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Induction of Pulmonary Allergic Responses by Antigen-Specific Th2 Cells

Xiu-Min Li,²* Brian H. Schofield,† Qian-Fei Wang,* Kawn-Hyoung Kim,* and Shau-Ku Huang*

The development of pulmonary allergic responses was examined in mice following pulmonary transfer of Ag (conalbumin)-specific Th2 cells. The levels of serum-specific IgE, cellular infiltrates, airway mucus goblet cells, and airway responsiveness were analyzed and compared with those in Ag-sensitized and -challenged mice. Pulmonary transfer of the conalbumin-specific Th2 clone (D10) induced, in an Ag-specific manner, high levels of the Th2 cytokines IL-4 and IL-5 in the bronchoalveolar lavage fluids and mucosal eosinophils, concomitant with an increase in airway responsiveness. The D10 cell-induced responses were seen in the absence of serum specific IgE. In the presence of Ag, the transferred D10 cells not only remained in the lungs, but also increased in number 72 h post-cell transfer. Although significantly higher levels of IL-4 and IL-5 in the bronchoalveolar lavage fluids were found in D10-transferred mice, the levels of pulmonary eosinophilia, mucus goblet cells, and airway responsiveness were significantly lower than those in Ag-sensitized and -challenged mice. These results demonstrate that although Ag-specific activation of Th2 cells at mucosal sites is able to mediate the recruitment of eosinophils and the subsequent induction of airway hyper-responsiveness, the more severe pulmonary allergic responses were observed only in mice sensitized and challenged with Ag. The Journal of Immunology, 1998, 160: 1378–1384.

A g-induced IgE and inflammatory responses have been implicated in the pathogenesis of a variety of allergic diseases, including asthma (for review, see Refs. 1 and 2). Human atopic subjects, when exposed to an appropriate Ag, suffer an acute IgE-dependent response, often followed by an inflammatory late phase response (LPR)³ 6 to 12 h later (3, 4). It has been postulated that the expression of an LPR is associated with the release of cytokines and mediators from a variety of cell types that are attracted to the site as a result of an immediate IgE-dependent inflammatory response (4, 5). Recently, a considerable body of evidence suggests that activation of T cells and eosinophilic infiltration of the airways are important in the pathophysiology of allergic asthma (6–9). Activated CD4⁺ T cells with predominant Th2-type cytokine (IL-4 and IL-5) expression are present in both the bronchoalveolar lavage fluids (BALFs) and lung biopsies of atopic asthmatic patients (6–8). It has been demonstrated that the cell sources for Th2 cytokines are multiple. In addition to T cells, basophils, mast cells, and eosinophils may be sources of Th2 cytokines in the lung (10–12). The expression of these cytokines could represent activation of T cells, mast cells, and eosinophils, but the relative contribution of each cell type to allergic inflammation and its relationship to the IgE-dependent activation event in allergic asthma remain to be defined.

Similar features of pulmonary allergic inflammation have also been documented in various murine models (13–18). These studies have provided important information regarding the role of cytokines in the regulation of allergic inflammation. For example, it has recently been shown that following challenge of mice with Ag, there is a significant increase in both IL-4 and IL-5, but not IFN-γ, expression in the Ag-challenged lung, which is associated with a significant enhancement of eosinophilia and airway hyper-responsiveness (AHR) (18, 19). Gavett et al. (20) have recently shown that the eosinophil infiltration, induction of airway mucus goblet cells, and AHR are dependent on the expression of IL-4. Temann et al. (21) have also demonstrated that mucus cells and eosinophils are present in the airways of IL-4-transgenic mice, suggesting that excess production of mucus is also mediated by IL-4. The importance of CD4⁺ T cells in the development of Ag-induced pulmonary eosinophilia and AHR was demonstrated by in vivo depletion of murine CD4⁺ T cells (22) and by adoptive transfer of allergic responses with Ag-primed CD4⁺ T cells in a rat model (23). Furthermore, Kaminuma et al. (24) reported that transfer of IL-5-producing Th2 cell clones induces a late phase eosinophilic infiltration in the mouse lung. Although these studies are informative, the relative contribution of T cells and cytokines to the induction of airway inflammatory responses is still unclear. While it has been demonstrated that AHR can be transferred by allergen-specific IgE and IgG1 (25), and that mast cell activation can enhance airway responsiveness in mice (26), airway allergic responses can occur in IgE-deficient as well as in mast cell-deficient mice (27, 28). These latter results suggest that an IgE-independent pathway for airway allergic responses may exist in mice.

We hypothesized that Th2 cells are able to directly induce late allergic airway responses similar to those seen in Ag-sensitized and -challenged mice. In this study we performed pulmonary cell transfer experiments using an Ag-specific Th2 clone (D10), and the D10 cell-induced allergic responses, following activation in...
situ, were analyzed and compared with those of Ag-sensitized and -challenged mice.

Materials and Methods

Mice, immunization, and analysis of airway response

Male AKR/J mice (6–8 wk old; The Jackson Laboratory, Bar Harbor, ME) were sensitized i.p. with 100 μg of conalbumin (CA; Sigma Chemical Co., St. Louis, MO) absorbed in 2 ng of alum in 0.4 ml of PBS on days 0 and 7. Sham-immunized mice received two injections of either PBS or alum alone. Seven days after the second sensitization, the mice were anesthe-
tized and challenged twice (1 wk apart) intratracheally with 100 μg of CA or an irrelevant Ag, short ragweed (RW; 100 μg/mouse), in 0.05 ml of PBS. Three days after the last challenge, airway responsiveness was measured as previously described (19, 29). Airway reactivity was estimated as the peak airway pressure in peak airway pressure measurement, referred to the time-integrated change in peak airway pressure index (centimeters of H2O per second). Following the measurement of airway responsiveness, lungs were lavaged once with 1 ml of HBSS, and the BALF cell differential counts and percentages were determined by Diff-Quik (Baxter, McGaw Park, IL) staining of cytospin slides. Five hundred cells per slide were enumerated. Aliquots of BALF were stored for the cytokine measurement. For histology, the lavaged lungs of mice from various groups were fixed in 10% buffered formaldehyde, embedded in paraffin, and sectioned. Three- to five-micron sections were stained with hematoxylin and eosin, periodic acid-Schiff’s reagent (PAS), and Alcian blue.

Cell transfer

An Ag-specific T cell clone, D10.G4.1 (D10; purchased from American Type Culture Collection, Rockville, MD), is a classic CA-specific Th2 clone derived from AKR/J mice (30). D10 cells were stimulated periodically with CA (100 μg/ml) in the presence of irradiated AKR spleen cells as APCs. Before the cell transfer, D10 cells were kept in culture for 8 days after stimulation, and the viable cells were isolated and suspended in medium containing Ag (CA, 4 mg/ml), an irrelevant Ag (RW, 4 mg/ml) or PBS. In addition, spleen cells from naive AKR mice were suspended in PBS containing Ag (CA, 4 mg/ml). Aliquots of the suspensions (5 × 106 cells in 0.05 ml) from various conditions were transferred intratracheally into the mouse lungs. Other control mice received Ag alone or PBS, or remained untreated. At various time points after cell transfer, airway responsiveness and BAL cell differential counts were determined as described above.

Measurement of Ag-specific IgE and BALF cytokines

Blood was obtained via the vena cava immediately following the airway responsiveness measurement and before lavage. Levels of CA-specific IgE were measured by ELISA. Immulon II round-bottom plates (Dynatech Laboratories, Inc., Chantilly, VA) were coated with 25 μg/ml CA in PBS and incubated overnight at 4°C. Plates were washed three times with PBS/0.05% Tween-20 and blocked with 1% BSA-PBS for 1 h at 37°C. After three washings, serum samples were diluted fivefold in 1% BSA-PBS and incubated overnight at 4°C. Plates were then washed three times, and 100 μl of goat anti-mouse IgE Ab (0.3 μg/ml; Sigma Chemical Co.) was added to the wells for an additional 2-h incubation at 37°C. After three washings, 100 μl of donkey anti-goat IgG Ab conjugated with peroxidase (0.3 μg/ml) was added for an additional 1-h incubation at 37°C. The reaction was developed with 3,3′,5,5′-tetramethylbenzidine (Bio-Rad Laboratories, Hercules, CA) for 30 min at room temperature, stopped with 1 N H2SO4, and read at 450 nm. The level of IgE was calculated by comparison with a reference curve generated by using a mouse mAb, anti-DNP IgE (Sigma Chemical Co.). Briefly, DNP-conjugated BSA (DNP-BSA) was coated at the same concentration as CA. After overnight incubation at 4°C, the plates were washed and blocked as described above. Ten serial 1/2 dilutions of mouse anti-DNP IgE starting from 1 ng/ml were added. Quantitation of cytokine proteins, IL-4, IL-5, and IFN-γ was determined by ELISA according to the manufacturer’s instructions (PharMingen, San Diego, CA) and as described previously (29).

Monitoring of transferred Th2 cell in vivo

The polycationic molecule, 4,6-diamidino-2-phenylindole (DAPI; Sigma Chemical Co.) was used to label the D10 cells. DAPI is a fluorescent tag that strongly binds to adenosine-thymidine-rich regions of nuclear DNA through electrostatic interactions. It has been used to stain both intact and live cells (31). An initial in vitro study demonstrated that DAPI-labeled D10 cells proliferated at the same rate as nonlabeled D10 cells (data not shown), suggesting that DAPI is nontoxic to the T cells. Briefly, D10 cells were maintained using the protocol described above. Viable cells were isolated 7 days after the last stimulation and cultured at a density of 1 × 106 in RPMI 1640 complete culture medium containing 10 μg/ml of DAPI overnight. This time period was determined by our previous kinetic study, which was optimized so that 100% of the cells were stained brightly. DAPI-stained D10 cells were then harvested, washed twice with PBS, and resuspended in PBS containing CA. Trypan blue exclusion showed that >99% of the DAPI-stained cells were viable. Cells (5 × 106) in 0.05 ml of PBS containing 200 μg of CA were transferred into naive mouse lungs by intratracheal instillation. DAPI-stained D10 cells in PBS without specific Ag were also transferred.

To evaluate the location of labeled D10 cells within the lungs and to determine whether the transferred cells migrated to extrapulmonary lymphoid tissues, lungs, spleens, brachial, axillary, and inguinal lymph nodes were fixed in 10% formalin, and 10-μm frozen sections were examined by fluorescence microscopy. Samples from naive animals were also examined. To determine the numbers of transferred D10 cells within the lung at various time points after transfer, lung cells were isolated as described by Augustin et al. (32) with slight modifications. The lungs were perfused via the right atrium with PBS, minced, and stirred in 0.5 mM EDTA in PBS at 4°C for 40 min. The cells were then pelleted and resuspended in RPMI medium. Aliquots of each cell suspension from lung isolates were placed in a hemocytometer and examined by fluorescence microscopy. Positive isolates and total cell numbers were counted in at least four aliquots of each sample. Results were expressed as the average of four counts of each sample. Isolates of spleens and lymph nodes from the same animals were counted in the same manner.

Statistical analysis

Differences in inflammatory cell number and airway responsiveness among groups of mice were determined using analysis of variance (StatView, Brain Power, Inc., Calabasas, CA). When differences among groups were significant (p < 0.05), Fisher’s protected least significant difference test was used to distinguish between pairs of groups.

Results

Induction of pulmonary eosinophilic inflammation and AHR by Ag sensitization and challenge

We first demonstrated that intratracheal challenge of sensitized mice with Ag (CA) produces significant increases in the total numbers of BALF cells and in the percentage of eosinophils (on the average, 73% of the BALF cells; Fig. 1A, group CA/CA). In contrast, in mice challenged with an irrelevant Ag (Fig. 1A, group CA/RW), there was a significant increase in neutrophils in BALFs. These findings demonstrate that the eosinophil infiltration is an Ag-specific event. Airway reactivity was also determined by measuring airway pressure changes following i.v. injection of mice with acetylcholine (Ach). A significant increase in the airway response to Ach was observed in mice sensitized and challenged with CA (Fig. 1B, group CA/CA), which was associated with the increase in eosinophils in the BALF. In contrast, when sensitized mice were challenged with RW, there was no increase in airway responsiveness (Fig. 1B, group CA/RW) even though a significant neutrophil influx was observed.

Increased levels of serum Ag-specific IgE and BALF cytokines following Ag sensitization and challenge

Significant levels of serum CA-specific IgE were found in all CA-sensitized mice. No CA-specific IgE was detected in sera from unsensitized or sham-sensitized mice at any time point. There was no significant difference in CA-specific IgE levels between Ag- and sham-challenged groups (Fig. 2A). The levels of both IL-4 and IL-5, but not IFN-γ, expression were also significantly increased at 24 h postchallenge in the BALFs from Ag-challenged lungs, but declined markedly 48 h after challenge (Fig. 2B). No IL-4 or IL-5 was detected in samples challenged with saline or RW at any time point. These results are consistent with previous findings that the expression of Th2 cytokines and an elevated level of serum Ag-specific IgE are associated with airway eosinophilic inflammation.
and AHR in Ag-sensitized and -challenged mice. It is not clear, however, to what extent the Th2 cells and cytokines contribute to the process of eosinophil influx and AHR. To provide direct evidence for the role of Th2 cells in the development of pulmonary allergic response, we performed a series of pulmonary adoptive transfer experiments using a well-characterized CA-specific Th2 clone (D10). The D10 cell is a classic Th2 clone generated from AKR mice, providing an ideal cytokine delivery vehicle following activation in situ.

Induction of airway allergic responses by pulmonary D10 cell transfer

To examine the degree of pulmonary eosinophilia and hyper-reactivity, D10 cells (5 × 10^6 cells/mouse) were mixed with or without CA (200 μg/mouse) or RW (200 μg/mouse) and transferred intratracheally into naive AKR mice. At 24, 48, and 72 h after cell transfer, airway reactivity to Ach was examined, and BALF differential cell counts were performed. Results showed that after transfer of D10 + CA, only a few eosinophils (<3%) were found in the BALF at 12 h. A significant increase in BALF eosinophils was observed at 24 h (Fig. 3A), peaked at 48 h, and decreased slightly at 72 h. A significant increase in AHR was observed at 48 and 72 h, and peaked at 48 h (Fig. 3B). However, pulmonary adoptive transfer of <5 × 10^5 D10 cells did not induce significant eosinophil infiltration (<5%).

Like Ag-sensitized and -challenged mice, increased BALF eosinophils and airway reactivity were Ag specific, since mice that received D10 cells and an irrelevant Ag (RW; Fig. 3A, group D10 + RW; Fig. 3B, group D10 + RW) showed no increase in eosinophils or AHR, but, rather, showed a predominant neutrophil influx at 48 h, which decreased by twofold at 72 h after cell transfer. The initial neutrophil influx may be a nonspecific response to foreign materials, since neutrophils were present at the early time period in BALF of naive mice treated with Ag only or with D10 cells only, but not int those treated with PBS. In addition, all control groups except the D10 + RW group showed no significant changes in the cell differential counts, and all control groups showed no significant changes with time in the level of airway responsiveness.

Local activation of the transferred D10 cells was evident by the finding of a significant increase in both IL-4 and IL-5 (peak at 24 h), but not IFN-γ, secretion in the BALFs at all three time points following pulmonary transfer of mice with D10 cells and CA (Fig. 4A). The levels of Th2 cytokines, particularly IL-5, were significantly higher in D10-transferred mice than in Ag-sensitized and -challenged mice (p < 0.001, for differences in the levels of both cytokines; see also Fig. 2B, group CA/CA (24 h)). Also of significance is the finding that no serum CA-specific IgE was detected in mice transferred with D10 and CA compared with that in Ag-sensitized and -challenged mice (Fig. 4B). The decreased levels of cytokines at 72 h is probably due to the decrease or cessation of T cell secretion or to the consumption by other cells, such as macrophages and eosinophils, in vivo. These results demonstrate that following Ag-specific activation of D10 cells at the mucosal sites, there is an induction of Th2 cytokines and eosinophil infiltration that is associated with an increase in airway responses. However, the degrees of BALF eosinophilia and AHR were significantly lower than those in Ag-sensitized and challenged mice (p < 0.001 and p < 0.01, respectively).
Localization of DAPI-labeled D10 cells in the lung

To localize the transferred T cells in situ, resting D10 cells were labeled in vitro with DAPI, a fluorescence tag (see Materials and Methods). DAPI-labeled D10 cells were easily identified by fluorescence microscopy at 6, 24, 48, and 72 h following transfer by means of their intense blue color. Endogenous cells and nonlabeled D10 cells exhibited no blue autofluorescence. Fluorescence microscopy demonstrated that 6 h following intratracheal instillation, the majority of D10 cells in the lung were localized in the vicinity of the terminal airways and appeared to be in contact with alveolar and bronchiolar epithelium (Fig. 5A); a small number of D10 cells appeared to have migrated into the terminal bronchioles and alveolar walls. By 72 h, D10 cells had migrated into bronchi and bronchioles, and some of these cells were present in the airway epithelium (Fig. 5B). A small number of the D10 cells was also present within the alveolar walls. Very few D10 cells were found in either spleen or lymph node sections at any time point (one to two cells per section) or in spleen or lymph node isolates. The latter finding was not unexpected, since fully activated T cells recirculate poorly (33). Quantitative analysis of DAPI-labeled D10 cells transferred with Ag showed that the number of D10 cells in whole lung isolates was increased 2.5-fold at 24 h, 3.5-fold at 48 h, and 4.2-fold at 72 h compared with that at 6 h (Fig. 5C). In contrast, there was no increase in the number of D10 cells in the...
absence of Ag at any time point (data not shown). These results showed that in the presence of Ag, transferred D10 cells were able to proliferate in the lung.

**Histologic analysis of airway allergic responses in the lungs**

Histologic examinations of the lungs revealed distinct differences between different groups of mice. Lungs from sham-challenged mice contained few, if any, inflammatory cells (Fig. 6A), and no PAS- or alcian blue-positive mucus cells were present in the bronchial epithelium (Fig. 6E). CA-sensitized, RW (an irrelevant Ag)-challenged lungs, on the other hand, showed a predominantly neutrophil infiltrate in the perivascular and peribronchial regions (Fig. 6B), but no PAS- or alcian blue-positive bronchial epithelial mucus cells. In contrast, CA-sensitized, and -challenged mice exhibited perivascular and peribronchial eosinophil infiltration. Numerous eosinophils were also observed adhering to the endothelium of intrapulmonary veins. Eosinophils were present in the smooth muscle layer of the larger bronchi (Fig. 6C), and numerous PAS- and alcian blue-positive mucus cells were present in the bronchial epithelium (Fig. 6F). A similar histologic pattern was observed in D10-cell transferred mice (Fig. 6D). Numerous perivascular and peribronchial eosinophils were present, some of which were located within the airway smooth muscle layer. However, while significantly higher levels of Th2 cytokines, including IL-4, were found in D10-transferred mice (see Fig. 5A), much fewer mucus-containing airway epithelial cells were observed in D10-transferred mice than in CA-sensitized and -challenged mice (Fig. 6G). In addition, no mast cells were present in either bronchial or alveolar tissues of Ag-sensitized and -challenged mice or D10 cell-transferred mice.

**Discussion**

To provide direct evidence for the role of Th2 cells and cytokines in the development of pulmonary allergic responses, we demonstrated that transfer of Th2 cells (D10) into naive mouse lungs in the presence of Ag resulted in significant induction of IL-4 and IL-5 cytokines and a predominant eosinophil influx, which was associated with an increase in AHR. Although less pronounced, the Th2 cell-induced responses resemble those in mice sensitized and challenged with Ag, but are IgE independent. It is known that in addition to Th2 cells, IL-4 and IL-5 are secreted by other cell types, such as mast cells and eosinophils (10–12). It is unlikely that either mast cells or eosinophils contribute to the IL-4 or IL-5 secretion in this experimental model, because extensive histologic examinations of the lungs from D10-transferred mice revealed that mast cells are absent, the D10 cell-induced pathologic changes were observed in the absence of specific IgE, and the number of eosinophils peak 48 h post-cell transfer, at which time the levels of IL-4 and IL-5 were dramatically decreased.

To monitor the trafficking of transferred T cells, we labeled D10 cells with DAPI before the cell transfer. As a vital stain, DAPI is relatively nontoxic and allows identification of labeled cells in vivo. In this study we demonstrated that in the presence of Ag, intratracheal transferred DAPI-labeled D10 cells not only remain in the lung for at least 72 h, but also increase in number. This finding demonstrates that the elevation of Th2 cytokines (IL-4 and IL-5) in the BALFs results from the activation of transferred D10 cells in vivo. Although the type of APCs in the lungs of these mice is presently unknown, resident macrophages and airway epithelial cells may serve as APCs when D10 cells and CA were introduced via the airways. Pulmonary transfer of T cells into the lung may thus provide a useful model to examine the detailed mechanisms of T cell- and cytokine-mediated airway inflammation and AHR. Intravenous injections of D10 cells combined with intratracheal Ag challenge did not induce either marked eosinophil infiltration or AHR (data not shown). It is likely that the transferred D10 cells do not remain in the lung long enough, or an insufficient number of cells respond to intratracheally administered Ag, since only 10% of i.v. transferred DAPI-labeled D10 cells were present in the lung at 24 h post-cell transfer.

IL-5 is an important proinflammatory cytokine controlling the proliferation, migration, and activation of eosinophils (34, 35). IL-4 is not only critical in the regulation of IgE synthesis (36), but may account, at least in part, for selective eosinophil recruitment to sites of allergic reactions (37) and induction of bronchial mucus cells (20, 21, 38). IL-4 is also important in the differentiation of...
Th2 cells, both in vitro and in vivo (39, 40). Studies in mice have shown that IL-4-deficient mice do not develop eosinophilia or AHR when sensitized and challenged with Ag (41). In addition, blockade of the IL-4R suppressed lung eosinophilia, AHR, and increased numbers of mucus goblet cell (20). Kaminuma et al. (23), however, reported that an IL-5-producing T cell clone, but not an IL-4-producing T cell clone, induces BAL eosinophils. Airway mucus cells were not examined. Studies of cytokine gene knockout mice have found that both IL-5-dependent and -independent inflammatory responses are associated with AHR (42, 43). Some of these seemingly contradictory results may be due to differences between the strain and genetic background of the mice used (44). Our study demonstrated that although the levels of BALF IL-4 and IL-5, in particular IL-5, are significantly higher in D10-transferred mice than those in Ag-sensitized and -challenged mice, the levels of BALF eosinophils, airway mucus cells, and AHR are significantly lower. These findings suggest that there is no quantitative association between the levels of Th2 cytokines and the degree of airway allergic responses. Additional studies are needed to determine the relative contributions of IL-4 and IL-5, and other cytokines to the development of pulmonary allergic responses. It is of interest to note, however, that mucosal IFN-γ gene transfer inhibits both Th2 cell- and Ag-induced eosinophil infiltration and the development of AHR (29).

The role of IgE in mast cell degranulation and mediator release in immediate allergic responses has been well established. However, the role of IgE in the late allergic inflammatory responses remains controversial (1). Several investigators have demonstrated that the development of a late asthmatic response is related to the increased level of allergen-specific serum IgE (45, 46), and that there is a significant correlation between the level of serum-specific IgE and airway responses in adults with asthma (47, 48). These observations are of particular relevance to the findings that mast cells, basophils, and tissue macrophages secrete cytokines following activation through their respective IgE receptors (1, 10, 11). However, it has been demonstrated that mucus hypersecretion is not related to serum IgE levels in adults with bronchial asthma (49). Pulmonary allergic responses in mice occur in the absence of detectable IgE (27) and mast cells (28).

Our studies of Ag- and Th2 cell-induced airway responses demonstrated significant features similar to those observed in the Ag-challenged lung LPR of atopic asthmatic patients (3, 4). The Th2-induced responses in naive mice showed, however, a lesser degree and extent of allergic responses compared with those in mice sensitized and challenged with Ag. The comparative study of Ag- and Th2 cell-induced airway responses demonstrated that neither the degree of eosinophil-associated AHR nor the induction of airway mucus cells is directly correlated to the levels of pulmonary IL-4 and IL-5. Thus, the greater responses in sensitized and challenged mice may be attributed to IgE-mediated activation of other cell types, such as macrophages, perhaps by secretion of eosinophil chemotactic macrophage inflammatory protein-1α (50). These results suggest that both IgE-dependent and -independent activation events, either operative sequentially or in concert, are critical to confer expression of severe allergic responses. Thus, it is likely that activation of other cell types together with Th2 cells may contribute to greater and sustained allergic inflammatory responses in the airways.

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References
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