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STAT4 Signal Pathways Regulate Inflammation and Airway Physiology Changes in Allergic Airway Inflammation Locally Via Alteration of Chemokines

Kavita Raman,* Mark H. Kaplan, † Cory M. Hogaboam,* Aaron Berlin,* and Nicholas W. Lukacs1*

Mice homozygous for the STAT4-null mutation were sensitized to cockroach Ag, challenged intratracheally 21 days later, and compared with STAT4-competent allergic mice. The STAT4−/− mice showed significant decreases in airway hyperreactivity (AHR) and peribronchial eosinophils compared with wild-type controls. In addition, pulmonary levels of chemokines were decreased in the STAT4−/− mice, including CC chemokine ligand (CCL)5, CCL6, CCL11, and CCL17. However, levels of Th2-type cytokines, such as IL-4 and IL-13, as well as serum IgE levels were similar in the two groups. Transfer of splenic lymphocytes from sensitized wild-type mice into sensitized STAT4−/− mice did not restore AHR in the mutant mice. Furthermore, chemokine production and peribronchial eosinophilia were not restored during the cellular transfer experiments. Thus, it appears that STAT4 expression contributes to a type 2 process such as allergen-induced chemokine production and AHR. In additional studies, competent allergic mice were treated with anti-IL-12 locally in the airways at the time of allergen rechallenge. These latter studies also demonstrated a decrease in AHR. Altogether, these data suggest that STAT4-mediated pathways play a role locally within the airway for the exacerbation of the allergen-induced responses.

Materials and Methods

Mice

BALB/c (wild-type (wt)) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). STAT4−/− mice were obtained and backcrossed on a BALB/c background as previously described (32).

Cockroach Ag challenge

Allergic mice were immunized with sterile clinical grade cockroach allergen (Bayer, Elkhart, IN) with no endotoxin contamination as previously described (33–35). Briefly, mice were immunized with 10 μg of cockroach allergen in IFA on day 0. On day 14, the mice were given an intranasal challenge of 10 μg of cockroach allergen in 10 μl of diluent to localize the response to the airway. This initial intranasal challenge with Ag induced little cellular infiltrate into the lungs of the mice upon histological examination. Mice were then rechallenged 6 days later by intratracheal administration of 10 μg of cockroach allergen in 50 μl of sterile PBS or with PBS alone (vehicle).

Measurement of airway hyperreactivity (AHR)

AHR in anesthetized mice was measured as previously described (33–35) with a mouse plethysmograph (Buxco, Troy, NY) using a direct ventilation method specifically designed for low tidal volumes. A single optimal methacholine dose was used, which elicited a minimal response in control BALB/c mice but gave a significant hyperreactive response in allergic mice. There was no difference in background methacholine responses in STAT4−/− vs wt BALB/c mice (data not shown).

ELISAs

Whole lungs were homogenized in 1 ml of lysis buffer (PBS with 0.01% Triton X-100 nonionic detergent) containing protease inhibitors. Debris-free supernatants were isolated and the cytokines were measured by ELISA as described (35, 36). Ab pairs from R&D Systems (Minneapolis, MN) were used for ELISAs. The sensitivity of the analyses was ~10 pg/ml. No cross-reactivity to any other chemokine or cytokine was detected in individual assays.

Cell transfer

Spleens were harvested from sensitized wt or STAT4−/− mice and teased to a single-cell suspension. After lysing the RBCs, the adherent cell populations were depleted by incubating in tissue culture dishes for 1 h at 37°C. The isolated lymphocytes (~70% CD3+ and ~26% CD19+) were then transferred into fully allergen-sensitized STAT4−/− mice (2 × 107 cells/mouse). The mice were then challenged intratracheally with allergen at 24 h post-cell transfer and then analyzed for AHR at 24 h post-allergen rechallenge.

Anti-IL-12 treatment of mice

Sensitized allergic mice were given anti-IL-12 polyclonal Ab intranasally (150 μg of purified IgG in 15 μl) within 15 min of intratracheal allergen challenge to anesthetized mice. Control mice were given appropriate control purified IgG Ab. Both the anti-IL-12 and control IgG were harvested from specific pathogen-free rabbits in the University of Michigan facility. The serum was harvested, the IgG fraction was collected over protein G columns, and the Ab was tested for endotoxin contamination. The IL-12 IgG Ab was tested for specificity by direct ELISA titters and reacted significantly only to IL-12. Treated mice were checked for AHR at ~18 h post-allergen challenge.

Real-time PCR of lungs from allergic mice for IL-12

Five micrograms of total RNA from specific samples was reverse transcribed into cDNA using a prescribed reverse transcriptase kit from PE Biosystems (Foster City, CA). Primers and probe sets for IL-12 and GADPH were developed by PE Biosystems using a patented technique for optimal and specific amplification. Briefly, during PCR, a fluorogenic probe, consisting of an oligonucleotide with both a reporter and a quencher dye attached, anneals specifically between the forward and reverse primers. When the probe is cleaved by the 5′ nuclease activity of the DNA polymerase, the reporter dye is separated from the quencher dye, and a sequence-specific signal is generated. With each cycle, additional reporter dye molecules are cleaved from their respective probes, and the fluorescence intensity is monitored during the PCR. This real-time detection gen-

2 Abbreviations used in this paper: wt, wild type; AHR, airway hyperreactivity; CCL, CC chemokine ligand.

\[ \text{Change in AHR (cmH}_2\text{O/m}\text{sec) vs time after allergen challenge} \]

FIGURE 1. Alteration of AHR in STAT4−/− mice. BALB/c mice with and without STAT4 expression were immunized and rechallenged with cockroach allergen (see Materials and Methods). Twenty-four and 48 h post-allergen challenge, the change in airway resistance was measured after an i.v. methacholine challenge (100 μg/kg). The data represent the mean ± SE from eight mice per group. *p < 0.05.
although modest, may have contributed to the decrease in peribronchial eosinophil accumulation. In this Th2 model, the IFN-γ levels are relatively low, and therefore it may not be surprising that they were not altered. In addition to the Th1/Th2 cytokine profiles, we also examined an additional aspect of allergic disease, serum IgE levels. Isotype switching to IgE in B cells is dependent upon IL-4-mediated switching, clearly a Th2-type response. The levels of IgE were not altered in the STAT4−/− mice (Fig. 4), which followed the findings in Fig. 3 of equivalent levels of IL-4 in the STAT4−/− and wt mice. Thus, the overall cytokine environment and sensitization of the animals appeared to be quite similar, both having a sufficient Th2-related response.

**Alteration of chemokines in the STAT4−/− mice**

Although the cytokine profiles and serum IgE levels appeared to be generally intact, the pathophysiology and inflammation were significantly altered. Therefore, we next examined the chemotactic mediators to determine whether altered chemokine levels could explain the reduced leukocyte recruitment and activation within the lungs of the mice. The data in Fig. 5 clearly indicates that a number of critical CC chemokines were significantly lower in the STAT4−/− animals. In particular, CC chemokine ligand (CCL)5, CCL6, CCL11, and CCL17 were all significantly reduced in the STAT4−/− animals. Thus, although the Th2 cytokine environment appeared to be largely maintained, the local production of chemokines was significantly altered, suggesting that STAT4 plays a role in the production of these chemokines.

**FIGURE 2.** Histologic examination of STAT4−/− demonstrates a reduction in peribronchial inflammation. Lungs from STAT4−/− and STAT4+/+ allergic mice were examined 48 h after allergen challenge, processed for histology, and stained with H&E. Representative airways depict decreased inflammation in the STAT4−/− mice.

**FIGURE 3.** Absence of STAT4 during allergen challenge has only a marginal effect on cytokine levels in the lungs of allergic mice. Whole lung homogenates were examined for levels of cytokines, IL-4, IL-5, IL-12, IL-13, and IFN-γ, using specific ELISAs. The data represents mean ± SE in lungs from six mice per group. *p < 0.05.

**FIGURE 4.** STAT4−/− mice have no alteration in serum IgE levels. Total serum IgE levels were compared in STAT4−/− mice and in allergic wt mice. There were negligible levels of IgE found in nonallergic wt and STAT4−/− mouse serum. Data represent the mean ± SE from six mice per group.
Transfer of STAT4-sufficient lymphocytes does not restore allergic airway responses

To further examine the possible mechanism of the response, we addressed the question of whether the STAT4−/− animals had an altered lymphocyte response that reduced the overall activation of the lung environment. Although the cytokine phenotype, Th1/Th2, appeared to be intact, it was possible that, in the absence of STAT4, there would be a lymphocyte defect. To initially examine this aspect, we used splenic lymphocyte populations from sensitized wt or STAT4−/− mice that contained both B and T lymphocytes (70% CD3+ and 26% CD19+). In this way, we would be able to examine whether reconstitution of sensitized lymphoid populations would be sufficient to regain the expected allergen-mediated response. The transfer of splenic lymphocytes (2 × 10^7 /mouse) 24 h before final rechallenge demonstrated no reconstitution of the response. Neither the AHR (Fig. 6) nor the peribronchial eosinophil accumulation (Fig. 7A) and chemokine levels (Fig. 7B) were restored with the transfer of sensitized lymphocytes from STAT4−/− animals. Transfer of lymphocytes from sensitized STAT4−/− mice into STAT4−/− mice also did not reconstitute any of the responses. In addition, studies, transfer of splenic lymphocytes from sensitized wt mice into naive wt mice induced a significant AHR response upon allergen challenge compared with that of control mice (15.3 ± 2.3 cm H_2O/ml/s vs 3.2 ± 0.8 cm H_2O/ml/s, respectively). Thus, although the transfer of lymphocytes from sensitized mice can effectively induce an allergic response, it was not sufficient to reconstitute the STAT4−/− mice. Overall, it appeared that there was a dysregulation of the local activation of chemokines.

FIGURE 5. Alteration of chemokines in STAT4+/− mice. Chemokine levels in whole lung homogenates were examined in wt and STAT4+/− allergic mice using specific ELISAs. The data represent the mean ± SE of chemokine levels from six mice per group. *p < 0.05.

FIGURE 6. Transfer of STAT4-sufficient lymphocytes does not reconstitute allergen-induced AHR responses. Splenic lymphocytes, T and B cells, were isolated from STAT4-sufficient and -deficient animals and transferred via i.v. tail vein injection (2 × 10^7 /mouse) into STAT4-deficient mice. After 24 h, the mice were challenged with cockroach allergen as previously described. The AHR responses were measured at 24 h postchallenge. The data were combined from two repeat experiments. *p < 0.05.

FIGURE 7. Transfer of STAT4-sufficient lymphocytes does not reconstitute eosinophil accumulation or chemokine levels. The association of the inflammatory response with changes in airway physiology was examined by enumeration of the peribronchial eosinophils (A) as well as by determining the level of chemokines within the lungs of the allergic animals (B), as previously described. The data were combined from two repeat experiments. *p < 0.05.

FIGURE 8. IL-12 mRNA expression in allergic mice during the allergen response assessed by real-time PCR. At various time points, whole lung mRNA was isolated from allergic mice challenged with cockroach allergen or saline. Reversed-transcribed cDNA samples were subjected to real-time PCR analysis and the experiment, and the fold-increase of cockroach-challenged animals was determined compared with controls. The data represent the mean ± SE from three to five mice per time point.
This latter view has been supported in the present studies of allergen challenge compared with that of control-treated animals. To address whether IL-12 produced during the response had any effect on the pulmonary function, we administered purified IgG polyclonal anti-IL-12 or control IgG (150 μg/mouse) was administered intranasally 15 min post-allergen challenge. The animals were examined for changes in airway physiology at 24 h post-allergen challenge. The data represent the mean ± SE from five mice per group.

Neutralization of IL-12 in allergic mice alters AHR similar to STAT4−/− mice

A number of previous studies have clearly established IL-12 as a potent inducer of Th1 responses and administration of high doses of rIL-12 (100–1000× physiologic levels) can alter allergen-induced responses. Furthermore, systemic inhibition of IL-12 appears to significantly increase the Th2-mediated responses (27). However, the role of IL-12 produced locally in an established allergic response has not been thoroughly investigated. Because the STAT4−/− mice display an altered allergic response, we hypothesized that IL-12 produced during the allergic response may contribute to the local pulmonary pathogenesis. Initially, we assessed the expression pattern of IL-12 p40 in allergen-challenged mice using quantitative real-time PCR (Fig. 8). These data demonstrated that expression was increased by >15 fold at 4 h post-challenge compared with that of control-treated animals. To address whether IL-12 produced during the response had any effect on the pulmonary function, we administered purified IgG polyclonal anti-IL-12 or control IgG by intranasal delivery (150 μg in 15 μl saline/mouse). The data in Fig. 9 demonstrate that local administration of anti-IL-12 during the allergic response significantly attenuated the AHR. The magnitude of the reduced response was not equal to that observed in the STAT4−/− mice. When local cytokine and chemokine production was assessed, there were no significant differences (data not shown). Altogether, these results indicate that IL-12 and STAT4 have a role locally in the lung during allergic airway responses.

Discussion

The activation and coordination of allergic inflammatory/immune responses depend upon a complex interaction of multiple cell populations and diverse mechanisms. According to dogma, allergic airway responses are dependent upon Th2-type cytokines, including local production of IL-4, IL-5, and IL-13 (39–41). In contrast, a number of investigations have demonstrated that addition of superphysiologic levels of exogenous Th1-associated cytokines, such as IL-12 and IFN, down-regulate the allergic response and AHR in murine models of asthmatic disease (23–26). Several studies have suggested that type 1 cytokines actually contribute to the overall allergic environment and play a significant role in the development of the detrimental responses (30, 42). In fact, a recent study demonstrated that IL-12−/− animals had reduced airway inflammation, especially eosinophils, suggesting that IL-12 may have a role during the development of allergic airway responses (30). This latter view has been supported in the present studies where the absence of STAT4, a major type 1 immune signal, significantly alters the development of an allergic airway response via dysregulation of chemokine production, airway inflammation, and attenuation of increased airway physiology. Interestingly, the IL-12 and IL-5 levels were reduced but not those of IFN-γ, IL-4, or IL-13. The reduction observed in IL-12 is consistent with previous observations in the STAT4−/− mice (32). The alteration appeared to be centered primarily upon chemokine production. The chemokines that were reduced have been shown to be regulated by STAT6 signal transduction pathways (18, 43); however, in the absence of STAT4 signals, the chemokines appeared to be reduced. Given the fact that lymphocyte transfer did not reconstitute any of the responses, one must assume that the source of the STAT4-related chemokines could be from structural cells or non-lymphoid leukocytes, such as airway epithelial cells and macrophages. Both airway epithelial cells and resident macrophages may be the most significant source of chemokines in the lungs during local responses (44). Additional studies are under way to further investigate the role of these two cell populations in the lung for their role in this STAT4-related response.

A previous study indicated that systemic neutralization of IL-12 48 h before and during allergen challenge resulted in a significant increase in the Th2-mediated OVA-induced response (27). Whereas our neutralization protocol targeted local airway IL-12 only at the time of allergen challenge, the previous study altered systemic IL-12 that includes lymph nodes and may have altered lymphocyte activation/differentiation (45–47). Furthermore, other Th2-mediated models have found that local IL-12 significantly contributes to the pathogenesis of the response possibly by altering chemokines (30, 42, 48). Interestingly, in the present studies, although anti-IL-12 delivered to the airway reduced AHR, the treatment had no significant effect on cytokine or chemokine production. The STAT4−/− mice would have a complete signal blockade, whereas anti-IL-12 would have only a partial inhibition because other cytokines, such as type I IFNs, IL-18, and IL-23, can signal via STAT4 (49–54). We have previously shown that IL-18 can locally induce CCL11, one of the chemokines reduced in the STAT4−/− mice (35). The fact that other STAT4-associated cytokines are also present during the response may help explain the anti-IL-12 results.

STAT4 has been extensively studied in T lymphocyte activation for the development of Th1-type cells related to IFN-γ production. Original studies have identified that the STAT4 protein is a major signaling molecule required for the development of Th1 responses both in vitro and in vivo through IL-12R signaling (32, 37, 47, 55). However, other cell populations have not been examined as closely for STAT4 signal transduction responses. Recent studies have indicated that macrophages can also have STAT4-mediated responses associated with activation of anti-pathogen and inflammatory responses (56, 57). Although chemokine production in lymphocytes does not appear to be dependent upon STAT4 activation (18), chemokine production may be one aspect of macrophage activation regulated by STAT4-mediated pathways. Other studies have indicated that IL-12, a specific STAT4 activator, may be an effective component in sepsis and during antifungal responses in vivo via its ability to up-regulate chemokine production (58, 59). Although STAT4 has not been reported to induce epithelial cell activation, airway epithelial cells can produce several of the chemokines that were attenuated in this model. Thus, one must consider that STAT4-mediated mechanisms may be relevant for epithelial cell-derived chemokine responses. Interestingly, IL-12 production has recently been described from airway epithelial cells (60) possibly contributing to the local activation within the airway. In addition, type I IFNs, IL-18, and IL-23 can activate STAT4 and...
may have a role within the allergic airway responses for activation of local cell populations (49–54, 61).

Previous studies on STAT4 protein responses in models of allergic inflammation have concentrated primarily on STAT6, which provides a major stimulus for not only development of a complete Th2-type response with significant eosinophilia and AHR but also mucus production within the airway (38, 62–66). As STAT6 is the primary signal induced by Th2 cytokines, these studies verified the requirement for IL-4/IL-13 receptor signaling during asthmatic-type inflammation. Other investigators have also found that constitutive STAT1 activation, normally associated with IFN-α, -β, and -γ, also appears to be related to the severity of the asthmatic response (67). This corresponds well with cell transfer experiments that failed to regulate, but rather enhanced Th2-type allergic responses when Th1-type cells were transferred into challenged animals (29). Thus, the Th2-type cytokines during asthmatic/allergic responses may not be the only cytokines needed for inflammatory responses in the development of severe airway asthma. Support of these concepts can be found during viral infections, which are the most common cause of asthma exacerbations and induce significant eosinophilia and AHR but also provides a major stimulus for not only development of a complete Th1-type T helper cells (Th1) and Th2 cells. J. Exp. Med. 202:1297.


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