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

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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 eotaxin 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.


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 (SFERFEIFPK) 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 naïve lymph node T cells (sorted CD4+ MEL-14+/45+) (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) spleen 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-2 (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-phycoerythrin and Vß8.1/8.2-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 naïve BALB/c mice. Mice were challenged i.n. at 24 h postransfer 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 three times with 5 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, Grand Island, NY) at 2°C to 8°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.

In cases of virus infection, Th1 or Th2 cells (6 × 10^6/mouse) or Th1 plus Th2 cells (6 × 10^6/mouse of each type) were transferred i.v. into naïve BALB/c mice. These mice were infected i.n. with 25 µl (2.5 hemagglutinating units) of influenza virus (influenza A/PR/8/34/Mt. Sinai, as described in Ref. 20) at 24 h after cell transfer. The mice were sacrificed after 3 days, and BAL and tissue were processed as described above.

**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. Bouins’ solution-fixed lung tissues were embedded in paraffin, and sections were stained with hematoxylin and eosin (Sigma). Frozen lung sections were fixed with methanol and stained with 3-amino-9-ethylcarbazole (AEC) substrate (Sigma). Eosinophils were stained for cytidine-resistant eosinophil peroxidase activity as described previously (21). Briefly, frozen lung sections were fixed with 1% formalin (Fisher) in acetone. Tissues were subsequently stained for 10 min with 0.4 mg/ml sodium cyanide (Sigma), 3 µl/ml H_2O_2 (Fisher), and 0.75 mg/ml diaminobenzidine (Sigma) substrate in PBS and then counterstained with hematoxylin.

**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).

**RNase protection assay**

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]dCTP-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.
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+ Vβ8.1/8.2+ cells were CD25+ in Ag-stimulated Th1- or Th2-injected mice compared with only 4 to 12% (average 8%) in control mice.

Without Ag stimulation, neither Th1 nor Th2 cells induced lung infiltration. However, after three daily SFE peptide challenges, increased numbers of infiltrating mononuclear cells and neutrophils were detected in the BAL of Th1- and Th2-injected mice (Fig. 2). On average, ~1.2 × 10^6 and 1.4 × 10^6 mononuclear cells (mainly alveolar macrophages) vs 4.4 × 10^5 and 3.1 × 10^5 neutrophils were harvested from the BAL of Th1- and Th2-injected mice, respectively, compared with only 0.5 × 10^6 mononuclear 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-cell-type-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.
Th2 cells were transferred alone (Fig. 5, combination of those that were expressed in the lungs when Th1 or Th2 cotransferred lungs were a Th2 cells in the lung induced different patterns of chemokine pro-
report (17). These results indicated that the activation of Th1 and
tectable eotaxin (Fig. 5 C). Th1 and Th2 cells, but neither Th1 nor Th2 cells expressed de-
MIP-1). The major source of eotaxin does not appear to be the injected
lung inflammation, and Th1 and Th2 cyto-
kines were coexpressed in the BAL of mice that had been transferred with both
cell types, Th1 or Th2 cells (5 x 10^6 mouse) or Th1 plus Th2 cells (5 x 10^6 of
each type per mouse) were transferred into
naive BALB/c mice and challenged with peptide. BAL cytokines were detected by
ELISA as described in Materials and
Methods. This figure combines results from two independent experiments; each
point represents one mouse.

which may provide an explanation for the lack of cross-inhibition
between Th1- and Th2-induced lung inflammatory responses.

**Th1 and Th2 cells induce different patterns of chemokine production during lung inflammation**

Chemokines are directly responsible for cellular recruitment in infl-
ammatory responses. Using RNase protection assays, we tested a
panel of chemokines that were potentially involved in Th1 and Th2
cell-mediated lung inflammations. Two specific patterns of chem-
okines were found in Th1- vs Th2-transferred lungs (Fig. 5). Eotaxin, which is an eosinophil-specific chemokine, was mainly
expressed in Th2-transferred lungs; only low levels were seen in
Th1-transferred lungs (Fig. 5A). This pattern correlated well with
the eosinophilia that was induced by Th2 but not Th1 cells. By
contrast, lymphotactin (Ltn) was mainly expressed in Th1-injected
lungs, and higher levels of IFN-γ-inducible protein (IP-10),
RANTES, MIP-1β, and MCP-1 and variably low levels of MIP-1α
were found in Th1- compared with Th2-transferred lungs (Fig.
5B). The major source of eotaxin does not appear to be the injected
Th cells, because equivalent numbers of T cells produced almost
undetectable amounts of chemokines when activated in vitro by
Con A. When using higher numbers of cells, similar levels of Ltn,
MIP-1α, MIP-1β, and T cell activation gene 3 were produced by
Th1 and Th2 cells, but neither Th1 nor Th2 cells expressed de-
tectable eotaxin (Fig. 5C); this finding is in contrast to a recent
report (17). These results indicated that the activation of Th1 and
Th2 cells in the lung induced different patterns of chemokine pro-
duction, most likely by parenchymal cells in the lung. Finally,
the chemokines found in Th1 plus Th2 cotransferred lungs were a
combination of those that were expressed in the lungs when Th1 or
Th2 cells were transferred alone (Fig. 5, A and B), which correlated
well with the additive effects on granulocyte recruitment.

**Th2 cells mediate both lung eosinophilia and eotaxin expression after influenza virus infection, and this response cannot be inhibited by Th1 cells**

Respiratory virus infection is often a trigger of allergic asthma,
develop the fact that Th1 responses are usually induced by viral
infection (25). To study the potential role of virus infection in lung
inflammation in mice that were biased to Th1 or Th2 responses,
influenza virus was used to infect mice that had been transferred
with Th1, Th2, or Th1 plus Th2 cells. In control mice that were
given no effector T cells, influenza infection produced significant
neutrophil inflammation at 3 days after infection (Fig. 6A) and
induced the expression of chemokines such as RANTES, IP-10,
and MCP-1 (Fig. 6B) 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 x 10^5 and 11.9 x 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 ef-
effects in lung inflammation that correlate with the different patterns
of chemokines induced in the lung. This observation provides a
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
results of either the lung parenchymal cells or other recruited cell
types after stimulation by Th1 or Th2 cytokines. In the Th1 and
Th2 cotransferred mice, the chemokines produced in the lung
showed a combination of those patterns that were detected from
mice that had been given Th1 or Th2 cells alone, which suggests
an additive influence of Th1 and Th2 cells on the ultimate chem-
okine-producing cells. Interestingly, in some experiments, the
levels of RANTES and IP-10 were lower in Th1 and Th2 cotrans-
ferred mouse lung when compared with those levels that were
expressed in mice that had been given Th1 cells alone; this finding
suggests a possible inhibition of Th1 cell-induced chemokines by
Th2 cells or their cytokines. By contrast, there was no detectable
suppression of eotaxin in Th1 plus Th2 cotransferred mice, despite
the fact that high levels of IFN-γ were detected in the lungs of
those mice. Thus, IFN-γ may not inhibit eotaxin production; in-
deed, two recent studies suggest that in the presence of IL-1 or
TNF-α, IFN-γ may even serve as a stimulator of the eotaxin that
is expressed by human dermal fibroblasts (26) and lung epithelial
cells (27). The observed production of both Th1 and Th2 cytokines
in asthma (12, 28) might therefore suggest a contributory role for
Th1 cells in disease pathogenesis.
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+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 eosinotaxis 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 eosinotaxis in vivo (13) and the targeted disruption of the eosinotaxis 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 eosinotaxis, which suggests that several factors contribute to eosinophil recruitment (our unpublished observations).

Our study, when taken together with two recent reports (10, 11), shows the critical role of allergen-specific Th2 cells in late-phase asthma responses. The effect of the injected Th2 cells is even more impressive when the dilution of these cells by a large excess of
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 relevant 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 infiltration. Given these observations, it is possible that although not all atopic individuals are asthmatic, they may all still be susceptible 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 depletion of Th2 effector cells, since it may not be possible to establish regulatory interactions to suppress such cells.

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References


