Interferon-γ Is Necessary for the Expression of Hypersensitivity Pneumonitis

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Abstract

Farmers lung disease is a common form of hypersensitivity pneumonitis (HP) and is characterized by inflammation and granuloma formation in the lung. Interferon-γ is important for the expression of granulomatous diseases caused by infectious agents; however, the role this mediator in regulating expression of the granulomatous response to inhaled antigen is not known. To evaluate this, we compared the response to inhaled antigen of mice that do not express the gene coding for interferon-γ (GKO) with that of their normal littermates (WT). GKO and WT mice on a BALB/c background were exposed to 150 μg of the thermophilic bacteria *Saccharopolyspora rectivirgula* or saline alone, for three consecutive days a week, for 3 wk. After exposure to antigen, WT mice developed a marked granulomatous inflammation associated with an increase in lung weight and numbers of cells in bronchoalveolar lavage fluid (BAL). Although GKO mice also exhibited an increase in lung weight and numbers of cells in BAL fluid, they developed minimal inflammation and no granulomas after a similar exposure to antigen. To further evaluate if the lack of a response to antigen in GKO mice was due to lack of IFN-γ, we replaced this mediator via intraperitoneal injections. When given replacement IFN-γ, the GKO mice developed granulomatous inflammation in the lung. These studies show that IFN-γ is essential for the expression of hypersensitivity pneumonitis. (*J. Clin. Invest.* 1997. 99:2386–2390.) Key words: knockout-mice • granuloma • lung disease • farmers lung disease • histopathology

Introduction

Hypersensitivity pneumonitis (HP)[1] is a syndrome caused by sensitization to and repeated inhalation of an inhaled antigen (1–8). The clinical and laboratory features of the disease have been well defined, but many of the immune mechanisms involved in the development of the disease have not been well defined. The most common antigens are thermophilic actinomycetes (2). There is widespread exposure to these antigens but the number of individuals who develop the disease is relatively low. This observation strongly suggests that host factors are important in the expression of clinical disease (1).

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1. *Abbreviations used in this paper:* BAL, bronchoalveolar lavage; GKO, disruption of the IFN-γ gene; HP, hypersensitivity pneumonitis, SR, *Saccharopolyspora rectivirgula*; WT, wild-type.

Because precipitating antibodies against specific antigens are found in patients with HP, an early conclusion was made that it was an immune complex–mediated process (9). However, later studies showed that cell-mediated immunity is more important (10, 11). The first response to antigen is an increase in PMNs in the alveoli and small airways. This is followed by an influx of mononuclear cells and formation of granulomas (1). Bronchoalveolar lavage (BAL) reveals an increase in T lymphocytes (12, 13). In very early disease, most T cells are of the CD4+ type, but during the recovery from acute disease more are of the CD8+ subset (12, 13). Increased numbers of CD8+ T cells can also be seen in exposed but asymptomatic individuals (13). It is not known if these asymptomatic subjects develop a transient CD4+ response after each exposure to antigen. Cytokine release from T lymphocytes is an important part of the pathogenesis of HP and studies in humans have shown increased concentration of IFN-γ in BAL fluid from patients with HP (14).

A well-described murine model of HP has often been used to study the immune mechanisms that cause HP (15–21). In this model, mice exposed to the actinomycete *Saccharopolyspora rectivirgula* (SR) (previously named *Micropolyspora faeni*) or *Thermatoactinomyces vulgaris*, via nasal inhalation, develop diffuse bronchoalveolitis and form granulomas in the lung (21). Previous studies, using this model of HP to evaluate the role of IFN-γ in the pathogenesis of the disease, used monoclonal antibodies to block the function of IFN-γ (22). In these studies, there was little change in the number of cells in the BAL fluid; however, less of a lung leak developed after exposure to antigen. These studies did not evaluate histologic changes in the lung. In our studies, we found that disruption of the IFN-γ gene (GKO) mice do not develop significant amounts of granuloma formation in the lungs after exposure to antigen. They do develop HP, however, if IFN-γ is replaced. These studies show that IFN-γ is necessary for the expression of HP.

Methods

Animals. BALB/c mice with a targeted disruption (at exon 2 with the neomycin resistance gene) of the IFN-γ gene (GKO) and wild-type (WT) littermates (23) were purchased from Jackson Laboratories (Bar Harbor, ME). They were bred in the Animal Care Facility at the University of Iowa. Female BALB/c mice weighing 18–24 grams were used for these studies. They were housed in an antigen-free and virus-free environment and maintained on standard mouse chow and water ad libitum. All procedures used in this study were in compliance with Animal Welfare Act Regulations, and with the Guide for the Care and Use of Laboratory Animals.

Antigen and antibodies. Antigen was prepared from a strain of *S. rectivirgula* that was obtained from American Type Culture Collection (Rockville, MD). It was grown in a trypticase soy broth in a 55°C shaking incubator for 4 d, centrifuged, and rinsed with distilled water. It was then homogenized and lyophilized. Antigen was resuspended in pyrogen-free saline. A limulus amebocyte lysate assay from Sigma Chemical Co. (St. Louis, MO) showed that this material contained < 20 ng of endotoxin/mg.

BAL fluid was evaluated for the presence of anti-SR antibodies. This was done by adding 100 μl of BAL fluid to 100 μg of antigen and incubating for 1 h at room temperature. After three washes with PBS, 100 μl of a 1:100 dilution of FITC-conjugated goat anti–mouse-Ig (Sigma Chemical Co.) was added and incubated for 1 h at room temperature. After three washes with PBS, the samples were examined under a fluorescent microscope.

Induction of hypersensitivity pneumonitis. HP was induced by installing 150 μg of SR antigen in saline, intranasally, under light anesthesia. The material was applied at the tip of the nose and inhaled involuntarily. This was done for three consecutive days per week for 3 wk. This dose and timing was chosen based on previous work done by others (16, 19, 20). We also confirmed that this was the optimal dose schedule in preliminary studies. Previous studies have shown that 15 and 60% of the antigen administered reaches the lung under these conditions (24). Mice were killed 4 d after the last exposure with pentobarbital injection. Previous studies by others have shown that the inflammatory response, including granuloma formation in the lung, resolves in 3–4 wk after antigen exposure is terminated (16). We also confirmed these observations in preliminary studies. Prior studies have shown that antibody production occurs both in serum and BAL fluid (15, 16). We evaluated BAL fluid for antibodies to SR antigen. We were able to detect antibodies to SR antigen both in GKO and WT mice exposed to antigen but not in saline-treated animals. We did not detect any differences between GKO and WT mice.

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

Lung index (weight). Lungs were removed, trimmed of extraneous tissue, rinsed, and weighed. Lung indexes were calculated, as described by Wilson et al. (25).

$$Lung\ index\ =\ \frac{(Lung\ weight/body\ weight)\ test\ animal}{(Lung\ weight/body\ weight)\ control\ animal}.$$

Histological 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-μm–thick sections and stained with hematoxylin and eosin. The sections were evaluated by light microscopy. A histological 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 10–30% of the lung; 3 = lesions involving 30–50% of the lung; and 4 = lesions involving > 50% of the lung (17, 18). The slides were evaluated without knowledge of the type of mouse or exposure to antigen. The area covered by an eyepiece grid (0.99 × 0.99 mm using 100 magnification) was judged to be normal or abnormal. An average of 200 fields was evaluated from each mouse.

Reverse transcriptase polymerase chain reaction. Total lung RNA was prepared as described by Chirgwin et al. (26) and modified by Maiatis et al. (27). It was then DNAase-treated and reverse transcribed using MMLVRT enzyme in a total reaction volume of 50 μl. PCR was performed on the resultant cDNA, using primers specific for IFN-γ or IFN-α, purchased from Clontech (Palo Alto CA), and the PCR products were analyzed on a 3% agarose gel and visualized with an ethidium bromide stain. The PCR product was then trans-
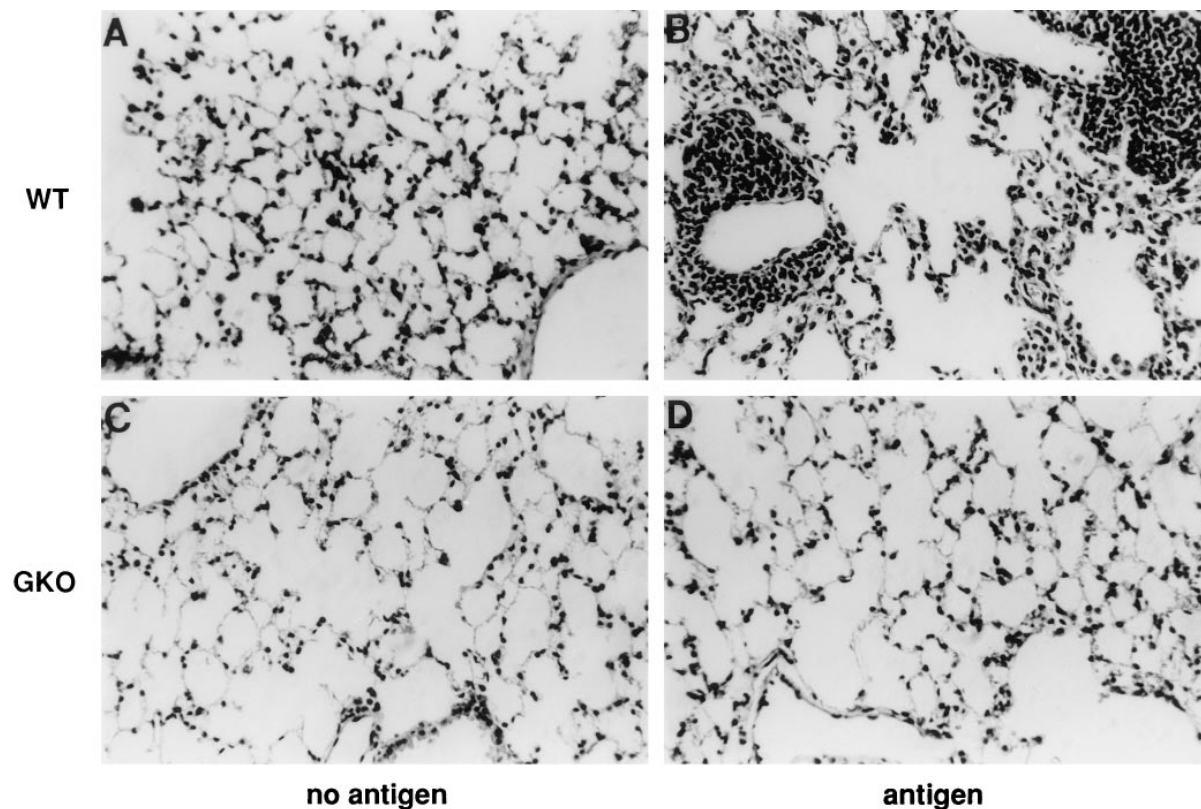

Figure 1. Expression of HP in GKO and WT mice. (*A*) WT mice exposed to saline alone. (*B*) WT mice exposed to antigen. (*C*) GKO mice exposed to saline alone. (*D*) GKO mice exposed to antigen. Representative hematoxylin and eosin–stained histology sections. ×140.
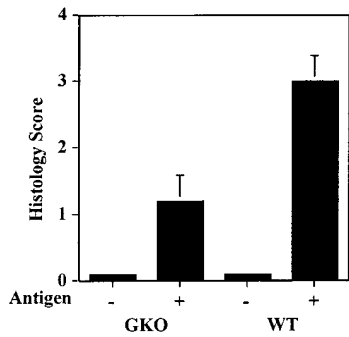
Figure 2. Quantitation of inflammation and granulomas in WT and GKO mice. GKO and WT mice were exposed to saline alone or antigen as described in Methods. Amounts of inflammation and granulomas were quantitated as described in Methods and are quantitated on the ordinate. The types of mice and exposure to antigen are indicated on the abscissa. Data are expressed as the mean±SEM for *n* = 4–6 animals in each group. There was a significant difference between antigen-treated WT and antigen-treated GKO mice (*P* = 0.008).

Figure 4. Changes in lung weight (*lung index*) in GKO and WT mice. WT and GKO mice were exposed either to antigen or saline alone, as described in Methods. 4 d after the last exposure to saline or antigen, mice were killed and lung index quantitated, as described in Methods. On the ordinate is a quantitation of the lung index and on the abscissa is indicated the types of mice and their exposure to saline or antigen. Data are expressed as the mean±SEM for *n* = 5–6 animals in each group. There was a statistically significant difference between antigen (*P* = 0.001) and saline (*P* < 0.005) treated mice in both groups and also between antigen-treated GKO and WT mice (*P* = 0.027).

ferred from the agarose gel to a membrane, hybridized with the appropriate ^{32}P-labeled cDNA probe, and analyzed following autoradiography.

IFN-γ replacement therapy. Recombinant murine IFN-γ was purchased from Genzyme (Boston, Massachusetts). It had a specific activity of 4.5×10^6 U/mg and contained < 1 ng/ml of endotoxin. The material was diluted in PBS with 1% bovine albumin. Groups of mice were given an i.p. injection of 1,000 U in 0.2 ml of diluent 24 h before the first exposure each week and then daily just before exposure.

Statistics. Statistical analysis was performed using an unpaired (two-tailed) *t* test. Data are reported as means±SEM. The 95% confidence limit was taken as significant (*P* < 0.05) (28).

Results

Comparison of WT and GKO mice. Antigen exposure resulted in a marked inflammatory response with granulomas in the WT mice but it caused only a minor inflammatory response without granulomas in the GKO mice, as demonstrated in Fig. 1. Saline exposed controls had no inflammation or granulomas. The histology score after antigen exposure was significantly higher in the WT mice than in the GKO mice, as shown in Fig. 2. Exposure to antigen led to an increase in the total number of cells in BAL fluid as demonstrated in Fig. 3. This occurred both in the GKO and WT mice and there was not a significant difference between them. Most of the cells in both WT and GKO mice were macrophages, but there was also an increase in lymphocytes. Both the GKO and WT mice had an
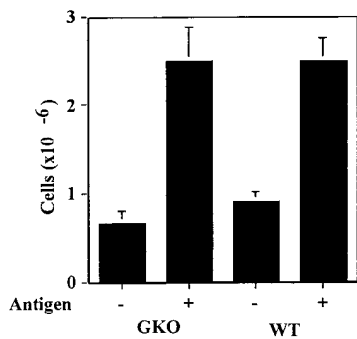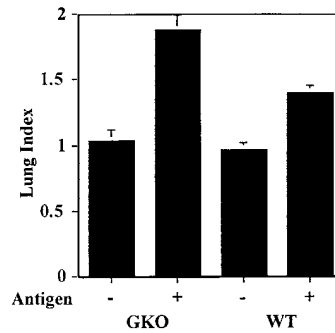
increase in lung index when exposed to SR, as shown in Fig. 4. The GKO mice had a significantly higher lung index than the WT mice. There was an increase in IFN-γ mRNA in WT mice exposed to antigen compared with saline-treated animals but no IFN-γ mRNA was detected in the GKO mice (Fig. 5). The GKO mice, exposed to antigen, exhibited increased amounts of IFN-α mRNA, compared with both saline-treated GKO mice and WT mice, as shown in Fig. 6. Thus, although GKO mice respond to antigen with an increase in numbers of inflammatory cells in BAL and an increase in lung permeability, they do not develop significant histologic evidence of granulomas in the lung. These observations strongly suggest that IFN-γ is necessary for the development of granulomatous inflammation in response to inhaled antigens. The studies further suggest that a compensatory increase in IFN-α cannot replace IFN-γ for the expression of the disease.

IFN-γ replacement therapy. When GKO mice were given replacement therapy with IFN-γ, they developed granulomatous inflammation in their lungs after exposure to antigen, as demonstrated in Fig. 7. This also led to an increase in their histology score compared with the mice that were not given replacement therapy, as demonstrated in Fig. 8. These observations show that the lack of response to inhaled antigen in GKO mice is due to the absence of IFN-γ, and not another developmental process.

Figure 3. Cells in BAL fluid. WT and GKO mice were exposed either to antigen or saline alone, as described in Methods. 4 d after the last exposure to saline or antigen, bronchoalveolar cells were isolated and quantitated, as described in Methods. On the ordinate is a quantitation of the cells and on the abscissa is indicated the types of mice and their exposure to saline or antigen. Data are expressed as the mean±SEM for *n* = 5–6 animals in each group. There was a significant difference between the antigen- and saline-exposed mice in both GKO (*P* = 0.002) and WT mice (*P* < 0.005).
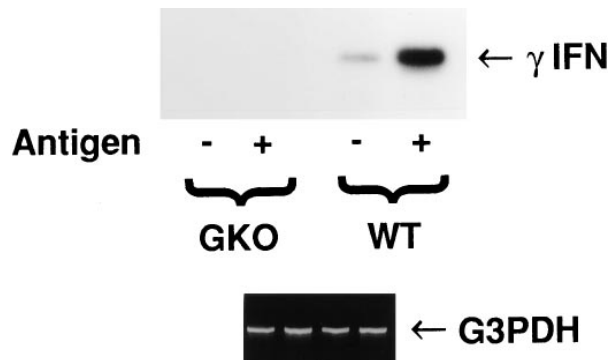

Figure 5. Quantitation of IFN-γ mRNA in GKO and WT mice treated with antigen or saline. Quantitation of GPDH mRNA demonstrates equal loading of RNA; *n* = 3.
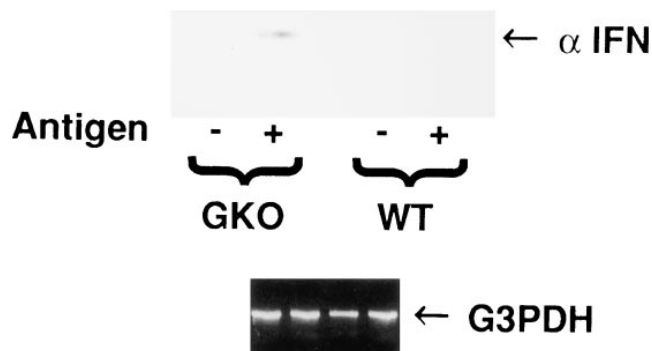
Figure 6. Quantitation of IFN-α mRNA in GKO and WT mice treated with antigen or saline. Quantitation of GPDH mRNA demonstrates equal loading of RNA; *n* = 3.

Figure 8. Quantitation of inflammation and granulomas in GKO mice with and without IFN-γ replacement therapy. GKO mice were exposed to antigen as described in Methods and given IFN-γ replacement therapy as described in Methods. Amounts of inflammation and granulomas were quantitated as described in Methods and are quantitated on the ordinate. Replacement therapy is indicated on the abscissa. Data are expressed as the mean±SEM for *n* = 3 animals in each group. There is a statistically significant difference between the two groups (*P* = 0.03).

Discussion

The goal of this study was to evaluate the role of IFN-γ in the expression of HP. To address that question, we compared the development of HP in mice that can express the IFN-γ gene and mice that cannot express this gene. We found that IFN-γ is essential for the expression of this disease, since GKO mice did not develop granulomatous lung disease in response to antigen. This was not due to an unrelated defect in these animals since they did develop HP, if IFN-γ was replaced. The absence of IFN-γ did not prevent the migration of inflammatory cells into the lung in response to antigen, nor did it prevent the development of lung permeability in response to antigen.

GKO mice exhibit normal development and growth (23). However, they have a markedly reduced resistance to intracellular pathogens, especially mycobacteria (29, 30). They often have increased production of other types of interferon, like IFN-α, to partially compensate for the absence of IFN-γ. We also observed this effect in the present study. We also found that this compensatory increase in IFN-α did not replace IFN-γ in the process of granuloma formation.

Kamijo et al. showed that mice with a disruption of the IFN-γ receptor gene, that were inoculated with the BCG strain of *Mycobacterium bovis*, had a reduction in the formation of characteristic granulomas in the liver, compared with WT controls (31). No studies have been reported that have examined the response of GKO mice to antigens that cause HP or to agents that cause other noninfectious granulomatous diseases.
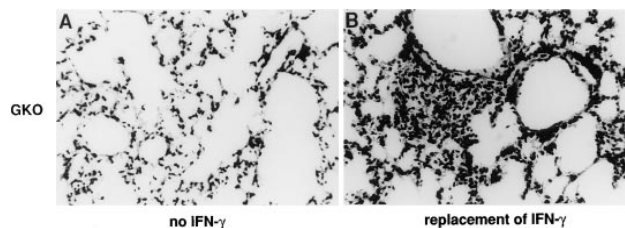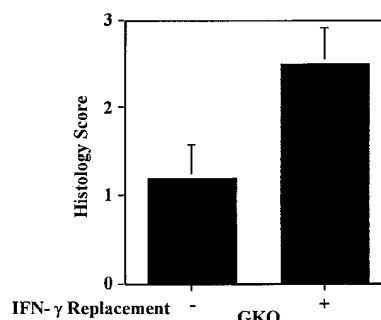

Figure 7. Expression of HP in GKO mice with and without IFN-γ replacement therapy. (*A*) GKO mice exposed to antigen without IFN-γ replacement therapy. (*B*) GKO mice exposed to antigen with IFN-γ replacement therapy. Representative hematoxylin and eosin–stained histology sections. ×140.

Studies in a murine model of HP have shown that if mice are treated with antibodies against IFN-γ, there is little change in the number of cells in the BAL fluid but there is less lung permeability and less production of TNF-α (22). One of the potential problems of studies using antibodies to cytokines is that they may not neutralize all of the cytokine and they may trigger immune complex disease. Our observation that IFN-γ–deficient mice have similar numbers of cells in BAL fluid after an antigen challenge are similar to those of previous studies (22). Our finding of higher lung index in GKO mice treated with antigen, compared with WT mice, is in contrast to previous studies (22). The reason for this difference between the studies is not clear. However, we speculate that the increase in permeability in our studies might be due to the generation of more immune-complexes in the GKO mice. When no granulomas are formed, more antigen might escape the lung and be available for formation of circulating immune complexes. Previous studies have suggested that immune complexes increase lung permeability (32).

One of the concerns that arises when using genetically altered animals is that it is not clear if the changes seen in these animals are due to the lack of production of the product of the deleted gene or if it is the result of a compensatory developmental process. Therefore, we gave the GKO mice replacement therapy with IFN-γ. This resulted in a granulomatous inflammation in these animals when they were exposed to antigen. This is strong evidence that the lack of granuloma formation that we observed in the GKO mice is due to lack of IFN-γ.

The Th1 and Th2 subsets of CD4+ T cells were first described by Mosman et al. in mice (33). These subsets of T cells were defined on the basis of their pattern of production of inflammatory mediators. Th1 cytokines include IL-2, IL-12, and IFN-γ. Th2 cytokines include IL-4, IL-5, IL-6, and IL-10. Expression of the Th1 or Th2 phenotypes may depend, in part, on the type of exposure to antigen. For example, exposure to helminthic antigens often promotes a Th2 type of inflammation (34), and exposure to mycobacterial antigen often leads to a Th1 type of response (23, 29, 30). The categorization of CD4+ cells into these groups appears to have functional relevance. Th1 cells regulate delayed type hypersensitivity responses (35) and Th2 responses are prominent in allergic reactions

(36). Th1 and Th2 cells can interact in a counterregulatory fashion. For example, IL-10 downregulates cytokine production by Th1 cells (37) and IFN-γ inhibits the proliferation of Th2 cells (38). Although these subsets of T cells were first characterized in mice, they also appear to regulate immune responses in humans (39, 40). IFN-γ is one of the most important cytokines in Th1 responses. The fact that GKO mice do not develop granulomas suggests that Th1 responses are an important part of the immune response in HP, and in granuloma formation in general.

Acknowledgments

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