

## NEW Biosimilar Antibodies

PD-1 | Nivolumab Biosimilar  
PD-L1 | Atezolizumab Biosimilar  
CTLA-4 | Ipilimumab Biosimilar  
and more

DISCOVER

BioCell



## Modulation of Airway Inflammation by Passive Transfer of Allergen-Specific Th1 and Th2 Cells in a Mouse Model of Asthma

This information is current as of December 5, 2021.

David A. Randolph, Cynthia J. L. Carruthers, Susanne J. Szabo, Kenneth M. Murphy and David D. Chaplin

*J Immunol* 1999; 162:2375-2383; ;  
<http://www.jimmunol.org/content/162/4/2375>

**References** This article cites 29 articles, 14 of which you can access for free at:  
<http://www.jimmunol.org/content/162/4/2375.full#ref-list-1>

### Why *The JI*? Submit online.

- **Rapid Reviews! 30 days\*** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

\*average

**Subscription** Information about subscribing to *The Journal of Immunology* is online at:  
<http://jimmunol.org/subscription>

**Permissions** Submit copyright permission requests at:  
<http://www.aai.org/About/Publications/JI/copyright.html>

**Email Alerts** Receive free email-alerts when new articles cite this article. Sign up at:  
<http://jimmunol.org/alerts>

*The Journal of Immunology* is published twice each month by  
The American Association of Immunologists, Inc.,  
1451 Rockville Pike, Suite 650, Rockville, MD 20852  
Copyright © 1999 by The American Association of  
Immunologists All rights reserved.  
Print ISSN: 0022-1767 Online ISSN: 1550-6606.



# Modulation of Airway Inflammation by Passive Transfer of Allergen-Specific Th1 and Th2 Cells in a Mouse Model of Asthma<sup>1</sup>

David A. Randolph,\* Cynthia J. L. Carruthers,<sup>‡</sup> Susanne J. Szabo,<sup>†</sup> Kenneth M. Murphy,<sup>†‡</sup> and David D. Chaplin<sup>2\*‡</sup>

Although evidence is strong that Th cells play a major role in mediating the airway inflammation observed in asthma, the relative contributions of the Th cell subsets, Th1 and Th2, are unclear. It has been suggested that asthma is driven by Th2 predominant responses in the lung, but other data suggest a role for Th1 cells as well. Here we show by intracellular cytokine staining and flow cytometric analysis that in the murine model of OVA-induced airway inflammation, both Th1 and Th2 cells are recruited to the airways. Th1 cells predominate early in the response and Th2 cells predominate late. We further show that increasing the number of Th1 cells by passive transfer of OVA-specific Th1 cells results in increased inflammation. This effect is observed regardless of whether the T cells are transferred before sensitization or after airway inflammation is already in progress. Transfer of Th1 cells also results in increased recruitment of host T cells of both Th1 and Th2 phenotypes. Passive transfer of Th2 cells results in little change in the inflammatory response. These results demonstrate that Ag-specific Th1 cells are not protective in this model of asthma, but rather may potentiate the inflammatory response. *The Journal of Immunology*, 1999, 162: 2375–2383.

Inflammation of the lower airways is central in the pathogenesis of asthma. Histologically, asthmatic airways generally show leukocytic infiltrates consisting variably of eosinophils, lymphocytes, neutrophils, monocytes, and other cells. Although the specific events that initiate the inflammatory response are unknown, the inflammatory cells are believed to be recruited as a consequence of an immune response to inhaled Ags, with CD4<sup>+</sup> Th cells playing a major role (1–6). When the airways of asthmatic patients are analyzed by bronchoalveolar lavage (BAL)<sup>3</sup> (7) or by endotracheal biopsy (8), activated T cells are found and there is a strong correlation of asthma with T cell-dependent IgE responses to allergens.

CD4<sup>+</sup> Th cells can be divided into phenotypic subsets based on the cytokines they produce (reviewed in Ref. 9). Th1 cells secrete IFN- $\gamma$ , IL-2, and lymphotoxin and promote immunity to intracellular pathogens such as *Listeria* as well as Ig class switching to IgG2a. Th2 cells secrete IL-4, IL-5, IL-6, IL-10, and IL-13 and promote immune responses to helminthic infections as well as Ig class switching to IgG1 and IgE. Once an immune response begins to deviate toward a Th1 or a Th2 response, there is potential for further polarization of the Th response mediated by the cytokines

produced by the Th cells themselves. IFN- $\gamma$  from Th1 cells promotes IL-12R expression on T cells and activates macrophages to produce IL-12 that promotes differentiation of naive T cells into Th1 cells (10). IFN- $\gamma$  also inhibits proliferation of Th2 cells. IL-4 and IL-10 promote differentiation of naive T cells into Th2 cells and also down-regulate macrophage function and Th1 activation (11). Thus, each subset has the potential to promote its own expansion while inhibiting the differentiation of the other.

Because of the associations of asthma with IgE and eosinophil predominant inflammation, it has been suggested that a relative imbalance of Th2 responses over Th1 responses drives the pathogenesis of the disease. It has been further suggested that Th1 responses in the lung may protect against asthma (2–6, 12). In support of this model, BAL T cells from human asthmatics have been reported to express elevated levels of IL-4 and IL-5 mRNA (8, 13). In addition, an inverse association between Th1 tuberculin responses and asthma has been observed (12, 14). In mouse models of asthma, CD4<sup>+</sup> cells, IL-4, and IL-5 are all essential for the development of eosinophilic lung inflammation (15–19). Other studies have demonstrated that the Th1-associated cytokines, IL-12 and IFN- $\gamma$ , can inhibit allergic inflammation (20–22). However, additional experiments have shown increased numbers of IFN- $\gamma$ -producing T cells in BAL fluid from human asthmatics (23, 24). Also, viral infections of the respiratory tract, which can induce Th1 responses, are one of the most commonly recognized triggers of asthmatic exacerbations (reviewed in Ref. 25).

To better elucidate the respective roles of Th1 and Th2 cells in allergic lung inflammation, we have applied intracellular cytokine staining and flow cytometry to study T cells in a mouse model of Ag-induced asthma. In addition, we have tested the ability of passively transferred Ag-specific Th1 and Th2 cells to modulate allergic lung inflammation. Here we show that both Th1 and Th2 cells are recruited to the lung in sensitized mice after challenge. Skewing toward a Th1 response by passive transfer of Th1 cells increases lung inflammation, regardless of whether the cells are transferred before sensitization or after airway inflammation is established. Transfer of Th2 cells has little effect. Transferring Th1

Center for Immunology and Departments of \*Internal Medicine and <sup>†</sup>Pathology and <sup>‡</sup>Howard Hughes Medical Institute, Washington University School of Medicine, St. Louis, MO 63110

Received for publication July 23, 1998. Accepted for publication November 5, 1998.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

<sup>1</sup> This work was supported by a National Institutes of Health Medical Scientist Training Program grant (to D.A.R.), and National Institutes of Health Grants HL56419 and AI34580 (to D.D.C.). K.M.M. and D.D.C. are investigators of the Howard Hughes Medical Institute.

<sup>2</sup> Address correspondence and reprint requests to Dr. David D. Chaplin, Howard Hughes Medical Institute and Department of Internal Medicine, Washington University School of Medicine, 660 S. Euclid Avenue, Box 8022, St. Louis, MO 63110. E-mail address: chaplin@im.wustl.edu

<sup>3</sup> Abbreviations used in this paper: BAL, bronchoalveolar lavage; H&E, hematoxylin and eosin; PE, phycoerythrin.

cells promotes the recruitment of endogenous Th1 cells, but also increases the number of infiltrating endogenous Th2 cells and other inflammatory cells. These data indicate that Th1 cells are inefficient at inhibiting inflammation in this model. Rather, they may augment the inflammatory response.

## Materials and Methods

### Mice

BALB/c mice were obtained from Harlan Sprague-Dawley (Indianapolis, IN). DO11.10 TCR transgenic mice (26) were backcrossed >10 times to the BALB/c background before the initiation of these experiments. The transgenic TCR was maintained in a heterozygous fashion by backcrossing to BALB/c. All mice used in these experiments were females, 4–6 wk of age at the time of initial immunization. Mice were housed in a specific pathogen-free facility with food and water provided ad libitum.

### Induction of airway hypersensitivity

Mice were sensitized to OVA (Sigma-Aldrich, St. Louis, MO) and challenged according to a modification of the method of Kung et al. (27). Groups of three to six mice were immunized i.p. with 8  $\mu$ g OVA adsorbed to 2 mg alum (Sigma-Aldrich) in 0.5 ml PBS. The mice received an identical booster immunization 7 days later. On day 15 and day 21 after the initial sensitization, mice were challenged with an aerosol of 1% (w/v) OVA in PBS in a plexiglass chamber for 20 min both in the morning and afternoon of each challenge day. The aerosol was generated by a DeVilbiss Ultra-Neb 99 nebulizer (De Vilbiss, Somerset, PA). Vehicle control mice received mock sensitization with i.p. injections of alum in PBS and were challenged with an aerosol of PBS without OVA.

### Analysis of airway and lung inflammation

When indicated, airway inflammatory cells were obtained by BAL. Mice were anesthetized by i.p. injection of ketamine/xylazine and sacrificed by cervical dislocation. The trachea was cannulated and the lungs were flushed with four 0.8 ml aliquots of ice cold 2% FCS in PBS. RBCs in the lavage were lysed using ammonium chloride, and nucleated cells were counted by a hemacytometer. Lavage samples from three mice were pooled for analysis of intracellular cytokines. Lung parenchymal T cells were prepared from minced tissue that had been digested for 30 min at 37°C with collagenase/dispase (1 mg/ml; Boehringer Mannheim, Indianapolis, IN) and DNase I (0.1 mg/ml, Boehringer Mannheim) in complete medium. The tissue was then homogenized between ground glass slides and strained through a 70- $\mu$ m nylon mesh to generate a single cell suspension. CD4<sup>+</sup> cells were purified from the suspension using anti-mouse CD4 Dynabeads and mouse CD4 DETACHaBEAD (DynaL, Lake Success, NY) according to the manufacturer's instructions. Cells from three mice were pooled for analysis of intracellular cytokines.

### Analysis of intracellular cytokines

Total BAL or purified parenchymal CD4<sup>+</sup> T cells were cultured at 37°C in Iscove's medium supplemented with 10% FCS (HyClone, Logan, UT), 0.01 mM nonessential amino acid mix, 2 mM sodium glutamate, 1 mM sodium pyruvate, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 5.5  $\mu$ M 2-ME (all from Life Technologies, Grand Island, NY) (T cell medium) for 6 h in the presence of 2  $\mu$ M monensin (Sigma-Aldrich) with or without 10 ng/ml PMA (Sigma-Aldrich) and 1  $\mu$ M ionomycin (Sigma-Aldrich). CD4<sup>+</sup> cells were marked using allophycocyanin-anti-CD4 (PharMingen, San Diego, CA). Cells were fixed for 30 min in 4% paraformaldehyde in PBS, permeabilized with 0.1% saponin, and then stained with phycoerythrin (PE) anti-IFN- $\gamma$  (PharMingen) and either FITC-anti-IL-4 (PharMingen) or FITC-anti-IL-5 (PharMingen). Anti-CD4 and anti-IFN- $\gamma$  were used at 1:100 dilution; anti-IL-4 and anti-IL-5 were used at 1:50. Transgenic DO11.10 T cells were stained with biotinylated anti-clonotypic Ab KJ1-26 (26) followed by streptavidin-CyChrome (PharMingen). Samples were analyzed using a FACScalibur Becton Dickinson flow cytometer (Mountain View, CA). The forward scatter and side scatter properties of the cells were used to exclude dead cells from analysis. For BAL cells, one-fourth of the cells collected were analyzed; for purified CD4<sup>+</sup> parenchymal cells, 10,000 cells were analyzed.

### Preparation of differentiated CD4<sup>+</sup> T cells

OVA-specific Th1 and Th2 cells were generated in vitro as described previously (28). Briefly, FACS-sorted CD4<sup>+</sup> L-selectin<sup>+</sup> T cells from lymph nodes and spleens of DO11.10 TCR transgenic mice were cultured with irradiated splenocytes at a ratio of 1:20 for 3 days in the presence of 0.3

$\mu$ M OVA<sub>323–339</sub> peptide. For Th1 cultures, IL-12 (2 ng/ml; R&D Systems, Minneapolis, MN) and anti-IL-4 (10  $\mu$ g/ml, 11B11) were added. For Th2 cultures, IL-4 (1000 U/ml; R&D Systems) and anti-IL-12 (TOSH; 10  $\mu$ g/ml, courtesy of Dr. E. Unanue, Washington University) (31) were added. On day 3, cells were expanded 1:4 and IL-2 (40 U/ml; R&D Systems) was added. On day 7, cells were frozen in FCS containing 10% DMSO. Before transfer into recipient mice, cells were thawed and restimulated with irradiated APCs and 0.3  $\mu$ M OVA peptide. Supernatants for ELISA were collected at 48 h. After 72 h, the cells were expanded 1:4 in medium with 40 U/ml IL-2. IFN- $\gamma$  and IL-4 concentrations in the supernatants were measured using Quantikine ELISA kits (R&D Systems) according to the manufacturer's instructions. Supernatants from the Th1 cells contained <0.04 ng IL-4/ml and 1200 ng IFN- $\gamma$ /ml. Th2 supernatants contained 4.2 ng IL-4/ml and only 2.5 ng IFN- $\gamma$ /ml. The cells were also analyzed by flow cytometry. Th1 cells expressed IFN- $\gamma$  but little IL-4 or IL-5, whereas Th2 cells expressed IL-4 and IL-5 but little IFN- $\gamma$  (see Fig. 4). In addition, surface expression of membrane lymphotoxin was demonstrated on Th1, but not Th2, cells by staining with biotinylated soluble lymphotoxin  $\beta$  receptor fusion protein (kindly provided by J. Browning, Biogen, Cambridge, MA).

### Passive transfer of Th cells

Cells differentiated as above were harvested 8 or 9 days after restimulation with the OVA peptide in the presence of APCs, washed in sterile PBS, and transferred to recipient mice by i.v. injection in 0.5 ml of PBS. For transfers to unsensitized mice, groups of four animals received  $25 \times 10^6$  or  $5 \times 10^6$  Th1 cells, mock transfers, or  $25 \times 10^6$  or  $5 \times 10^6$  Th2 cells the day before being sensitized with i.p. injections as described above. The mice were challenged 7 days after the booster injection and sacrificed 3 days later. For transfers after challenge, groups of five mice received  $25 \times 10^6$  Th1, mock transfers, or  $25 \times 10^6$  Th2 cells the day after the first challenge. The following day the mice were rechallenged, and 3 days later they were sacrificed.

### Histology

For staining with hematoxylin and eosin (H&E), lungs were inflated and fixed with 10% buffered formalin after BAL cells were collected. Samples were embedded in paraffin, sectioned, and stained with H&E.

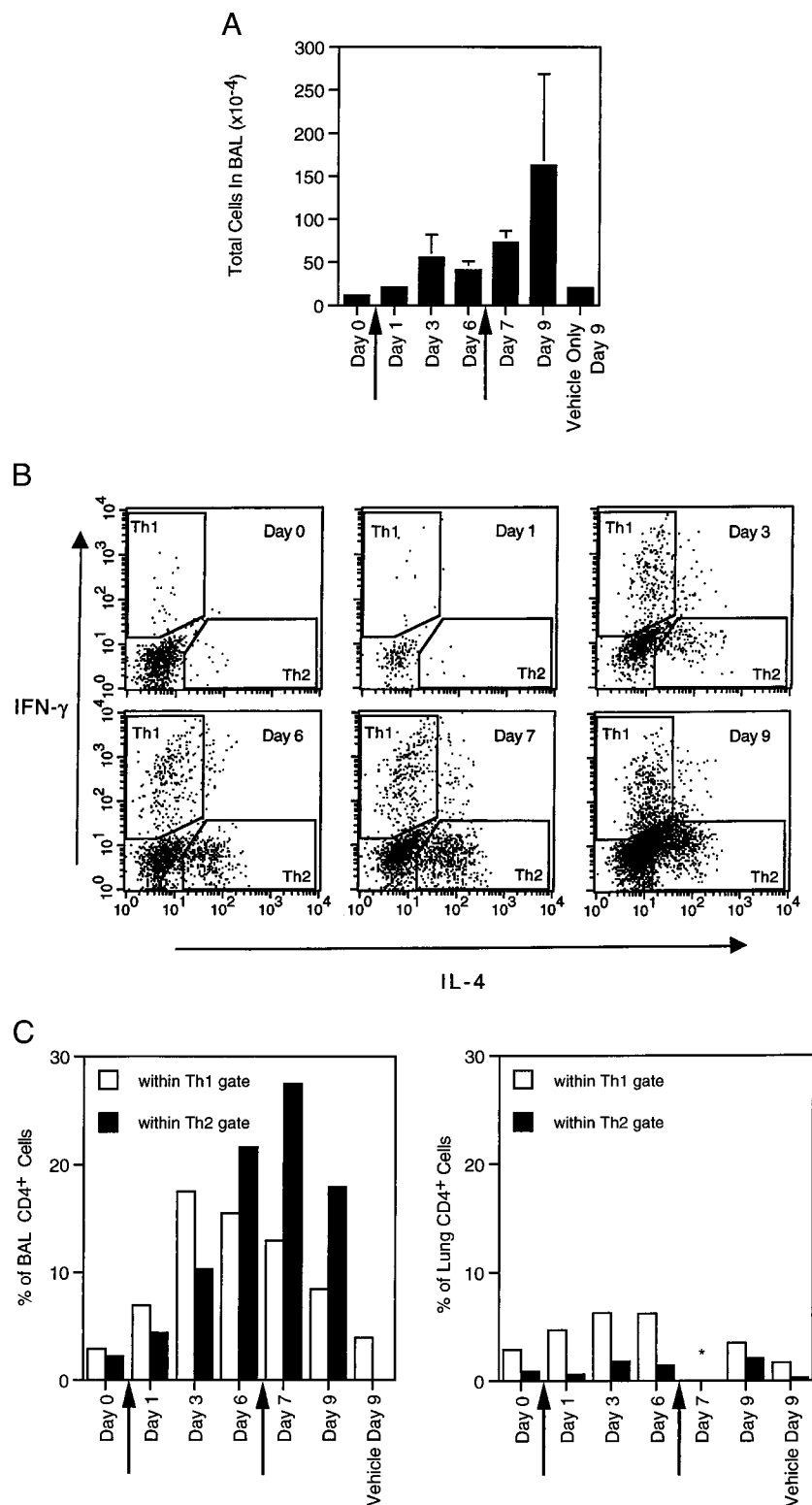
## Results

### Analysis of Th cells and inflammation in OVA-induced airway hypersensitivity

To better define the nature of the infiltrating Th cells in the murine model of asthma, we used intracellular cytokine staining and flow cytometry to analyze the phenotype of CD4<sup>+</sup> T cells in the BAL and lung parenchyma over time following airway challenge. Mice received primary and booster immunizations with i.p. injections of OVA adsorbed to alum in PBS. Control animals were treated with alum alone in PBS. The mice were then challenged on day 0 and day 6 with an aerosol of 1% OVA in PBS. Control mice were challenged with an aerosol of PBS alone. After challenge, mice were sacrificed and BAL fluid and lung tissues were collected. Intracellular IFN- $\gamma$  and IL-4 or IL-5 in BAL cells and purified lung parenchymal T cells were analyzed by flow cytometry.

The total numbers of inflammatory cells increased with time and with repeated challenges in the airways of mice challenged with 1% OVA but not saline (Fig. 1A). The inflammatory cells contained both Th1 and Th2 cells, as defined by intracellular IFN- $\gamma$  and IL-4 staining, respectively (Fig. 1, B and C). Similar results were obtained in three separate experiments. The data were also similar when IL-5 staining was used as a Th2 marker. Interestingly, Th1 cells predominated early in the development of inflammation, but Th2 cells predominated later. Also, the fraction of CD4<sup>+</sup> cells that was producing Th1 or Th2 cytokines was larger in BAL than in the cells recovered from lung tissue (Fig. 1C). This result suggests that cytokine-producing cells are preferentially recruited to the airways compared with the parenchyma.

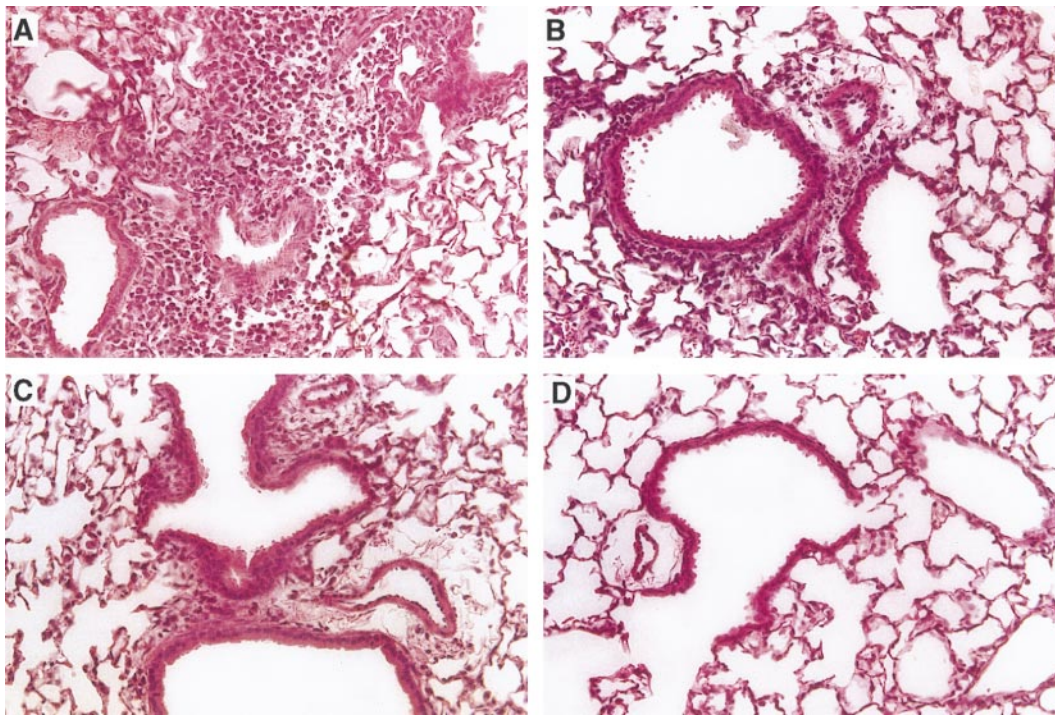
**FIGURE 1.** Analysis of airway inflammation over time. Mice were sensitized with i.p. injections of 8  $\mu\text{g}$  OVA adsorbed to 2 mg alum 15 days and 8 days before challenge. On days 0 and 6, mice were challenged with an aerosol of 1% OVA in saline (arrows). At the indicated times, groups of three mice were sacrificed. BAL fluid was collected by flushing the lungs four times with 2% FCS in saline. *A*, Total cell numbers in BAL increase with time and with repeated airway challenges. The mean number of nucleated BAL cells ( $\pm\text{SE}$ ) is shown. *B*, Analysis of intracellular cytokine expression in BAL  $\text{CD4}^+$  T cells demonstrates that both Th1 and Th2 populations increase in the lung after airway challenge. BAL cells from groups of three mice were pooled and the cells were stimulated for 6 h with PMA and ionomycin in the presence of monensin. One-fourth of the cells were stained with allophycocyanin-anti- $\text{CD4}$ , fixed, permeabilized, stained with PE-anti- $\text{IFN-}\gamma$  and FITC-anti-IL-4, and analyzed by flow cytometry. Plots show all live-gated,  $\text{CD4}^+$  cells in the sample. Very few cytokine-producing cells could be detected at any of the time points without *in vitro* stimulation with PMA and ionomycin. Data shown are representative of three separate experiments. Similar results were obtained when IL-5 was used instead of IL-4 as a marker for Th2 cells. *C*, Cytokine-expressing  $\text{CD4}^+$  T cells are preferentially recruited to the airways compared with the lung parenchyma. The percentages of cytokine-expressing  $\text{CD4}^+$  cells falling within the Th1 and Th2 gates are shown for BAL cells (*left panel*) and lung parenchymal T cells (*right panel*). Parenchymal  $\text{CD4}^+$  T cells were purified from single cell suspensions of lung tissue using magnetic beads, then stimulated and stained for cytokines. \*, Data not available for this sample.



#### Passive transfer of Th1 and Th2 cells before sensitization

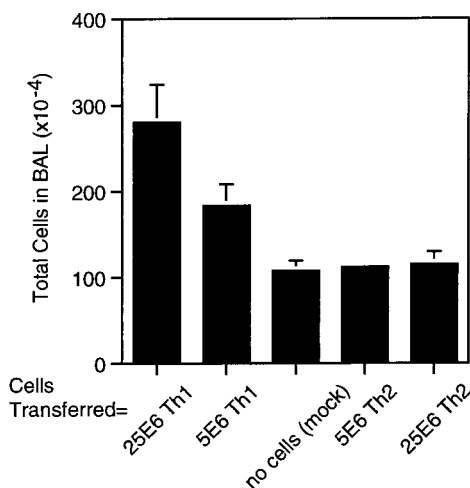
To investigate whether altering the relative balance of Ag-specific Th1 and Th2 cells in mice could modulate the airway inflammatory response, we passively transferred 25 or 5 million DO11 Th1 or Th2 cells into naive BALB/c female mice. The mice were then sensitized and boosted with i.p. injections of OVA adsorbed to alum 14 and 7 days before challenge. On day 0, the mice were challenged with an aerosol of 1% OVA in PBS for 20 min in the

morning and afternoon. On day 3, the mice were sacrificed and BAL fluid, lung tissue, and serum were collected. OVA-sensitized and -challenged mice showed increased levels of serum IgE above unchallenged controls, although the levels were lower in recipients of transferred Th1 cells (OVA-specific IgE levels by ELISA were: unsensitized mice, 0.01 arbitrary units (AU); recipients of  $25 \times 10^6$  Th1 cells, 0.08 AU; sensitized controls, 0.20 AU, recipients of  $25 \times 10^6$  Th2 cells, 0.17 AU). OVA-challenged mice showed



**FIGURE 2.** Lung inflammation in recipients of transferred allergen-specific T cells. Groups of four mice received  $25 \times 10^6$  or  $5 \times 10^6$  Th1 or Th2 cells or mock transfers. The next day, the mice received an i.p. injection of OVA adsorbed to alum followed by a booster injection 1 wk later. One week after the booster, the mice were challenged with an aerosol of 1% OVA in saline. Vehicle control mice received i.p. alum without OVA and were challenged with saline alone. The mice were sacrificed 3 days after challenge and BAL fluid was collected. The lungs were then inflated with 10% buffered formalin, embedded in paraffin, sectioned, and stained with H&E. Shown here are examples of recipients of (A)  $25 \times 10^6$  Th1 cells, (B) a mock transfer, (C)  $25 \times 10^6$  Th2 cells, and (D) a vehicle control. Note the perivascular and subepithelial inflammation in the challenged mice, particularly in the recipient of OVA-specific Th1 cells.

accumulation of inflammatory cells in both the perivascular and subepithelial regions, but the inflammation in the recipients of OVA-specific Th1 cells was much more severe (Fig. 2). This result was accompanied by dramatically increased numbers of cells re-



**FIGURE 3.** Effects of T cells transferred before sensitization on cell numbers in the bronchoalveolar lavage after challenge. T cells were transferred to mice and then the mice were sensitized and challenged as described in Fig. 2. Three days after aerosol challenge, the mice were sacrificed and their lungs were flushed four times with 0.8 ml of saline containing 2% FCS. Nucleated cells in the recovered lavage were counted after lysis of RBC. Data represent the average number of BAL cells ( $\pm$ SD,  $n = 4$  mice per group).

covered by BAL from mice that had received Th1 cells (Fig. 3), whereas mice that received Th2 cells showed total cell numbers indistinguishable from controls. Passively transferred Th2 cells failed to mobilize an inflammatory response in the lungs or airways even when as many as  $10^8$  Th2 cells were transferred (data not shown). The largest increases of infiltrating cells in mice that received Th1 cells were in the lymphocyte and monocyte compartments (Table I). Interestingly, the average number of eosinophils increased as well. Although this increase did not reach statistical significance in this experiment, a trend toward increased numbers of eosinophils was observed in similar experiments, and eosinophils were clearly visible in the lung tissue as demonstrated by eosinophil peroxidase staining and Biebrich scarlet staining (data not shown). In mice that received Th2 cells, the only significant change was a modest decrease in infiltrating lymphocytes. Similar data were obtained in four separate experiments. Thus, transfer of OVA-specific Th1 cells increased the airway inflammation whereas transfer of Th2 cells had little effect.

Under certain culture conditions, Th1 and Th2 populations have been observed to switch phenotype *in vitro* (9). To confirm that the transferred cells survived and were stable *in vivo*, we analyzed BAL and lung T cells by flow cytometry. Before injection, the transferred cells were  $>99\%$  KJ1-26 $^+$  and CD4 $^+$  (data not shown). When stained for intracellular cytokines, the majority ( $>90\%$ ) of Th1 cells expressed IFN- $\gamma$ , but very few were positive for IL-4 or IL-5 (Fig. 4). In contrast,  $<1\%$  of the Th2 cells stained for IFN- $\gamma$ , whereas 43% were IL-4 positive and 35% were IL-5 positive. After transfer and airway challenge, transgenic (KJ1-26 $^+$ ) Th1 and Th2 cells were easily detected in the BAL of recipient mice, indicating that both Th1 and Th2 populations could

Table I. Differential cell counts in BAL of mice that received transferred T cells prior to sensitization<sup>a</sup>

Cells Transferred/Sensitization	BAL Eosinophils ( $\times 10^{-3}$ )	BAL Lymphocytes ( $\times 10^{-3}$ )	BAL Monocytes + Macrophages ( $\times 10^{-3}$ )
$25 \times 10^6$ Th1 cells/OVA	440 (95)	1200 (280)*	1100 (180)*
$5 \times 10^6$ Th1 cells/OVA	650 (230)	480 (62)*	700 (160)
Mock transfer/OVA	360 (38)	210 (17)	490 (42)
$5 \times 10^6$ Th2 cells/OVA	390 (39)	150 (8)	580 (83)
$25 \times 10^6$ Th2 cells/OVA	300 (68)	81 (15)*	770 (160)
Mock transfer/vehicle	2 (2)*	18 (5)	270 (55)*

<sup>a</sup> Mice received transfers of  $25 \times 10^6$  or  $5 \times 10^6$  Th1 or Th2 cells or mock transfers by i.v. injection. The mice were then sensitized by i.p. injection of OVA adsorbed to alum and challenged with an aerosol of 1% OVA in PBS. Vehicle control mice were injected with alum alone and challenged with PBS alone. Three days after challenge, BAL fluid was collected from the mice. Total cells in the BAL were counted by hemacytometer and differential cell counts were performed on cytospins of BAL cells stained with Wright stain. Data shown are the average number of cells in the BAL ( $\pm$ SE; n, four mice per group).

\*  $p < 0.05$  compared to mock transfer/OVA.

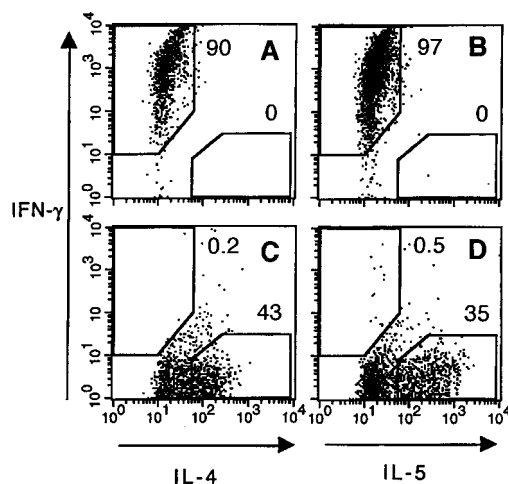
persist in vivo for the length of the protocol, although the total number of transferred Th1 cells recovered was much higher than the number of Th2 cells (Fig. 5). When transgenic Th1 cells were infused, less than 2% of the recovered KJ1-26<sup>+</sup> cells expressed IL-4. Conversely, when transgenic Th2 cells were transferred, less than 2% of the recovered KJ1-26<sup>+</sup> cells expressed IFN- $\gamma$  (Fig. 6, A–C). Therefore, both Th1 and Th2 phenotypes appeared to be stable in vivo throughout the course of the experiments.

Because of the potential for cross-regulation between Th1 and Th2 subsets, we next analyzed the phenotypes of the recruited host BAL T cells. Regardless of whether Th1 or Th2 cells were transferred, the endogenous BAL T cells contained both Th1 and Th2 populations (Fig. 6, D–F). Of interest, infusion of transgenic Th1 cells resulted in increased accumulation of host Th1 cells relative to host Th2 cells in the airways (Figs. 6, D–F, and 7A). However, this did not result in fewer absolute numbers of Th2 cells in the

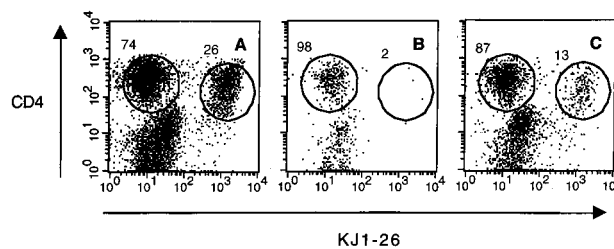
lungs of these mice. Rather, the absolute number of Th2 cells actually increased in Th1 recipients relative to controls (Fig. 7B).

#### Passive transfer of Th1 and Th2 cells after Ag challenge

To test whether Ag-specific Th1 or Th2 cells could alter the allergic inflammatory infiltrate in the airway of an already challenged mouse, we immunized and challenged BALB/c female mice to establish airway inflammation, and 1 day later passively transferred DO11 Th1 or Th2 cells. On the next day, the mice were rechallenged and then sacrificed 3 days later. Addition of Ag-specific Th1 cells early in the course of an airway inflammatory response lead to increased inflammation whereas addition of Th2 cells had little effect (Fig. 8A). In mice treated with Th1 cells, the total numbers of lymphocytes and macrophages increased as shown in Table II. The average number of eosinophils also increased although this did not reach statistical significance in any of three separate experiments ( $p = 0.10, 0.06,$  and  $0.20$ ). Similar to Figs. 5–7, both transferred and host derived BAL CD4<sup>+</sup> T cells were analyzed by flow cytometry. Both transferred Th1 and Th2 cells were detected in the BAL, and both maintained their original phenotype. Again infusion of host Th1 cells resulted in a dramatic increase in recruitment of host Th1 cells, and a lesser increase in the recruitment of host Th2 cells (Fig. 8B). Transferring Th2 cells had little effect, except in one experiment of three in which transferring

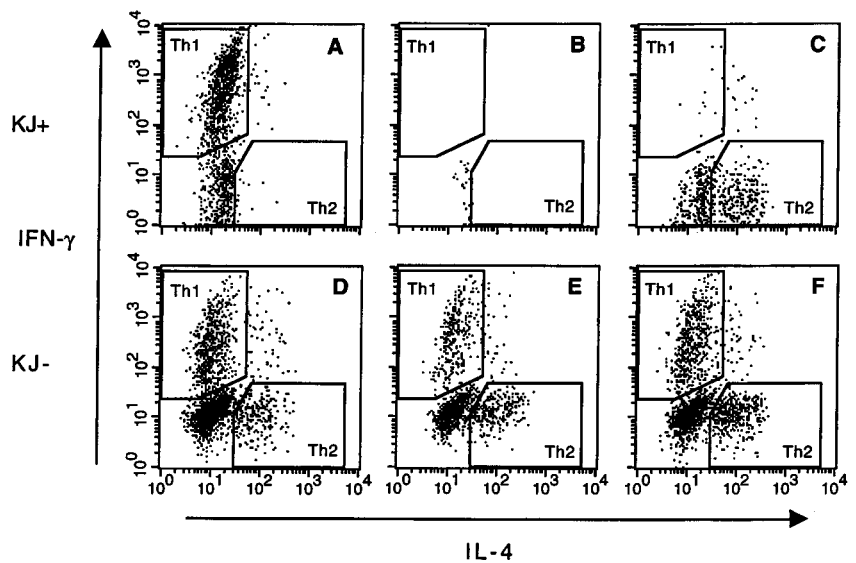


**FIGURE 4.** Intracellular cytokine analysis of cultured OVA-specific Th1 and Th2 cells before transfer. Th1 and Th2 cells were cultured as described in *Materials and Methods*. Seven days after stimulation, aliquots of cells were restimulated for 6 h with PMA and ionomycin in the presence of monensin. The cells were stained with allophycocyanin-anti-CD4, fixed, permeabilized, and stained with PE-anti-IFN- $\gamma$  and FITC-anti-IL-4 (A and C) or with PE-anti-IFN- $\gamma$  and FITC-anti-IL-5 (B and D). Plots show live-gated, CD4<sup>+</sup> cells. Numbers indicate percentages of CD4<sup>+</sup> cells within the adjacent gate. A and B, Th1 cells. C and D, Th2 cells.



**FIGURE 5.** Flow cytometric analysis of transferred and endogenous T cells in BAL of mice after sensitization and challenge. Groups of four mice were treated as described in Fig. 2. Aliquots from pooled BAL cells were stimulated for 6 h with PMA and ionomycin in the presence of monensin. The cells were then stained with anti-CD4 and the clonotypic Ab KJ1-26. As examples, FACS plots of live-gated BAL cells from recipients of  $5 \times 10^6$  Th1 cells, mock transfers, or  $5 \times 10^6$  Th2 cells are shown. Populations of KJ1-26<sup>+</sup> CD4<sup>+</sup> cells are seen in the BAL fluid from mice that received either Th1 or Th2 cells, indicating that the transferred cells have survived in vivo and are present in the lung. The percentages of CD4<sup>+</sup> KJ1-26<sup>-</sup> and CD4<sup>+</sup> KJ1-26<sup>+</sup> cells in the sample are indicated. A, Th1 transfer; B, mock transfer; C, Th2 transfer.

**FIGURE 6.** Cytokine profiles of transferred and endogenous CD4<sup>+</sup> cells. BAL cells from Fig. 5 were fixed, permeabilized, and stained for intracellular IFN- $\gamma$  and IL-4. A–C represent transferred cells (KJ1–26<sup>+</sup>), whereas D–F represent host cells (KJ1–26<sup>−</sup>). Note that the transferred cells maintain their phenotypes *in vivo*, and that both Th1 and Th2 cells are found in the endogenous cells, regardless of the type of cells that was transferred. Data for IL-5 and IFN- $\gamma$  were similar. A and D, Th1 transfer; B and E, mock transfer; C and F, Th2 transfer.



$50 \times 10^6$  Th2 cells resulted in a 50% decrease in total inflammatory cell recruitment (data not shown).

## Discussion

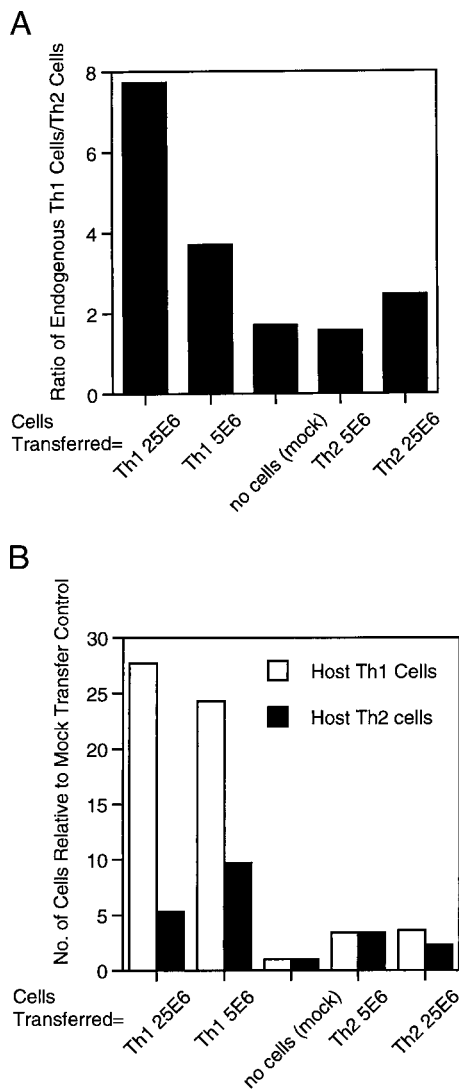
Although the evidence is strong for involvement of CD4<sup>+</sup> T cells in human asthma, the relative contributions of Th1 and Th2 subsets as inducers of the inflammatory response remain undefined. Several studies have suggested a central role for Th2 cells. For example, Robinson et al. (13) used *in situ* hybridization to study cytokine transcripts in BAL cells. Compared with controls, human asthmatics had more cells expressing mRNA for IL-2, IL-3, IL-4, IL-5, and granulocyte-macrophage CSF, but equivalent numbers of cells expressing IFN- $\gamma$  mRNA. Similarly, Bentley et al. (8) used immunohistochemistry and *in situ* hybridization to evaluate cytokine production in human bronchial mucosa after allergen challenge. Airway challenge induced increases in mRNA for IL-5 and granulocyte-macrophage CSF, and to a lesser extent IL-4 and IL-2, but again IFN- $\gamma$  mRNA was not increased. However, other data suggest an inductive role for Th1 cells. For example, in a study using intracellular cytokine staining to evaluate BAL T cell cytokine profiles, asthmatic patients had increased numbers of IFN- $\gamma$ -producing T cells compared with nonasthmatic controls (23). In this study, increased numbers of IL-4-producing T cells were not observed. Also, asthma attacks resulting in visits to hospitals are more likely to be associated with viral respiratory infections, presumably usually enhancing Th1 cytokine production, than with exposure to allergens (2).

Similarly, in mouse models of asthma, the majority of the data supports a critical role for Th2 cells, whereas the role of Th1 cells remains less well defined. At the level of cytokine expression, the Th2 cytokines IL-4 and IL-5 have each been shown to be important. Mice rendered IL-4-deficient either by gene targeting or by administration of neutralizing anti-IL-4 Abs fail to develop either eosinophilic inflammation or airway hyper-reactivity after allergen sensitization and challenge (17–19). Similarly, IL-5 knockout mice develop neither eosinophilic inflammation nor airway hyper-reactivity (16). Additional studies have analyzed the role of cytokines from T cells. Using DO11 T cells differentiated *in vitro* in a fashion similar to ours, Cohn et al. (30) showed that unsensitized mice that received OVA-specific Th2 cells followed by daily aerosol challenges for 7–10 days developed lung inflammation character-

ized by eosinophilia and mucus over-production. Mice that received OVA-specific Th1 cells developed lung inflammation dominated by neutrophils and lymphocytes and without mucus over-production. In another study, Corry et al. (17) demonstrated using enzymatically dispersed lung tissue and an ELISPOT assay that both IL-4- and IFN- $\gamma$ -producing cells were increased after challenge of sensitized animals.

In the studies described here, we tested the potential roles of Th1 and Th2 cells in the OVA-induced model of allergic inflammation. We used intracellular cytokine staining to demonstrate that both Th1 and Th2 cells are recruited to the lung in the OVA model of experimental asthma. Although the percentage of total inflammatory cells that express Th2 cytokines increases as a function of time and with successive challenges, Th1 cells remain in significant numbers. The technique of intracellular cytokine staining is particularly useful in that it can relatively rapidly offer cytokine analyses at the single cell level and with limited possibility for cloning artifacts. It thereby provides a “snapshot” of a T cell population at any given time. However, the technique has some limitations. In particular, it is not easy to determine whether the cytokines detected are the products of cells responding in an Ag-specific fashion. To accumulate easily detectable quantities of cytokine within cells, it is necessary to culture them *in vitro* for several hours in PMA, ionomycin, and monensin. This is thought not to induce Th phenotype differentiation, but rather to elicit increased cytokine production and accumulation from already committed cells; however, increased cytokine production is expected to occur in all Th cells regardless of their Ag specificity. In our experiments, it seems likely that many of the cells in the airway are OVA-specific. We base this judgment on results shown in Fig. 1C in which the T cells recruited to the airways contain a higher proportion of cytokine-producing cells than the parenchymal T cells, and also on experiments with passively transferred cells in which OVA-specific Th1 and Th2 cells were both enriched in the BAL compared with the lung parenchyma. However, it remains a possibility that mature Th1 and Th2 cells are recruited more efficiently than undifferentiated cells regardless of their Ag specificity.

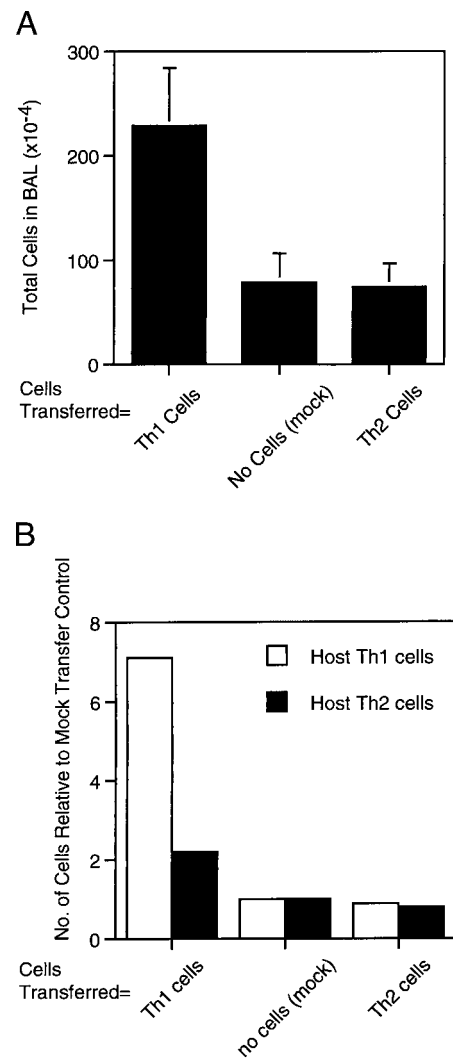
Other investigators have tried to modulate allergic responses with the Th1-promoting cytokines, IL-12 and IFN- $\gamma$ . Using sheep RBCs as the Ag, Gavett et al. (21) showed that intratracheal and



**FIGURE 7.** Analysis of the endogenous T cell response. *A*, Effect of transferred cells on the quality of the endogenous T cell response. The number of cells falling within the Th1 and Th2 gates shown in Fig. 6 were counted for mice receiving  $25 \times 10^6$  or  $5 \times 10^6$  Th1 or Th2 cells or mock transfers. Data are plotted as the ratio of Th1 cells to Th2 cells for each sample. *B*, Effect of transferred cells on total numbers of endogenous Th1 and Th2 cells in BAL relative to controls. Bars represent the number of cells within the Th1 or Th2 gates divided by the number of Th1 or Th2 cells in mice that received mock transfers. To count the total cell numbers, equal volumes of BAL samples were analyzed.

i.p. administration of IL-12 at the time of challenge reduced airway hyper-reactivity and eosinophilia. This coincided with an increase in IFN- $\gamma$  and a decrease in IL-4 mRNA and protein as measured by RT-PCR using total lung RNA and by ELISA using BAL fluid. IL-5 levels were also reduced. In a system of aerosol sensitization and challenge with OVA, Lack et al. (22) showed that nebulized IFN- $\gamma$  could decrease OVA-specific IgE and prevent airway hyper-reactivity even when the IFN- $\gamma$  was administered after sensitization. Furthermore, CD4<sup>+</sup> T cells from IFN- $\gamma$  treated mice were able to inhibit IgE production in vitro.

In these studies, we have additionally tested the ability of transferred Th1 and Th2 cells to modulate the allergic inflammatory response in the airway. Our results show clearly that addition of Ag-specific Th2 cells had little effect on the airway inflammatory



**FIGURE 8.** Evaluation of BAL cells in mice receiving transfers of T cells after airway inflammation was established. Groups of five mice were sensitized and challenged as described in Fig. 1. After challenge, mice received  $25 \times 10^6$  Th1 or Th2 cells, or a mock transfer. Vehicle control mice received no cells, were immunized with alum, and challenged with saline. *A*, Total cell numbers in BAL. Shown are average number of nucleated cells in the BAL ( $\pm$ SD). Vehicle control mice had an average of  $14 \times 10^4$  BAL cells. Similar data were obtained in three separate experiments. *B*, Influence of transferred cells on the total numbers of endogenous Th1 and Th2 cells in BAL. Similar to Figs. 5–7, pooled BAL cells from mice that had received Th1, Th2, or mock transfers were stained and analyzed for CD4, KJ1–26, and intracellular cytokines. As in Fig. 7*B*, the influence of transferred cells on the accumulation of endogenous (KJ1–26<sup>+</sup>) T cells in BAL is shown by plotting the number of endogenous CD4<sup>+</sup> cells within the Th1 or Th2 gates for each sample divided by the number of Th1 or Th2 cells in the mock transfer sample.

response, but that addition of Ag-specific Th1 cells dramatically increased the inflammatory response. This was regardless of whether the cells were transferred before sensitization or after challenge. By gating specifically on endogenous cells in our FACS analysis, we were able to monitor the effect of the transferred cells on the host T cell response. Transferred Th1 cells promoted increased-host Th1 responses (Figs. 7, *A* and *B*, and 8*B*) and partially inhibited OVA-specific IgE production; however, the proinflammatory properties of the Th1 cells apparently outweighed any counter-regulatory effects they may have on Th2 cells, and the end



Table II. Differential cell counts in BAL of mice that received transferred T cells after challenge<sup>a</sup>

Cells Transferred/Sensitization	BAL Eosinophils ( $\times 10^{-3}$ )	BAL Lymphocytes ( $\times 10^{-3}$ )	BAL Monocytes + Macrophages ( $\times 10^{-3}$ )
$25 \times 10^6$ Th1 cells/OVA	420 (110)	1000 (110)*	860 (63)*
Mock transfer/OVA	180 (53)	140 (61)	470 (59)
$25 \times 10^6$ Th2 cells/OVA	130 (39)	76 (15)	540 (130)
Mock transfer/vehicle	0.47 (0.3)*	12 (2)*	130 (9)*

<sup>a</sup> Mice were sensitized by i.p. injections of OVA adsorbed to alum and challenged with an aerosol of 1% OVA in PBS. Vehicle control mice were injected with alum alone and challenged with PBS alone. The day after challenge, mice received transfers of  $25 \times 10^6$  Th1 or Th2 cells or mock transfers by i.v. injection. The mice were rechallenged the following day. Three days after rechallenge, BAL fluid was collected from the mice. Total cells in the BAL were counted by hemacytometer, and differential cell counts were performed on cytospins of BAL cells stained with Wright stain. Data shown are the average number of cells in the BAL ( $\pm$ SE; n, five mice per group).

\*  $p < 0.05$  compared to mock transfer/OVA.

result of transferring Th1 cells was that increased numbers of host cells were recruited to the lung. These data argue that in studies using IFN- $\gamma$  and IL-12 to inhibit airway inflammation, the inhibition seen was not mediated solely by the promotion of Th1 responses. Both IFN- $\gamma$  and IL-12 are pleotropic cytokines and could be altering airway inflammation via several different mechanisms that might involve CD8<sup>+</sup> T cells, NK cells, and/or macrophages.

It is curious that transferring Th2 cells did not increase the number of eosinophils in the Ag-induced inflammatory response. In fact, the only observable effect that the Th2 cells had was to decrease the number of lymphocytes recruited to the BAL. The transferred Th2 cells survive in the recipient animal over the course of the experiment (Fig. 5), and they are still capable of producing cytokines (Fig. 6C). Although it remains possible that transferred Th2 cells could have greater effects on the inflammatory response at later time points after challenge, our data suggest more simply that the generation of Th2 cells may not be rate limiting for the development of eosinophilic inflammation in this model. Perhaps the i.p. sensitization with OVA and alum is sufficiently efficient in generating host Th2 cells that a maximal Th2 effect has already been elicited and that the addition of exogenous Th2 cells cannot increase the response above this maximum. This might also explain the differences between our data and those of Cohn et al. (30). In their system, passive transfer of Th2 cells into unsensitized animals followed by multiple airway challenges resulted in airway eosinophilia whereas transfer of naive T cells followed by the same airway challenge protocol resulted in no inflammation. This suggested that the aerosol challenges were inefficient at priming naive T cells.

In summary, we have shown that both Th1 and Th2 cells are recruited to the lung in the OVA-induced mouse model of asthma. Passively transferred OVA-specific Th1 cells can neither prevent the development of a Th2 response nor extinguish an established Th2 response. In fact, transfer of Th1 cells results in increased inflammation. This finding indicates that Th1 cells are inefficient at inhibiting Th2 responses in vivo, and that in this model the proinflammatory effects of Th1 cells outweigh any counter-regulatory effects they may have on the inflammatory response. These results further suggest a possible mechanism for the role of viral respiratory infections in triggering asthma attacks. In this setting, proinflammatory cytokines from virus-specific T cells could cooperate with allergen-specific Th2 cells without inhibiting them to increase the eosinophilic airway inflammatory reaction. These results also raise a warning flag regarding certain proposed immunotherapies for asthma, because therapies designed to enhance Th1 responses may actually increase airway inflammation.

## Acknowledgments

We thank Teresa Tolley for assistance with histological evaluations and James Verbsky and Michael Holtzman for thoughtful discussions.

## References

- Holtzman, M. J., D. Sampath, M. Castro, D. C. Look, and S. Jayaraman. 1996. The one-two of T helper cells: does interferon- $\gamma$  knock out the Th2 hypothesis for asthma? *Am. J. Respir. Cell Mol. Biol.* 14:316.
- Boushey, H. A., and J. V. Fahy. 1995. Basic mechanisms of asthma. *Environ. Health Perspect.* 103(Suppl. 6):229.
- Kline, J. N., and G. W. Hunninghake. 1994. T-lymphocyte dysregulation in asthma. *Proc. Soc. Exp. Biol. Med.* 207:243.
- Kay, A. B. 1997. T cells as orchestrators of the asthmatic response. *Ciba Found. Symp.* 206:56.
- Krug, N., and A. J. Frew. 1997. The Th2 cell in asthma: initial expectations yet to be realized. *Clin. Exp. Allergy* 27:142.
- Borish, L., and L. Rosenwasser. 1997. TH1/TH2 lymphocytes: doubt some more. *J. Allergy Clin. Immunol.* 99:161.
- Wilson, J. W., R. Djukanovic, P. H. Howarth, and S. T. Holgate. 1992. Lymphocyte activation in bronchoalveolar lavage and peripheral blood in atopic asthma. *Am. Rev. Respir. Dis.* 145:958.
- Bentley, A. M., Q. Meng, D. S. Robinson, Q. Hamid, A. B. Kay, and S. R. Durham. 1993. Increases in activated T lymphocytes, eosinophils, and cytokine mRNA expression for interleukin-5 and granulocyte/macrophage colony-stimulating factor in bronchial biopsies after allergen inhalation challenge in atopic asthmatics. *Am. J. Respir. Cell Mol. Biol.* 8:35.
- Abbas, A. K., K. M. Murphy, and A. Sher. 1996. Functional diversity of helper T lymphocytes. *Nature* 383:787.
- Hsieh, C. S., S. E. Macatonia, C. S. Tripp, S. F. Wolf, A. O'Garra, and K. M. Murphy. 1993. Development of TH1 CD4<sup>+</sup> T cells through IL-12 produced by *Listeria*-induced macrophages. *Science* 260:547.
- Hsieh, C. S., A. B. Heimberger, J. S. Gold, A. O'Garra, and K. M. Murphy. 1992. Differential regulation of T helper phenotype development by interleukins 4 and 10 in an  $\alpha\beta$  T-cell-receptor transgenic system. *Proc. Natl. Acad. Sci. USA* 89:6065.
- Cookson, W. O., and M. F. Moffatt. 1997. Asthma: an epidemic in the absence of infection? *Science* 275:41.
- Robinson, D. S., Q. Hamid, S. Ying, A. Tscopoulos, J. Barkans, A. M. Bentley, C. Corrigan, S. R. Durham, and A. B. Kay. 1992. Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. *N. Engl. J. Med.* 326:298.
- Shirakawa, T., T. Enomoto, S. Shimazu, and J. M. Hopkin. 1997. The inverse association between tuberculin responses and atopic disorder. *Science* 275:77.
- Coyle, A. J., G. Le Gros, C. Bertrand, S. Tsuyuki, C. H. Heusser, M. Kopf, and G. P. Anderson. 1995. Interleukin-4 is required for the induction of lung Th2 mucosal immunity. *Am. J. Resp. Cell Mol. Biol.* 13:54.
- Foster, P. S., S. P. Hogan, A. J. Ramsay, K. I. Matthaie, and I. G. Young. 1996. Interleukin 5 deficiency abolishes eosinophilia, airways hyperreactivity, and lung damage in a mouse asthma model. *J. Exp. Med.* 183:195.
- Corry, D. B., H. G. Folkesson, M. L. Warnock, D. J. Erle, M. A. Matthay, J. P. Wiener-Kronish, and R. M. Locksley. 1996. Interleukin 4, but not interleukin 5 or eosinophils, is required in a murine model of acute airway hyperreactivity. *J. Exp. Med.* 183:109.
- Brusselle, G. G., J. C. Kips, J. H. Tavernier, J. G. van der Heyden, C. A. Cuvelier, R. A. Pauwels, and H. Bluethmann. 1994. Attenuation of allergic airway inflammation in IL-4 deficient mice. *Clin. Exp. Allergy* 24:73.
- Kips, J. C., G. G. Brusselle, G. F. Joos, R. A. Peleman, R. R. Devos, J. H. Tavernier, and R. A. Pauwels. 1995. Importance of interleukin-4 and interleukin-12 in allergen-induced airway changes in mice. *Int. Arch. Allergy Immunol.* 107:115.

20. Coyle, A. J., S. Tsuyuki, C. Bertrand, S. Huang, M. Aguet, S. S. Alkan, and G. P. Anderson. 1996. Mice lacking the IFN- $\gamma$  receptor have impaired ability to resolve a lung eosinophilic inflammatory response associated with a prolonged capacity of T cells to exhibit a Th2 cytokine profile. *J. Immunol.* 156:2680.
21. Gavett, S. H., D. J. O'Hearn, X. Li, S. K. Huang, F. D. Finkelman, and M. Wills-Karp. 1995. Interleukin 12 inhibits antigen-induced airway hyperresponsiveness, inflammation, and Th2 cytokine expression in mice. *J. Exp. Med.* 182:1527.
22. Lack, G., K. L. Bradley, E. Hamelmann, H. Renz, J. Loader, D. Y. Leung, G. Larsen, and E. W. Gelfand. 1996. Nebulized IFN- $\gamma$  inhibits the development of secondary allergic responses in mice. *J. Immunol.* 157:1432.
23. Krug, N., J. Madden, A. E. Redington, P. Lackie, R. Djukanovic, U. Schauer, S. T. Holgate, A. J. Frew, and P. H. Howarth. 1996. T-cell cytokine profile evaluated at the single cell level in BAL and blood in allergic asthma. *Am. J. Resp. Cell Mol. Biol.* 14:319.
24. Krouwels, F. H., B. E. Hol, B. Bruinier, R. Lutter, H. M. Jansen, and T. A. Out. 1996. Cytokine production by T-cell clones from bronchoalveolar lavage fluid of patients with asthma and healthy subjects. *Eur. Respir. J. Suppl.* 22:95s.
25. Busse, W. W., J. E. Gern, and E. C. Dick. 1997. The role of respiratory viruses in asthma. *Ciba Found. Symp.* 206:208.
26. Murphy, K. M., A. B. Heimberger, and D. Y. Loh. 1990. Induction by antigen of intrathymic apoptosis of CD4<sup>+</sup>CD8<sup>+</sup> TCR<sup>lo</sup> thymocytes in vivo. *Science* 250:1720.
27. Kung, T. T., H. Jones, G. K. Adams, S. P. Umland, W. Kreutner, R. W. Egan, R. W. Chapman, and A. S. Watnick. 1994. Characterization of a murine model of allergic pulmonary inflammation. *Int. Arch. Allergy Immunol.* 105:83.
28. Hsieh, C. S., S. E. Macatonia, A. O'Garra, and K. M. Murphy. 1995. T cell genetic background determines default T helper phenotype development in vitro. *J. Exp. Med.* 181:713.
29. Korsgren, M., J. S. Erjefalt, O. Korsgren, F. Sundler, and C. G. Persson. 1997. Allergic eosinophil-rich inflammation develops in lungs and airways of B cell-deficient mice. *J. Exp. Med.* 185:885.
30. Cohn, L., R. J. Homer, A. Marinov, J. Rankin, and K. Bottomly. 1997. Induction of airway mucus production by T helper 2 (Th2) cells: a critical role for interleukin 4 in cell recruitment but not mucus production. *J. Exp. Med.* 186:1737.
31. Tripp, C. S., O. Kanagawa, and E. R. Unanue. 1995. Secondary response to *Listeria* infection requires IFN- $\gamma$  but is partially independent of IL-12. *J. Immunol.* 155:3427.