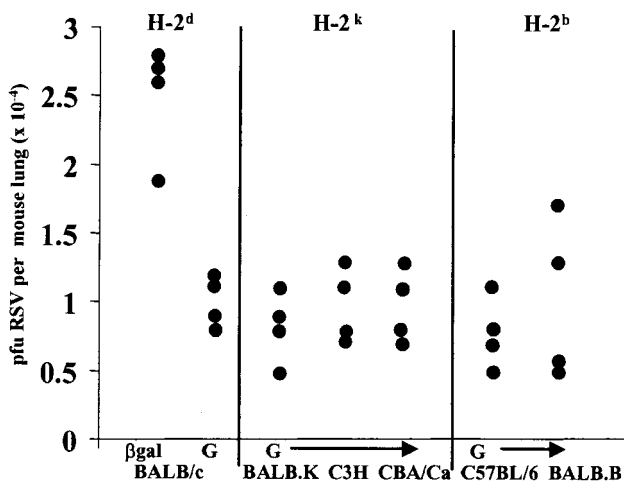
### Statistical analysis

The significance of differences was assessed by Student's *t* test.

## Results

### Eosinophil induction in inbred strains of mice

The development of pulmonary eosinophilia in G-primed BALB/c mice after RSV challenge has been extensively documented (for a review, see Ref. 23). However, when other inbred strains of mice were treated in a similar manner, distinct patterns of pathology were observed (Fig. 1). Similar to BALB/c mice, other strains with MHC H-2<sup>d</sup> haplotype developed an eosinophilic response to G priming after intranasal RSV challenge. Though variable percentages of eosinophils were noted in different H-2<sup>d</sup> strains, there was little difference in the absolute number of eosinophils among these strains. However, no eosinophils were recovered from the BAL of mouse strains with an H-2<sup>k</sup> haplotype, despite developing an enhanced infiltrate compared with control-primed mice after challenge. Mice with a MHC H-2<sup>b</sup> haplotype showed various responses, some strains developing eosinophilia and others not. The



**FIGURE 2.** RSV titers recovered from different inbred mouse strains 4 days after challenge. Mice were scarified with rVV-G at day 0 and challenged intranasally on day 14 with RSV. Virus titers from lung homogenates were assessed 4 and 7 (not shown) days after challenge by plaque-forming assay. Data from four individual mice are shown. The data is representative of two experiments containing four mice.

lack of eosinophilia in two mouse strains with H-2<sup>b</sup> haplotype was associated with a C57BL background (C57BL/6 and C57BL/10). Eosinophilia in sensitive mouse strains was specifically associated with a secondary immune response to RSV, as no mouse strain tested developed eosinophilia after RSV challenge when previously sensitized with the control construct (rVV-β-gal, data not shown).

*Vaccinia and respiratory syncytial virus replication in different strains of mice*

To examine whether the differential induction of eosinophilia might result from altered replication of vaccinia virus or RSV, we measured virus replication by plaque assay using lung homogenates or skin from different inbred strains of mice. All mice cleared vaccinia virus by day 14 after scarification. In addition, in all of the mouse strains tested, the inguinal lymph nodes (draining the site of scarification) expanded at a similar rate, confirming that efficient priming had occurred (data not shown). As in previous studies, higher titers of RSV were recovered from mice undergoing a primary RSV infection (β-gal-primed mice) than from those pre-sensitized with rVV-G. RSV replication was similar in mouse strains previously sensitized to G, regardless of whether they developed eosinophilia or not ( $p > 0.05$  for day 4 and day 7, respectively, by Student's *t* test; Fig. 2).

*Immune responses in the BAL of mice during primary and secondary infections*

The differential induction of eosinophils in the various inbred mouse strains may have reflected differences in the extent, rather than the type, of immune response to the virus. Therefore, total cell recovery, lymphocytic infiltrate, and the proportion of CD4:CD8 T cells from the BAL of mice were examined during primary and secondary infections. There were no significant differences in total cells ( $p > 0.05$ ) or lymphocytic responses ( $p > 0.05$ ) between mouse strains of different MHC haplotype (data not shown). Pre-sensitization with the G protein resulted in enhanced cell accumulation in H-2<sup>d</sup> and H-2<sup>b</sup> mice after viral challenge compared with mice undergoing a primary RSV infection, indicating that all mice developed an immune response to vaccination with rVV-G ( $p < 0.01$  comparing primary and secondary cell accumulation for each mouse strain). The increase in cell accumulation after G protein sensitization also occurred in all H-2<sup>k</sup> mouse strains but was less marked ( $p \leq 0.039$  comparing primary and secondary cell recruitment).

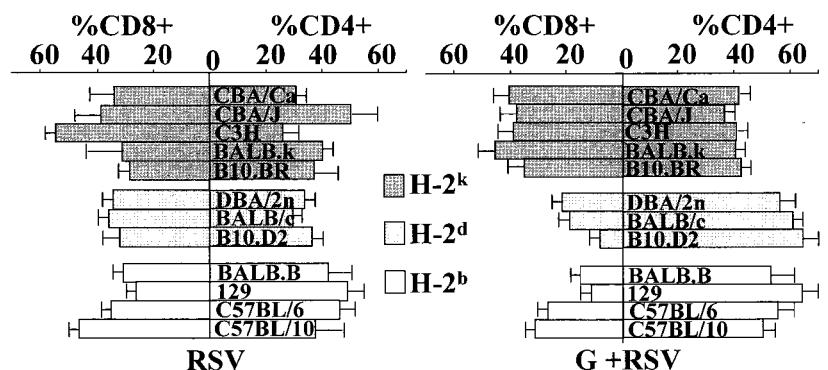
As CD4<sup>+</sup> T cells are necessary for eosinophil recruitment in this model, we then examined the proportion of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in BAL samples by flow cytometry. No significant difference was observed in CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses between mice of different MHC haplotype during primary RSV infection ( $p > 0.05$ ; Fig. 3). However, during secondary infection after G protein priming, mice that developed lung eosinophilia had either enhanced CD4<sup>+</sup> T cells, reduced CD8<sup>+</sup> T cells, or both, compared with mice of the same strain undergoing a primary immune response (Fig. 3). In addition, CD8<sup>+</sup> T cells were significantly decreased in mice that developed eosinophilia compared with other G-vaccinated mice that did not ( $p < 0.01$ ). In H-2<sup>k</sup> mice, which did not develop eosinophilia, there was no significant difference in the proportion of T cell subsets present during primary and secondary infection in three strains (BALB.K, CBA/J, and B10.BR;  $p > 0.05$ ). However, CBA/Ca and C3H mice did show enhanced CD4<sup>+</sup> T cell recruitment after G priming (Fig. 3).

These effects were not observed in mediastinal lymph nodes, which showed similar changes during primary and secondary RSV infection (data not shown). Using the data from mediastinal lymph nodes, taken from all G-primed mouse strains in two experiments with five mice per group, B cells were always more abundant ( $40 \pm 1.34\%$ ) than CD4<sup>+</sup> ( $34.9 \pm 2.44\%$ ) or CD8<sup>+</sup> ( $18 \pm 0.97\%$ ) T cells.

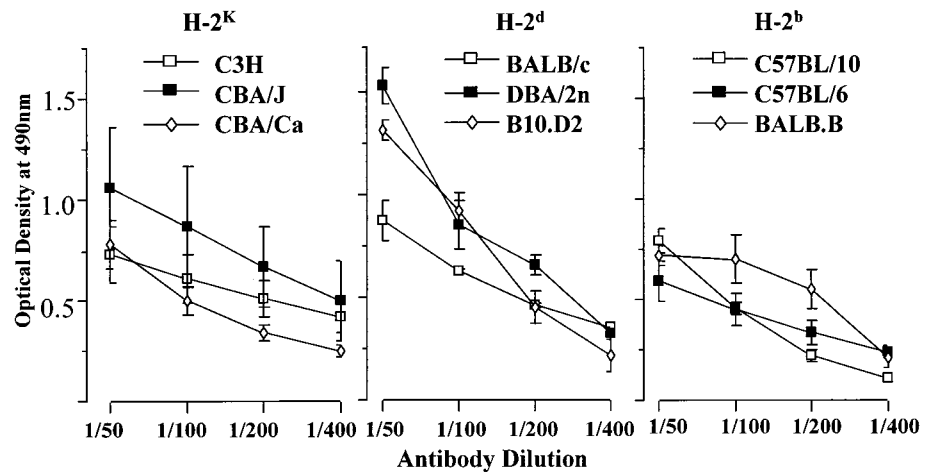
*G-specific Ab production*

CD4<sup>+</sup> T cells are required for induction of RSV-specific Ab, whereas CD8<sup>+</sup> T cells are inhibitory. The presence of RSV-specific Ab therefore reflects the relative balance of these two T cell

**FIGURE 3.** T cell responses in different inbred mice during primary RSV infection and after G protein vaccination. Bronchoalveolar lavage was removed from G-vaccinated or untreated mice 7 days after RSV challenge. Cells were stained on ice with Quantum red conjugated Abs to CD4 and CD8 and analyzed by flow cytometry. Results shown are the mean and SE from five separate mice in two experiments.



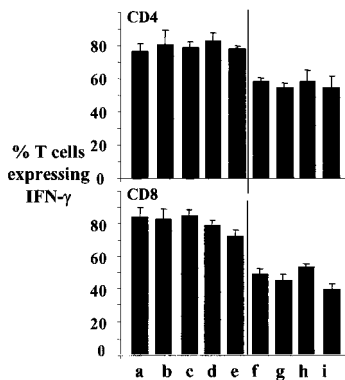
**FIGURE 4.** Induction of G-specific Ab during secondary infections. Mice were primed with rVV-G and challenged 14 days later with RSV. Serum was removed from five individual mice per group after 7 days and G-specific Ig tested by ELISA. The results shown are the mean  $\pm$ SE of five individual mice per mouse strain. The graphs have been divided based on haplotype for clarity. Similar results were obtained in two independent experiments containing five mice per group.



subsets. All G protein-sensitized mice generated specific Ab after RSV challenge (Fig. 4), which was higher than that obtained for the same mouse strain undergoing a primary immune response to RSV. For clarity only, Ab generated to G priming followed by RSV challenge is shown. All mice displayed G-specific Ab production. Though two of the H-2<sup>d</sup> mice gave higher optical density readings at low serum dilutions, the end point titer was similar in all mouse strains tested.

#### Intracellular cytokine production by CD4<sup>+</sup> and CD8<sup>+</sup> T cells in G-primed mice after intranasal virus challenge

To investigate the possible mechanisms of eosinophil induction or inhibition we examined intracellular IFN- $\gamma$ , IL-4, IL-5, and IL-10 expression in CD4<sup>+</sup> and CD8<sup>+</sup> T cells by flow cytometry. Mice that developed eosinophilia to G had significantly reduced IFN- $\gamma$  in both CD4<sup>+</sup> and CD8<sup>+</sup> BAL T cells ( $p < 0.001$  for both) (Fig. 5). IFN- $\gamma$  expression in CD4<sup>+</sup> and CD8<sup>+</sup> mediastinal lymph node T cells was similar in all groups (not shown). No significant IL-4 or IL-5 was detected in any T cell population from the lung or any



**FIGURE 5.** Intracellular IFN- $\gamma$  expression in lung CD4<sup>+</sup> and CD8<sup>+</sup> T cells during RSV challenge of G-primed mice. Cells recovered from the BAL after 7 days were stained for cytokines and surface markers. CD4<sup>+</sup> (top) and CD8<sup>+</sup> (bottom) T-cells expressing IFN- $\gamma$  were determined by flow cytometry. The results represent the mean and SE from four individual mice. Similar results were observed in a second experiment containing five mice per group. Lane a, CBA/Ca; lane b, CBA/J; lane c, BALB.K; lane d, B10.BR; lane e, C57BL/6; lane f, BALB/c; lane g, B10.D2; lane h, BALB.B; lane i, DBA/2n. Strains a–e do not get eosinophilia, whereas f–i do.

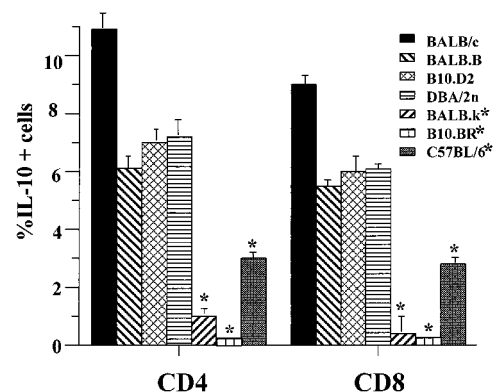
other site. IL-10, however, was detected in CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the BAL, more so in mouse strains that developed eosinophilia than in those that did not (Fig. 6,  $p < 0.05$  by Student's *t* test). The majority of IL-10 expression was within cells that also co-expressed IFN- $\gamma$ .

#### F<sub>1</sub> generation responses to G-induced eosinophilia to G

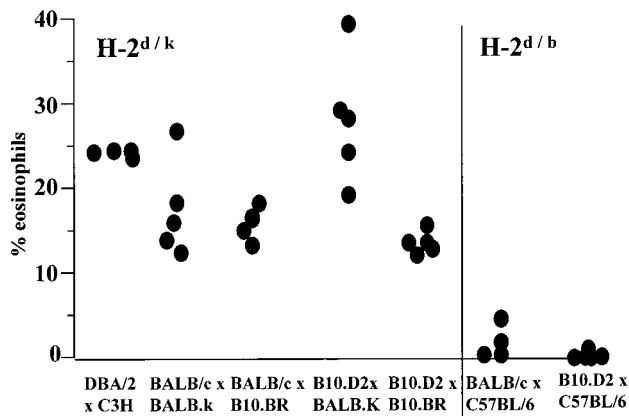
To determine the influence of background genes and MHC haplotype on eosinophil induction by the G protein of RSV, first generation crosses of mice that developed eosinophilia were bred with strains of mice that do not. All H-2<sup>d</sup>  $\times$  H-2<sup>k</sup>, but no H-2<sup>d</sup>  $\times$  H-2<sup>b</sup>, first generation mice tested developed eosinophilia (Fig. 7).

#### Depletion of CD8<sup>+</sup> T cells from eosinophil-resistant strains of mice

Mice resistant to eosinophil induction by G protein priming had higher levels of IFN- $\gamma$  in CD8<sup>+</sup> T cells compared with sensitive strains. We therefore depleted CD8<sup>+</sup> T cells using neutralizing Abs before and during challenge. Removal of CD8<sup>+</sup> T cells from C57BL/6, but not BALB.K, mice resulted in lung eosinophilia (Fig. 8). Similar observations were observed by depletion of IFN- $\gamma$  (data not shown). Eosinophil induction in C57BL/6 mice was not due to the Ab treatment, as depletion of both CD4<sup>+</sup> and CD8<sup>+</sup> T



**FIGURE 6.** Intracellular expression of IL-10 in lung CD4<sup>+</sup> T cells. Mice were vaccinated with rVV-G and challenged intranasally 14 days later. After 7 days, the percent of IL-10<sup>+</sup>/CD4<sup>+</sup> T cells was determined by flow cytometry. The mean and SE of results from five mice per mouse strain that do or do not (designated by \*) develop eosinophilia are shown.



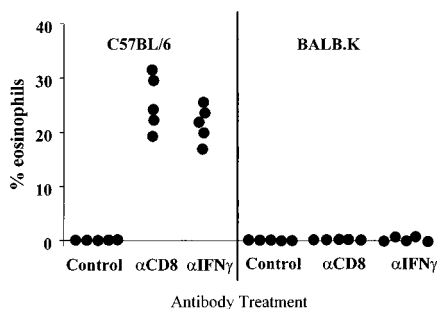
**FIGURE 7.** The inheritance of eosinophilia to G protein vaccination. BAL eosinophils were evaluated by counting cells that had typical morphology and eosinophilic granules in Wright/Geimsa-stained cytocentrifuge preparations. At least 300 cells were counted for each individual mouse.

cells abolished BAL eosinophilia. In addition, we have shown previously that C57BL/6 mice with CD8 $\alpha^{-/-}$ ,  $\beta_2$ -microglobulin $^{-/-}$ , and TAP1 $^{-/-}$  gene deletions similarly develop eosinophilia to the G protein (13). The present results suggest that elements responsible for resistance to eosinophilia are different in mice of H-2<sup>b</sup> and H-2<sup>k</sup> haplotype.

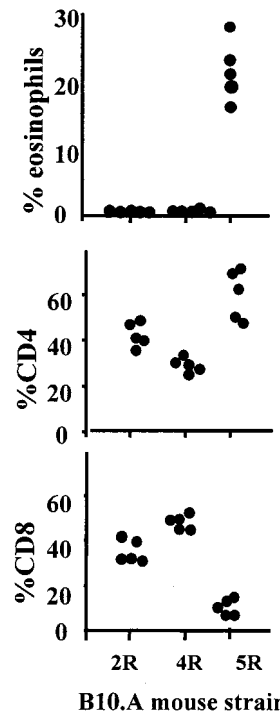
The induction of lung eosinophilia in BALB.B was abrogated by depletion of CD4<sup>+</sup> T cells before and after RSV challenge (data not shown). Since CD4<sup>+</sup> T cells are necessary for eosinophilia, it appears that, unlike H-2<sup>k</sup> mice, T cell responses in H-2<sup>b</sup> mice are influenced by background genes.

*Immune responses to the G protein in recombinant mice*

As CD8<sup>+</sup> T cells appeared an important regulator of eosinophilic pathology, we examined the effect of G priming and RSV challenge in mice with a B10 background but expressing different MHC alleles. G-primed B10.A(5R) mice expressing H-2D<sup>d</sup> developed lung eosinophilia after intranasal virus challenge, whereas B10.A(4R) and B10.A(2R) mice, which both express H-2D<sup>b</sup>, did not (Fig. 9, top). CD4<sup>+</sup> T cells were enhanced (Fig. 9, middle), and CD8<sup>+</sup> T cells effectively abrogated (Fig. 9, bottom) in B10.A(5R) mice. In addition, CD4<sup>+</sup> T cells from these mice were highly activated based on the increased loss of CD45RB staining compared with the same cells from B10.A(2R) and (4R) mice (Fig. 10).



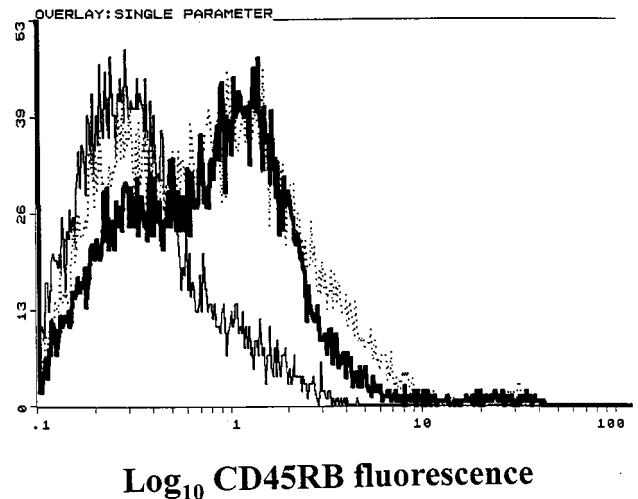
**FIGURE 8.** The differential effect of CD8 T cell or IFN- $\gamma$  depletion on immune responses to the G protein in BALB.K and C57BL/6 mice. Five mice per group were treated with neutralizing Abs to CD8 or IFN- $\gamma$  during RSV challenge of G-sensitized mice. BAL samples were collected from individual mice 7 days after RSV challenge and eosinophils enumerated in Wright/Geimsa-stained cytocentrifuge preparations.



**FIGURE 9.** Immune response to the G protein of RSV in MHC recombinant mice. Five mice per group were scarified with rVV-G and challenged intranasally 14 days later. Lung lavage was recovered after 7 days and the percent eosinophils (A), CD4<sup>+</sup> T cells (B), and CD8<sup>+</sup> (C) T cells were evaluated.

**Discussion**

Since the 1970s, development of an effective vaccine to prevent RSV infection has been held back by the fear of causing augmented disease. The available evidence points to T cell over-activity and, in particular, Th2 cells as the cause of vaccine-augmented bronchiolitis. Broadly, there are two mechanisms that can



**FIGURE 10.** CD45RB expression on CD4<sup>+</sup> T cells from MHC recombinant mice. Lung lavage from G-vaccinated and RSV-challenged mice was stained with Abs to CD4 (Quantum Red) and CD45RB (FITC) and analyzed by flow cytometry. CD4<sup>+</sup> lymphocytes were assessed for CD45RB expression. The overlay histogram shows CD45RB expression in B10.A 2R (dotted line), B10.A 4R (bold line) and B10.A 5R (thin line) mice.

be evoked: first, that various RSV Ags (which resist formalin treatment) prime harmful class I or class II restricted T cells; second, RSV protein(s) have some unusual properties that cause aberrant T cell behavior. The first explanation would be expected to cause effects in some individuals but not in others, depending on the possession of specific MHC proteins. The second could operate in a range of individuals of diverse genetic background. To develop safe and effective vaccines, it is essential to differentiate between these alternatives. The present studies show that possession of certain MHC genes results in the development of eosinophilia in RSV-infected mice previously sensitized to G.

To summarize current knowledge of the BALB/c mouse model of RSV infection, induction of lung eosinophilia requires priming of CD4<sup>+</sup> T cells that make IL-4 and IL-5. Depletion of these cells with anti-CD4 mAbs prevents eosinophilia. Eosinophilia can be transferred to naïve recipients with RSV specific CD4<sup>+</sup> T cells that make Th2 cytokines in vitro. The effects of these Th2 cells can be antagonized by virus-specific CD8<sup>+</sup> T cells, which make IFN- $\gamma$  (5, 7, 13), by administration of IL-12 during vaccination (20), or by the removal of immunodominant CD4<sup>+</sup> T cell epitope(s) from the G protein (24). According to this model, G protein primes for eosinophilia because it induces only CD4<sup>+</sup> T cells. Its failure to prime CD8<sup>+</sup> T cells (5, 6) is responsible for allowing the CD4<sup>+</sup> T cells to default to a Th2 pattern of cytokine production, which leads to eosinophilia.

Our initial studies concentrated on comparison of C57BL/6 and BALB/c mice. These are the most common inbred mouse strains used to study infectious agents and often show contrasting patterns of immunity and disease. For example, during *Leishmania major* infection, BALB/c mice show a strong CD4<sup>+</sup> Th2 cell response and are incapable of clearing infection, whereas C57BL/6, B10.D2, and C3H mouse strains that resolve infection show strong Th1 responses (25, 26). By contrast, mouse strains that show a strong Th2 response during infection with *Trichuris muris* are resistant to infection, whereas those that mount a Th1 response fail to expel these worms (27–29). It is said that C57BL/6, B10.D2, and C3H mice tend to develop Th1 responses, but that a BALB background results in Th2 response to many Ags (30, 31). However, previously published studies have generally been confined to small numbers of inbred and congenic mice, limiting the interpretation that can be placed on the results presented in our study.

Our results with H-2<sup>d</sup> mice are compatible with the hypothesis that eosinophilia is simply a result of unbalanced CD4<sup>+</sup> T cell induction. Eosinophils were observed in G protein primed DBA/2n and B10.D2 (DBA/2n MHC on a BL/10 background) mice after RSV infection. In these mice, it seems that H-2<sup>d</sup> presents appropriate epitopes to CD4<sup>+</sup> T cells, but that CD8<sup>+</sup> T cell responses are not induced. Failure of IFN- $\gamma$  production by CD8<sup>+</sup> T cells allows Th2 cells to develop, and lung eosinophilia therefore ensues. The results in H-2<sup>b</sup> mice were more complex. C57BL/6 and C57BL/10 mice were resistant to G-induced eosinophilia, but on a BALB background (BALB.B) H-2<sup>b</sup> renders mice susceptible. In BALB.B mice, CD4<sup>+</sup> T cell depletion prevents lung eosinophilia, showing that H-2<sup>b</sup> can induce a pathogenic CD4<sup>+</sup> T cell response. We suggest that the variation in eosinophilia in H-2<sup>b</sup> mice must be due either to background genes or to variations in the levels of T cell cytokines in different strains. Indeed, CD4<sup>+</sup> and CD8<sup>+</sup> T cells produced high levels of IFN- $\gamma$  and lower levels of IL-10 in C57BL/6 and C57BL/10 mice compared with BALB.B and 129 mice primed with the G protein. Previous studies have classified C57BL/6 mice as high IFN- $\alpha/\beta$  producers (If-1<sup>b</sup>), whereas BALB/c mice are low producers (If-1<sup>l</sup>). This effect has been

demonstrated during Newcastle disease virus infection where C57BL/6 mice show 10- to 15-fold higher circulating levels of IFN than BALB/c mice (32). Although innate immunity (such as IFN- $\alpha/\beta$  production) may vary with background genes, there is insufficient evidence to judge whether the variations in eosinophilia in MHC H-2<sup>b</sup> mouse strains could be explained by such factors.

C57BL/6 and 129 mice also differ in MIs alleles that may influence immune responses to G. 129 mice express the MIs2<sup>a</sup> allele similar to BALB/c mice, whereas C57BL/6 mice express MIs2<sup>b</sup> (33, 34). Such differences, and the distribution of mouse mammary tumor virus variants, may affect the TCR V $\beta$  repertoires and subsequent immunity to the G protein.

Using recombinant B10 mouse strains, we were able to show that expression of D<sup>b</sup> protected mice from eosinophilia after G protein priming, whereas expression of D<sup>d</sup> did not. In B10 mice with H-2 D<sup>d</sup>, BAL CD8<sup>+</sup> T cells were dramatically reduced, and CD4<sup>+</sup> T cell recruitment and activation (assessed by CD45RB expression) were increased. Such mice developed extensive lung eosinophilia when primed with the G protein. This result underscores the premise that CD8<sup>+</sup> T cell activation has a critical influence on CD4<sup>+</sup> T cell activation and subsequent eosinophilia.

The absence of eosinophilia in G-primed H-2<sup>k</sup> mouse strains is not explained by background genes, because simple substitution of H-2<sup>k</sup> onto the BALB background (BALB.K) does not induce lung eosinophilia. In contrast to the effects in C57BL/6 mice, removal of CD8<sup>+</sup> T cells using depleting Ab did not allow eosinophilia to develop in BALB.K mice. We have not yet determined whether CD8<sup>+</sup> T cell depletion induces eosinophilia in other mouse strains of H-2<sup>k</sup> haplotype. Our previous work showed that CD4<sup>+</sup> T cells are necessary for eosinophil induction (13), and, therefore, an alternative explanation for the lack of eosinophilia is that H-2<sup>k</sup> mice fail to generate CD4<sup>+</sup> T cell responses to G protein. Our finding that three out of the five H-2<sup>k</sup> mouse strains did not display enhanced CD4<sup>+</sup> T cell recruitment, which we have observed to be a reliable indicator of secondary CD4<sup>+</sup> T cell responses, supports this possibility. However, we cannot rule out the possibility that T cells secreting type 1 rather than type 2 cytokines are induced. Although CD4<sup>+</sup> T cell recruitment was less prominent in H-2<sup>k</sup> than in H-2<sup>d</sup> mice, those T cells that were present in the lungs of H-2<sup>k</sup> mice expressed higher levels of intracellular IFN- $\gamma$ .

First generation crosses between mice with MHC H-2<sup>d</sup> and H-2<sup>k</sup> were susceptible to G-induced lung eosinophilia. Whether H-2<sup>k</sup> mice recognize the G protein or not, the H-2<sup>d</sup> response (which we know recognizes a CD4<sup>+</sup> T cell epitope in G) is dominant. Such differential induction of eosinophilia in H-2<sup>d</sup> (BALB/c), but not H-2<sup>k</sup> (B10.BR), mice has previously been shown during sensitization with *Dermatophagoides farinae* (35). In this study, the only noticeable difference between the two strains was the lack of an early type hypersensitivity response in B10.BR mice compared with BALB/c. It was surprising that F<sub>1</sub> crosses between H-2<sup>d</sup> mice and C57BL/6 (H-2<sup>b</sup>) did not result in lung eosinophilia. We know that both of these mouse strains induce a CD4<sup>+</sup> T cell response and that H-2<sup>d</sup> cannot support CD8<sup>+</sup> T cell response (13). Therefore, this first generation cross includes CD4<sup>+</sup> T cell responses via both MHC haplotypes but CD8<sup>+</sup> T cell responses from only the H-2<sup>b</sup> MHC. As depletion of CD8<sup>+</sup> T cells renders C57BL/6 mice susceptible to G-induced eosinophilia, we believe that induction of such cells is responsible for the result obtained with the first generation cross. Other possible influences in this F<sub>1</sub> include competition between d and b alleles for expression on the cell surface,

presentation of an alternative epitope that does not induce eosinophils, tolerization to eosinophilic epitope(s) in G, and processing and presentation alterations.

Though the differential induction of CD4<sup>+</sup> and CD8<sup>+</sup> T cells explains most of the results presented in this study, numerous parameters have been highlighted in other mouse models of infectious diseases. Airway responsiveness (36, 37), the number of T cell epitopes recognized (38), the avidity or affinity of ligand:T cell interaction (39–42), eosinophil precursor frequency (27, 43), and the dose of Ag (44, 45) are all consistent with the mouse strain profiles presented in our study.

The lack of IL-4 and IL-5 production by T cells *ex vivo* using intracellular cytokine staining is interesting. We and others have previously observed Th2 cytokine production in T cells from G-primed mice using either RT-PCR or ELISA (5, 46, 47). Th2 cytokine mRNA is only transiently expressed very early after RSV challenge in G-primed mice, and the source of this mRNA is undetermined (46). Therefore, we used intracellular cytokine staining in this study. We have previously shown that IFN- $\gamma$  and IL-10 accumulation detected by intracellular staining correlates well with cytokine mRNA (22). We believe that the results presented in this paper are an accurate reflection of events occurring *in vivo*. Though we have detected IL-4 and IL-5 by cytokine staining (20), future studies would benefit from an analysis of cytokines by several complementary methods.

The differential induction of eosinophilia does not reflect variable replication of either the vaccinia virus construct or RSV in these mice. Though the study by Prince et al. (48) shows that different strains of 3-day-old mice vary in the titer of RSV recoverable from the lungs, we did not observe differences of this type in 8- to 10-wk-old mice. Furthermore, the results do not reflect differences in the severity of the inflammatory reaction, since total cell recovery and lymphocytic infiltrate did not significantly differ between mouse strains. What did correlate with eosinophilia was the level of CD4<sup>+</sup> and CD8<sup>+</sup> T cell induction and the level of cytokines expressed by these cells.

Since the G protein is a surface protein and a target of neutralizing Ab, its inclusion in a vaccine would be logical. The present study indicates that a safe vaccine might be one that induces a strong CD8<sup>+</sup> T cell response. Despite the possible influence of non-MHC genes in some mouse strains, the effects seen in inbred, crossbred, and recombinant mice and the effect of T cell depletions shows that MHC is the primary determinant of exuberant T cell responses and eosinophilia during RSV challenge. Our results are consistent with a simple model of T cell induced disease, which may help explain the variability in response to vaccination and RSV infection in humans.

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