# IL-12 Modulates Expression of Hypersensitivity Pneumonitis[1]

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**Hypersensitivity pneumonitis (HP) is a granulomatous, inflammatory lung disease caused by inhalation of organic Ags, most commonly thermophilic actinomycetes. Only a minority of individuals exposed to these Ags develops disease, suggesting that host factors are important for the expression of HP. We compared the expression of HP in a sensitive strain of mice, C57BL/6, and in a resistant strain of mice, DBA/2. They were exposed to the thermophilic bacteria *Saccharopolyspora rectivirgula* (SR) or to saline alone for 3 consecutive days/week for 3 wk. After exposure to Ag, C57BL/6 mice, but not DBA/2 mice, developed granulomatous inflammation with an increase in lung index (lung weight). Both strains had similar amounts of Ag delivered to the lungs after intranasal installation, as determined with $^{14}$C-labeled Ag. Both also had similar increases in total bronchoalveolar cells after Ag exposure, but the C57BL/6 mice had more lymphocytes. Compared with the resistant strain, the sensitive strain had a significantly greater Ag-induced increase in IL-12 and IFN-$\gamma$ gene expression. DBA/2 mice resembled sensitive, C57BL/6 mice if they received IL-12 augmentation therapy at the time of Ag exposure. These findings were not limited to lung, since both unstimulated and SR-stimulated spleen cells from C57BL/6 mice released significantly more IL-12 than cells from DBA/2 mice. However, spleen cells from DBA/2 mice made more IFN-$\gamma$ when exposed to IL-12, than cells from C57BL/6 mice. These results suggest that the IL-12 response to Ag may modulate in part the expression of HP.** ***The Journal of Immunology,*** **1998, 161: 991–999.**

Hypersensitivity pneumonitis (HP)[3] is a syndrome caused by repeated inhalation of and sensitization to an organic Ag (1–6). The most common Ags are thermophilic actinomycetes that cause farmer's lung disease, but multiple other organic Ags can cause HP (1). There is widespread exposure to these Ags, but the number of individuals who develop the disease is relatively low. It has been estimated that between 5 and 15% of an exposed population will develop HP (2). This observation strongly suggests that host factors are important in the expression of clinical disease. The reason for individual sensitivity is unknown.

In prior studies, we used a well-described murine model to study HP. In this model, mice exposed to the actinomycete, *Saccharopolyspora rectivirgula* (SR; previously named *Micropolyspora faeni* and *Faeni rectivirgula*), via nasal inhalation, develop diffuse bronchoalveolitis and form granulomas in the lung (7,34). Using this model, we evaluated the role of IL-10 in modulating the effect of the proinflammatory cytokines, IFN-$\gamma$, IL-1, and TNF. Mice that could not produce IL-10 had more severe inflammatory changes in the lung than wild-type littermates, and they exhibited increased Ag-induced expression of the IFN-$\gamma$, IL-1, and TNF genes. When IL-10 was replaced, IL-10 knockout mice resembled their wild-type littermates (34). In another study, using IFN-$\gamma$ knockout mice (GKO), we showed that IFN-$\gamma$ is necessary for granuloma formation in HP. The GKO mice did not develop the granulomatous inflammation. Replacement of IFN-$\gamma$ in the GKO mice resulted in expression of HP in lung similar to that in wild-type littermates (7). The observations of these studies suggested that expression of a Th1-type immune response might determine the clinical expression of HP.

To further evaluate factors that might determine the expression of a Th1-type immune response and expression of lung disease in HP, we took advantage of prior studies that showed that various strains of mice have differences in susceptibility to developing HP (8–11). C57BL/6 mice are sensitive and DBA/2 mice are resistant to developing HP. Our hypothesis for these studies was that these mouse strains differ in their expression of one or more Th1-type cytokines in response to inhaled Ag and that this determines in part the expression of HP. We confirmed that, compared with resistant mice, a sensitive strain develops a more severe granulomatous inflammatory response in the lung with higher lung weights. The sensitive mice also express increased amounts of lung IL-12 and IFN-$\gamma$ mRNA. Associated with these changes, they exhibit more lung TGF-$\beta$, collagen I, and fibronectin mRNAs and greater amounts of lung hydroxyproline. When given augmentation therapy with IL-12, the resistant mice resemble sensitive mice. The strain differences observed in these mice were not limited to lung, since spleen cells from sensitive mice make more IL-12 than cells from the resistant mice, but DBA/2 mice make more IFN-$\gamma$ when stimulated with IL-12. These observations suggest that the IL-12 response to Ag determines in part the expression of HP in this murine model.

## Materials and Methods

### *Animals*

C57BL/6 and DBA/2 mice were purchased from Harlan Sprague-Dawley Laboratories (Indianapolis, IN). Female mice, weighing 18 to 24 g, were used for these studies. They were housed in an Ag-free and virus-free environment at the University of Iowa Animal Care Unit and maintained on standard mouse chow and water ad libitum. All animal care and housing

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[3] Abbreviations used in this paper: HP, hypersensitivity pneumonitis; SR, *Saccharopolyspora rectivirgula*; GKO, interferon-$\gamma$ knockout; BAL, bronchoalveolar lavage; GVHD, graft-versus-host disease.



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requirements set forth by the National Institutes of Health Committee on Care and Use of Laboratory Animal Resources were followed, and animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee.

### *Antigen*

Ag was prepared from a strain of SR obtained from American Type Culture Collection (catalogue no. 29034, Rockville, MD). It was grown in a trypticase soy broth in a 55°C shaking incubator for 4 days, centrifuged, and rinsed with distilled water three times. It was then homogenized and lyophilized. Ag was resuspended in pyrogen-free saline. A limulus amebocyte lysate assay from Sigma (St. Louis, MO) showed that this material was endotoxin free. Ag was also prepared, as described above, and grown in medium containing $^{14}$C-labeled amino acids (DuPont-New England Nuclear Research Products, Boston, MA).

### *Induction of HP*

HP was induced by installing 30 $\mu$g of SR Ag in saline, intranasally, under light anesthesia, as we previously described (7,34). The material was applied at the tip of the nose and inhaled involuntarily. This was performed for 3 consecutive days/week for 3 wk. This dose of Ag and the timing of exposures were chosen after preliminary studies showed that these methods were optimal for distinguishing between sensitive and resistant strains. To evaluate whether resistance to Ag was dose dependent, groups of mice were also treated with 90 $\mu$gof Ag. Mice were sacrificed by pentobarbital injection at various time points after the last exposure.

### *Bronchoalveolar lavage*

After death, a 20-gauge catheter was inserted into the trachea. Bronchoalveolar lavage (BAL) samples were obtained by washing the lungs with three 1-ml aliquots of 0.9% saline. After centrifugation, BAL cell pellets were washed and resuspended in HBSS, and total cell counts were enumerated using a Coulter counter (Coulter, Hialeah, FL). Cytospin preparations were fixed and stained using Diff-Quick staining (Baxter, McGaw Park, IL). Differential cell counts were made on 200 cells using standard morphologic criteria to identify the cells as neutrophils, eosinophils, lymphocytes, or macrophages (7).

### *Flow cytometry*

Flow cytometry was performed with a Becton Dickinson FACScan (Becton Dickinson, San Jose, CA). CellQuest software (Becton Dickinson) was used for acquisition and analysis of data. A bitmap gate was placed around the lymphocyte population on the basis of forward and orthogonal light scatter. Cells falling within the lymphocyte gate were then analyzed in a FITC vs phycoerythrin dual parameter histogram, and percentages were generated with quadrant statistics. FITC-conjugated rat anti-mouse CD4 Ab and phycoerythrin-conjugated rat anti-mouse CD8a Ab were obtained from PharMingen (San Diego, CA). IgG isotype standards were also obtained for controls.

### *Lung index (weight)*

Lungs were removed, trimmed of extraneous tissue, rinsed, and weighed. Lung indexes were calculated as described by Wilson et al. (8): lung index = [(lung weight/body weight) $\times$ test animal]/[(lung weight/body wight) $\times$ control animal].

### *Histologic evaluation*

Lungs were perfused with 2% paraformaldehyde through the heart and trachea and fixed in 2% paraformaldehyde-PBS. The sections were embedded in paraffin, cut in 5-$\mu$m-thick sections, and stained with hematoxylin and eosin. The sections were evaluated by light microscopy. A histologic score for each lung was determined according to the following criteria: 0 = no lung abnormality, 1 = presence of inflammation and granulomas involving <10% of the lung parenchyma; 2 = lesions involving 10 to 30% of the lung; 3 = lesions involving 30 to 50% of the lung; and 4 = lesions involving >50% of the lung (7). The slides were evaluated without knowledge of the type of mouse or exposure to Ag. The area covered by an eyepiece grid (0.99 $\times$ 0.99 mm using $\times$100 magnification) was judged to be normal or abnormal. An average of 200 fields were evaluated for each mouse (7).

### *Northern analysis*

Total lung RNA was prepared using the guanidine thiocyanate (GITC) extraction and cesium chloride centrifugation method of Chirgwin et al. (12), as modified by Maniatis et al. (13). The RNA was fractionated on a 1.5% agarose gels containing 2.2 M formaldehyde by the method of Lehrach and co-workers (14). The gels were stained with ethidium bromide and destained overnight in 0.1% ammonium acetate to assess RNA integrity and equivalent loading. *Escherichia coli* 23S and 16S mRNA served as standards. Subsequently, the RNA was transferred to GeneScreen Plus (New England Nuclear, Boston, MA) following the manufacturer's specifications and then UV cross-linked to the nylon membrane. Fixed membranes were prehybridized for 12 to 24 h at 42°C in a solution of 50% formamide, 1 M NaCl, 1$\times$ Denhardt's solution, 0.05 M Tris (hydroxymethyl) aminomethane (Tris), 1% SDS, and 10% dextran sulfate. The membranes were hybridized with fresh solution at 42°C with a $^{32}$P-labeled cDNA probes for murine TNF, IL-1, and IL-12, obtained from Clontech (Palo Alto, CA). TGF-$\beta$1 probe was obtained from American Type Culture Collection (Rockville, MD). Other probes were generously provided as noted: murine $\alpha$1(I) procollagen provided by Dr. Benoit deCrombrugghe and rat fibronectin by Dr. Richard Hynes. Following hybridization, the filters were washed twice at room temperature in 1$\times$ SSC, followed by two rinses at 65°C in 1$\times$ SSC/1% SDS, and a final room temperature wash in 0.1$\times$ SSC. Hybridized membranes were then exposed to radiographic film.

### *RT-PCR*

In prior studies (7), we noted that IFN-$\gamma$ mRNA cannot be detected in mouse lung by Northern analysis. Thus, we used the method of RT-PCR to detect this mRNA. Total lung RNA was prepared as described above. It was then treated with DNase and reverse transcribed using murine Moloney leukemia virus reverse transcriptase (MMLVRT) enzyme in a total reaction volume of 50 $\mu$l. PCR was performed on the resultant cDNA, using primers specific for IFN-$\gamma$ (purchased from Clontech) and the PCR products were analyzed on a 3% agarose gel and visualized with an ethidium bromide stain. The PCR product was then transferred from the agarose gel to a membrane, hybridized with the appropriate $^{32}$P-labeled cDNA probe, and analyzed following autoradiography.

### *Hydroxyproline studies*

The total lung hydroxyproline was determined as previously described (15). Briefly, lung was homogenized with cold TCA followed by hydrochloric acid. Chloramine-T was added, followed by perchloric acid and *p*-dimethyl-aminobenzaldehyde. Color change was assessed by spectrophotometry and compared with hydroxyproline standard.

### *IL-12 augmentation therapy*

Recombinant murine IL-12 was purchased from Genzyme (Cambridge, MA). It had a sp. act. of 5 $\times$ 10$^5$ U/mg. The material was diluted in PBS with 1% BSA. Groups of DBA/2 mice were given an i.p. injection of 200 ng of IL-12 (or diluent) in 0.2 ml of diluent daily just before each exposure to Ag for the first 2 wk of the 3-wk period that the animals were exposed to Ag.

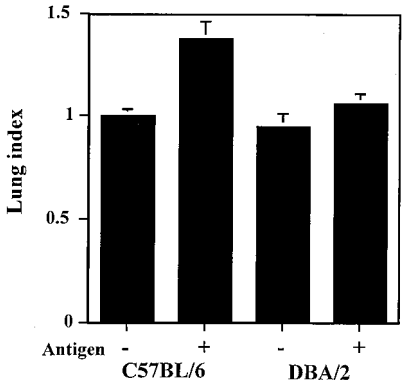


**FIGURE 1.** Changes in lung weight (lung index). Mice were exposed to either low dose Ag or saline as described in *Materials and Methods*. Four days after the last exposure, mice were sacrificed, and lung index was quantitated as described in *Materials and Methods*. On the ordinate is a quantitation of the lung index, and the type of mouse is indicated on the abscissa. Data are expressed as the mean $\pm$ SEM for four to six animals in each group. There was a significant difference between Ag-exposed DBA/2 mice and C57BL/6 mice ($p < 0.003$).

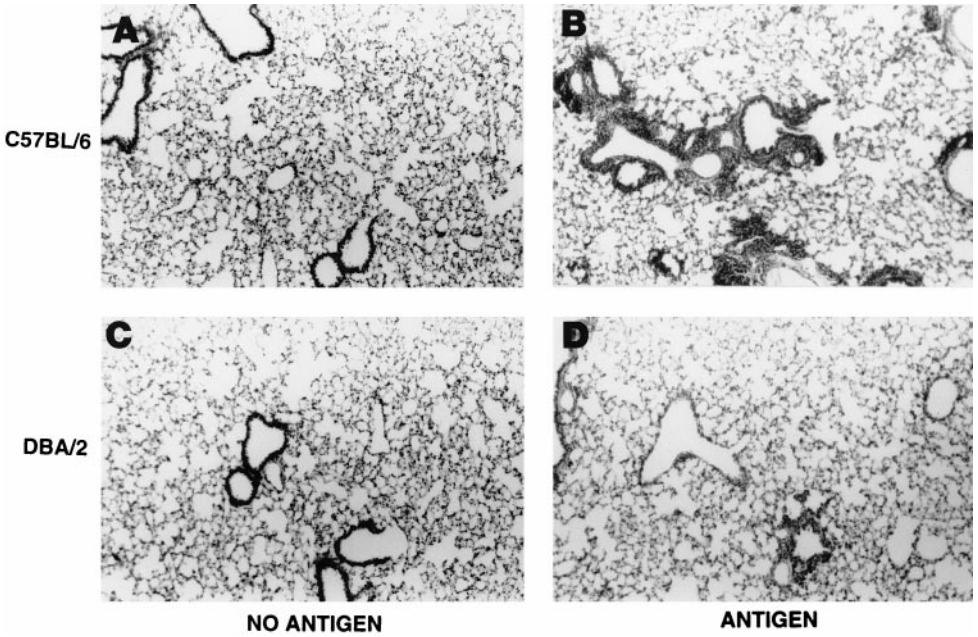


**FIGURE 2.** Expression of hypersensitivity pneumonitis in C57BL/6 and DBA/2 mice exposed to low dose Ag or saline as described in *Materials and Methods*. *A*, C57BL/6 mice exposed to saline alone. *B*, C57BL/6 mice exposed to Ag. *C*, DBA/2 mice exposed to saline alone. *D*, DBA/2 mice exposed to Ag. Representative hematoxylin- and eosin-stained histology sections are shown (magnification, ×100).

### *IL-12 and IFN-γ release from spleen cells*

Spleens were removed from naive C57BL/6 and DBA/2 mice and single cell suspensions isolated in RPMI 1640 medium (Life Technologies, Grand Island, NY) containing 1% FCS (HyClone, Logan, UT). Cells were washed twice, suspended at $5 \times 10^6$ cells/ml in RPMI 1640 medium containing 5% FCS, and subsequently plated in aliquots of 1 ml in 12-well plates. To induce cytokine release, cells were stimulated with 1 μg/ml of SR Ag. As a positive control, we used Con A (25 μg/ml). In some instances, cells were primed with 100 U/μl of rIFN-γ (Genzyme) for 24 h and then stimulated with 1 μg/ml of LPS obtained from Sigma as an additional positive control. Abs to IL-10 (Genzyme; 1 μg/ml) and IL-4 (Genzyme; 1 μg/ml) were also used. Plates were then incubated for 24 h at 37°C in 5% carbon dioxide. Supernatants were harvested, and IL-12 was measured by ELISA (Genzyme). Cells were also stimulated with 5 μg/ml of SR Ag, 1 μg/ml of recombinant mouse IL-12 (Genzyme), or both. Supernatants were harvested, and IFN-γ was measured by ELISA (Genzyme). To insure that there were equal numbers of adherent cells in the spleen populations of C57BL/6 and DBA/2 mice, these experiments were repeated using only adherent cells. In these studies, $15 \times 10^6$ cells were allowed to adhere to the plate for 1 h. Subsequently, nonadherent cells were removed. The adherent cells were then stimulated as described above, and supernatants were harvested after 24 h. The protein content of the wells was determined with the Coomassie blue method (16). For these studies, IL-12 and IFN-γ release is expressed as picograms of IL-12 or IFN-γ per microgram of protein.

### *Statistics*

Statistical analysis was performed using an unpaired (two-tailed) *t* test. Values are expressed as the mean ± SEM. The 95% confidence limit was taken as significant ($p < 0.05$). Calculations were performed using the StatView 4.01 (Abacus Concepts, Berkeley, CA) statistical analysis program.

## Results

### *Comparison of histology and lung index in sensitive and resistant strains of mice*

C57BL/6 mice, but not DBA/2 mice, had an increase in lung index after exposure to low dose Ag (30 μg), as shown in Figure 1. They also had a greater amount of granulomatous inflammatory changes after exposure to Ag, as illustrated in Figure 2. The C57BL/6 mice had more diffuse inflammatory changes with granuloma formation, while the DBA/2 mice tended to have peribronchial lymphocytic infiltration, as shown in Figure 3. These results are quantitated in Figure 4. These differences between C57BL/6 and DBA/2 mice were dose dependent. When C57BL/6 and DBA/2 mice were exposed to 90 μg of SR, no change was seen in the histology score for C57BL/6 mice compared with that using the lower dose of Ag.

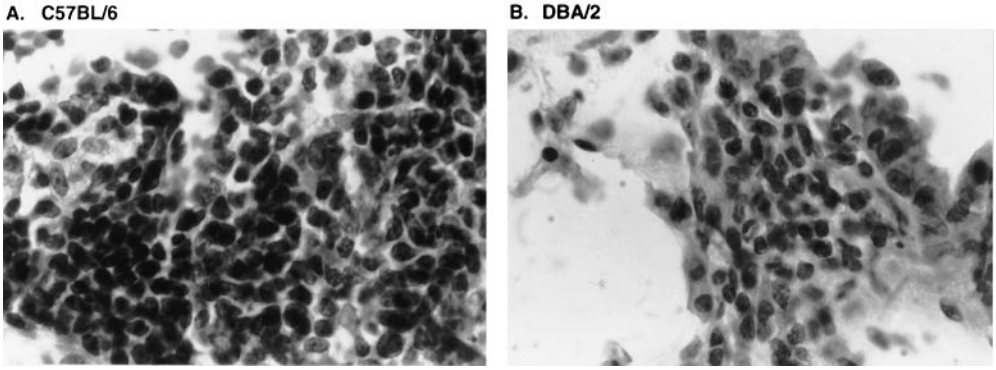


**FIGURE 3.** Higher magnification of lesions in C57BL/6 and DBA/2 mice exposed to low dose Ag, as described in *Materials and Methods*. *A*, C57BL/6 mice exposed to Ag. *B*, DBA/2 mice exposed to Ag. Representative hematoxylin- and eosin-stained histology sections are shown (magnification, ×600).

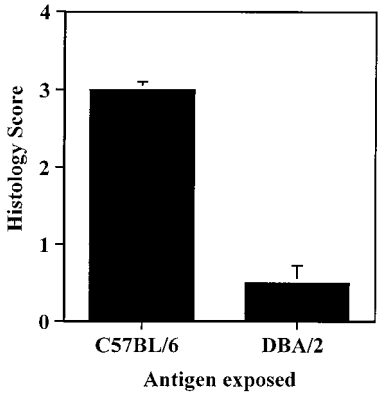


**FIGURE 4.** Quantitation of inflammation and granulomas. Mice were exposed to saline alone or low dose Ag as described in *Materials and Methods*. Amounts of inflammation and granulomas were evaluated as described in *Materials and Methods* and are quantitated on the ordinate. The type of mouse and exposure to Ag are indicated on the abscissa. Data are expressed as the mean ± SEM for four to six animals in each group. There was a statistically significant difference between Ag-treated DBA/2 mice and C57BL/6 mice ($p < 0.005$).

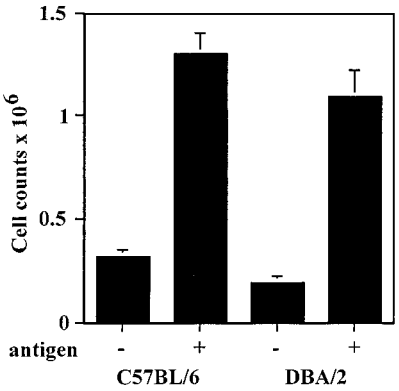


**FIGURE 5.** Cells in BAL fluid. C57BL/6 and DBA/2 mice were exposed to either low dose Ag or saline alone as described in *Materials and Methods*. Four days after the last exposure, bronchoalveolar cells were isolated and quantitated as described in *Materials and Methods*. On the ordinate is a quantitation of the cells, and the type of mouse and the exposure to saline or Ag are indicated on the abscissa. Data are expressed as the mean ± SEM for six animals in each group. There was a significant difference between the Ag-treated and the saline-exposed mice in both C57BL/6 mice ($p < 0.001$) and DBA/2 mice ($p < 0.002$). There was no difference between Ag-exposed C57BL/6 and DBA/2 mice.

However, DBA/2 mice expressed more granulomatous inflammation in the lungs with the higher dose of Ag than with the lower dose of Ag. With the higher dose of Ag, the histology score of the DBA/2 mice was not different from that of the C57BL/6 mice (not shown).

### *Comparison of Ag delivery to lungs of sensitive and resistant strains of mice*

To exclude the possibility that the differences in susceptibility to HP seen in the mice were due to different amounts of Ag delivered to the lungs, we exposed the sensitive and resistant mice to Ag labeled with $^{14}C$ and counted the radioactivity in lung homogenates. There was not a significant difference between the two strains of mice (not shown).

### *Comparison of BAL cells in sensitive and resistant strains of mice*

Both C57BL/6 and DBA/2 mice had an increase in inflammatory cells in BAL fluid 4 days after exposure to low dose Ag, as shown in Figure 5. The number of cells in BAL fluid in the two strains was not different. After Ag exposure, most of the cells were macrophages, but the C57BL/6 mice had more lymphocytes, as shown in Table I. These lymphocytes were more commonly $CD4^+$ than $CD8^+$ cells. These observations suggest that both strains of mice respond to low dose Ag, but the response to Ag in the resistant mice does not trigger a granulomatous inflammatory response that can be detected histologically. In Figure 6*A* is shown the time course for cells in BAL fluid from the day of exposure to SR until 7 days after exposure. Both strains had the greatest number of cells on the day of exposure, and then the numbers of cells decreased with time. As shown in Figure 6*B*, neutrophils were most prominent immediately after exposure, and the numbers decreased over time. There was a more sustained neutrophil response in the DBA/2 mice during the first 3 days after exposure. There were also significantly more lymphocytes in the C57BL/6 mice on days 1 and 3 after the last exposure (results not shown). These observations suggest that more lymphocytes are recruited into the lungs of sensitive mice than resistant ones.

### *Comparison of peripheral blood leukocytes in sensitive and resistant strains of mice*

Table II compares total leukocyte counts and differential counts in C57BL/6 and DBA/2 mice. There was no difference in the numbers of cells, including lymphocytes, between the two strains nor was there any difference in the percentages of $CD4^+$ cells and $CD8^+$ cells.

Table I. *Comparison of cells in BAL fluid*[a]

| | Macrophages | Neutrophils | Eosinophils | Lymphocytes | CD4 (%) | CD8 (%) |
|---|---|---|---|---|---|---|
| C57BL/6:sa[b] | 3.1 ± 0.2 (98)[c] | 0.05 ± 0.02 (0.6) | 0 ± 0 (0) | 0.05 ± 0 (0.4) | | |
| C57BL/6:ag | 9.6 ± 0.2 (74) | 0.8 ± 0.1 (6) | 0.3 ± 0.05 (2) | 2.3 ± 0.1[d] (18) | 33 ± 7 | 4.7 ± 0.7[d] |
| DBA/2:sa | 1.8 ± 0.1 (98) | 0.05 ± 0.01 (1) | 0 ± 0 (0) | 0.05 ± 0.01 (1) | | |
| DBA/2:ag | 8.5 ± 0.3 (78) | 1.1 ± 0.1 (10) | 0.1 ± 0.1 (1) | 1.2 ± 0.25 (11) | 43 ± 1.5 | 8 ± 0.4 |

[a] Mean number of cells × $10^4$ ± SEM derived from six individual mice per group.
[b] Sa = animals exposed to saline and ag = animals exposed to SR antigen.
[c] Mean percentage of cells.
[d] $p < 0.05$ between ag-exposed C57BL/6 and DBA/2 mice.

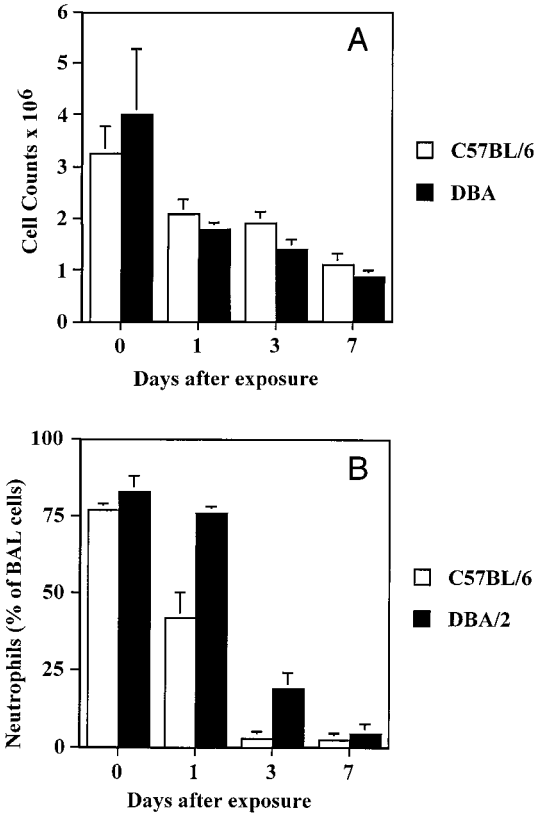


**FIGURE 6.** *A*, Cells in BAL fluid. C57BL/6 and DBA/2 mice were exposed to low dose Ag as described in *Materials and Methods*. On the day of the last exposure and up to 7 days after the last exposure, bronchoalveolar cells were isolated and quantitated as described in *Materials and Methods*. On the ordinate is a quantitation of the cells, and the time since last exposure and the type of mouse are indicated on the abscissa. Data are expressed as the mean ± SEM for three animals in each group. There was not a significant difference between the C57BL/6 mice and the DBA/2 mice for any time point. *B*, Percentage of neutrophils in BAL fluid. C57BL/6 and DBA/2 mice were exposed to low dose Ag as described in *Materials and Methods*. Four days after the last exposure, bronchoalveolar cells were isolated and quantitated as described in *Materials and Methods*. On the ordinate is the percentage of cells, and the time since last exposure and the type of mouse are indicated on the abscissa. Data are expressed as the mean ± SEM for three animals in each group. There was a significant difference between the C57BL/6 mice and the DBA/2 mice for days 1 and 3 ($p < 0.05$).

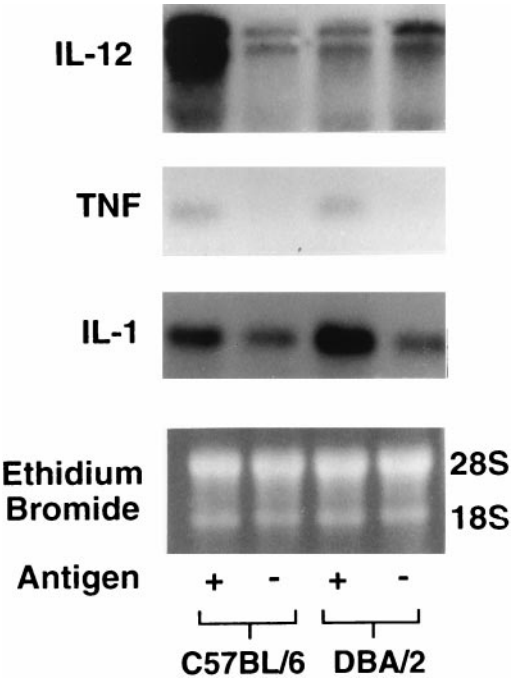


**FIGURE 7.** Northern analysis of IL-12, IL-1, and TNF mRNA in C57BL/6 and DBA/2 mice treated with low dose Ag or saline. C57BL/6 mice expressed more IL-12 mRNA after exposure to Ag than did DBA/2 mice, but both C57BL/6 and DBA/2 mice expressed comparable amounts of IL-1 and TNF mRNAs after exposure to Ag. Ethidium bromide staining demonstrates equal loading of RNA ($n = 3$).

### *Proinflammatory cytokine gene expression in sensitive and resistant mice*

Both C57BL/6 and DBA/2 mice had similar increases in lung TNF and IL-1 mRNAs after low dose exposure to Ag. In contrast, the C57BL/6 mice had a greater increase in IL-12 and IFN-$\gamma$ mRNAs compared with DBA/2 mice (Figs. 7 and 8). These cytokine mRNAs were also evaluated at other time points from 1 day after the last exposure to 7 days after the last exposure. Only the time point with the peak expression of mRNAs is shown for both strains of mice. These results show that both sensitive and resistant strains of mice respond to Ag by increasing expression of mRNAs for the proinflammatory cytokines IL-1 and TNF. This by itself does not appear to trigger granuloma formation in the resistant strain of mice. Sensitive, C57BL/6 mice develop a greater up-regulation of IL-12 and IFN-$\gamma$ mRNAs compared with DBA/2 mice, and this is associated with the development of a granulomatous inflammatory response in the lung. These observations are consistent with our prior studies, which showed that IFN-$\gamma$ is necessary for granuloma formation in HP (7). With higher doses of Ag (90 $\mu$g), the IL-12 responses are comparable in the two strains of mice (Fig. 9), suggesting that DBA/2 mice are able to make IL-12.

### *Differences in expression of extracellular matrix proteins in sensitive and resistant strains of mice*

C57BL/6 mice had greater amounts of hydroxyproline in the lungs after low dose Ag exposure compared with DBA/2 mice as shown in Figure 10. They also had increased expression of mRNAs for collagen I, fibronectin, and TGF-$\beta$ (Fig. 11). These observations

Table II. *Peripheral blood leukocyte counts*[a]

| | Total Cells | Neutrophils | Monocytes | Eosinophils | Lymphocytes | CD4 (%) | CD8 (%) |
|---|---|---|---|---|---|---|---|
| C57BL/6 | (3.40 ± 0.18) | 26 ± 1.7 (0.9) | 12 ± 0.9 (0.4) | 1 ± 0.4 (0.03) | 61 ± 2.3 (2.07) | 19.4 ± 1.8 | 10.2 ± 1.2 |
| DBA/2 | (3.62 ± 0.55) | 26 ± 2.2 (0.94) | 11 ± 1.25 (0.4) | 0.5 ± 0.3 (0.02) | 62.5 ± 3.0 (2.26) | 25.4 ± 4.4 | 9.3 ± 2.8 |

[a] Numbers represent mean percentages ± SEM derived from four individual mice per group. Numbers in parentheses represent the mean numbers of cells × $10^9$/L.

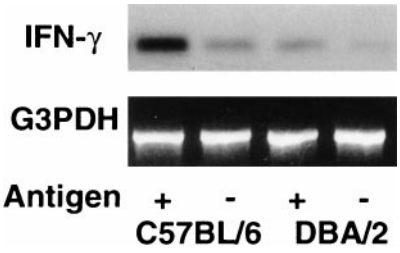


**FIGURE 8.** Quantitation of IFN-γ mRNA in C57BL/6 and DBA/2 mice treated with low dose Ag or saline. C57BL/6 mice have increased IFN-γ mRNA after exposure to Ag, while DBA/2 mice do not. Quantitation of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA demonstrates equal loading of RNA ($n = 3$).

suggest that the response to Ag in sensitive mice, but not that in resistant mice, results in changes in extracellular matrix proteins.

*IL-12 augmentation therapy*

IL-12 is a potent inductor of IFN-γ production. Therefore, we evaluated whether administration of exogenous IL-12 would permit a resistant strain of mice to respond to low dose Ag and develop HP. Groups of DBA/2 mice were treated (as described in *Materials and Methods*) with IL-12 during Ag exposure. DBA/2 mice that received IL-12 developed significantly more granulomatous inflammation than DBA/2 mice exposed to Ag but not given IL-12 (Fig. 12). Mice exposed to saline alone or given IL-12 alone did not have detectable amounts of inflammation. These observations suggest that IL-12 is important for the development of a granulomatous inflammatory response in this murine model of HP.

*IL-12 and IFN-γ production by spleen cells*

We next determined whether there were systemic differences in production of IL-12 in sensitive and resistant mice. When spleen cells from naive C57BL/6 and DBA/2 mice were exposed to SR Ag, the cells from the C57BL/6 mice produced significantly more IL-12 than those from the DBA/2 mice (Fig. 13*A*). Baseline, unstimulated release of IL-12 was also greater in the C57BL/6 mice. Both also responded to stimulation with Con A and to LPS after cells had been primed with IFN-γ. However, the absolute amount of IL-12 was, again, significantly lower in the DBA/2 mice. This

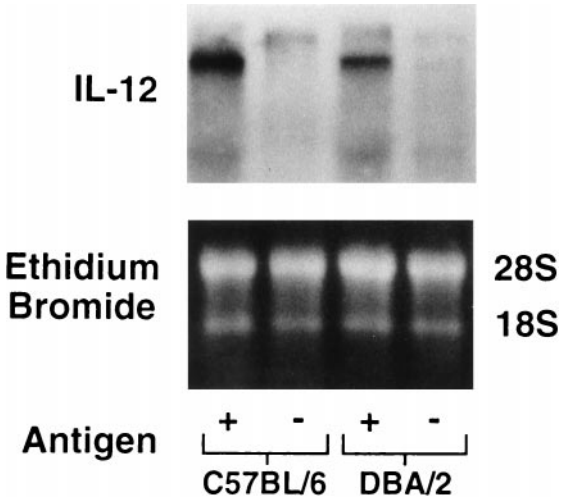


**FIGURE 9.** Northern analysis of IL-12 mRNA in C57BL/6 and DBA/2 mice treated with high dose Ag or saline. Both C57BL/6 and DBA/2 mice expressed comparable amounts of IL-12 mRNAs after exposure to Ag. Ethidium bromide staining demonstrates equal loading of RNA ($n = 3$).

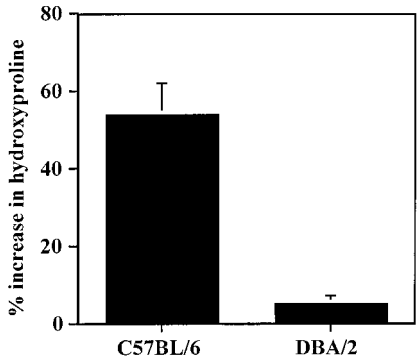


**FIGURE 10.** Amounts of hydroxyproline in C57BL/6 and DBA/2 mice after exposure to low dose Ag. On the ordinate is the percent increase in hydroxyproline in animals exposed to Ag compared with the control value. The type of mouse is indicated on the abscissa. Data are expressed as the mean ± SEM for five animals in each group. There is a significant difference between the two groups ($p < 0.001$).

was also true if only adherent spleen cells were evaluated, and the data were expressed as IL-12 per milligrams of cell protein as shown in Figure 13*B*.

When spleen cells were exposed to IL-4 Abs in addition to SR, there was no significant difference between cells from DBA/2 mice or C57BL/6 mice in IL-12 production (Fig. 14*A*). When cells were exposed to Abs to IL-10 along with SR, cells from C57BL/6 mice made more IL-12 as shown in Figure 14*B*. This further supports the importance of IL-10 as an anti-inflammatory cytokine in C57BL/6 mice as we have previously shown.

As shown in Figure 15, cells from both C57BL/6 and DBA/2 mice responded to stimulation by IL-12 by making IFN-γ. These observations suggest that the production of IL-12 itself, and not responses downstream of IL-12, distinguishes the sensitive mice from the resistant mice.

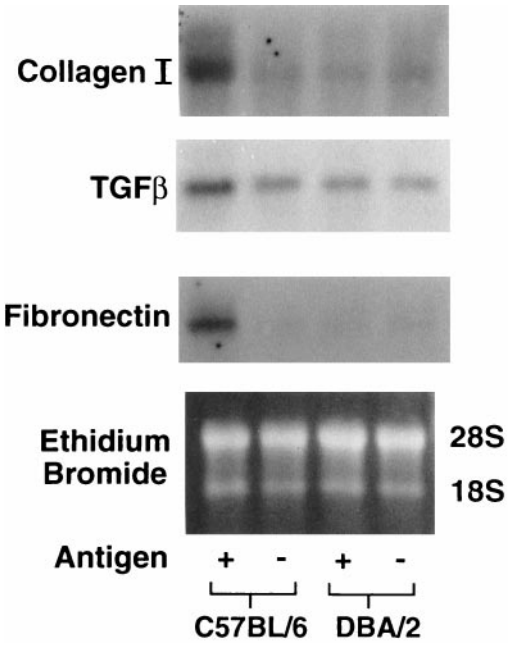


**FIGURE 11.** Northern analysis of collagen I, fibronectin, and TGF-β mRNAs in C57BL/6 and DBA/2 mice. C57BL/6 mice express increased amounts of collagen I, fibronectin, and TGF-β mRNAs compared with DBA/2 mice after exposure to low dose Ag. Ethidium bromide staining demonstrates equal loading of RNA ($n = 3$).

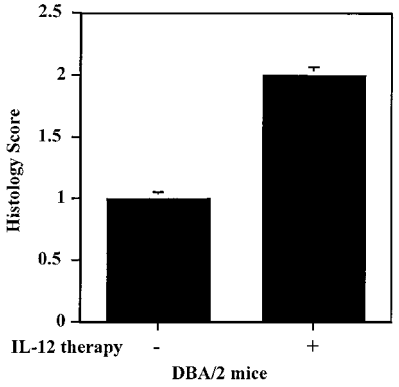


**FIGURE 12.** Quantitation of inflammation and granulomas in DBA/2 mice with and without IL-12 therapy. DBA/2 mice were exposed to Ag and given IL-12 replacement therapy as described in *Materials and Methods*. Amounts of inflammation and granulomas are quantitated on the ordinate. Replacement therapy is indicated on the abscissa. Data are expressed as the mean ± SEM for three animals in each group. There is a significant difference between the two groups ($p < 0.02$).

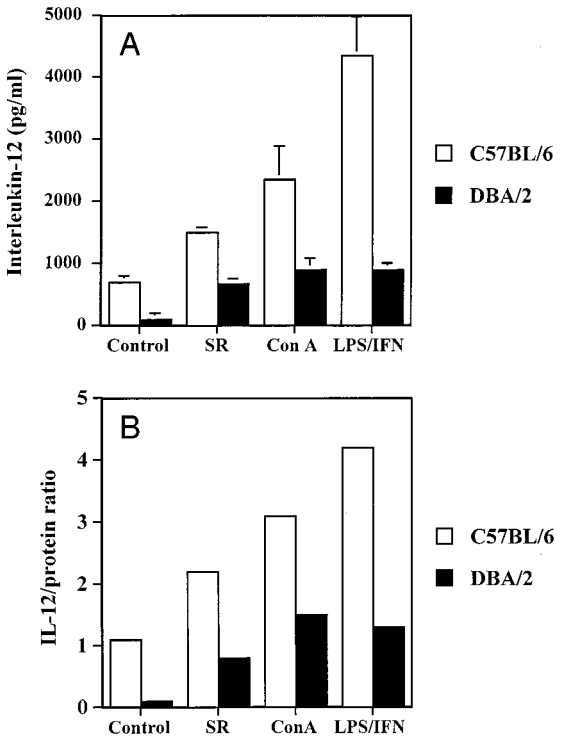


**FIGURE 13.** *A*, IL-12 production by spleen cells from C57BL/6 and DBA/2 mice. Cells were unstimulated or stimulated with SR, Con A, or both IFN-γ and LPS as indicated on the abscissa. IL-12 release is quantitated on the ordinate. Data are expressed as the mean ± SEM for three animals in each group. There is a significant difference between the two strains for all groups ($p < 0.02$). *B*, IL-12 production by adherent spleen cells from C57BL/6 and DBA/2 mice. Cells were unstimulated or stimulated with SR, Con A, or both IFN-γ and LPS as indicated on the abscissa. On the ordinate is shown the ratio of IL-12 release per milligram of protein. Data are expressed as the mean for three animals in each group. There is a significant difference between the two strains for all groups ($p < 0.05$).

## Discussion

In these studies we used a murine model of HP to compare the responses to low doses of inhaled Ag in sensitive and resistant strains of mice. The sensitive strain (C57BL/6) had a higher lung index, suggesting increased permeability, and a more severe granulomatous inflammatory response in the lung compared with the resistant strain of mice (DBA/2). When we further compared sensitive and resistant strains of mice, we found that similar amounts of Ag are delivered to the lungs in sensitive and resistant strains of mice; both strains had a similar increase in BAL cells, excluding lymphocytes; and both strains had similar increases in lung IL-1 and TNF mRNAs. These observations suggest that both strains of mice respond in some manner to Ag. The sensitive, C57BL/6 mice differed from the resistant, DBA/2 mice in that they had a greater increase in lung IL-12 and IFN-γ mRNAs with Ag exposure. The sensitive mice also expressed more TGF-β, collagen I, and fibronectin mRNAs in their lungs after Ag exposure. There also was more hydroxyproline in the lungs of Ag-exposed, sensitive mice. When given augmentation therapy with IL-12, the resistant mice resembled sensitive mice in their response to low dose Ag. With larger doses of Ag, there was a similar response to Ag in the two strains of mice. To determine whether the differences between sensitive and resistant strains were limited to lung, we performed similar studies with spleen cells. Spleen cells from DBA/2 mice produced less IL-12 than cells from C57BL/6 mice, but they produced more IFN-γ when stimulated with IL-12. These observations, as an aggregate, suggest that the IL-12 response to Ag determines at least in part the development of HP in these two strains of mice.

It is well documented in humans that there are different susceptibilities to developing HP. It has been estimated that in farmers lung disease between 5 and 15% of individuals that are exposed to Ag develop clinical disease (1, 2). Many individuals have precipitating Abs against thermophilic bacteria, suggesting exposure, but they are asymptomatic (1–4). Some of these individuals and some that do not have precipitating Abs have lymphocytic alveolitis when BAL is performed (17). These observations suggest that exposed, asymptomatic subjects also respond in some manner to Ag. The reasons for the differences in clinical susceptibility to Ag are not well understood, but they are important in understanding the pathogenesis of HP.

Previous studies have identified differences in susceptibility to HP in various strains of mice. Wilson and colleagues used a lung index to screen several mouse strains for susceptibility to HP. They found that several mouse strains had an increase in lung index, suggesting the development of HP. Among these were C57BL/6 and C3H/He strains. DBA/2 mice had no change in lung index, suggesting resistance (8). Histology of lung tissue was not performed in this study. The findings of this study were used to select strains of mice for the present study. Donnelly and associates studied the influence of MHC genes on the expression of HP in mice. They found that mice expressing the k and b MHC haplotypes developed more severe lesions than mice expressing d or q haplotypes even when the MHC genes were expressed on a common genetic background (9). A study by Rossi et al. showed similar results with OVA-induced lung disease in mice (10). C57BL/6 mice have a b haplotype, and DBA/2 mice carry the d haplotype (11).

The Th1 and Th2 subsets of T cells are defined on the basis of their pattern of production of cytokines (18). Th1 cytokines include IL-2, IL-12, and IFN-γ. Th2 cytokines include IL-10, IL-4, IL-5, and IL-6. In a murine model of experimental HP, Schuyler and associates showed that Th1 cells were able to adoptively transfer HP (19). We have previously shown that IFN-γ is important in the pathogenesis of HP (7). When mice that do not produce IFN-γ are exposed to Ag, they do not develop granulomatous inflammation. In addition, IL-10 dampens the expression of IFN-γ and the development of HP in wild-type mice (34). Thus, Th1 responses are likely to be important in HP.

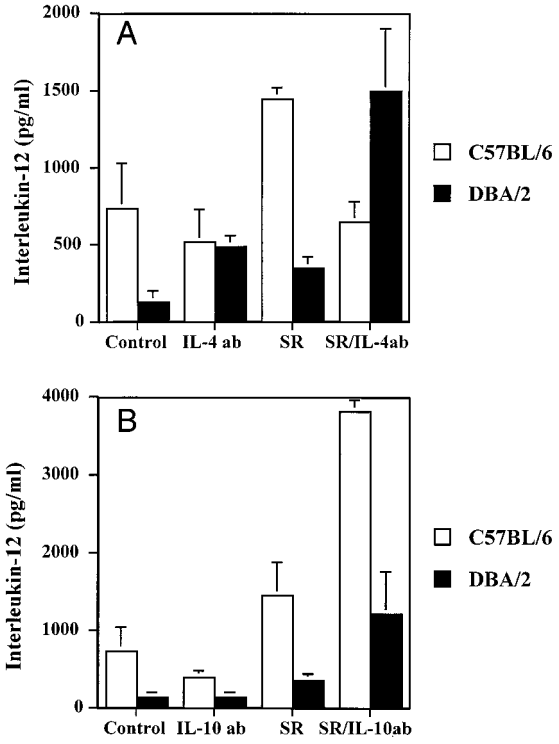


**FIGURE 14.** *A*, Effects of IL-10 Abs on IL-12 production by spleen cells from C57BL/6 and DBA/2 mice. Cells were unstimulated or were stimulated with SR, and IL-10 Abs were added as indicated on the abscissa. IL-12 release is quantitated on the ordinate. Data are expressed as the mean ± SEM for three animals in each group. There is a significant difference between the two strains for IL-10 Abs alone or with SR ($p < 0.005$). *B*, Effects of IL-4 Abs on IL-12 production by spleen cells from C57BL/6 and DBA/2 mice. Cells were unstimulated or were stimulated with SR, and IL-4 Abs were added as indicated on the abscissa. IL-12 release is quantitated on the ordinate. Data are expressed as the mean ± SEM for three animals in each group. There is a significant difference between the two strains for the control group and the SR-stimulated group ($p < 0.05$).

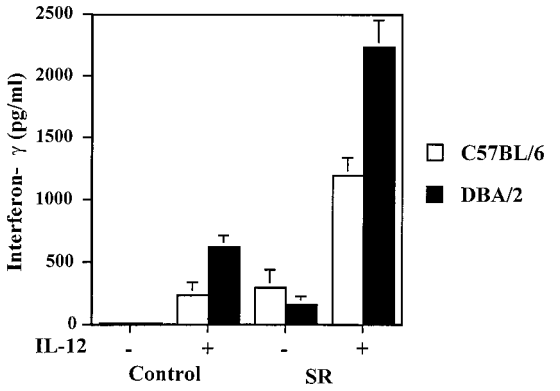


**FIGURE 15.** IFN-γ production by spleen cells from C57BL/6 and DBA/2 mice. IFN-γ is quantitated on the ordinate, and the type of stimulation is indicated on the abscissa. Data are expressed as the mean ± SEM for three animals in each group. There is a significant difference between the two strains when spleen cells were stimulated with SR and IL-12 ($p < 0.001$) or with IL-12 alone ($p < 0.02$).

IL-12 is important for regulating many Th1 responses (20, 21). It can be produced by T cells, macrophages/monocytes, and neutrophils and is important for both the induction and the maintenance of Th1 responses (20–22). It is also produced by APCs, such as dendritic cells and skin Langerhans' cells (21). The major cellular targets of IL-12 are T cells, NK cells, and B cells (22). A major effect of IL-12 on these cells is augmentation of the production of IFN-γ (23). One consequence of a dampened IL-12 response to Ag might be insufficient IFN-γ to support the development and/or maintenance of granulomatous lung disease. The observations of this study would be consistent with this hypothesis. In support of this hypothesis is a study by Caruso and colleagues (24). They observed, using the hapten trinitrophenol, that T cells from mice with the k MHC haplotype produce high levels of IFN-γ, while T cells from mice with the d haplotype produce low levels of IFN-γ, on re-exposure to the Ag.

The reason for the lack of an IL-12 response to low dose Ag in DBA/2 mice is not clear. It does not appear to be due to a total absence of a cellular response to Ag, because these mice do develop a lymphocytic alveolitis. They, therefore, resemble asymptomatic humans who have lymphocytic alveolitis after Ag exposure (17). The level of response in the DBA/2 mice is clearly dose related, since the DBA/2 mice develop granulomatous inflammation when exposed to higher concentrations of Ag. A total lack of response by macrophages to low dose Ag also does not appear to be a reason for the difference, since both strains of mice express similar amounts of IL-1 and TNF mRNAs in response to Ag. IL-1 and TNF are both known to be important components of the granulomatous response to Ag (25). It is likely that the lack of a granulomatous inflammatory response in DBA/2 mice is due to a defect in the production of IL-12, since DBA/2 mice develop lung disease similar to that in sensitive mice when given IL-12 augmentation therapy. In addition, spleen cells from DBA/2 mice are able to make IFN-γ when stimulated with IL-12, and combined stimulation with both IL-12 and SR leads to greater IFN-γ production than stimulation with IL-12 alone. Our findings that spleen cells from DBA/2 mice release less IL-12 in response to stimulation with SR, Con A, or combined stimulation with IFN-γ and LPS support the hypothesis that the response to Ag in DBA/2 mice is related to an intrinsic defect in the production of IL-12. This could be due to intrinsic differences in the IL-12 gene itself that result in different levels of expression (26) or to an alteration in upstream factors that regulate expression of the gene.

There also may be a relationship between the type of Ag and the induction of a Th1- or Th2-type response. This is suggested by a study by Rempel and associates, who observed that C57BL/6 mice develop a Th2 response when immunized with OVA. When given IL-12, these animals increase the production of IFN-γ and modify the response to Ag (27). In other studies, treatment with IL-12 has also been shown to change the response to Ag. In murine models of bacterial and fungal infections, treatment with IL-12 increased morbidity and mortality (28). The same has been shown in autoimmune disease models (29).

The cytokine environment at the time of stimulation is also thought to be important for the differentiation of the type of Th cell response. To support this, Williamson and associates have recently shown, in a model of graft-vs-host disease (GVHD), that C57BL/6 mice develop an acute, lethal Th1-mediated GVHD, but DBA/2 mice develop a Th2-dependent chronic GVHD. If C57BL/6 mice are treated with anti-IL-12 Abs, they switch to a Th2 response (30). The dose of Ag can also play a role in determining the type of immune response. This has been demonstrated in a model of *Leishmania major* infection (31) and in a model of herpes simplex virus infection (32, 33). Our studies, which show that exposure to IL-12 at the time of Ag exposure or an increase in the dose of Ag can change the response of resistant mice to Ag, are consistent with these prior studies.

Whatever the mechanisms that determine sensitivity to HP in these strains of mice, the present studies suggest that this murine model will be useful to further dissect the genetics and/or mechanisms of susceptibility to HP. The observations are highly relevant to human disease, since there are marked individual differences in the expression of clinical disease in individuals exposed to inhaled organic Ags.

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## References

1. Sharma, O. P., and N. Fujimura. 1995. Hypersensitivity pneumonitis: a noninfectious granulomatosis. *Semin. Respir. Infect. 10:96.*
2. Fink, J. N. 1992. Hypersensitivity pneumonitis. *Clin. Chest Med. 13:303.*
3. Krasnick, J., H. J. Meuwissen, M. A. Nakao, A. Yeldandi, and R. Patterson. 1996. Hypersensitivity pneumonitis: problems in diagnosis. *J. Allergy Clin. Immunol. 97:1027.*
4. do Pico, G. A. 1992. Hazardous exposure and lung disease among farm workers. *Clin. Chest Med. 13:311.*
5. Salvaggio, J. E., and B. W. Millhollon. 1993. Allergic alveolitis: new insights into old mysteries. *Respir. Med. 87:495.*
6. Salvaggio, J. E. 1994. Inhaled particles and respiratory disease. *J. Allergy Clin. Immunol. 94:304.*
7. Gudmundsson, G., and G. W. Hunninghake. 1997. Interferon-gamma is necessary for expression of hypersensitivity pneumonitis. *J. Clin. Invest. 99:2386.*
8. Wilson, B. D., J. L. Sternick, Y. Yoshizawa, and A. Katzenstein. 1982. Experimental murine hypersensitivity pneumonitis: multigenic control and influence by genes within the I-B subregion of the H-2 complex. *J. Immunol. 129:2160.*
9. Donnelly, K. B., B. O. Brooks, E. S. Cruz, and D. L. Wassom. 1996. Major histocompatibility complex class II genes control susceptibility to hypersensitivity pneumonitis in the mouse. *Chest 109(Suppl.):73S.*
10. Rossi, G. A., S. Szapiel, V. J. Ferrans, and R. G. Crystal. 1987. Susceptibility to experimental interstitial lung disease is modified by immune- and nonimmune-related genes. *Am. Rev. Respir. Dis. 135:448.*
11. Klein, J., F. Figeroa, and C. S. David. 1983. H-2 haplotypes, genes and antigens: second listing. *Immunogen 17:553.*
12. Chirgwin, J. M., A. E. Pryzbala, R. J. MacDonald, and W. J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry 18:5294.*
13. Manatis, T., E. F. Fritsch, and J. Sambrook. 1982. Extraction, purification, and analysis of messenger RNA from eukaryotic cells. In *Molecular Cloning: A Laboratory Manual.* T. Manatis, E. F. Fritsch, and J. Sambrook, eds. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, p. 7.3.
14. Lehrach, D., D. Diamond, J. Wosney, and H. Boedtker. 1977. RNA molecular weight determinations by gel electrophoresis under denaturing conditions, a critical reexamination. *Biochemistry 16:4743.*
15. Laurent, G. J., R. J. McAnulty, B. Corrin, and P. Cockerill. 1981. Biochemical and histological changes in pulmonary fibrosis induced in rabbits with intratracheal bleomycin. *Eur. J. Clin. Invest. 11:441.*
16. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem. 72:248.*
17. Cormier, Y., J. Belanger, and A. Tardif. 1986. Relationship between radiographic change, pulmonary function, and bronchoalveolar lymphocytosis in farmer's lung disease. *Thorax 41:28.*
18. Fiorentino, D. F., M. W. Bond, and T. R. Mosman. 1989. Two types of murine helper T cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *J. Exp. Med. 170:2081.*
19. Schuyler, M., K. Gott, A. Cherne, and B. Edwards. 1997. Th1 $CD4^+$ cells adoptively transfer experimental hypersensitivity pneumonitis. *Cell. Immunol. 177: 169.*
20. Trinchieri, G. 1993. Interleukin-12 and its role in the generation of Th1 cell. *Immunol. Today 14:335.*
21. Kennedy, M. K., K. S. Picha, K. D. Shanebeck, D. M. Anerson, and K. H. Grabstein. 1994. Interleukin-12 regulates proliferation of Th1, but not Th2 or Th0 clones. *Eur. J. Immunol. 24:2271.*
22. Ma, X., M. Aste-Amezaga, and G. Trinchieri. 1996. Regulation of interleukin-12 production. *Ann. NY Acad. Sci. 795:13.*
23. Chensue, S. W., J. H. Ruth, K. Warmington, P. Lincoln, and S. L. Kunkel. 1995. In vivo regulation of macrophage IL-12 production during type 1 and type 2 cytokine-mediated granuloma formation. *J. Immunol. 155:3546.*
24. Caruso, C., G. Candore, M. A. Modica, C. T. Bonanno, G. Sireci, F. Dieli, and A. Salerno. 1996. Major histocompatibility complex regulation of cytokine production. *J. Interferon Cytokine Res. 16:983.*
25. Tsuji, M., V. B. Dimov, and T. Yoshida. 1995. In vivo expression of monokine and inducible nitric oxide synthase in experimentally induced pulmonary granulomatous inflammation: evidence for sequential production of interleukin-1, inducible nitric oxide synthase, and tumor necrosis factor. *Am. J. Pathol. 147:1001.*
26. Plevy, S. E., J. H. M. Gemberling, S. Hsu, A. J. Dorner, and S. T. Smale. 1997. Multiple control elements mediate activation of the murine and human interleukin 12 p40 promoters: evidence of functional synergy between C/EBP and Rel proteins. *Mol. Cell. Biol. 17:4572.*
27. Rempel, J. D., M. Wang, and K. T. HayGlass. 1997. In vivo IL-12 administration induces profound but transient commitment to T helper cell type 1-associated patterns of cytokine and antibody production. *J. Immunol. 159:1490.*
28. Seder, R. A., B. L. Kelsall, and D. Jankovic. 1996. Differential roles for IL-12 in the maintenance of immune responses in infectious versus autoimmune disease. *J. Immunol. 157:2745.*
29. Leonard, J. P., K. E. Waldburger, and S. J. Goldman. 1995. Prevention of experimental autoimmune encephalomyelitis by antibodies against interleukin 12. *J. Exp. Med. 181:381.*
30. Williamson, E., P. Garside, J. A. Bradley, I. A. R. More, and A. M. Mowat. 1997. Neutralizing IL-12 during induction of murine acute graft-versus-host disease polarizes the cytokine profile toward a Th2-type alloimmune response and confers long term protection from disease. *J. Immunol. 159:1208.*
31. Bretscher, P. A., G. Wei, J. N. Menon, and H. Bielefeldt-Ohmann. 1992. Establishment of stable, cell mediated immunity that makes 'susceptible' mice resistant to *Leishmania major. Science 257:539.*
32. Brenner, G. J., N. Cohen, and J. A. Moynihan. 1994. Similar immune response to nonlethal infection with herpes simplex virus-1 in sensitive (BALB/c) and resistant (C57BL/6) strains of mice. *Cell. Immunol. 157:510.*
33. Altmann, D. M., and W. A. Blyth. 1985. Protection from herpes simplex virus-induced neuropathology in mice showing delayed hypersensitivity tolerance. *J. Gen. Virol 66:1297.*
34. Gudmundsson, G., A. Bosch, B. L. Davidson, D. J. Berg, and G. W. Hunninghake. 1998. IL-10 modulates the severity of hypersensitivity pneumonitis. *Am. J. Respir. Cell Mol. Biol. in press.*