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*J Immunol* 1998; 161:3128-3135; 

http://www.jimmunol.org/content/161/6/3128

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Th2-Induced Eotaxin Expression and Eosinophilia Coexist with Th1 Responses at the Effector Stage of Lung Inflammation

Li Li, Yiyang Xia, Andrea Nguyen, Lili Feng, and David Lo

The T cell-mediated lung inflammation that is associated with allergic asthma is characterized mainly by massive eosinophil infiltration, which induces airway injury and the subsequent late-phase reactivity. Because Th2 cells are often isolated from asthmatic subjects, these cells are postulated to play a role in asthma pathogenesis. We report that adoptively transferred, influenza hemagglutinin-specific Th1 and Th2 cells induced different patterns of chemokines leading to different types of cellular infiltration. Th2 cells were sufficient to induce dramatic Ag-dependent lung eosinophilia and eotaxin expression; by contrast, Th1 transfer primarily induced neutrophil recruitment with little eosinophilia production. To determine whether Th1 cells show inhibitory effects on Th2 cell-mediated responses, Th1 and Th2 cells were cotransferred. Hemagglutinin-specific Th1 cells did not inhibit Ag-induced lung eosinophilia, nor did they inhibit eotaxin expression. Furthermore, influenza virus infection of the lung in mice receiving hemagglutinin-specific Th2 cells also induced eotaxin expression and eosinophilia that could not be inhibited by the cotransfer of Th1 cells. Our results show that Th2-mediated allergic lung inflammation coexists with the Th1-mediated responses that are stimulated by diverse forms of Ags. The Journal of Immunology, 1998, 161: 3128–3135.

A llergen-specific CD4+ Th cells are critical regulators of the inflammatory cascade in airway eosinophilia and the pathogenesis of asthma (1, 2). The effector CD4+ Th cells can develop into at least two types according to their cytokine profiles (3), and it is the Th2 cells (producing IL-4 and IL-5) rather than the Th1 cells (producing IL-2 and IFN-γ) that are more often associated with asthma. In an experimental animal model of allergic inflammation, IL-4 and IL-5 but not IFN-γ mRNAs were up-regulated among bronchoalveolar lavage (BAL)3 cells, and the increase of IL-4 and IL-5 was T cell-dependent (4). Furthermore, both the constitutive transgenic expression of IL-4 in the lung (5) and a tracheal injection of high levels of IL-5 (6) are associated with lung eosinophilia. In humans, allergen-specific T cell clones that were isolated from atopic subjects produced Th2 cytokines when stimulated in vitro (7), and Th2 cells were present among the BAL cells and airway mucosa of asthma patients (8, 9). Furthermore, it was demonstrated recently that adoptively transferred Th2 cells, either through i.v. (10) or intranasal (i.n.) (11) administration, induced asthma-like lung inflammatory responses when stimulated with Ag. Taken together, these results support the concept that Th2 cells and their cytokines play a central role in inflammatory responses in asthma; however, an increase of IFN-γ-producing T cells has also been reported in a clonal analysis of activated T cells in asthma (12). This finding raises the possibility that Th1 cells are also involved in asthma, although the specific role of Th1 cells in lung inflammation during asthma is not clear.

It is evident that T cell-produced cytokines are not necessarily the primary mediators of inflammatory cell recruitment; eosinophils and other leukocytes are more directly drawn into tissues by the production of chemokines by monocytes and parenchymal cells (13). The chemokines that have been implicated in asthma include RANTES, macrophage inflammatory protein (MIP)-1α, and monocyte chemotactic protein (MCP)-3 (14). In a lung granuloma model, MIP-1α and RANTES were associated with eosinophil accumulation (15). Furthermore, the recently identified eosinophil chemottractant, eotaxin, has been detected in rodent models of allergic inflammation (16, 17) and was determined to be one of the molecules linking T cell activation and the recruitment of eosinophils into the airway (18). The potential direct regulation of chemokine production in the lung by Th1 and Th2 cytokines has not been elucidated; therefore, the present study was initiated to establish the relationship between Th subsets and the Ag-dependent induction of chemokine production leading toward eosinophilia.

We adoptively transferred Ag-specific Th1 and/or Th2 cells into naive mice and challenged the mice i.n. with Ag. We found that the Ag-specific activation of Th1 and Th2 cells induced different patterns of chemokines leading to different types of cellular infiltration. The activation of Th2 cells was sufficient to induce both eotaxin expression and lung eosinophilia; however, the cotransfer of Th1 cells did not significantly inhibit Th2 cell activation, nor did it suppress Th2 cell-induced eotaxin expression and eosinophilia in the lung. These results were obtained regardless of whether Ag was provided as synthetic peptide or by infectious influenza virus infection. We conclude from these studies that Th1 and Th2 cell-mediated responses are codominant at the effector stage of lung inflammation regardless of the nature of the Ag stimulus.

Materials and Methods

Mice

TCR-SFE×BALB/c transgenic mice have been described previously (19). These mice are transgenic for a TCR that is specific for influenza PR8 hemagglutinin peptide 110–119 (SFERFEIPPK) presented on I-E4. Naive
BALB/c mice (6–8 wk of age) were provided by the Rodent Breeding Colony of The Scripps Research Institute (TSRI). The BALB/c and TCR-SFE×BALB/c mice were maintained under specific pathogen-free conditions in the TSRI rodent colony in accordance with National Institutes of Health (Bethesda, MD) and TSRI institutional guidelines.

**Generation and testing of Th1 and Th2 cells**

Th1 and Th2 cells were generated from naive lymph node T cells (sorted CD4+ McI-I-4+57) (PharMingen, San Diego, CA) that had been obtained from TCR-SFE×BALB/c mice. A total of 10^6 T cells were cultured with 5 × 10^5 irradiated (2500 rad) splenic cells under Th1 (20 ng/ml IL-12; Genzyme, Cambridge, MA) or Th2 (25 ng/ml IL-4; PeproTech, Rocky Hill, NJ) and 100 μg/ml of anti-IL-12 (monoclonal rat IgG, clone C17.8.20; a kind gift of Dr. G. Trinchieri, The Wistar Institute Philadelphia, PA) conditions with 0.5 μg/ml of SFE peptides. The media was changed every 2 to 3 days, and 50 U/ml of IL-2 (PeproTech) was added to all the culture from day 3 on. Cells that had been cultured for 9 days were used for adoptive transfer. At 7 days after the culture, an aliquot of Th1 or Th2 cells (10^5/well) was stimulated with Con A (5 μg/ml), and cytokines in the supernatants were tested by ELISA (PharMingen). Aliquots of T cells were also stained for CD4-phycocerythrin and V beta8.1/8.2-FITC (PharMingen) and analyzed by flow cytometry. Th1 or Th2 cells (5 × 10^5/well) were stimulated with irradiated (2500 rad) syngeneic spleen cells (1.5 × 10^5/well) in the presence of different concentrations of SFE peptide, and their in vitro proliferation was assessed by [3H]thymidine incorporation.

**Induction of lung inflammations**

Th1 or Th2 cells (5 × 10^6/mouse) or Th1 plus Th2 cells (5 × 10^6 of each type per mouse) were transferred i.v. into naive BALB/c mice. Mice were challenged i.n. at 24 h posttransfer with 100 μg of SFE peptide daily for 3 days. Control mice were either transferred with Th1 or Th2 cells but challenged with PBS or challenged with SFE without cell transfer. Mice were sacrificed at 3 h after the last challenge, and their lung was taken for the testing of serum IgE levels. The lung was then perfused from the right ventricle using PBS until it turned white, and BAL was collected by washing the lung through the trachea with 1 ml of RPMI 1640 plus 2% horse serum. Cytospins were prepared for BAL cells from each mouse. The upper right lobe of the lung was then frozen in Trizol reagent (Life Technologies) and 24 μg/ml of RNase A (Sigma) for 1 h at 30°C.

**Histology**

Cytospins of BAL cells were fixed with methanol and stained with eosin and methylene blue (Fisher, Pittsburgh, PA). Leukocytes were analyzed by a differential count of 200 to 300 cells on coded slides. Bouin’s solution-fixed lung tissues were embedded in paraffin, and sections were stained with hematoxylin and eosin (Sigma). Frozen lung sections were fixed with 2% horse serum. Cytospins were prepared for BAL cells from each mouse. The upper right lobe of the lung was then frozen in Trizol reagent (Life Technologies, Grand Island, NY) at −70°C for RNA extraction. One-half of the left lung was fixed in Bouin’s solution for hematoxylin and eosin staining, while the other half was frozen in OCT compound (Miles, Elkhart, IN) for immunohistochemical staining.

**Detection of cytokines from BAL**

Both mice that had been transferred with Th1 and/or Th2 cells and control mice that did not receive any cells were sacrificed and perfused at 2 days after SFE challenge. The BAL was subsequently collected by washing the lung through the trachea with 1 ml of RPMI 1640 plus 2% horse serum. BAL was kept at 37°C for 45 min, and the media were collected by spinning down the cells. Levels of IL-4, IL-5, and IFN-γ levels in the BAL media were detected by ELISA (PharMingen).

**Detection of cytokines from BAL**

Total RNA was isolated using Trizol reagent. The probes for a panel of chemokines have been described previously (22) or were purchased from PharMingen. The assay was performed as described previously (23). Briefly, RNA was dissolved in 80% formamide, 0.4 M NaCl, 1 mM EDTA, and 40 mM PIPES; heated to 85°C for 5 min; and hybridized for 10 h with corresponding [α-32P]uridine triphosphate-labeled antisense probes at 55°C. The unhybridized RNA was digested with 50 U/ml of RNase T1 (Life Technologies) and 24 μg/ml of RNase A (Sigma) for 1 h at 30°C.

**FIGURE 1.** Th1 and Th2 cells used for adoptive transfer. A. Th1 and Th2 cells produced high levels of corresponding cytokines when stimulated in vitro. Th1 or Th2 cells were stimulated with Con A, and the cytokines in the supernatants were tested by ELISA. Each bar represents the mean ± SD of triplicate wells. Cytokine patterns were tested for each batch of Th1 and Th2 cells. B. Th1 and Th2 cells were CD4+ and expressed the Vβ8.2 TCR transgene. Aliquots of T cells were stained for CD4 and Vβ8.1/8.2 and analyzed by flow cytometry. The expression of these cell surface markers was tested for each experiment. C. Both Th1 and Th2 cells showed similar in vitro proliferation to Ag. Th1 or Th2 cells were stimulated with irradiated syngeneic spleen cells in the presence of SFE peptide, and the proliferation of Th1 and Th2 cells was assessed by [3H]thymidine incorporation. Each point represents the mean ± SD of triplicate wells. This figure represents three independent experiments.
After phenol-chloroform extraction and sodium acetate-ethanol precipitation, the protected, hybridized RNA was denatured and electrophoresed on 10% polyacrylamide gel. The gel was dried and exposed to film.

Results

Ag-specific Th1 and Th2 cells induce different patterns of cellular infiltration in the lung after activation: Th2 but not Th1 cells mediate lung eosinophilia

To characterize the lung inflammatory responses induced by Th1 or Th2 cells, bulk Th1 and Th2 cells were generated from naive CD4 T cells of influenza hemagglutinin-specific TCR-SFE×BALB/c transgenic mice under Th1- and Th2-favoring conditions. In vitro, the established Th1 and Th2 cell lines produced their corresponding cytokines when stimulated with either SFE peptide (data not shown) or Con A (Fig. 1A). Both Th1 and Th2 cells were CD4+ and expressed the Vβ8.2 chain of the TCR transgene (Fig. 1B). Furthermore, Th1 and Th2 cells showed similar Ag-specific proliferative responses after stimulation with syngeneic APCs plus SFE peptides (Fig. 1C). These Th1 or Th2 cells were adoptively transferred into unmanipulated naive BALB/c mice, and the mice were given three daily i.n. challenges with SFE peptide. Similar numbers of CD4+Vβ8.1/8.2+ T cells were detected in the BAL of Th1- and Th2-injected mice after the last challenge (12.4 ± 2.4 × 10^4 in Th1 mice vs 14.8 ± 7.2 × 10^4 in Th2 mice; n = 8), which suggests a similar recruitment of these lymphocytes to the lung. In both cases, a high proportion of these cells appeared to be activated, based on the expression of the T cell activation marker CD25: 20 to 60% (average 43%) of total CD4+ cells and 0.3 × 10^5 neutrophils in the control mice. Interestingly, significant numbers of eosinophils were detected in the BAL of Th2-injected mice but not in the BAL of Th1-transferred mice (Fig. 2). An average of 8.7 × 10^5 eosinophils were isolated from the BAL of Th2-transferred mice compared with only 5 × 10^4 eosinophils in Th1-transferred mice or 7 × 10^3 in control mice. Furthermore, despite active inflammation, total serum IgE levels were not induced above background (data not shown). The failure to induce IgE production under these conditions is presumably due to the fact that Ag stimulation only used a short peptide that was administered over a 3-day course. Histologically, perivascular and peribronchial infiltration by mononuclear and polymorphonuclear cells was detected, including CD4+ T cells, neutrophils, and eosinophils (Fig. 3). Similar patterns of CD4+ T cell infiltration were found in Th1- and Th2-transferred mice. Eosinophils were absent or scattered in most of the infiltrated areas in Th1 mice (Fig. 3 shows one of the most heavily infiltrated areas), whereas massive eosinophil infiltration was present in all of the infiltrated areas that were studied in Th2 mice (Fig. 3). These results demonstrate that Th1 and Th2 cells cause different patterns of Ag-dependent lung inflammation, and the activation of Th2 cells was sufficient to induce lung eosinophilia. Interestingly, we were able to generate significant effects in these experiments by transferring small numbers of T cells; it was not necessary to reduce the pool of host T lymphocytes. This is in striking contrast to other studies adoptively transferring inflammatory diseases such as diabetes and graft-vs-host disease, in which it was required that recipient mice be depleted by sublethal irradiation.

Th1 cells do not inhibit the lung eosinophilia induced by Th2 cells

Th1 and Th2 cells often show a reciprocal inhibition of functions during immune responses; such inhibition has been observed in Leishmania infection, experimental allergic encephalomyelitis, and autoimmune diabetes (24). To study the effect of Th1 cells on the lung eosinophilia induced by Th2 cells, Th1 and Th2 cells were cotransferred into naive BALB/c mice. After three daily SFE challenges, mice that had received a mixture of Th1 and Th2 cells at a 1:1 ratio showed eosinophil infiltration in both the BAL and lung
tissue that was similar to that seen in mice that had received Th2 cells alone (Figs. 2 and Fig. 3). Approximately $6.5 \pm 5.3 \times 10^5$ ($n = 8$) eosinophils were collected from the BAL of Th1 plus Th2 cotransferred mice compared with $8.7 \pm 6.2 \times 10^5$ ($n = 11$) eosinophils in the mice that had been transferred with Th2 alone. The difference is not significant when analyzed using ANOVA. In one experiment, in which Th1 cells were cotransferred with Th2 cells at both a 1:1 and 2:1 ratio, the total number of eosinophils collected from the BAL were $3.7 \times 10^5$ and $6.8 \times 10^5$ per mouse, respectively, compared with an average of $4.9 \times 10^5$ eosinophils in the Th2-transferred mice. These results suggested that at the effector stage, Th1 cells did not significantly inhibit the lung eosinophilia induced by Th2 cells.

To confirm the absence of counterinhibition between Th1 and Th2 cell activation in the lung, representative Th1 (IFN-γ) and Th2 (IL-4 and IL-5) cytokines were assayed from the BAL of mice that had been transferred with Th1 or Th2 cells alone or Th1 plus Th2 cells. IFN-γ was detected from the BAL of Th1-transferred mice but not from the BAL of Th2-transferred mice, while IL-4 and IL-5 were only found from Th2 cell transfer BAL (Fig. 4). This result further confirmed that the transferred Th1 and Th2 cells were active in vivo, and they retained their in vitro cytokine phenotypes in the inflamed lung. Interestingly, a mixture of Th1 and Th2 cytokines were detected from the BAL in the mice that had been cotransferred with both Th1 and Th2 cells. Th2 cytokine levels were not inhibited compared with the results obtained for single-celltype-injected mice (Fig. 4). Although the IFN-γ levels in mice that were given both Th1 and Th2 cells appeared to be slightly reduced in comparison with the levels seen for mice given Th1 only, these effects were not statistically significant. This finding suggested that Th1 and Th2 cells did not efficiently counterinhibit each other’s cytokine production at the effector phase of lung inflammation.

**FIGURE 3.** CD4 and eosinophil infiltration in the lung parenchyma of mice that had been transferred with Th1 or Th2 cells or Th1 plus Th2 cells. Frozen sections were stained either with anti-CD4 or with sodium cyanide and diaminobenzidine for cyanide-resistant eosinophil peroxidase activity. Photographs were taken at ×400 magnification.
Th2 cells were transferred alone (Fig. 5, combination of those that were expressed in the lungs when Th1 or Th2 cells, but neither Th1 nor Th2 cells expressed detectable eotaxin (Fig. 5b) compared with uninfected mice (Fig. 2). This neutrophil recruitment was similarly evident in mice that were given Th1 or Th2 cells alone and Th1 plus Th2 cells. However, the most striking observation was that in mice given Th2 cells, eosinophilia and eotaxin production were also induced (Fig. 6C). This was true regardless of whether Th2 cells were administered alone or in a mix with Th1 cells. On average, $8.7 \times 10^5$ and $11.9 \times 10^5$ eosinophils were collected from the BAL of both Th2 and Th1 plus Th2 cell-transferred mice, respectively. Thus, with both forms of the stimulus (either free peptide or infectious virus), Th2 activation by Ag in the lung results in eotaxin production and eosinophilia that cannot be inhibited by Th1 cells.

**Discussion**

We have demonstrated that Th1 and Th2 cells show different effects in lung inflammation that correlate with the different patterns of chemokines induced in the lung. This observation provides direct link between Ag stimulation of different subsets of Th cells and the patterns of granulocyte recruitment. Interestingly, most of the chemokines that were detected from the inflamed lung, such as RANTES, IP-10, MCP-1, and eotaxin, were not produced by the injected T cells. Therefore, the major chemokines were likely the chemokines that were detected from the inflamed lung, such as RANTES, IP-10, MCP-1, and eotaxin. Overall, we have shown that Th1 and Th2 cells mediate lung eosinophilia and eotaxin expression after influenza virus infection, and this response cannot be inhibited by Th1 cells.
Curiously, the patterns of granulocyte recruitment described here were quite distinct from the lymphoid infiltration that was observed in a closely related model in which autoimmune diabetes was induced using the same Ag (expressed in islet β cells) and TCR-transgenic T cells (19). This contrast illustrates the important principle that while Ag-mediated inflammation may be driven by a local activation of circulating T cells, tissue-specific responses by parenchymal cells (e.g., chemokine expression) can have a major influence on the character of the local inflammatory response. Further studies are in progress to identify these tissue-specific factors.

IgE induction and eosinophil rich inflammation are often associated with allergic asthma. The in vivo IgE depletion (29) and passive transfer of IgE (30) studies in mouse models suggest an important role for IgE in inducing lung eosinophilia and airway hypersensitivity. But despite the eosinophilia, IgE was not induced above background in Th2 mice or Th1 plus Th2 transferred mice (data not shown), presumably due to the brief duration of Ag challenge (3 days), and the fact that the Ag was only a very short (10 aa) synthetic peptide. This result suggested that IgE and IgE-mediated mast cell activation was not required for the induction of lung pathology in our model. Other recent studies have also shown that airway eosinophilic inflammation can occur in IgE- or B cell-deficient mice (31–33). It is important to note that our results may have no bearing on the issue of airway hypersensitivity in allergic responses; since airway hypersensitivity and eosinophilia can be considered two separate aspects of asthma (5, 34), our results only suggest that IgE may not be required in eosinophilia and late-phase airway injury in asthma.

In our model, the cotransfer of Th1 cells did not produce a significant inhibition of Th2 cell activation and eosinophil expression in response to Ag. This finding appears to contradict studies showing that the administration of IL-12 (35, 36) or IFN-γ (37) can inhibit Ag-induced Th2 responses, airway hypersensitivity, and lung inflammation. In those studies, the most effective inhibition occurred when IL-12 was administered at the immunization stage of the response or given at least a few days before the challenge, at a point before the activation of the Th2 cells (35–37). This timepoint presumably provided enough time for the major effector of the suppression, IFN-γ, to effectively inhibit the proliferation and activation of Th2 cells. In contrast, when Th1 and Th2 cells were activated simultaneously, IFN-γ may have little effect on the activated Th2 cells; thus Th1 cells would not inhibit Th2 responses. Curiously, in mice that were treated with IL-12 during initial Ag challenge, both Th1 and Th2 cytokines were detected from the BAL (36), suggesting that Ag-specific Th1 and Th2 cells may already coexist in those animals. It is tempting to speculate that if those mice were further challenged with Ag in the absence of any cytokine treatments, Th1 and Th2 responses might both expand, leading to the situation presented in our model.

It should be noted that, although the present paper provides some interesting correlations between Th1/Th2 cell activation in vivo and various patterns of chemokines, the correlations do not conclusively establish causative links between specific cytokines/chemokines and granulocyte recruitment. Thus, while eosinax is consistently associated with eosinophilia, it is probably not exclusively required for the recruitment of eosinophils. For example, in recent studies, both an Ab blockade of eosinax in vivo (13) and the targeted disruption of the eosinax gene (38) caused only a 50% reduction in eosinophil recruitment in models of tissue eosinophilia. Moreover, in our preliminary studies, the neutralization of some Th2 cytokines was able to inhibit eosinophilia without blocking the induction of eosinax, which suggests that several factors contribute to eosinophil recruitment (our unpublished observations).
recipient T cells that were not specific for the Ag is considered. 
Our results suggest that, while concomitant Th1 responses may be 
present in most situations, preexisting Th2 responses are most rel-

evant to the generation of eosinophilia and associated pathology. 
Infection with influenza virus did not affect the activation of Th2 
cells and eosinophilia despite its previously described preferential 
induction of Th1 cells and its regular induction of neutrophil in-
filtration. Given these observations, it is possible that although not 
all atopic individuals are asthmatic, they may all still be suscep-
tible to some degree of Ag-induced lung eosinophilia; asthmatic 
patients might be distinguished only by unusual airway sensitivity 
to the eosinophilia. In sum, these results suggest that the treatment 
of allergic lung inflammation may depend upon the direct deple-
tion of Th2 effector cells, since it may not be possible to establish 
regulatory interactions to suppress such cells.

Acknowledgments

We thank Dr. A. Wu for discussions and Dr. M. Carson for helpful com-
ments on the manuscript.

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