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Ras Activation in T Cells Determines the Development of Antigen-Induced Airway Hyperresponsiveness and Eosinophilic Inflammation

Youichi Shibata,†‡ Tohru Kamata,* Motoko Kimura,* Masakatsu Yamashita,** Chront-Reen Wang,†** Kaoru Murata,* Masaru Miyazaki,‡ Masaru Taniguchi,*¶† Naohiro Watanabe,# and Toshinori Nakayama‡*†¶

The central role for Th2 cells in the development of Ag-induced airway hyperresponsiveness and eosinophilic inflammation is well documented. We have reported a crucial role for TCR-induced activation of the Ras/extracellular signal-regulated kinase mitogen-activated protein kinase cascade in Th2 cell differentiation. Here, we show that the development of both OVA-induced airway hyperresponsiveness and eosinophilic airway inflammation in a mouse asthma model are attenuated in transgenic mice by the overexpression of enzymatically inactive Ras molecules in T cells. In addition, reduced levels of IL-5 production and eosinophilic inflammation induced by nematode infection (Nippostrongylus brasiliensis or Heligmosomoides polygyrus) were detected. Thus, the level of Ras activation in T cells appears to determine Th2-dependent eosinophilic inflammation and Ag-induced airway hyperresponsiveness. The Journal of Immunology, 2002, 169: 2134–2140.

On the basis of their cytokine production profiles, CD4+ helper T cells can be divided into two distinct subpopulations, i.e., Th1 and Th2 cells (1). Th1 cells produce IFN-γ, and Th2 cells produce IL-4, IL-5, and IL-13. The development of Th1 and Th2 cells is a critical issue in understanding Th cell-dependent immune responses in infectious, allergic, and autoimmune diseases (2–4). Th1 cells play a central role in cellular immunity, whereas Th2 cells are crucial for humoral immunity and are involved in allergic and helminthic diseases.

The direction of differentiation toward Th1 and Th2 cells is dependent on the exogenous cytokines present during primary antigenic stimulation of naive T cells (4–6). IL-12-induced STAT4 activation promotes Th1 cell differentiation, whereas IL-4-mediated signaling, including STAT6 activation, is required for Th2 cell differentiation. Ag recognition by TCR is also indispensable for both Th1 and Th2 cell differentiation (4). Flavell and colleagues (7–9) showed that Th1 cell differentiation and Th1 cytokine production are dependent on c-Jun N-terminal kinase and the p38 mitogen-activated protein kinase (MAPK) cascade, respectively. We have reported that there is a preferential requirement for the TCR-induced activation of p56Lck, calcineurin, and the Ras/MAPK cascade in the differentiation of Th2 cells (10–12). We analyzed the efficiency of Th1/Th2 cell differentiation in naive T cells from H-ras-dominant-negative Ras (dnRas) transgenic (Tg) mice, in which enzymatically inactive Ras molecules were overexpressed, and found that TCR-induced MAPK cascade activation was strongly compromised. Severely impaired Th2 cell differentiation and increased Th1 cell differentiation were observed, whereas other functions, such as anti-TCR-induced proliferative responses and IL-2 production, were within normal ranges (12). In addition, in vivo Ag-induced Th2 responses, such as Ag-specific IgG1 and IgE production, were impaired, whereas Th1-dependent IgG2a immune responses were enhanced. However, it has not been elucidated whether IL-5 production and Th2-dependent eosinophilic inflammation are also dependent on the activation of the Ras/MAPK cascade in T cells.

Airway inflammation is a central issue in the pathogenesis of asthma. The airway of asthma patients demonstrates chronic inflammation characterized by leukocyte infiltration in the peribronchial and perivascular regions of the lung, hypersecretion of mucus, obstruction of airways, epithelial damage, and basement membrane thickening (13). Recent clinical and experimental investigations revealed crucial roles for CD4+ Th2 cells and eosinophils in allergic airway inflammation and hyperresponsiveness (14–16). Th2 cytokines, including IL-4, IL-5, and IL-13, appear to play a crucial role in the development of allergic airway inflammation (17, 18). IL-4 has been suggested to be important for the generation of allergen-specific Th2 cells during sensitization (19),

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3 Abbreviations used in this paper: MAPK, mitogen-activated protein kinase; BAL, bronchoalveolar lavage; dnRas, H-ras-dominant negative Ras; LM, littermate; Tg, transgenic; ERK, extracellular signal-regulated kinase.
whereas IL-13 is thought to be more important in the induction of airway hyperresponsiveness (20–22). In contrast, IL-5 appears to be critical for the induction of eosinophilic inflammation in bronchial tissues (23–25). However, the differential roles for these Th2 cytokines in the development of allergic asthma remain ambiguous.

Here, we used dnRas Tg mice to assess the role of Ras activation in OVA-induced allergic airway inflammation in a mouse asthma model. Our results suggest that the levels of Ras activation in T cells determine the development of Th2-dependent eosinophilic inflammation and Ag-induced airway hyperresponsiveness.

Materials and Methods

Mice

A Tg mouse with T cell-specific dnRas expression driven by the lck proximal promoter was characterized previously (12, 26). In this report, 6- to 8-wk-old heterozygous (Tg+/−) dnRas Tg mice with a (C56BL/6 (B6) × BALB/c)F1, background were used. All mice used in this study were maintained under specific pathogen-free conditions. Animal care was in accordance with the guidelines of Chiba University (Chiba, Japan).

Immunization and airway challenge with OVA

dnRas Tg mice 6–8 wk old were immunized i.p. with 100 μg of OVA (chicken egg albumin; Sigma-Aldrich, St. Louis, MO) in 1 mg of aluminum hydroxide gel (alum) on days 0 and 7. On days 14, 15, and 16, the sensitized mice inhaled aerosolized OVA for 30 min in a chamber (31 × 23 × 13 cm) connected to a nebulizer that generates a 1% w/v OVA aerosol mist. Control mice inhaled 0.9% saline.

Cytokine production in vitro

Two weeks after the last OVA or Nippostrongylus brasiliensis injection, the spleens were removed. Whole spleen cell populations were cultured for 3 days with 6.25–50 μg/ml DNP-N. brasiliensis Ag or 3–100 μg/ml OVA (27). The concentrations of cytokines (IL-4, IL-5, IL-13, and IFN-γ) in the culture supernatant were quantified by standard ELISA as described previously (28).

Collection and analysis of bronchoalveolar lavage (BAL) fluid

Two days after the last OVA inhalation, BAL was performed as described (29). Total BAL fluid was collected, and the cells in 100-μl aliquots were counted. One hundred thousand viable BAL cells were cytocentrifuged onto slides by a Cytospin 3 (Shandon, Pittsburgh, PA), and stained with May-Grünwald-Giemsa solution (Merk, Rahway, NJ) as described (24). Two hundred leukocytes were counted on each slide. Cell types were identified based on morphological criteria. The percentages and absolute numbers of each cell type were calculated.

Lung histology

Two days after the last OVA inhalation, the lungs were fixed in 4% paraformaldehyde-PBS, dehydrated in 50–100% ethanol-propanol-xylene, and embedded in paraffin. Then the samples were sectioned and stained with H&E or Luna (30) and examined for pathological changes under a light microscope at ×80 and ×160.

Measurement of airway responsiveness

Airway responsiveness was assessed by methacholine-induced airflow obstruction of conscious mice placed in a whole body plethysmograph (model PLY3211; Buxco Electronics, Troy, NY) as described (29). The respiratory parameters were obtained by exposing mice to 0.9% saline aerosol, followed by incremental doses (3–50 mg/ml) of aerosolized methacholine. Airflow obstruction was monitored and analyzed by system XA software (model SFT1610; Buxco Electronics). Results are expressed as the percentage of baseline enhanced pause values after 0.9% saline exposure.

Helminthic infection and the measurement of Th2 responses.

dnRas Tg mice were injected s.c. with 750 third-stage N. brasiliensis larvae on days 0 and 21 or inoculated orally with 300 third-stage Heligmosomoides polygyrus larvae (27, 31). To determine the number of eosinophils in the blood, mice were bled 14 days after primary and 7 days after secondary N. brasiliensis infection and 21 days after H. polygyrus infection. The number of eosinophils was counted under a microscope after staining with Hinkelman’s solution. The number of eosinophils before primary or secondary infection was <50/ml.

Statistical analysis

Student’s t test was used to evaluate the significance of the differences.

Results

OVA-induced IL-5 production is decreased in dnRas Tg mice

The goal of this study was to evaluate the roles for Ras activation in T cells in allergic airway inflammation and airway hyperresponsiveness. We initiated the analysis by assessing Ag-induced Th2 cytokine production in dnRas Tg mice, where Ras activation is inhibited specifically in T cells. Eight-week-old dnRas Tg mice with a (B6 × BALB/c)F1 background were immunized with OVA in alum. Two weeks later, spleen cells were individually prepared, and in vitro Ag-induced cytokine production was measured (Fig. 1). As can be seen, OVA Ag dosage-dependent increases in the production of Th2 cytokines (IL-4, IL-5, and IL-13) were observed in littermate (LM) mice and, as expected, significantly reduced responses in the dnRas Tg cultures were detected. In contrast, a slight enhancement of IFN-γ production was detected. The production of IL-2 appeared to be slightly decreased. These results are consistent with the previous finding that Th2 cell differentiation is impaired in dnRas Tg mice (12), as is Ag-specific IgE production. Here, Ag-induced IL-5 and IL-13 production are revealed to be attenuated in dnRas Tg mice.

Decreased OVA-induced eosinophilic infiltration in the airway of dnRas Tg mice

Two weeks after OVA immunization, dnRas Tg mice were exposed to inhaled OVA for 3 consecutive days, and a further 2 days later BAL fluid was examined for eosinophilic infiltration. As can be seen in Fig. 2A, the majority of infiltrated cells were eosinophils.
in LM mice. The percentages and absolute numbers of eosinophils, macrophages, and neutrophils were calculated as described in Materials and Methods. Fig. 2B shows significantly decreased frequencies of eosinophils and increased frequencies of macrophages. As shown in Fig. 2C, a substantial reduction in the absolute number of eosinophils was revealed. The number of leukocytes in the BAL fluid was significantly reduced in dnRas Tg mice (see total). According to morphological criteria, the number of lymphocytes was negligible in the cells designated as “others.” They appeared to be blast cells (data not shown). We also measured cytokines (IL-2, IL-4, IFN-γ, and IL-5) in the BAL fluid. Under our conditions, only IL-5 was detectable by ELISA, and slightly decreased concentrations were consistently detected in the dnRas Tg groups (Fig. 2D).

Concurrently, changes in the lung were evaluated histologically by H&E staining (Fig. 3). Leukocyte infiltration in peribronchial regions was significantly milder in dnRas Tg mice than in control mice (Fig. 3A, compare a and b). Bronchiolar mucus hyperproduction and airway obstruction were often seen in littermate controls (Fig. 3Aa). A summary of infiltrated leukocyte numbers is shown in Fig. 3B, where a significant reduction was noted. Simultaneously, the sections were stained with Luna, and perivascular and peribronchial regions were assessed for eosinophil infiltration (Fig. 3C). Although some perivascular and peribronchial regions showed the presence of significant numbers of leukocytes in dnRas Tg lung (Fig. 3C, c and d), the number of eosinophils was clearly fewer in dnRas Tg mice than in LM controls. Taken together, these results suggest that OVA-induced IL-5 and eosinophilic inflammation in the lung is dependent on the levels of Ras activation in T cells.

Reduced airway hyperresponsiveness to methacholine in dnRas Tg mice

To assess the levels of airway hyperresponsiveness in OVA-sensitized mice, dnRas Tg mice were immunized and exposed to inhaled OVA. Then airway hyperresponsiveness was assessed by measuring methacholine-induced airflow obstruction in a whole body plethysmograph. As shown in Fig. 4A, airway hyperresponsiveness was diminished in the dnRas Tg mice. The levels of airway hyperresponsiveness in nonsensitized dnRas Tg mice were similarly assessed and did not reveal a significant difference between normal and dnRas Tg mice (Fig. 4B), suggesting that the baseline level of airway hyperresponsiveness was not altered in dnRas Tg mice. Thus, the Ras activation level was found to control the development of airway hyperresponsiveness in a mouse OVA-induced asthma model.

Eosinophilia induced by nematode infection is less severe in dnRas Tg mice

Finally, we assessed the role of Ras activation in eosinophilia induced by nematode infection. Eight-week-old dnRas Tg mice were infected with N. brasiliensis or H. polygyrus, both of which are known to induce eosinophilic inflammation in experimental mice (32, 33). The number of eosinophils in the peripheral blood of uninfected normal mice was lower than 50/mm³ (data not shown). As shown in Fig. 5A, the number of eosinophils increased greatly after primary or secondary infection with N. brasiliensis in wild-type LM (B6 × BALB/c)F₁ mice. The levels of eosinophilia were significantly lower in the dnRas Tg groups. In H. polygyrus infection, the numbers of eosinophils in the blood of dnRas Tg mice

![Image](http://www.jimmunol.org/Downloadedfrom/5620.png)
were reduced to about one-half to one-third of those in LM controls (experiment 1 or 2). Concurrently, we prepared spleen cells from *N. brasiliensis*-infected animals, and *N. brasiliensis* Ag-induced cytokine production in vitro was assessed. As can be seen in Fig. 5B, the production of IL-5 and IL-13 was significantly reduced in dnRas Tg cultures. A slight increase in the production of IFN-γ was observed. The decrease in the production of IL-4 in dnRas groups was not as prominent (Fig. 5B, IL-4 panel). The reason for this apparent disparity from the OVA challenge data (see earlier) is not known, but the *N. brasiliensis* Ag-specific response may have been masked by a high background level of IL-4 production (50 pg/ml) by spleen cells from *N. brasiliensis*-infected mice (see Fig. 5B, IL-4 panel, extreme left). Only at the highest in vitro DNP-*N. brasiliensis* Ag dose of 50 μg/ml does the difference in IL-4 production become evident where the highest level of IL-4 detected reached ~150 pg/ml. In any event, IL-5 production and eosinophilia induced by nematode infection also appear to be dependent on the levels of activation of Ras in T cells.

**Discussion**

Clinical and biological manifestations of bronchial asthma are heterogeneous, reflecting a multitude of causative factors and the presence of complex pathophysiological mechanisms. Murine
models of allergic lung diseases have been used to dissect the underlying complex pathogenesis in asthma (15). In this report, we used the OVA-induced allergic asthma model to demonstrate that the development of both Ag-induced eosinophilic airway inflammation and airway hyperresponsiveness depend largely on the levels of Ras activation in T cells. We reported previously that Th2 cell differentiation is impaired in dnRas Tg mice (12). In the present study, the decreased production of Th2 cytokines (IL-4, IL-5, and IL-13) in Ag-primed dnRas Tg T cells is confirmed (Fig. 1). Thus, it is conceivable that the block in the development of eosinophilic airway inflammation and airway hyperresponsiveness is a consequence of the reduced generation of Ag-specific Th2 cells during OVA sensitization. Undoubtedly, however, there are effects of dnRas downstream of Th2 priming.

IL-5 is critical for the development of eosinophilic inflammation (34). Airway eosinophilic inflammation and airway hyperresponsiveness have been demonstrated to be reduced by anti-IL-5 Ab treatment and IL-5 gene deletion in mice (24, 25). Furthermore, ectopic Tg expression of IL-5 in the lung epithelium results in eosinophilic invasion of airways comparable with that seen in asthmatic patients (35). Interestingly, the IL-5 Tg mice displayed airway hyperresponsiveness in the absence of Ag-induced inflammation. Thus, IL-5 appears to be most critical for the development of airway eosinophilic inflammation and also the development of airway hyperresponsiveness. In dnRas Tg mice, significantly reduced IL-5 levels were seen (Fig. 1). Because the production of IL-4 and IL-13 was also reduced in dnRas Tg mice, it is not so easy to estimate the contribution of the IL-5 deficit to the attenuated airway inflammation. However, it is now clear that the activation of Ras is involved in the pathogenesis of both eosinophilic inflammation and airway hyperresponsiveness.

Our previous results indicate that the efficiency of Th2 cell differentiation depends on the activation level of the Ras/extracellular signal-regulated kinase (ERK) MAPK cascade (12). Consistent with this observation, the production of Th2 cytokines (IL-4, IL-5, and IL-13) was impaired in dnRas Tg T cells (Fig. 1). In naive CD4 T cells, stimulation with a very large amount of IL-4 (100 U/ml) did not induce detectable levels of ERK MAPK cascade activation (our unpublished observations). In contrast, TCR stimulation induced a substantial increase in the level of ERK phosphorylation (12). These results suggest that activation of the Ras/ERK MAPK cascade in naive CD4 T cells is a consequence of TCR-mediated signaling. The downstream functional target molecules of ERK-MAPK in Th2 cell differentiation are not clarified very well at this time. One candidate is the IL-4R signaling complex (12). IL-4-induced tyrosine phosphorylation of Jak1 and

![FIGURE 5](http://www.jimmunol.org/Downloadedfrom/)

The levels of eosinophilia and Th2 cytokine response in dnRas Tg mice infected with nematodes. Eight-week-old dnRas Tg mice were injected s.c. with 750 third-stage *N. brasiliensis* (Nb) larvae on days 0 and 21 or inoculated orally with 300 third-stage *Heligmosomoides polygyrus* larvae. A. The numbers of eosinophils 14 days after primary and 7 days after secondary *N. brasiliensis* infection were determined (left). For *H. polygyrus* infection, two representative results (Exp. 1 and Exp. 2) are shown. Values are means ± SD for five mice in each group. Values of *p* were determined by Student’s *t* test. Values of *p* in A: after primary infection, 0.017; after secondary infection, 0.027. Values of *p* in B: Exp. 1, 0.05; Exp. 2, 0.0023. B. dnRas Tg mice were immunized with *N. brasiliensis* as in A. Two weeks after the last immunization, whole spleen cells (1 × 10^6^) were prepared and cultured with the indicated concentrations of DNP-*N. brasiliensis* Ag. Values are means ± SD of cytokine production in the culture supernatant determined by ELISA. Three independent experiments were done with similar results. □, LM mice; ■, dnRas Tg mice. Values of *p* were determined by Student’s *t* test and were as follows: IL-4, 0 μg/ml: 0.5, < 0.001, 6.25 μg/ml: 0.5, < 0.001, 12.5 μg/ml: 0.5, < 0.001, 25 μg/ml: 0.5, < 0.001, 50 μg/ml: 0.004. IL-5, 6.25 μg/ml: 0.001, 12.5 μg/ml: < 0.0001, 25 μg/ml: < 0.0001, 50 μg/ml: 0.05. IL-13, 6.25 μg/ml: 0.04, 12.5 μg/ml: 0.005, 25 μg/ml: < 0.001, 50 μg/ml: 0.5, < 0.001. IFN-γ, 0 μg/ml: 0.03, 6.25 μg/ml: 0.33, 12.5 μg/ml: 0.22, 25 μg/ml: 0.003, 50 μg/ml: 0.01.
NF-κB and its role in asthma. NF-κB is a key transcription factor that regulates the expression of genes involved in inflammation and immune responses. It plays a critical role in the activation of Th2 cells, which are involved in the allergic response in asthma.

The activation of NF-κB in Th2 cells is mediated by the Ras-Raf/ERK pathway, which controls the development of Th2-dependent eosinophilic inflammation. Thus, NF-κB regulation is a promising target for the development of new therapeutic strategies for asthma.

The accumulated results implicating the involvement of the activation of Th2 cells in the development of asthma support several new strategies that could attenuate Th2-induced airway inflammation in the asthmatic airway. Most of the strategies aim to block Th2 cell differentiation and the production of Th2 cytokines (37, 38). One of the potential strategies is to develop drugs that block the signal transduction pathway required for Th2 cell differentiation. Inhibition of the Ras pathway, as shown in the present study, could alter the cytokine pattern by suppressing IL-4, IL-5 and IL-13 and enhancing IFN-γ production. In fact, a mitogen-activated protein/extracellular signal-related kinase inhibitor, U0126, has been shown to reduce IL-13-induced cell stiffness in cultured human airway smooth muscle cells, a model of airway narrowing observed in patients with asthma (39). In addition, several other independent pathways downstream of Ras have been reported and appear to mediate the distinct biological functions of Ras. The activation of Ras results in the phosphorylation of p70S6 kinases (40). Rapamycin, a p70S6 kinase inhibitor, has been reported to inhibit the proliferation of T lymphocytes from patients with chronic asthma, and rapamycin has been suggested to be useful in clinical trials for asthmatic patients given their modes of action in T cells (41). Thus, specific blockade of the Ras-Raf/ERK MAPK pathway may have therapeutic value in asthma.

In conclusion, a mouse allergic asthma model, the activation of Ras in T cells controls the development of Th2-dependent eosinophilic airway inflammation and airway hyperresponsiveness. Thus, a search for specific inhibitors focusing on Ras-mediated signaling pathways would be helpful for establishing a new approach to the treatment of inflammatory diseases such as bronchial asthma.

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