

Recall Helper T Cell Response

T Helper 1 Cell-resistant Allergic Susceptibility without Biasing Uncommitted CD4 T Cells

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Effector and memory T lymphocytes differ significantly, and there is no experimental evidence that memory cells are sufficient to render an otherwise normal individual susceptible to localized allergic inflammation. Furthermore, nothing is known about the kinetics of memory responses after inhalation of antigen or interplay between an allergen-specific memory helper T (Th) cell Th2 population and uncommitted or competing Th1 cells. To study these processes, T cell receptor-transgenic CD4⁺ effector cells were generated *in vitro*, transferred into naive recipients, and allowed to resume a quiescent state. Inhalation of protein antigen reactivated these Ag-specific Th2 donor cells, leading to allergic pulmonary inflammation and airway hyperreactivity. Susceptibility was correlated with the size of the input Th2 population, but Th1 cells neither enhanced nor reduced inflammation in this model. Importantly, the reactivation of these antigen-experienced cells by inhaled antigen did not skew the cytokine balance of recipient-derived T cells recruited to the lung nor did it inhibit the development of donor-derived Th1 cells from uncommitted antigen-experienced cells that form a normal part of immune responses. These data indicate that a quiescent memory Th2-cell population can create susceptibility to allergic pulmonary inflammation in a manner refractory to inhibition by Th1 cells or endogenous inhibitory mechanisms.

Keywords: mucosal antigen; helper T cell 1 / helper T cell 2; immunoregulation; allergic lung disease

Atopic asthma arises from combinations of genetic predisposition and environmental factors that lead to allergic airway inflammation in response to inhaled antigens (1–4). However, inhaled soluble proteins normally do not cause allergic inflammation or airway hyperreactivity in experimental models (5–8). Instead, they may evoke a release of interleukin (IL)-10 by dendritic cells and lead to tolerance among the antigen-specific T cells (9). In contrast, certain forms of antigen exposure (e.g., parenteral immunization with alum as an adjuvant) induce the helper T cell (Th) 2 cells critical to a first effector phase. Thus, protein inhalation causes allergic airway disease and bronchial hyperreactivity mediated by the Th2-derived cytokines IL-4 and IL-13 through their actions on B lymphocytes, mast cells, smooth

muscle, and epithelia (10–18). IL-5, acting on eosinophils as part of allergic inflammation (19, 20), and IL-9 may also contribute to asthma pathophysiology (21), whereas allergic inflammation can result in airway remodeling and alterations in the antigen-presenting cell (APC) populations represented in the lung (22–24). After this initiation phase of allergic airways disease, the recrudescence of symptoms on exposure to a previous sensitizing antigen is a hallmark of atopic asthma. Indeed, effector/memory-phenotype T cells have been detected in normal and asthmatic airways; nevertheless, relatively little evidence is available about mechanisms that are sufficient for flares of allergic airways diseases.

Although it is likely that some antigen-induced effector T cells survive as a mixture of memory Th1 and Th2 cells after termination of environmental exposure (25–27), specific analyses of the recall responses of memory T cells triggered by inhaled protein and their role in allergic airway diseases are lacking. Recent findings establish that the properties of memory T cells differ substantially from those of activated effectors (28, 29), and thus, it is unclear whether the presence of such memory cells is sufficient to render an individual with otherwise normal lungs susceptible to localized allergic inflammation or hyperreactive to bronchoconstrictors. Moreover, it is not known whether an initial subset of Ag-reactive T cells serves to bias the frequencies of Th1 or Th2 cells developing from naive CD4 cells on later antigen exposures, and the interplay between memory Th1 and Th2 cells is not understood. The ability to investigate such questions in an individual subject is complicated by the existence of several mechanisms that do not require T-cell memory to mediate asthma flares (1, 18, 22–24, 30–32). Thus, an important unanswered question about the pathogenesis of atopic asthma is whether antigen-specific memory T cells can drive an allergic response when all local factors are those of a naive animal at the time of initial antigen inhalation. (Because “memory T cells” are sometimes confused with a steady-state memory-phenotype or memory/effector (CD44^{hi} or CD45Rb^{lo}) population, which comprise a variety of subsets including currently activated effector T cells, this work refers to cells as rested [in the absence of antigen], antigen-experienced T cells and [currently activated] effectors.) To investigate these questions, we have developed a model in which T cell receptor (TCR)-transgenic CD4⁺ effector cells are generated *in vitro*, transferred into naive recipients, and then allowed to resume a quiescent state in which they have been termed a memory T-cell population as opposed to activated effectors (26, 33–35). To determine whether inhaled antigen would lead to tolerance (5–9) or whether instead the rested, antigen-experienced T cells would cause allergic airway inflammation, recipient mice were then exposed to aeroallergen.

METHODS

Animals, Antibodies, and Flow Cytometric Analyses

DO-11.10 mice (BALB/c) transgenic for a TCR recognizing the ovalbumin 323–339 peptide (OVA323–339) were bred in the Vanderbilt

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University mouse facility. Immunocompetent, nonirradiated BALB/c transfer recipients were 4- to 8-week old females from the Jackson Laboratory (Bar Harbor, ME). All mice were maintained in specific pathogen-free conditions using microisolator cages and were used in accordance with regulations after institutional approval. Unless otherwise indicated, monoclonal antibodies, cytokines, and immunofluorescent detection reagents were purchased from B-D Pharmingen (San Diego, CA). Other purified cytokine reagents were rIL-2 (BRMP program, National Cancer Institute, Frederick, MD) and mouse IFN- γ (R&D Systems, Minneapolis, MN). Samples were analyzed using a fluorescence-activated cell sorter-calibur flow cytometer and CellQuest software (Becton Dickinson, San Jose, CA), gating on forward-scatter and side-scatter properties of viable lymphoid cells. For fluorescence-activated cell sorter analysis of intracellular cytokines from lungs and peribronchial lymph nodes, mice were sacrificed 1 day after the final aerosol inhalation, and the lungs and peribronchial lymph nodes were removed separately. Single-cell suspensions were cultured for 6 hours in medium (36, 37) in the presence of Golgi Stop (B-D Pharmingen, San Diego CA), 10-ng/ml phorbol myristate acetate, and 1- μ M ionomycin (Sigma Chemical Co., St. Louis, MO). Immunofluorescent staining was performed using allophycocyanin anti-CD4 and biotinylated anti-clonotypic KJ1-26 monoclonal antibody with streptavidin-PerCP to detect DO-11.10 T cells. Stained cells were then washed, fixed (30 minutes in 4% paraformaldehyde in phosphate-buffered saline [PBS]), permeabilized with 0.1% saponin, and further stained with phycoerythrin-anti-IFN- γ plus fluorescein isothiocyanate-anti-IL-4.

Differentiated CD4⁺ Effector T-cell Populations and Adoptive Transfers

CD4⁺ T cells from spleen and lymph nodes of TCR transgenic mice were isolated by negative selection using anti-CD8 and anti-MHC II microbeads (Miltenyi, Auburn, CA) according to manufacturer's instructions. Naive (CD62L^{hi}) cells were then positively selected using bead-conjugated anti-CD62L monoclonal antibody at an antibody:buffer ratio of 1:50. Of the resultant cells, more than 95% were CD4⁺, CD44^{lo} and more than 90% CD4⁺, CD62L^{hi} by fluorescence-activated cell sorter. These naive cells were cultured at 5×10^6 cells/ml together with APCs from BALB/c nontransgenic mice obtained by panning and plated at a 1:1 ratio with T cells and OVA peptide (1 μ g/ml; Research Genetics, Birmingham, AL). Cells were maintained in medium as described (36, 37). For development of Th1-polarized populations, IFN- γ (20 U/ml), anti-IL-4, (1 μ g/ml), and recombinant IL-2 (10 U/ml) were added (Day 1 and Day 3). For Th2-polarized cells, rIL-4 (10 ng/ml), anti-IL-12 (2 μ g/ml), anti-IFN- γ (1 μ g/ml), and rIL-2 (10 U/ml) were used. After further expansion in medium with IL-2, cells were harvested at Day 7, centrifuged on a Ficoll step gradient, rinsed, and resuspended in sterile PBS for transfer into BALB/c recipients. To measure polarization, a portion of each population was restimulated using BALB/c APCs and peptide (1 μ g/ml of OVA323-339) followed by ELISA to measure IL-4 and IFN- γ in the culture supernatants. To analyze the phenotype of cells recovered from recipient mice, $5-20 \times 10^6$ activated cells were introduced, followed by parking for 24 days. At the time of these transfers, control BALB/c mice received a similar number of naive (CD62L^{hi}) CD4⁺ T cells isolated from syngeneic BALB/c mice. Recovered cells (from a mixture of spleen and peripheral lymph nodes) were analyzed by flow cytometry and by *ex vivo* peptide stimulation followed by measurements of proliferation and cytokine production as described (32, 33, 37). Similar results are obtained when spleen and lymph nodes are analyzed separately. For proliferation assays, cells were plated (3.5×10^5 cells in 100 μ l) in the presence of OVA323-339 peptide for 24 hours, after which tritiated thymidine was added to each well (18 hours) followed by determination of radioisotope incorporation into DNA. To measure the cytokines produced on short-term restimulation of memory and naive T cells, recovered cells were plated (3×10^6 cells in 1 ml) in the presence of OVA peptide (1 μ g/ml) for 24 hours. Supernatants from these cultures were assayed for IFN- γ and IL-4 by ELISA. For ELISA spot analyses, single-cell suspensions were prepared from the lungs and plated (1×10^5 /100 μ l) in 96-well ImmunoSpot M200 plates (Cellular Technology, Cleveland, OH) previously coated capture antibodies for IFN- γ (R4-6A2; eBioscience, San Diego, CA) (100 μ L per well in PBS overnight at 4°C), blocked with PBS-1% bovine serum albumin (Sigma), and washed three times with

PBS. Syngeneic APCs, prepared by treating adherent splenocytes with mitomycin C (50 μ g/ml; 20 minutes at 37°C), were used at 2×10^5 APCs per 100 μ l. Antigen-specific responses were elicited by the addition of OVA323-339 (Research Genetics, Birmingham, AL) at a final concentration of 4 μ g/ml. After incubation (1 day at 37°C), the plates were washed, and biotinylated detection antibodies for IFN- γ (XMG1.2; eBioscience) in PBS-Tween-1% bovine serum albumin were added to the wells for 2 hours, followed by washing and incubation 2 hours with streptavidin-AP (DAKO, Carpinteria, CA). Spots were developed with a nitroblue tetrazolium chloride (Bio-Rad Laboratories, Hercules, CA) and 5-bromo-4-chloro-3-indolyl phosphate (Sigma Chemical Co.) mixture, visualized, and counted on an ImmunoSpot Series 1 Analyzer (Cellular Technology).

Sensitization and Lung Analyses

For induction of allergic airways disease after aeroallergen inhalation, $1-5 \times 10^6$ activated cells were transferred, as indicated, followed by 24 days in the absence of antigenic stimulation. Standard induction of allergic airways disease, anesthesia, lung physiology measurements, and bronchoalveolar lavage were performed as described (36, 37). In brief, BALB/c mice were immunized with OVA (Sigma Chemicals) (10 μ g, precipitated in Al[OH]₃). Two weeks later, a series of eight daily inhalations (40 minutes per day) was started, with mice placed as a group in a chamber whose atmosphere was kept saturated with nebulized OVA solution (1% wt/vol in sterile PBS). As described previously (36, 37), mechanical ventilation and a pressure transducer were used to measure lung resistance in anaesthetized mice at baseline and after each of a series of ascending doses of intravenous methacholine delivered by an indwelling internal jugular catheter. Immunizations with OVA in alum were timed so that sequential inhalations of Ag-containing aerosol and outcome measurements in each set of mice were performed on the same day as recipients of effector T-cell populations. Statistical analyses of differences between groups were performed with InStat software (GraphPad, Sorrentino, CA) using two-tailed *t* testing (unpaired) and post-test analyses of *t* test suitability.

To generate lymphocyte-enriched single-cell suspensions from lungs for intracellular cytokine staining, lungs from two mice were pooled in 2-ml culture medium and forced through a nylon strainer. Resultant suspensions were separated on a Ficoll-Hypaque step gradient from which cells at the interface were rinsed, counted, and used for intracellular cytokine staining. For histopathology, the left lung was excised after bronchoalveolar lavage and placed in 10% phosphate-buffered formaldehyde solution. Fixed lungs were paraffin embedded, cut in 6- μ m sections, mounted, and stained (hematoxylin and eosin, periodic acid-Schiff to assess mucus, a modified Wright-Giemsa stain to evaluate eosinophils). Slides were scored by a pathologist in a manner blind to sample identity, with assessments of alveolar spaces, airways at all levels, interstitium, and vessels (both arteries and veins), as previously described (38). In data not shown, mucus was evaluated as minimal, 1⁺; moderate, 2⁺; or maximal (circumferential), 3⁺. RNAs were measured by multiprobe RNase protection assays using lung RNAs and the mCK-5 kit (B-D Pharmingen) exactly according to instructions.

RESULTS

OVA Inhalation by Naive Mice Bearing a Rested Antigen-specific Th2-polarized Population Induces Eosinophil Recruitment and Airway hyperreactivity

OVA-specific effector T cells were generated from DO-11.10 TCR transgenic mice under conditions directing a portion of the cells into the Th1 or Th2 subsets. Populations containing these Th1 or Th2 effectors were transferred into naive, immunologically normal BALB/c recipients (Figure 1A and Table 1) and deprived of antigen during a 24-day period *in vivo* ("parking") so that a small portion survive and enter a quiescent state as memory T cells (25-29, 33-35). Mice were then exposed to serial inhalations of nebulized OVA in parallel with negative control subjects (no cells transferred) (Figure 1A). In parallel, a standard OVA-sensitization protocol (36, 37) was used as a positive control in additional BALB/c mice (Figure 1A). In all cases, lung

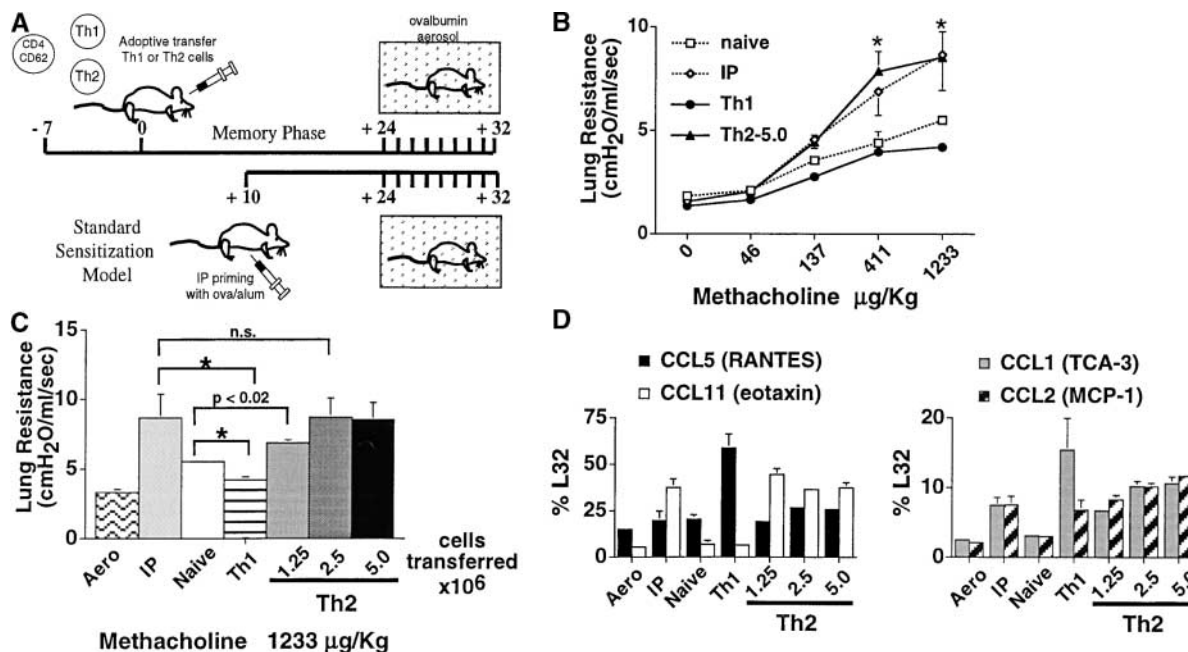


Figure 1. Susceptibility to airway hyperreactivity (AHR) because of a recall response of rested, antigen-experienced, ovalbumin (OVA)-specific Th2 cells. (A) Schematic experimental protocol. T-cell populations were transferred into BALB/c recipients (Day 0). Twenty-four days after cell transfer, mice started a series of eight daily OVA inhalations (Days 24 to 31). A standard sensitization protocol (one intraperitoneal injection of 10-μg OVA in alum) was initiated on Day 10 of the recall experiment, followed by eight sequential OVA inhalation sessions (Days 24–31) together with the mice that received transferred T cells. One day after the last OVA inhalation (Day 32), each mouse underwent measurements of lung resistance to airflow after each of six doses in an ascending dose series of intravenous methacholine injections. (B) Mean $\pm$ SEM lung resistance values after doses in a series of methacholine injections are shown. The means were derived from eight individual mice, in four separate experiments, for each data point; the asterisk represents a difference for which $p < 0.05$ in significance testing. (C) Each bar represents the mean $\pm$ SEM derived by averaging the peak airway response (reproducibly at the dose of 1,233 μg of methacholine per kg body weight) of each of eight mice in four independent experiments (two mice per group in each experiment); p values and the asterisk in the figure indicate that the observed differences were highly significant. (D) A chemokine code distinguishing the recall responses of Th1 versus Th2 populations after allergen inhalation. Naive recipient mice received transfers of the indicated cell populations or no transfer [positive control and negative control subjects as in (B)]. RNAs isolated from the indicated lung samples after OVA inhalation were analyzed using multiprobe RNase protection assays (RPA). Shown are the L32-normalized values for the induced chemokines that differed between Th1 and Th2 samples (in arbitrary image units). IP = intraperitoneal immunization.

resistance was measured in the mice at baseline and after each dose in an ascending series of challenges with a pharmacologic bronchoconstrictor. A statistically significant increase ($p < 0.05$) in airway responsiveness was observed in recipients of 5×10^6 Th2-polarized cells (Figure 1B) as compared with mice that received neither cells nor a priming injection of OVA in alum (Figure 1C, "AERO"). Little airway hyperreactivity (AHR) was observed in similar control experiments, where mice received

naive CD4⁺ TCR transgenic cells 24 days before inhalations of OVA (Figures 1B and 1C). In contrast to the effect of memory Th2 cells, parking OVA-specific Th1 cells did not confer sensitization to inhaled allergen (Figures 1B and 1C). Mice that received Ag-specific Th2 populations developed a dose-dependent increase in hyperreactivity, in that a lesser but statistically significant response at several bronchoconstrictor doses was noted with 1.25×10^6 cells transferred 24 days previously as compared

TABLE 1. SUMMARY OF EXPERIMENTAL GROUPS USED IN THE STUDY

Group	Number of OVA-specific Th1 Cells Transferred	Number of OVA-specific Th2 Cells Transferred	Number of OVA-specific Naive Cells Transferred	Priming	Aerosol
Aero				None	1% OVA
IP				i.p. OVA/alum	1% OVA
Naive			5.0×10^6	None	1% OVA or PBS
Th1	$1.25\text{--}5.0 \times 10^6$			None	1% OVA or PBS
Th2-1.25		1.25×10^6		None	1% OVA or PBS
Th2-2.5		2.5×10^6		None	1% OVA or PBS
Th2-5.0		5.0×10^6		None	1% OVA or PBS
Th1/2-1.25	0.625×10^6	0.625×10^6		None	1% OVA or PBS
Th1/2-2.5	1.25×10^6	1.25×10^6		None	1% OVA or PBS
Th1/2-5.0	2.5×10^6	2.5×10^6		None	1% OVA or PBS

Definition of abbreviations: i.p. = intraperitoneal; IP = intraperitoneal immunization; OVA = ovalbumin; PBS = phosphate-buffered saline; Th = helper T cell.

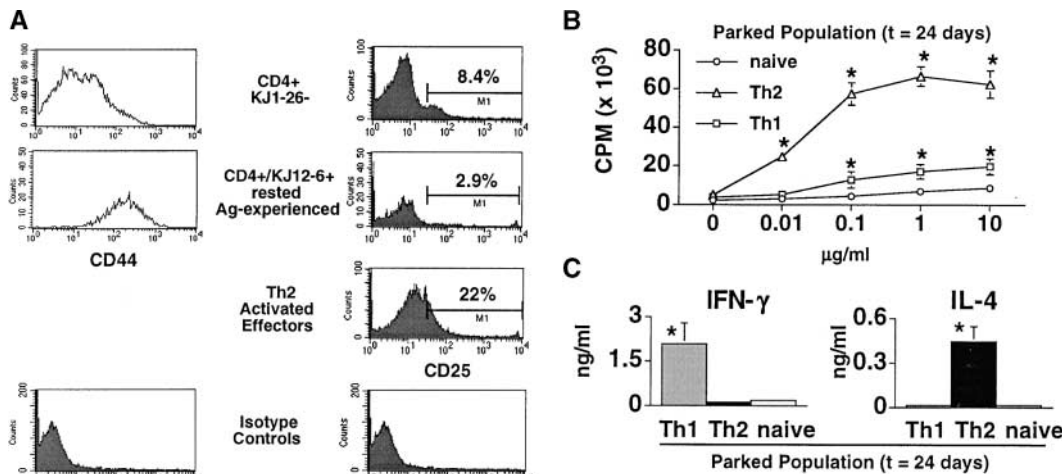


Figure 2. Transferred Th2 cells exhibit enhanced activation and cytokine production 24 days after parking in a naive recipient. Th2 populations (as in Figure 1) were transferred into recipients, as were naive CD4 T cells purified from DO-11.10 TCR transgenic mice. Twenty-four days after cell transfer, cells pooled from spleen and peripheral lymph nodes were analyzed. (A) Surface phenotype of cells stained with CD4-APC, KJ1-26-biotin/streptavidin-PerCP, and CD44-PE (panel set on left) or CD25-PE (panel set on right). Samples were gated on either CD4⁺

cells or CD4⁺/KJ1-26⁺ cells from the same mouse. A population of naive cells was activated and stained in parallel (CD4-APC, KJ1-26-biotin/streptavidin-PerCP and CD25-PE) after 1 week of culture ("polarized Th2 effectors"). Representative data from one of three separate experiments with similar results (two mice per group) are shown. (B) Enhanced proliferative response of rested, antigen-experienced Th2 cells reactivated *ex vivo* as compared with naive T cells. Proliferation of recovered cells (as in A) after restimulation with the indicated concentrations of OVA peptide, as detailed in the METHODS. Data are presented as mean $\pm$ SD from a representative experiment. Increased proliferation of the rested, antigen (Ag)-experienced population relative to naive cells was statistically significant for both the Th1 (* $p < 0.05$) and Th2 populations. (C) Enhanced production of effector cytokines by parked effector populations. Recovered cells (as in A) were restimulated with OVA peptide (1 μ g/ml) for 24 hours. Data shown represent the mean cytokine concentration $\pm$ SEM from three independent experiments. CPM = counts per minute.

with the response observed in recipients of 2.5×10^6 cells. Intracellular staining for IL-4 indicated that approximately 25% of cells in the activated population were of Th2 phenotype (data not shown). Thus, these data indicate that a rested subset derived from $2-3 \times 10^5$ antigen-experienced T cells led to the development of AHR long after antigen withdrawal and was sufficient to bypass whatever mechanisms account for the lack of allergic AHR after protein inhalation by the naive animal (5-9). The patterns of chemokines elicited in lung tissue by recall responses of Th1 and Th2 cells were determined by multiprobe RNase protection assay analyses (Figure 1D). No chemokine RNA induction was observed in the lungs of the recipients of naive OVA-specific T cells when these animals were later subjected to sequential inhalations of OVA. The analysis further showed that although comparable levels of several chemokine mRNAs were induced by Th1 and Th2 recall responses (macrophage inflammatory protein-1 α , -1 β , -2; data not shown), the recall Th2 response led to preferential increases in eotaxin and, to a lesser extent, MCP-1. Although regulated upon activation, normal T cell expressed and secreted and TCA-3 also were induced, these chemokine responses driven by a Th2 memory population were less than those evoked by Th1 memory cells. Together, these findings indicate that the preferential elicitation of eotaxin is a hallmark of the recall Th2 response to inhaled protein, but chemokines associated with Th1 effects are also elicited.

Rested Th1 and Th2 Cells Exhibit a Cell Surface Memory Phenotype and Enhanced Cytokine Responses

To determine whether previously described characteristics of memory T cells (26, 33-35) applied to this model, naive and polarized populations (Th1; Th2) generated from DO-11.10 mice were transferred into BALB/c recipients. Twenty-four days later, splenocytes were stained for the transgenic TCR, CD4, CD44, and CD25. Consistent with other analyses (26, 33-35), only a fraction of the input effector population (5-10%) survived; 90% or more of these CD4⁺/KJ1-26⁺ donor-derived cells were small, CD44^{hi}, and CD25^{lo} (Figure 2A). Similarly, the cells were CD44^{hi}

but negative for the early activation marker CD69 (data not shown). Because the activated effector populations were blastic and positive for CD25 and CD69 before transfer (data not shown), we conclude that the population remaining after the parking phase *in vivo* was consistent with previous characterizations of this population of resting cells. Splenocytes from recipients of effector Th2 cells proliferated at lower peptide concentrations and produced dramatically higher amounts of IL-4 than did freshly isolated naive transgenic cells or recipients of naive cells (Figures 2B and 2C). Parking the Th1-biased population also conferred greater IFN- γ production when compared with an equal number of adoptively transferred naive DO-11.10 T cells (Figure 2C) and a statistically significant increase in proliferation (Figure 2B), albeit one more modest than for Th2 cells (35). Thus, quiescent, antigen-experienced Th2 cells led to allergic airways disease susceptibility in a host free of preexisting pulmonary insult and never previously exposed to the allergen.

Allergic Lung Inflammation Promoted by a Resting Antigen-experienced Population Containing Th2 Cells is Refractory to Concurrent Th1 Population

Antigen-specific Th1 cells develop concurrently with the Th2 subset in the immune response to allergens. To determine whether cells derived from a concomitant Th1 effector population in an earlier response would block the effects of a recall response by memory Th2 cells, OVA-specific Th1 cells were transferred, alone or concurrently, with Th2 cells. There was no significant attenuation of AHR when a Th2 population was accompanied by an equal number of rested Ag-experienced Th1 cells (Table 1), as compared with mice bearing only an Ag-experienced Th2 population (Figures 3A and 3B) (p value not significant). Cell dose-dependent increases in the number of total cells, lymphocytes, and eosinophils in bronchoalveolar lavage fluid were observed for mice that carried rested Ag-specific Th2 cells at the time of aerosol challenge; these increases were not attenuated significantly ($p = NS$) by admixture of a Th1 population (Figure 3C). Increases in bronchovascular inflammation and

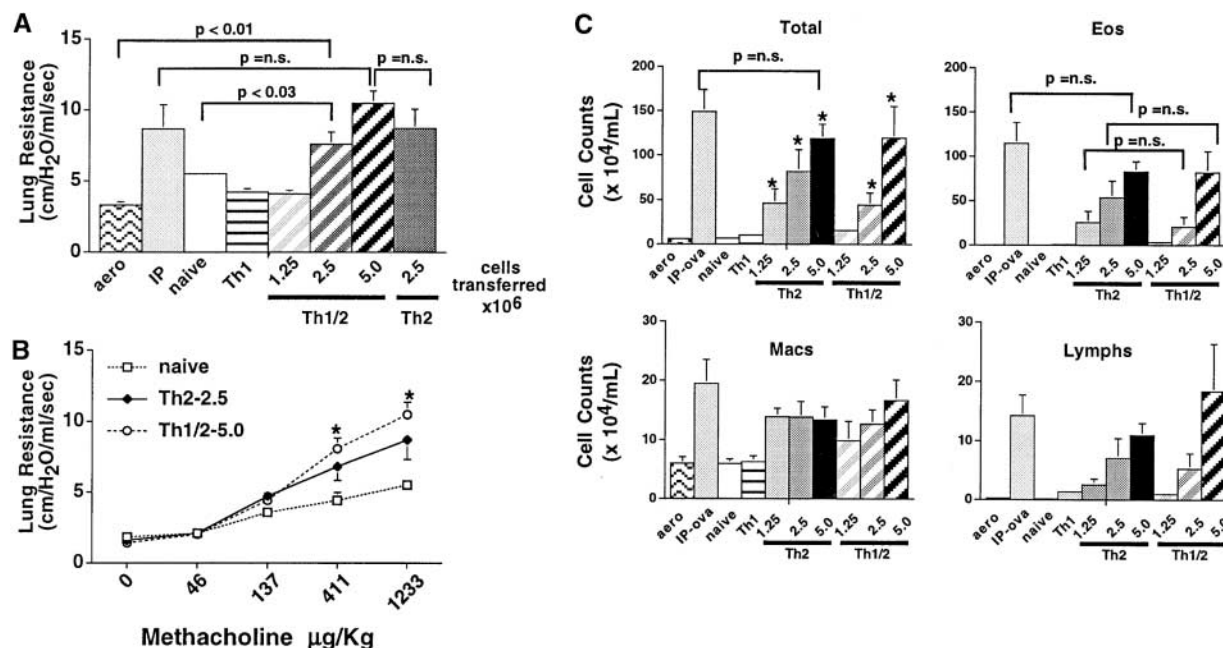


Figure 3. AHR induced by the rested Th2 population is refractory to inhibition by a coexistent Ag-specific Th1 population. Th1 and Th2 populations were transferred separately or together in equal numbers into recipients (5×10^6 , 2.5×10^6 , or 1.25×10^6 total cells per mouse, as indicated; Table 1), treated, and analyzed as in Figure 1. (A) Shown are the mean $\pm$ SEM peak airway responses (reproducibly at the highest dose of methacholine, 1,233 μ g/kg body weight) obtained in four independent experiments (two mice per group in each experiment). Results of statistical testing are shown as p values for comparisons between the indicated groups. (B) Full methacholine dose-response curves for the indicated samples, shown as mean $\pm$ SEM lung resistance values after each dose in a series of methacholine injections (* $p < 0.05$ for naive versus Th2 only and versus Th1/2-5.0; $p = NS$ for Th2 versus Th1/2). (C) Count and composition of cells obtained from bronchoalveolar lavage. Data represent the mean $\pm$ SEM values for the numbers of cells recovered from the airspaces in four separate experiments (two mice per sample per experiment). * $p < 0.05$ for groups compared with aero, naive, and Th1. No eosinophils were detectable in the samples aero, naive, and Th1; the difference from values after Th2-cell transfers was significant ($p < 0.05$), as determined from 95% confidence intervals.

eosinophil infiltration as a function of increasing Th2 cell dose were also observed in histopathology studies (Table 2). In contrast, recipients of naive CD4 T cells transferred 24 days before starting OVA inhalation exhibited no recruitment of eosinophils or lymphocytes measurable in bronchoalveolar lavage fluid, consistent with the lack of chemokine RNA induction (Figure 1D). Periodic acid-Schiff-positive mucus hyperexpression was observed in airway epithelial cells of antigen-challenged Th2 recipients but not control subjects (data not shown). Together, the

data indicate that soluble protein inhalation can reactivate an antigen-specific memory Th2 population and thereby trigger a cascade of events culminating in lung and air space inflammation as well as hyperreactivity to bronchoconstrictor.

Importantly, concurrent introduction of Ag-experienced Th1 cells neither abrogated nor enhanced the ability of rested, OVA-specific Th2 cells to cause allergic inflammation in the recall response. The numbers of eosinophils and other infiltrating cells in the lungs of mice that received a Th2-enriched population of 2.5×10^6 cells 24 days before their first OVA inhalation were similar to those recovered from mice that received a Th1-biased population of 2.5×10^6 cells along with this number of Th2 cells (5×10^6 total cells transferred) (Figure 3C and Table 2). Thus, in contrast to the effect of activated Th1 effector cells or their cytokine IFN- γ on the lung response to activated Th2 effectors (39–42), the allergic inflammation and AHR provoked by the recall response of memory Th2 cells after antigen inhalation was resistant to the memory Th1 population.

Does the Donor-derived Population of Rested Th2 Cells Bias Cytokine Production Profiles of Uncommitted Donor Cells or the Endogenous CD4 Subset?

To characterize the repertoire and functional characteristics of lymphocytes recruited after aerosol inhalation, lung tissue and peribronchial lymph nodes were harvested and analyzed by fluorescence-activated cell sorter after intracellular cytokine staining (Figures 4A and 4B). Time course analyses of the donor-derived (Ag-specific) and endogenous, recipient CD4 T cells (unknown Ag specificity) in lung and lymph nodes showed that although memory CD4 cells were present in both sites before the recall

TABLE 2. QUANTITATION OF INFLAMMATION AND EOSINOPHILS EVOKED AFTER OVALBUMIN INHALATION IN MICE BEARING OVALBUMIN-SPECIFIC MEMORY CD4 CELLS

Group	Bronchovascular Inflammation	Eos	Lymphs
Th1-5.0	0.3	0.0	1.0
BALB/c-IP	2.0	2.2	2.7
Th2-5.0	2.0	2.8	2.8
Th2-2.5	1.8	2.8	2.5
Th2-1.25	1.3	2.0	2.5
Th1/2-5.0	2.0	2.3	3.0
Th1/2-2.5	0.8	1.8	2.3
Th1/2-1.25	0.3	0.3	1.0

Definition of abbreviation: Th = helper T cell.

The left lung was excised and placed in 10% phosphate-buffered formaldehyde and processed; the entire slide was evaluated and then scored as described in the METHODS. Evaluation of eosinophils and lymphocytes was at $\times 40$: none = 0–1 cells/high-powered field (hpf); 1 = 1–3 cells/hpf; 2 = 4–11 cells/hpf; 3 = 12 or more cells/hpf.

response, their number increased in the bronchial lymph nodes after 2 days of antigen inhalation, and progressively increased in lungs from Day 0 through Day 6 (Figures 4A and 4B). Donor-derived memory $CD4^+$ T cells reactivated by inhaled protein retained their capacity to produce the effector cytokine IL-4. Notably, although the Th2 population contained virtually no IFN- γ -producing cells at the time of transfer (data not shown), a small percentage of Th1 cells had emerged during the parking phase and reproducibly increased during the course of the recall response detected in the lung. The OVA-specific endogenous repertoire could not be assayed due to the donor-derived cells, but it is noteworthy that a similar expansion of the Th1 population ($CD4^+$ IFN- γ^+ IL-4 $^-$) was observed among the $CD4^+$ KJ1-26 $^-$ subset recruited to the lung.

The impact of the recall response was also determined in experiments in which the antigen-specific effector population was polarized toward Th1, Th2, or an equal mixture of these subsets (Figure 5). In such experiments, $CD4^+$ KJ1-26 $^+$ cells recovered from the peribronchial lymph nodes maintained their Th1 or Th2 phenotype, producing either IFN- γ or IL-4, respectively, whereas such cells were absent from mice that received no donor cells (Figure 5). Importantly, these data provide additional evidence that donor-derived cells from both the Th1 and Th2 populations persisted in naive recipients long after transfer (Figure 2) (34, 35). Similarly, cotransfer experiments in which each subset was separately labeled showed efficient survival of the Th1-derived donor cells when Th2 cells were cotransferred (Y.H. Zhang and M. Boothby, unpublished observations). Mice that received a Th2-polarized population had a significant population of $CD4^+$ /KJ1-26 $^+$ cells, with many singly positive for either IFN- γ or IL-4 (Figure 5B). The largest difference in cytokine profiles for both donor (KJ1-26 $^+$) and recipient (KJ1-26 $^-$) $CD4^+$ cells was that in IFN- γ producing Th1 cells (Figures 5B and 5C).

Even when an OVA-specific memory Th2 population was present at the time of allergen inhalation, many of the uncommitted donor lymphocytes became IFN- γ -producing Th1 cells. This finding was especially true for cells that were recruited to the lung, inasmuch as the population of restimulated cells from peribronchial lymph nodes maintained a bias toward Th2 cells without a substantial Th1 subset (Figure 5, row A vs. row B). T cells from the naive, endogenous ($CD4^+$, KJ1-26 $^-$) repertoire (presumably expressing multiple antigen specificities) were acti-

vated, trafficked to the lung, and developed a Th1 cytokine profile despite the concurrent reactivation and recruitment of rested donor Th2 cells (Figure 5, row C, second panel). Indeed, donor-derived Th2 recall caused relatively little increase in the frequency of Th2-phenotype cells in the host-derived, polyclonal repertoire as inferred from the frequencies of IL-4 $^+$ cells in the Th1 and Th2 panels (Figure 5, row C). Only approximately 10% of the donor-derived T cells in the lung were IL-4 positive (Figures 4 and 5), indicating that a recall response in which few of the pulmonary $CD4$ cells were Th2 lymphocytes was sufficient for AHR and allergic inflammation. The finding that many donor Th2-derived $CD4$ T cells in the lung exhibited a Th1 cytokine profile after a recall response to inhaled antigen was reinforced by ELISPOT analyses of the frequency of peptide-specific Th1 (IFN- γ -producing) cells in the lung after OVA inhalation (Figure 6). Together, these findings show that aeroantigen inhalation caused the reactivation and pulmonary recruitment of donor-derived memory cells after 4 days. However, the memory Th2 population, although necessary and sufficient to engender allergic inflammation and AHR, did not inhibit the emergence of polarized Th1 cells or those that arise from the antigen-specific (donor-derived) repertoire.

DISCUSSION

Mucosal exposures (inhalation and ingestion) are the main routes by which individuals first encounter protein antigens. In considering the origins of allergic lung inflammation, it is noteworthy that these routes lead to inhibition of allergic responses when the lymphocyte repertoire consists of resting T cells (5–9). These findings show that unlike naive OVA-specific $CD4^+$ cells, a small, resting memory Th2 population is sufficient to induce eotaxin mRNA, allergic pulmonary inflammation, and AHR after antigen inhalation. The overall size of the memory pool that is created in our model system using adoptive transfer is similar to population estimates based on counting antigen-reactive T cells in the memory phase of a normal, endogenous repertoire after immunization or virus infection (25, 27, 43–46). This indicates that a resting pool of approximately 2×10^5 OVA-specific cells, of which perhaps 50,000 were antigen-experienced cells committed to IL-4 production, led to AHR after inhalation of antigen and activation of a recall response of the quiescent pool.

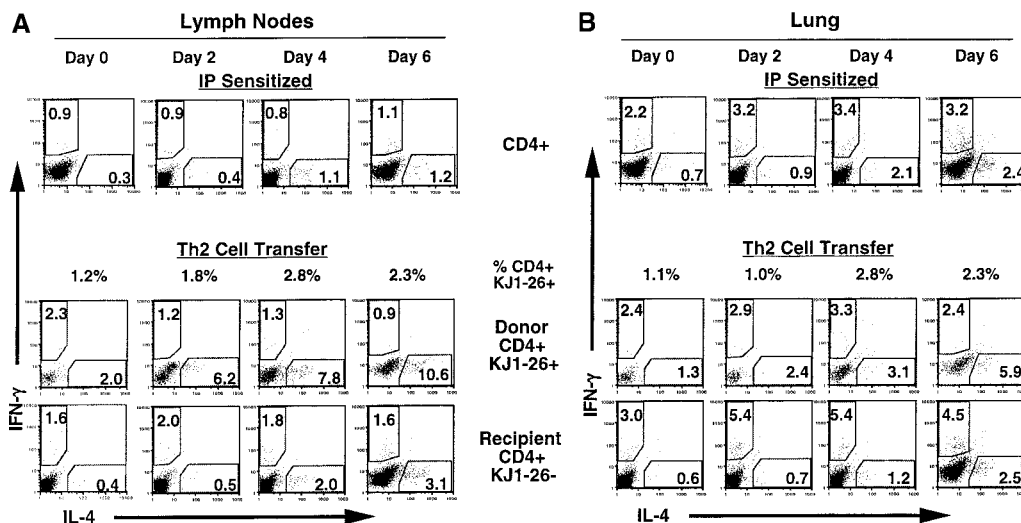
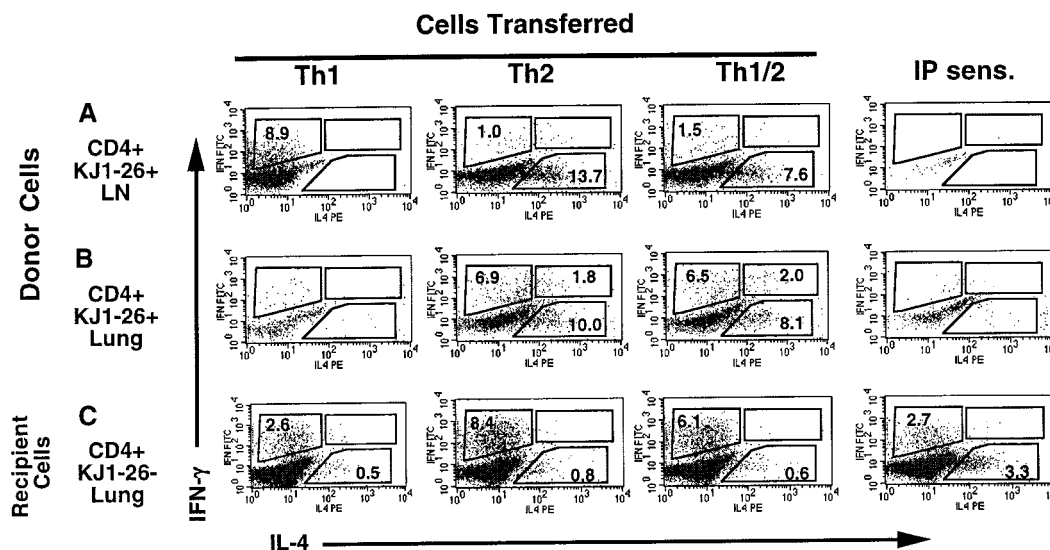


Figure 4. Time course of T-cell recruitment to lung and cytokine production profiles of donor and recipient $CD4$ T cells in the recall response elicited by protein inhalation. Th1 or Th2 populations were transferred into nonirradiated BALB/c recipients, which were then subjected to OVA inhalations for the indicated number of days starting 24 days later. In parallel, a standard sensitization protocol was employed (as in Figure 1A and the METHODS). One day after the last antigen inhalation, the peribronchial lymph nodes (LNs) (A) and lungs (B) were processed for intracellular cytokine staining. Data shown are representative of two independent experiments (two or more mice per recipient group in each experiment). Also shown are the percentages of the cells that were donor derived (KJ1-26 $^+$ $CD4^+$) at each time point. Total cell recoveries in the lungs of Th2 recipients (in millions) were 1.0 (Day 0), 0.8 (Day 2), 5.8 (Day 4), and 19 (Day 6). IL = interleukin.

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two independent experiments (two or more mice per recipient group in each). Mean recipient (R) and donor (D) CD4⁺ cell numbers recovered from lungs, respectively, were as follows (in thousands): Th1 recipients, 366 (R) and 5 (D); Th2 recipients, 361 (R) and 35 (D); mixed Th1/Th2 recipients, 462 (R) and 51 (D); conventional intraperitoneal sens, 367 (R) and fluorescence-activated cell sorter background. Total cell recoveries (in millions) were 1.2 (Th1), 1.4 (Th2), 1.8 (Th1 + Th2), and 1.3 (intraperitoneal/inhalation control, IP sens).

Notably, this recall response can provoke an episode of allergic lung inflammation even in an individual with no preexisting lung pathology, prior deposition of antigen in the lung, or pool of IgE-producing memory B cells specific for allergen. An implication of our data is that whatever tolerance induction is established when a naive animal inhales soluble protein antigen as its first encounter is bypassed if a small population of allergen-specific memory Th2 cells has been established through some prior immunologic experience. Intriguingly, this capacity of memory Th2 cells as compared with naive, antigen-specific CD4 T cells is the opposite of what has recently been found for graft-versus-host disease, in which memory T cells appeared incapable of causing disease, whereas naive cells could do so (47).

Transfers of activated, cycling CD4⁺ Th2 effectors generated *in vitro* can cause allergic inflammation and AHR when protein antigen inhalation is initiated immediately after the transfer of supraphysiologic numbers of Th2 cells (13, 14, 48). However, the requirements for reactivation of rested, antigen-experienced CD4⁺ T cells differ from those of activated, cycling populations of Th2 cells, and there are substantial molecular differences between these subsets (resting, memory-phenotype cells vs. activated effectors) (28, 29, 49, 50). This model of the recall response of quiescent memory T cells in allergic asthma has revealed the following points: (1) In contrast to findings when activated

effectors were immediately restimulated by antigen inhalation (39–42), a mixed population of antigen-specific Th1 and Th2 memory cells generated a recall response with AHR and allergic inflammation equal to that without Th1 effectors. (2) Inhaled antigen induced a recall response from a resting, Ag-experienced Th2 population without biasing the uncommitted cells, which comprised the great majority of the lymphocytes recruited to the lung, and (3) induction of RANTES, a chemoattractant for eosinophils, was triggered by the recall response of both memory Th1 and Th2 populations, yet eosinophil recruitment was observed only for the Th2 population. In addition to the fact that only the memory Th2 provides IL-5 delivery to the lung, the key discriminant between the chemokine induction pattern of the two recall responses (Th1 versus Th2) was the level of eotaxin mRNA.

An initially small population of allergen-specific Th2 cells could, in principle, mediate an atopic diathesis if this small population, when reactivated, biased later responses of uncommitted cells. Such a model of how T-cell populations could regulate disease susceptibility would be analogous to the pathophysiology of *Leishmania major* infection (51–53). In such a pathophysiologic process, uncommitted T cells of all Ag specificities would be prevented from becoming Th1 cells and exhibit an increased probability of becoming Th2 lymphocytes (51–54). A key finding of this study is that no such amplification of the Th2 phenotype among activated but uncommitted CD4⁺ T cells and no inhibition of Th1 differentiation were observed as an effect of the reactivated memory Th2 population (Figure 5C; data not shown). Instead, there was robust Th1 development from uncommitted CD4⁺ T cells (Figures 5B and 5C). During normal immune responses, the majority of activated antigen-specific CD4⁺ T cells appear not to commit to any polarized program of effector cytokine production (55, 56). When these uncommitted CD4⁺ lymphocytes regain a quiescent state, they are proposed to contribute to a “central memory” compartment in which they retain the capacity to differentiate into either Th1 or Th2 cells after reactivation. Fully committed Th1 and Th2 cells do not reverse their commitment (57, 58), and there were no IFN-γ-producing donor cells at the time of transfer (Figure 2; data

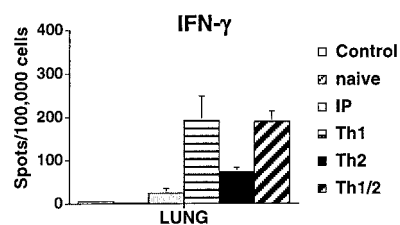


Figure 6. Antigen-specific IFN-γ production by lung lymphocytes is refractory to the recall response of a memory Th2 population after allergen inhalation. Frequencies of OVA323–339 peptide-specific, cytokine-producing cells in lung

dispersions from experimental sets (as in Figure 5) were measured by cytokine ELISA spot assays in duplicate wells. Results are presented as mean frequencies ± SEM of Ag-specific spots (peptide-induced minus background with no peptide) from a minimum of two mice per group.

not shown). Therefore, the KJ1-26⁺ Th1 cells must have arisen from activated but uncommitted donor cells in the transfer pool (56). As resting, uncommitted Ag-experienced T cells, it is likely that the CD4⁺ KJ1-26⁺ IFN- γ ⁺ cells in the lung after OVA inhalation reflect *in vivo* differentiation of central memory cells. As such, an inherent tendency toward IFN- γ -producing IL-4⁻ cells appears to influence the population of CD4 T⁺ cells, which appear in the lung after inhalation of this protein antigen in a memory response, a possibility that has been proposed on the basis of experiments with lung-derived APCs (32, 59). This admixture of Th1 and Th2 cells and the expansion of the IFN- γ -producing population match the findings from studies of asthma flares in humans (60–62).

It will be intriguing to specify further the mechanistic basis by which prior Th2 differentiation after antigen experience permits quiescent T cells to resist the processes by which inhaled antigen inhibits allergy-promoting responses (5–9). One proposed mechanism of inhibition is that regulatory T cells suppress the progress of allergic inflammation (63). Recent work suggests that, at least for memory CD8 T cells, recall responses can be blocked by regulatory populations (64); other recent work suggests that the CD4⁺CD25⁺ regulatory subset may simply divert some Th2 cells while enhancing Th1-mediated neutrophilic inflammation (65). In our experiments, such regulatory T-cell populations are similar to those confronting naive T cells because nonirradiated, wild-type mice not previously exposed to antigen were used as recipients. However, other recent work suggests that the CD4⁺CD25⁺ regulatory subset does not inhibit AHR even when attenuating Th2 effector responses (66). Instead, differences between naive and resting memory T cells in their requirement for costimulatory molecules (34) or their patterns of localization and recirculation (55), or other molecular changes that distinguish naive and memory T cells (28, 29), may be the reason that a recall Th2 response to inhaled antigen leads to allergic AHR, whereas a primary response of naive T cells does not.

Conflict of Interest Statement: M.A.A. has no declared conflict of interest; S.M. has no declared conflict of interest; S.S. has no declared conflict of interest; D.M. has no declared conflict of interest; M.G. has no declared conflict of interest; J.R.S. has no declared conflict of interest; M.B. has no declared conflict of interest.

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