IL-4-deficient Mice Aggravate Hypersensitivity Pneumonitis

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Background: Hypersensitivity pneumonitis (HP) comprises a group of lung diseases resulting from repeated inhalation of various antigens such as Saccharopolyspora rectivirgula (SR). HP is categorized as a Th1 disease. Therefore, it has been suggested that IL-4, Th2 type cytokine, plays a protective role in the development of HP. However, the functional role of IL-4 in HP has not been extensively investigated in vivo. Therefore, we investigated the functional role of IL-4 in HP using IL-4 knockout (KO) mice. Methods: HP was induced by repeated exposure to SR in C57BL/6 (B6) and IL-4 KO (C57BL/6 background) mice. Results: IL-4 KO mice aggravated HP in terms of histological alteration, SR-specific immune responses, and inflammatory cell infiltration in the lungs compared with B6 mice. IL-4 KO mice produced high levels of IFN-γ, TGF-β, and TNF-α in the lungs, whereas B6 mice showed the enhanced production of IL-4. Moreover, chemokines such as MIP-1α, MCP-1, and RANTES were highly expressed in IL-4 KO mice. IFN-γ-secreting CD4, CD8 T cells, and neutrophils were enhanced in the bronchoalveolar lavage fluid (BALF) of IL-4 KO mice than those of B6 mice. The administration of recombinant(r) IL-4 restored these immunologic parameters in IL-4 KO mice. Conclusion: These results indicate that IL-4 plays a suppressive role in SR-induced HP by attenuating Th1-dominant immune responses.

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INTRODUCTION

Hypersensitivity pneumonitis (HP) is a syndrome caused by repeated inhalation of and sensitization to organic antigens, Saccharopolyspora rectivirgula (SR), a thermophilic actinomycete, is the most common antigen that causes farmer’s lung disease, but multiple other organic antigens also can cause HP (1,2). Mice given SR antigen develop alveolitis where the inflammation is initially neutrophilic and sequentially more lymphocytic after exposure (3,4). Among the lymphocytes, T cells have been considered as critical cell subset for the development of HP since athymic nude mice develop a significantly less severe form of HP compared with their wild type mice (5-7). Moreover, several recent studies have suggested that HP is mediated by Th1 immune responses (8-10). Th1-biased B6 mice are more susceptible to SR-induced HP than Th2-biased DBA/2 mice (9), suggesting that the differential production of Th1 or 2-type cytokines in B6 and DBA/2 mice may determine their susceptibility or resistance to HP. IL-12, produced by activated alveolar macrophages, aggravates HP (11), and IFN-γ induces granulomatous inflammation in the murine models of HP (12). Furthermore, the adoptive transfer of Th1 clones has been shown to induce reversible HP in the mice (10). These results suggest that the development of HP in animals could be determined by the balance of Th1/Th2 immune responses in vivo.

Based on these findings, it has been suggested that IL-4 derive immune responses into Th2 type in HP, resulting in attenuating Th1-typed immune responses of HP. However, there is little direct evidence of protective effect of IL-4 on HP. Moreover, it has not been reported whether IL-4-deficient mice aggravate HP or not. Therefore, in this study, we investigated whether IL-4 plays the functional roles in the development of SR-induced HP using IL-4 knockout (KO) mice.
MATERIALS AND METHODS

Mice
C57BL/6 (B6) mice were purchased from Orient Company (Seoul, Korea), IL-4 KO (C57BL/6 background) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). These mice were bred and maintained in a specific pathogen-free environment at the AAALAC accredited Clinical Research Institute of Seoul National University Hospital. All procedures of animal experiments were approved by the Institutional Animal Care and Use Committee of the Clinical Research Institute of Seoul National University Hospital.

Antigens
SR antigen was prepared from a strain of SR obtained from the American Type Culture Collection (ATCC, catalog no. 29034, Manassas, VA), which was grown in a Trypticase soy broth in a 55°C shaking incubator for 4 days, centrifuged, and rinsed with distilled water three times. Next, the SR antigen was homogenized and lyophilized. The SR antigen was re-suspended in pyrogen-free saline. The SR antigen contained less than 20 ng/mg endotoxin, estimated using a limulus amebocyte lysate assay (Sigma Chemical, St. Louis, MO).

Induction of hypersensitivity pneumonitis
HP was induced by intranasal inoculation of 150 μg of SR antigen in saline into B6 or IL-4 KO mice under light anesthesia, B6 mice (wild type) were used as a control for IL-4 KO mice in SR-induced HP. This procedure was performed on 3 consecutive days per week for 3 weeks. The mice were sacrificed with a pentobarbital injection 4 days after the final treatment. For histological examination, paraffin-embed entire lungs were cut and stained with hematoxylin and eosin.

Hydroxyproline assay
The total hydroxyproline level of the lung was measured on 3 weeks after the first SR intranasal inoculation. The whole lung was excised, homogenized in PBS (2 ml), and dried for 6 h by vacuum dryer. The samples were added to 1 ml of 6 N HCl for 12 h at 110°C and then filtered. Aliquots (50 μl) of the samples were then examined by adding 50 μl of citrate acetate buffer and 1 ml of chloramines T solution followed by a reaction with Erlich’s solution at 65°C for 15 min. The absorbance was measured at 550 nm using a spectrophotometer. The increased hydroxyproline contents in the lungs of experimental groups were compared with the amount of hydroxyproline of the lungs from untreated B6 mice. The value of B6 control group is set to 1 and the relative increased values of hydroxyproline in each group were calculated. The results were statistically analyzed using t-test and presented as percentage.

Bronchoalveolar lavage and FACS analysis
The trachea was cannulated, and the lung was lavaged five times with 0.7 ml cold PBS. The BALF was centrifuged at 1,500 rpm for 10 min at 4°C, and the supernatant was removed. The total BALF cells were counted using a hemocytometer, and incubated for 15 min on ice with FeγRII/III blockade. After washing, the cells were stained in a 200 μl total volume with a 1 μg combination of the following mAbs: anti-CD8, -CD4, Ly-6G mAbs, which were purchased from BD Bioscience (SanDiego, CA).

Real-time PCR analysis
For quantitative real-time PCR, total RNA was isolated from lungs using RNeasy Mini Kit (Quiagen, Valencia, CA). Three micrograms of RNA was reverse transcribed with MMLV RT (Promega, Madison, WI) and PCR was performed. The following primers and probe were synthesized by Applied Biosystems (Foster City, CA): GAPDH, IFN-γ, IL-4, MIP-1α, MCP-1, IP-10, RANTES and Biosource (Camarillo, CA): TGF-β1: GCAACATGTGAAGCTTACGAGA (forward), GACGTGAAAAGACGCC ACTCA (reverse), and ACCCTGGTAACC-GGCTGCTGACCTAMRA; and, TNF-α (Biosource, Camarillo, CA). The results for each cytokine were normalized with respect to GAPDH expression.

ELISA and proliferation assay
BALF cells were taken from the lungs of B6 and IL-4 KO mice administered SR antigen 3 weeks after the SR antigen exposure, and cultured with filtered SR antigen. Blood collected from the orbital sinus was centrifuged, and the serum was analyzed for SR-specific IgG. SR-specific IgG in serum (dilution 1/100) and BALF was measured using ELISA. In addition, spleen cells were taken from B6 and IL-4 KO mice administered SR antigen 3 weeks after the SR antigen exposure, and cultured with filtered SR antigen, SR-specific immune cell proliferation was determined using MTS assay kits (Promega, Madison, WI).
Intracellular cytokine staining
For intracellular cytokine staining in CD4⁺, CD8⁺ T cells, and Ly-6G⁺ granulocytes, BALF cells were isolated from mice administered SR antigen in the first week after antigen exposure, and incubated overnight with Con A (2 μg/ml). The cells were surface stained with mAb specific for −CD4, −CD8 and -Ly-6G mAb fixed and permeabilized with Cytofix/Cytoperm (BD Pharmingen, San Diego, CA) according to the manufacturer's instructions. These cells were stained using PE-conjugated anti-IFN-γ mAb.

Administration of recombinant IL-4 in vivo
Recombinant murine IL-4 was purchased from R&D System (Minneapolis, MN) and diluted in PBS with 1% bovine albumin. Mice were given 100 μg intraperitoneally in 0.3 ml of PBS 24 h before the first SR antigen exposure each week.

Statistics
Statistical significance was analyzed using the program Prism 3.0. Student’s t-tests were used to determine the P-value for comparing two groups. p-values <0.05 were considered significant. For the hydroxyproline content in the lungs, one-way ANOVA was performed.

RESULTS
IL-4-deficient mice aggravate SR-induced HP
To address the functions of IL-4 in SR-induced HP, we induced and compared SR-induced HP in B6 and IL-4 KO mice. SR antigens induced more severe inflammatory response such as multifocal peribronchial lymphoid hyperplasia in the lungs of IL-4 KO mice compared with B6 mice (Fig. 1A). The immunological response to SR antigen in SR-induced HP was evaluated by measuring SR-specific IgG levels in serum, and proliferation of immune cells against SR antigen in IL-4 KO and B6 mice. IL-4 KO mice showed much higher levels of SR-specific IgG in serum and the immune cell proliferation against SR antigen (Fig. 1B and C) compared with B6 mice. IL-4 KO mice given recombinant(r) IL-4 reduced inflammation in their lungs and immune response against SR antigen compared with IL-4 KO mice (Fig. 1A-C). To explore fibrosis in the lung tissues of B6 and IL-4 KO mice during SR-induced HP, the amount of hydroxyproline (a collagen component) were measured 3 weeks after the first SR antigen exposure.

Figure 1. Hypersensitivity pneumonitis (HP) is aggravated in IL-4-deficient mice. In B6 and IL-4 KO mice, HP was induced by inoculating SR antigen nasally. These mice were sacrificed 3 weeks after HP induction. (A) The lungs were removed from the B6, IL-4 KO, and IL-4 KO mice given rIL-4 and fixed for paraffin sections that were stained with hematoxylin and eosin. Original magnification, ×100. The photographs are a representative of the 6 mice in each group. (B) The amount of SR-specific IgG in serum was measured by ELISA. (C) Immune cell proliferation against SR antigen was evaluated using the spleen cells by MTS assay. (D) The amounts of hydroxyproline in the lung tissues were measured. The number of mice in each group was three and the results were similar in three independent experiments. The results shown are a representative of three independent experiments. (*p<0.05, **p<0.01, B6 versus IL-4 KO mice or IL-4 KO versus IL-4 KO mice given rIL-4).
administration, the levels of hydroxyproline in the lungs were significantly higher in IL-4 KO mice than B6 mice (Fig. 1D), which was restored by administration of rhIL-4.

To investigate the subsets of infiltrated immune cells in the lungs following SR antigen exposure, we analyzed the numbers of CD4$^+$ and CD8$^+$ T cells, B cells, macrophages, and polymorphonuclear (PMN) leukocytes using BALF 7 days after the first SR antigen inoculation. The cell numbers of BALF were greater in IL-4 KO mice than B6 mice (Fig. 2A), suggesting that more immune cells were recruited into the lung tissues in IL-4 KO mice during SR-induced HP. Except for B cells, most of immune cells were significantly increased in BALF of IL-4 KO mice compared with B6 mice. In patients with HP, the lung involvement is characterized by a lymphocytic alveolitis with an increase in both the percentage and absolute number of CD8$^+$ T cells in the BALF. Therefore, the percentages of CD4$^+$ and CD8$^+$ T cells in the BALF from B6 and IL-4 KO mice were estimated during SR-induced HP. The percentages of CD8$^+$ T cells in the BALF of IL-4 KO mice was higher than those of B6 mice in SR-induced HP, whereas the percentages of CD4$^+$ T cells in BALF of IL-4 KO mice was similar to those of B6 mice. Therefore, the CD4/CD8 T cell ratio was significantly reduced in the BALF of IL-4 KO mice compared to B6 mice in SR-induced HP (Fig. 2B). These findings suggest that IL-4 contributes to regulating the influx or proliferation of immune cells and CD8$^+$ T cells in BALF during SR-induced HP.

Combined, these results indicate that IL-4-deficient mice aggravate SR-induced HP in terms of histological alteration, immune responses against SR antigen, fibrosis, recruitment of inflammatory cells, and CD4$^+/CD8^+$ T cell ratio.

IL-4-deficient mice predominantly produce Th1-type and proinflammatory cytokines during SR-induced HP

HP has been known to be Th1 type immune disease since high levels of Th1 cytokines and low levels of Th2 cytokines are produced in the lungs during SR-induced HP, and Th1 type immune responses in the lungs aggravates it (9-11, 13-15). In addition, it has been reported that the expression of TNF-α in the lung tissues correlates with the severity of HP and recruitment of inflammatory cells into the lung (16,17). Therefore, we measured expression levels of various cytokines including TNF-α in the lungs from B6 and IL-4 KO mice during SR-induced HP using semi-quantitative real time PCR. In general, semi-quantitative real time PCR is more sensitive methodology than ELISA, and relatively well reflects amount of target proteins. Moreover, the concentration of cytokines and chemokines in BAL is relatively low during SR-induced HP. Therefore, we used semi-quantitative real time PCR instead of ELISA for measuring concentration of cytokines and chemokines. The quantitative real time PCR assays revealed that the expression levels of IFN-γ, TNF-α, and TGF-β were much higher in the lung tissues of IL-4 KO mice than those of B6 mice (Fig. 3). The administration of rhIL-4...
reduced IFN-γ, TNF-α, and TGF-β in the lung tissues of IL-4 KO mice in SR-induced HP (Fig. 3). Therefore, these findings suggest that IL-4-deficient mice produce more Th1-type and proinflammatory cytokines, resulting in aggravation of SR-induced HP.

IL-4-deficient mice increase production of chemokines in the lung tissues during SR-induced HP

It has been reported that MIP-1α and MCP-1 are related to the recruitment of macrophages and monocytes (18,19). RANTES (regulated upon activation, normal T cell expressed and secreted) released by cytotoxic T lymphocytes (CTL) is...
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DISCUSSION

In this study, we demonstrated that IL-4 KO mice showed more severe histological alteration, SR-specific immune responses and inflammatory cell infiltration in the lungs than B6 mice. These findings indicate that absence of IL-4 in vivo induce more pulmonary inflammatory responses against SR antigens. Moreover, IL-4 KO mice expressed higher levels of Th1 cytokines than B6 mice, which might contribute to shifting immune responses into Th1-type in the lung tissues. The critical roles of IL-4 during the development of HP were further substantiated by the fact that the restoration of rIL-4 attenuated HP in IL-4 KO mice. However, the absence of IL-4 in vivo did not alter the number of B cells, although it increased the levels of SR-specific IgG of BAL in SR-induced HP. These findings suggest that IL-4 might contribute to differentiation of B cells into plasma cells producing SR-specific IgG but not significantly proliferation of B cells in the lung during SR-induced HP.

Several studies indicate that the immune responses in HP are predominantly Th1-type (9-11). The expression of IFN-γ was significantly enhanced in B6 mice after SR exposure (8,13,21,22), whereas inflammatory responses to SR antigen were less severe in GATA-3-transgenic mice than control mice (23). Moreover, CD4⁺ T cells or splenocytes from B6 mice expressed less IL-4 mRNA and protein compared with DBA/2 mice, resulting in more susceptibility to HP (24). These findings suggest that regulation of Th1/Th2 balance in vivo is critical in the development and progress of HP. Moreover, IL-4 has been suggested to be a protective cytokine for HP. In this study, we provided direct evidences to support this

Figure 5. The numbers of IFN-γ-producing CD4⁺, CD8⁺ T cells, and neutrophils in the lungs are increased in IL-4 KO mice during SR-induced HP. BALF cells were taken from the lungs of B6, IL-4 KO, and IL-4 KO mice given rIL-4 7 days after the first SR antigen injection and stained for intracellular IFN-γ production. The IFN-γ expression levels were plotted on gated CD4⁺, CD8⁺, and Ly-6G⁺ cells from BALF. The numbers of left upper quadrants represent the percentages of IFN-γ-positive cells among gated cell population.
suggestion. Recently, it was reported that NKT cells attenuated SR-induced HP by producing IL-4 rather than IFN-γ (21). Moreover, Ghadirian et al., reported that administration of rIL-4 into mice partially abrogated disease process (25). These findings support our results that IL-4 plays a protective role in the development of SR-induced HP. The majority of IFN-γ producing cells in the lung were Gr-1$^{hi}$-neutrophils and T cells in the SR-induced HP model (15,21,26). Our study demonstrated that the level of IFN-γ produced by Ly-6G$^+$ granulocytes, and CD4$^+$ and CD8$^+$ T cells was higher in the lung tissues of IL-4 KO mice than B6 mice in SR-induced HP. Moreover, the restoration of rIL-4 into IL-4 KO mice reduced the level of IFN-γ in CD4$^+$ and CD8$^+$ T cells, and Ly-6G$^+$ granulocytes of BALF in SR-induced HP. Ly-6G is a specific marker for neutrophils. Therefore, it is feasible that IL-4 might suppress IFN-γ production by both T cells and neutrophils in the lung during SR-induced HP.

It is invaluable to consider therapeutic implication or clinical relevance of suppressive role of IL-4 in treating the patients with HP. To address this issue, it should be necessary which cell subsets produce IL-4 during SR-induced HP. However, it has not been extensively investigated so far. A recent study demonstrated that NKT cells have been reported to protect SR-induced HP by producing IL-4, which suppress IFN-γ -producing neutrophils rather than T cells (21). Moreover, IL-4-producing NKT cells suppressed TGF-β production in the lung during SR-induced HP, suggesting that IL-4 might exert suppressive effect on TGF-β production in the lung during SR-induced HP. Consistent with this suggestion, IL-4 KO mice showed high level of TGF-β production in the lung during SR-induced HP. Nevertheless, it remains elusive which cell subset produces IL-4 to regulate IFN-γ -producing T cells in SR-induced HP. Therefore, we propose that NKT cells may be a target cell subset for developing therapeutic approaches based on their suppressive effects on HP by producing IL-4. Meanwhile, recent our experiments suggest that CD4$^+$CD25$^+$ regulatory T cells (Treg) suppress IFN-γ production by CD4$^+$ and CD8$^+$ T cells on direct cell contact manner in SR-induced HP (unpublished data). Combined, it is feasible that in SR-induced HP, NKT cells inhibit IFN-γ -producing neutrophils by producing IL-4, whereas CD4$^+$CD25$^+$ Treg cells directly suppress IFN-γ -producing CD4$^+$ and CD8$^+$ T cells.

In SR-induced HP, MIP-1α, MCP-1, and RANTES were highly expressed in the lung tissues of IL-4 KO mice compared with B6 mice, indicating that IL-4 contribute to suppressing chemokine expression in the lungs. In consistent with these results, it has been reported that IL-4 inhibits production of MIP-1α and MCP-1 (27,28). Recent studies showed that MIP-1α and MCP-1 played critical functions in SR-induced HP development (18,19) by recruitment of inflammatory cells into the lungs. Therefore, these findings suggest that IL-4 contribute to anti-inflammatory responses in the lungs by suppressing chemokine expression during SR-induced HP.

In conclusion, these results provide direct evidences that IL-4 attenuates SR-induced HP by attenuating Th1-dominant immune responses and suppressing chemokine expression in the lungs.

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