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Allergen-Specific Th1 Cells Counteract Efferent Th2 Cell-Dependent Bronchial Hyperresponsiveness and Eosinophilic Inflammation Partly Via IFN-γ[1]

Tung-Jung Huang,*† Paul A. MacAry,‡ Paul Eynott,* Artin Moussavi,‡ Kenn C. Daniel,‡ Philip W. Askenase,* David M. Kemeny,‡ and K. Fan Chung[2]*

Th2 T cell immune-driven inflammation plays an important role in allergic asthma. We studied the effect of counterbalancing Th1 T cells in an asthma model in Brown Norway rats that favors Th2 responses. Rats received i.v. transfers of syngeneic allergen-specific Th1 or Th2 cells, 24 h before aerosol exposure to allergen, and were studied 18–24 h later. Adoptive transfer of OVA-specific Th2 cells, but not Th1 cells, and OVA, but not BSA exposure, induced bronchial hyperresponsiveness (BHR) to acetylcholine and eosinophilia in a cell number-dependent manner. Importantly, cotransfer of OVA-specific Th1 cells dose-dependently reversed BHR and bronchoalveolar lavage (BAL) eosinophilia, but not mucosal eosinophilia. OVA-specific Th1 cells transferred alone induced mucosal eosinophilia, but neither BHR nor BAL eosinophilia. Th1 suppression of BHR and BAL eosinophilia was allergen specific, since cotransfer of BSA-specific Th1 cells with the OVA-specific Th2 cells was not inhibitory when OVA aerosol alone was used, but was suppressive with OVA and BSA challenge. Furthermore, recipients of Th1 cells alone had increased gene expression for IFN-γ in the lungs, while those receiving Th2 cells alone showed increased IL-4 mRNA. Importantly, induction of these Th2 cytokines was inhibited in recipients of combined Th1 and Th2 cells. Anti-IFN-γ treatment attenuated the down-regulatory effect of Th1 cells. Allergen-specific Th1 cells down-regulate efferent Th2 cytokine-dependent BHR and BAL eosinophilia in an asthma model via mechanisms that depend on IFN-γ. Therapy designed to control the efferent phase of established asthma by augmenting down-regulatory Th1 counterbalancing mechanisms should be effective. ***The Journal of Immunology,*** **2001, 166: 207–217.**

Asthma is an allergic inflammatory disease of the airways that is increasing throughout the westernized world. A central feature of asthma is bronchial hyperresponsiveness (BHR)[3] to airway challenge with nonspecific bronchoconstrictor stimuli. Recent findings in asthmatics and animal models point to a crucial role of T cells in mediating many manifestations of asthma, including BHR. Asthma patients have increased numbers of activated CD25$^+$ and HLA-DR$^+$ T cells in the bronchial mucosa that correlate with disease severity (1). Moreover, local recruitment of the Th2 subset of T cells may cause asthma, since airway CD4$^+$ T cells express a Th2 cytokine profile that can increase BHR and induce airway eosinophilia (2, 3). Animal models of asthma support these ideas in that actively sensitized and airway-challenged Brown Norway rats express Th2 and not Th1 cytokines in the lung (4, 5), and transfer of allergen-specific CD4$^+$ T cells into naive allergen-challenged recipients causes BHR and eosinophilic inflammation (6, 7). Furthermore, mice with deficiencies of Th2 cytokine expression induced by gene deletion, or by treatment with Th2 cytokine antagonists, have diminished BHR and eosinophilic inflammation (8–11).

Regulation of this effector Th2 asthmatic response by counterbalancing Thl cells has been the aim of several forms of therapy. Such Thl antagonism of Th2 effects is widely established in other related systems, such as IgE production and resistance to parasites, and is the preferred mode of deviating Th2 responses (12). One clinical example is the beneficial effects of allergen immunotherapy in modulating Th2 diseases, such as allergic rhinitis and IgE anaphylaxis resulting from insect stings, which may be due to induction of counterbalancing Thl responses (13). Such down-regulation of Th2 cells by Th1 cells is the goal of new immunotherapy procedures to combat allergic diseases and asthma, by employing DNA vaccines encoding allergens, along with bacterial CpG adjuvant sequences to promote Th1 responses (14, 15).

Animal models confirm this paradigm. Enhancement of the Thl pathway at immunization, by providing Thl cytokines such as IL-12 and IL-18 diminished the development of allergic asthma (16–18), and infection with Thl-promoting mycobacteria (19) or *Listeria* (20) attenuated allergy and asthma by inhibiting Th2-immune responses. In fact, a prominent theory about the current epidemic of asthma postulates that several Thl influences, such as bacterial infections, have diminished recently in affluent countries, allowing unbalanced Th2 reactivity and consequent development of allergic asthma (21). However, there is as yet no direct evidence in allergic asthma that specific Thl cells down-regulate the asthma-promoting Th2 effector cells responsible for established asthma.

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[3] Abbreviations used in this paper: BHR, bronchial hyperresponsiveness; ACh, acetylcholine; BAL, bronchoalveolar lavage; PC_{200}, provocative concentration of aerosolized ACh needed to increase lung resistance by 200% above the baseline; R_L, lung resistance; DAPI, 4,6-diamidino-2-phenylindole hydrochloride.

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The Brown Norway rat is a useful model for studying Th2 responses such as helminthic parasite infestations (22), IgE production (23), drug allergies (24), and asthma. Sensitization with OVA followed by OVA airway challenge leads to eosinophilic airway inflammation, local infiltration of activated Th2 cells, and BHR (4, 5, 25). The recent ability to develop allergen-specific, skewed Thl and Th2 T cell lines in rats (26, 27) provided the opportunity to examine the potential modulatory activity of Thl cells on efferent Th2 asthma. We report that OVA-specific Th2 cells induce IL-4 mRNA expression in the lung and transfer BHR and eosinophilic inflammation. Importantly, coadministration of OVA-specific Thl cells suppressed BHR, BAL eosinophilia, and IL-4 mRNA expression, and suppression was allergen specific. Furthermore, treatment with anti-IFN-γ Ab partially reversed Th1 down-regulation, suggesting that suppression of the effector phase of Th2 cell-mediated asthma depends in part on this prototypic Thl cytokine.

Materials and Methods

Animal sensitization and preparation of Th1 and Th2 cell lines

Pathogen-free inbred male Brown Norway rats (Harlan Olac, Bicester, U.K.; 200–250 g, 9–13 wk old) were immunized on day 0 by i.p. injection of 1 ml of 100 μg OVA (grade V, salt-free; Sigma, Dorset, U.K.), or with 100 μg BSA (Sigma) in 0.9% saline in 100 mg $Al(OH)_3$ suspension (BDH, Dorset, U.K.) on 3 consecutive days. On day 14, the rats were killed by lethal exposure to CO_2 and their parathymic and posterior mediastinal lymph nodes were removed aseptically into sterile PBS. Cell suspensions were obtained by pressing tissues through 70-μm nylon filters (Becton Dickinson, Cowley, U.K.) into chilled HBSS, washed three times in sterile PBS, and viable cell numbers were determined by trypan blue dye exclusion. Mononuclear cells were obtained by centrifugation over rodent Lymphoprep 1.077 (Nycomed, Denmark). $CD4^+$ T cells were isolated by positive selection by mixing with mouse anti-rat CD4 mAb (OX35; AMS Biotechnology, Whitney, U.K.)-coated magnetic beads generated as follows. Anti-mouse IgG beads (Dynabeads M450, sheep anti-mouse IgG; Dynal, Wirral, U.K.) previously were coated overnight with mouse anti-rat mAbs to CD4 (OX35) (Serotec, Oxford, U.K.) at 2 μg/ml in PBS/0.1% BSA at 4°C. The beads were washed four times in PBS/0.1% BSA, and 4.8×10^7 beads were added to 1.5×10^8 washed mononuclear cells in 2 ml PBS/0.1% BSA and incubated together at 4°C on a rolling mixer for 45 min. The attached CD4 cells were collected using a magnetic particle concentrator (Dynal) and then washed in PBS/0.1% BSA. Aliquots of the CD4 cells were saved and allowed to detach from the magnetic beads in RPMI 1640/10% FCS at 37°C overnight. The purity of these cells was then assessed by FACScan (Becton Dickinson, San Francisco, CA). The CD4 cells were consistently >95% pure as assessed by staining with PE-labeled anti-CD4 (w3/25) and also >95% cells stained with anti-rat CD3 (G4.18) (both mAbs from AMS Biotechnology). Purified $CD4^+$ T cells then were cultured at $1–2 \times 10^5$/ml with irradiated syngeneic lymph node cells as APCs with 100 μg/ml OVA for 7 days.

Then Th1 cells were generated in culture medium (50% AIM V serum-free medium, 50% Dulbecco's medium, 50 μM 2-ME, 2 mM L-glutamine, 1% sodium pyruvate and 1% nonessential amino acids, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B; all from Life Technologies, Paisley, Scotland) by adding IFN-γ (50 ng/ml; BioSource International, Watford, U.K.), IL-2 (50 U/ml; Euro-Cetus, Harefield, U.K.), anti-rat IL-4 (OX81 supernatant 1:100, from D. Mason, Oxford University, U.K.), and fresh APCs. Th2-like cells were generated by adding IL-4 (1:100 supernatant from rat IL-4 cDNA-transfected Chinese hamster ovary cell line, a kind gift from N. Barclay, Medical Research Council Cellular Immunology Unit, Oxford, U.K.) (28), and mouse anti-rat IFN-γ IgG1 Ab (DB1, 20 ng/ml; Serotec) (26). Every 7 days, cells were harvested, washed, and fresh APCs (normal irradiated Brown Norway splenocytes at a ratio of 2:1 for every cultured lymphocyte) along with the same cytokine and Ab mixture, in combination with IL-2, were added. The Ag specificity and cytokine phenotype of the resulting subpopulations were tested at 2 wk, respectively, by OVA-specific in vitro proliferation or incubation with APCs to derive supernatants for cytokine analysis.

ELISA quantitation of rat IFN-γ and IL-4 was measured in supernatants from T cell lines stimulated for 48 h with 100 μg/ml OVA and irradiated splenocyte APCs. ELISA microtiter plates (Nunc MaxiSorp; Life Technologies) were coated overnight with anti-rat IFN-γ mAb (DB1; BioSource International; 3 μg/ml in 0.1 M carbonate/bicarbonate buffer, pH 9.6). Then plates were washed in 0.05% PBS/Tween 20, and samples for assay and rat IFN-γ standards (BioSource International) were added and incubated for 2 h at 25°C. Then after washing the plates, rabbit anti-IFN-γ antiserum (from John Tite, Glaxo Wellcome, Ware, U.K.) was added at 1/1000 in PBS containing 1.5% rat serum and 0.5% Tween 20 for 1 h, followed by further washing and addition of goat anti-rabbit IgG mAb-alkaline phosphatase (1/10,000; Sigma) in the same diluent for 1 h. Then color was developed using 0.5 mg/ml *p*-nitrophenyl phosphate substrate in 0.05 M diethanolamine buffer (pH 9.8). The limit of detection was 0.25 ng/ml IFN-γ. The rat IL-4 was measured by an ELISA kit from BioSource International (supplied by Lifescreen, Watford, Herts, U.K.) according to the manufacturer's protocol. Briefly, microwell strips coated with anti-IL-4 were incubated with samples vs recombinant rat IL-4 standard, followed by biotinylated second mAb. After washing, avidin-peroxidase enzyme was added and color was developed after washing.

Th1 and Th2 cell lines were driven by OVA Ag and APCs and harvested at days 9–12 for use in adoptive transfers. When tested at day 12 with OVA and APCs added, the Th1 cells produced 6.5 ng/ml IFN-γ, whereas Th2 cells were 20-fold less IFN-γ (0.3 ng/ml), at the limit of detection of the ELISA of 0.3 ng/ml. The Th2 cells produced 0.5 ng/ml IL-4, whereas Th1 cells produced only 0.09 ng/ml IL-4 (i.e., 5-fold less), which also is at the limit of detection of the ELISA of 0.1 ng/ml.

In addition, we used a competitive quantitative PCR analysis to compare cytokines IL-4 and IL-5 and IFN-γ gene expression in the Th1 and Th2 cell lines using a technique described previously (29) following 6 h of stimulation with two different activating mixtures: 1) anti-CD3 (5 μg/ml) and anti-CD28 (5 μg/ml); and 2) with PMA (1 ng/ml) and plate-bound anti-CD3 (5 mg/ml). Th1 cells express high levels of IFN-γ mRNA, but no detectable levels of IL-4 or IL-5, while Th2 cells express IL-4 and IL-5 mRNA, with very low levels of IFN-γ under both conditions of stimulation (Fig. 1*A*). Following stimulation with anti-CD3 and anti-CD28 for 24 h, the levels of IL-4 and IFN-γ in the supernatants were: for Th1 cells, IFN-γ = 28.3 ± 1.6 ng/ml and IL-4 was not detectable; for Th2 cells, IL-4 = 5.4 ± 0.8 ng/ml and IFN-γ = 0.8 ± 0.1 ng/ml (triplicate measurements; mean ± SD).

Intracellular cytokine staining was also determined as a third measure of cytokine production of these lines in the presence of the protein transport inhibitor monensin (3 μM) after 6 h of activation. Cells were fixed with 4% formaldehyde in 1.5× PBS, washed, and permeabilized in 0.1% saponin/0.5% BSA in PBS before staining for IL-4 and IFN-γ (PE-labeled anti-IL-4 OX81; PharMingen, Oxford, U.K., and FITC-anti-IFN-γ DB-1; Serotec, 0.1 μg/sample each, with cells at 10^6/ml) and analyzed by flow cytometry (FACScalibur; Becton Dickinson). Isotype control mAbs and nonrestimulated T cells were used as negative controls to set quadrant markers. On restimulation with PMA (10 ng/ml) and ionomycin (400 ng/ml), 77.9% of the Th1 cells expressed IFN-γ and 0.5% IL-4, while 44.8% of the Th2 cells expressed IL-4 and 3.1% IFN-γ (Fig. 1*B*).

Adoptive transfer of T cell lines and anti-IFN-γ treatment

The same resting Th1 or Th2 cell lines were used at the end of their stimulation cycle throughout these studies. Following i.v. transfer, we determined the effect of OVA-specific Th2 cells alone, OVA-specific Th1 cells alone, and Th1 (10×10^6) and Th2 (5 or 10×10^6) cells administered together. Recipient rats were exposed to allergen aerosol challenge 24 h later. Rats were then studied for airway function and inflammation after an additional 24 h. In separate experiments, we determined the optimal number of Th1 cells to inhibit the positive Th2 cell effects and allergen specificity of the Th1 cell down-regulation, and whether inhibition by Th1 cells was dependent on IFN-γ by i.v. injecting mouse anti-rat IFN-γ IgG1 mAb (DB1; Serotec), 24 h before injection of T cells, at a dose of 0.3 mg/rat (30, 31). For the latter experiment, rats that were injected with an isotype control (mouse IgG1, 0.3 mg/rat; Serotec) were used as controls. In addition, the effects of the mouse anti-rat IFN-γ Ab (0.3 mg/rat) on the transferred effects of either OVA-specific Th1 or Th2 cells alone were examined. Rats were studied 18–24 h after airway allergen challenge by measuring bronchial responsiveness to $\log_{10}$ doses of acetylcholine (ACh; Sigma) and quantitation of inflammatory cells in bronchoalveolar lavage (BAL) fluid and airway tissues. In addition, lungs were kept for RT-PCR.

Allergen challenge

Aerosol exposure of cell transfer recipients was performed using a 6.5-liter plexiglass chamber connected to an ultrasonic nebulizer (model 2512; DeVilbiss Health Care, Middlesex, U.K.; 15 min, 1% allergen aerosol) generated by airflow supplied by a small animal ventilator (Harvard Apparatus, Kent, U.K.) at 60 strokes/min with a pumping volume of 10 ml.

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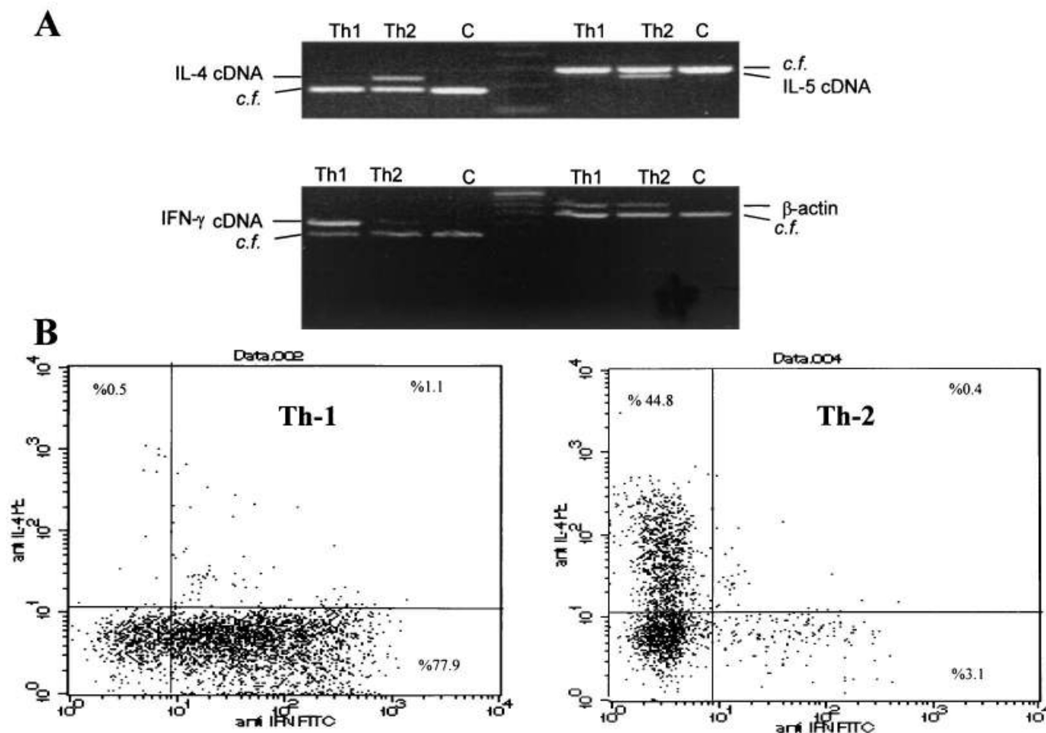

FIGURE 1. Cytokine expression in polarized Th1 and Th2 OVA-specific cell lines. *A,* Ethidium bromide-stained gel showing PCR products for IL-4, IL-5, IFN-γ, and β-actin in a competitive RT-PCR assay of Th1 and Th2 cell lines stimulated in vitro with anti-CD3 and anti-CD28. Controls (C) were devoid of cells. Th1 cells express high levels of IFN-γ mRNA, but no detectable levels of IL-4 or IL-5, whereas Th2 cells express IL-4 and IL-5 mRNA, with very low levels of IFN-γ. The competitive fragments are indicated as c.f. *B,* Flow cytometric analysis of intracellular expression of IFN-γ and IL-4 by Th1 and Th2 cell lines that were stimulated with PMA and ionomycin in the presence of the protein transport inhibitor monensin. Cells were analyzed for IFN-γ staining using FITC-anti-IFN-γ Ab, and for IL-4 staining using PE-labeled anti-IL-4 Ab. Under these conditions, 78% of Th1 cells show positive staining for IFN-γ with 1% only for IL-4, while 45% of Th2 cells stain for IL-4 but only 3% for IFN-γ.

Measurement of airway responsiveness to ACh

Anesthetized, tracheostomized, and ventilated rats were monitored for airflow with a pneumotachograph (model F1L; Mercury Electronics, Glasgow, Scotland) connected to a transducer (model FCO40, ±20 mm H_2O; Furness Controls, Sussex, U.K.) and for transpulmonary pressure via a transpleural catheter connected to a transducer (model FCO4O; ±1000 mm H_2O). Lung resistance (R_L) was calculated using software (LabView; National Instruments, Austin, TX) on a Macintosh II. Aerosol generated from increasing half-$\log_{10}$ concentrations of ACh ($10^{-3.5}$ mol/L to 10^{-1} mol/L) was administered in succession by inhalation (45 breaths of 10 ml/kg stroke volume). The concentration of ACh needed to increase R_L 200% above baseline (PC_{200}) was calculated by interpolation of the log concentration-lung resistance curve.

BAL and cell counting

After an anesthetic overdose, rats were lavaged via an endotracheal tube with 20 ml of 0.9% sterile saline in 2-ml aliquots. Total cell counts, viability, and differential counts of cytospin preparations stained by May-Grünwald stain were determined by microscopy. At least 500 cells were counted and identified as macrophages, eosinophils, lymphocytes, and neutrophils under ×400 magnification.

Collection of lung tissues

The left lung was inflated with 3 ml saline/OCT embedding medium (Tissue-Tek; Raymond A Lamb, London) (1:1) and two half-cm^3 blocks were cut around the major bronchus, embedded in OCT, and snap frozen in melting isopentane (BDH) and liquid N_2 (British Oxygen, Luton, U.K.). Cryostat sections (6 μm) were cut, air dried, fixed in acetone, air dried again, wrapped in foil, and stored at −80°C for later immunohistochemical study.

Immunohistochemistry

For detection of eosinophils, cryostat sections were incubated with a cross-reactive mouse IgG1 mAb against human major basic protein (BMK-13, Monosan; Bradsure Biologicals, Leicestershire, U.K.; 1:50; for 30 min at 25°C). After adding the second Ab, rabbit anti-mouse IgG positively stained cells were visualized by the alkaline phosphatase–anti-alkaline phosphatase method. Biotin-conjugated goat anti-mouse Ab (PharMingen) and avidin phosphatase (Dako, High Wycombe, U.K.), at a dilution of 1:200, were applied for 30 min in turn and to specificity controls. Alkaline phosphatase was developed as a red stain after incubation with Naphthol AS-MX phosphate in 0.1 M trismethylamine-HCl buffer (pH 8.2) containing levamisole to inhibit endogenous alkaline phosphatase and 1 mg/ml Fast Red-TR salt (Sigma). Sections were counterstained with Harris hematoxylin (BDH) and mounted in glycergel (Dako). Slides were read in a coded, randomized, blind fashion. Cells within 175 μm beneath the airway basement membrane were counted. The submucosal area was quantified with the aid of a computer-assisted graphic tablet visualized by a sidearm attached to the microscope. Counts were expressed as cells per mm^2 of the cross-sectional subepithelial area.

RT-PCR and Southern blotting

Total RNA from lung tissue of recipients was extracted (32) and the yield of RNA was measured by OD at 260 nm in a spectrophotometer. The RNA was analyzed on a 1.5% agarose/formaldehyde gel to check for degradation and stored at −80°C until later use. After denaturing at 70°C for 5 min, 1 μg of total RNA was used for reverse transcription in a 20-μl reaction volume containing 1× AMV buffer (50 mM Tris-HCl, pH 8.3, 50 mM KCl, 10 mM $MgCl_2$, 10 mM DTT, and 0.5 mM spermidine), 1 mM dNTPs, including dATP, dCTP, dGTP, and dTTP, ribonuclease inhibitor 32 U, 0.2 μg random primer pd(N)6 sodium salt (Pharmacia, Milton Keynes, U.K.), 8 U AMV reverse transcriptase (all apart from the random primer from Promega, Southampton, U.K.) at 42°C for 60 min. cDNA product was diluted to 100 μl in water. PCR was performed on 5 μl of diluted cDNA product in a total volume of 25 μl with a final concentration of 1× KCl or NH_4Cl buffer with 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.2 μg each of sense and antisense primers, and 1 U *Taq* polymerase (Bioline, London, U.K.) in a thermal cycler. The primers were designed according to published sequences (28, 33–35). The PCR reagents were overlaid with mineral oil and amplification was conducted using a multiwell thermal cycler through

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20–40 cycles of denaturation at 94°C for 30 s, annealing at individual temperature for 30 s, and extension at 72°C for 30 s, followed by final extension at 72°C for 10 min. The optimal PCR conditions, in terms of suitable buffer, annealing temperature, and number of cycles, were determined by PCR with pooled cDNA from all samples. Annealing temperatures were 62°C for GAPDH, IL-4, and IFN-γ, 65°C for IL-10, and 70°C for TGF-β. Serial sampling every two cycles through 20–42 cycles was used to determine the exponential phase of the product amplification curve. The cycle numbers we used for PCR were 26 for GAPDH, 32 for TGF-β, 35 for IL-4, and 34 for IL-10 and IFN-γ.

A total of 10 μl of each PCR product was size-fractionated and visualized with ethidium bromide (Sigma) on 1.5% agarose gel electrophoresis, followed by Southern blotting to Hybond-N membrane (Amersham, Bucks, U.K.) and hybridization to the appropriate cloned cDNA to confirm the identity of the product and, because all primer pairs cross at least one intron, to check for possible genomic contamination. Hybridizations were conducted at 65°C overnight with the appropriate cloned cDNA, which had been ^{32}P labeled in 6× SSC, 10× Denhardt's solution (0.2% w/v each of BSA, Ficoll, and polyvinylpyrrolidone), 5 mM EDTA, 0.5% SDS, 0.2% sodium pyrophosphate, and 100 μg/ml sonicated salmon sperm DNA. In addition, 5 μl of each PCR was dot blotted onto Hybond-N membrane and also hybridized to cDNA probe. Dot blots were excised and radioactivity was measured below the saturation level of a Packard 1900CA liquid scintillation analyzer (Packard Instrumentation, Groningen, The Netherlands). Results were generated from the counting of dot blots and expressed as a ratio of cytokine: GAPDH count, the latter used as an internal control.

Identification of transferred Th1 and Th2 cells in recipient lungs

To determine whether transferred OVA-specific Th1 or Th2 cells trafficked to the lungs, these cells were labeled with a fluorescent DNA ligand, 4,6-diamidino-2-phenylindole hydrochloride (DAPI; Sigma) before transfer. OVA-specific Th1 or Th2 cells were isolated 7 days after the last stimulation and cultured at a density of 1×10^6 cells in RPMI 1640 complete culture medium containing 10 μg/ml DAPI overnight and then washed. All cells showed intense nuclear staining, and >99% of the DAPI-labeled cells were viable as measured by trypan blue exclusion. OVA-specific Th1 or Th2 cells (10^7) were injected into a tail vein ($n = 3$ for each group) and, 24 h later, rats were exposed to OVA aerosol (1%, 15 min). Rats were sacrificed with an overdose of pentobarbitone at 12 h after exposure. A group of rats ($n = 3$) that did not receive DAPI-positive cells was also studied. DAPI-positive cells in lungs were examined under fluorescent microscopy on 10-μm frozen sections of the lungs. To determine the number of DAPI-positive T cells within the lungs, we counted 30–50 fields at ×400 magnification on at least three separate sections from each animal, and counts were determined within a 100- × 100-μm field in the airway wall and subepithelial mucosa or lung parenchyma separately.

Data analysis and statistics

Data were presented as means ± SEM. For multiple comparison of different groups, the Kruskal-Wallis test for ANOVA was used. If the Kruskal-Wallis test for ANOVA was significant, we then used the Mann-Whitney *U* test for comparison between two individual groups. Data analyses were performed using SPSS for Windows statistical software package. A p value of <0.05 was considered to be significant.

Results

Th1 cells inhibit Th2 cell-dependent BHR, BAL eosinophilia, and IL-4 mRNA

Adoptive transfer of OVA-specific Th2 cells caused a dose-dependent increase in bronchial responsiveness, when recipient rats were airway challenged with OVA, the relevant allergen, but not when exposed to BSA, an irrelevant allergen (Fig. 2, *A* and *B*). Also,

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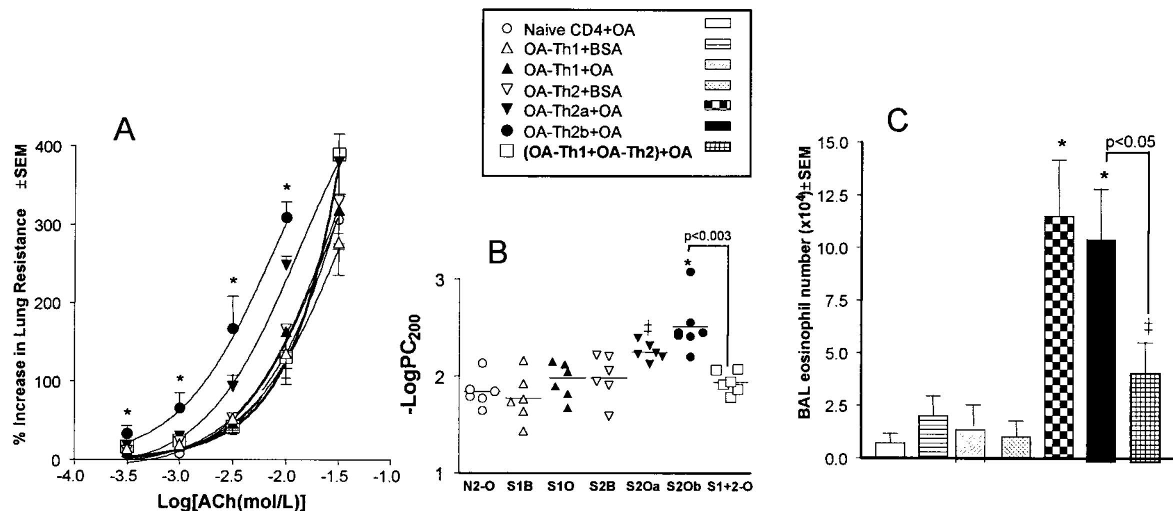

FIGURE 2. Th1 cells inhibit Th2 cell-dependent BHR and BAL eosinophilia. *A,* Mean percent increase in R_L to increasing concentrations of ACh was determined for seven different groups of six to eight rats each that received adoptive transfer of various T cell lines and then were airway challenged with allergens. Number of rats per experiment as indicated: ○, naive CD4 + OVA; negative control recipients of transferred naive CD4$^+$ T cells, then OVA airway challenged ($n = 7$); △, OVA-Th1 + BSA: recipients of OVA-specific Th1 cells, then BSA airway challenged ($n = 8$); ▲, OVA-Th1 + OVA: recipients of OVA-specific Th1 cells, then OVA challenged ($n = 7$); ▽, OVA-Th2 + BSA: recipients of OVA-specific Th2 cells, then BSA challenged ($n = 6$); ▼, OVA-Th2a + OVA: recipients of 5×10^6 OVA-specific Th2 cells, then OVA challenged ($n = 7$); ●, OVA-Th2b + OVA: recipients of 10 $\times 10^6$ OVA-specific Th2 cells, then OVA challenged ($n = 8$); and □, (OVA-Th1 + OVA-Th2) + OVA: recipients of 10×10^6 OVA-specific Th1 cells combined with 10×10^6 OVA-specific Th2 cells, then OVA challenged ($n = 8$). The ACh dose-response curve was significantly shifted leftward (hyperresponsive) in OVA-challenged rats that received OVA-specific Th2 cells in a cell number dose-dependent manner. Adoptive transfer of OVA-specific Th1 cells, along with the OVA-specific Th2 cells (group (OVA-Th1 + OVA-Th2) + OVA; □), resulted in reversal of the curve shift. *B,* Mean $-\log PC_{200}$, which is the negative logarithm of the provocative concentration of ACh needed to increase baseline R_L by 200%, is shown for the seven groups of rats detailed above. Adoptive transfer of OVA-specific Th2 cells, followed by OVA airway challenge, resulted in increased $-\log PC_{200}$ in a cell number dose-dependent manner (▼, ●). This increase was reversed when OVA-specific Th2 cells were transferred along with OVA-specific Th1 cells (□). *C,* Mean ± SEM of eosinophils in BAL fluid in the seven groups of rats. Transfer of OVA-specific Th2 cells, and then OVA challenge, induced increased numbers of eosinophils (bars second and third from the right). Transfer of OVA-specific Th1 cells, along with OA-specific Th2 cells, significantly reduced the increased BAL eosinophils induced by allergen challenge compared with recipients of OVA-specific Th2 cells alone (bar at far right). *, $p < 0.05$ comparing OVA-Th2b + OVA recipients (●) to three control groups: i.e., naive CD4 + OVA (○), OVA-Th1 + BSA (△), and OVA-Th2 + BSA (▽). ‡, $p < 0.05$ comparing OVA-Th1 + OVA-Th2 recipients (□) to other groups except OVA-Th1 + BSA.

significant BAL (Fig. 2*C*) and airway mucosal eosinophilia and increases in airway $CD2^+$, $CD4^+$, but not $CD8^+$ T cells (Fig. 3*A*), occurred in recipients of OVA-specific Th2 cells following airway challenge with OA. In contrast, naive $CD4^+$ T cells had no effect on bronchial responsiveness or airway inflammation in rats challenged identically with aerosol OVA (Fig. 2, *A–C*).

In contrast, transfer of OVA-specific Th1 cells, followed by OVA airway challenge, had no effect on BHR or BAL eosinophils, but induced some lung inflammation with an increase in mucosal eosinophils and also $CD2^+$, $CD4^+$, and $CD8^+$ T cells (Fig. 3*A*). Importantly, BHR induced by OVA-specific Th2 cells was markedly suppressed by concomitant transfer of the OVA-specific Th1 cells (Fig. 2, *A* and *B*). In addition, Th2-induced eosinophilia in BAL fluid was inhibited significantly (Fig. 2*C*), but increased eosinophils; $CD2^+$ and $CD4^+$ T cells persisted in the mucosa (Fig. 3*A*). Examination of different doses and ratios of Th1 cells injected along with 10×10^6 Th2 cells showed dose-dependent suppression of BHR and BAL eosinophilia, with maximal inhibition at a 1:1 ratio (Fig. 4, *A–C*), but no inhibition in tissue eosinophilia (Fig. 4*D*).

To determine whether the cytokine phenotypes of cultured cell lines were preserved in the target tissues following transfer and specific allergen challenge in vivo, we examined expression of IFN-γ, IL-4, and IL-10 mRNA in the lungs. Recipients of Th1 cells showed increased expression of IFN-γ, but not of IL-4 and IL-10, whereas recipients of Th2 cells had increased IL-4 mRNA, but not of IFN-γ in the lungs (Fig. 3, *B* and *C*). Importantly, rats receiving both Th1 and Th2 cells no longer showed increased Th2 cytokine expression of IL-4. In contrast, IL-10 mRNA expression in the lungs was not altered by these cell transfers. TGF-β mRNA expression was unchanged by Th1 or Th2 transfer alone or by combined Th1 and Th2 transfers.

Allergen specificity of Th1 inhibition of Th2-dependent BHR and BAL eosinophilia

To test for specificity of Th1 cell suppression, BSA-specific Th1 cells were mixed with OVA-specific Th2 cells, and aerosol challenge with OVA alone was compared with challenge with a mixture of OVA and BSA. BSA-specific Th1 cells were not able to suppress the positive effects of OVA-specific Th2 cells on BHR and BAL eosinophil influx when recipients were challenged with OVA, suggesting that Th1 cells were not activated to inhibit in the presence of OVA, a nonrelevant allergen for these Th1 cells. In contrast, when combined BSA-specific Th1 cell and OVA-specific Th2 cell recipients were challenged with a mixture of OVA and

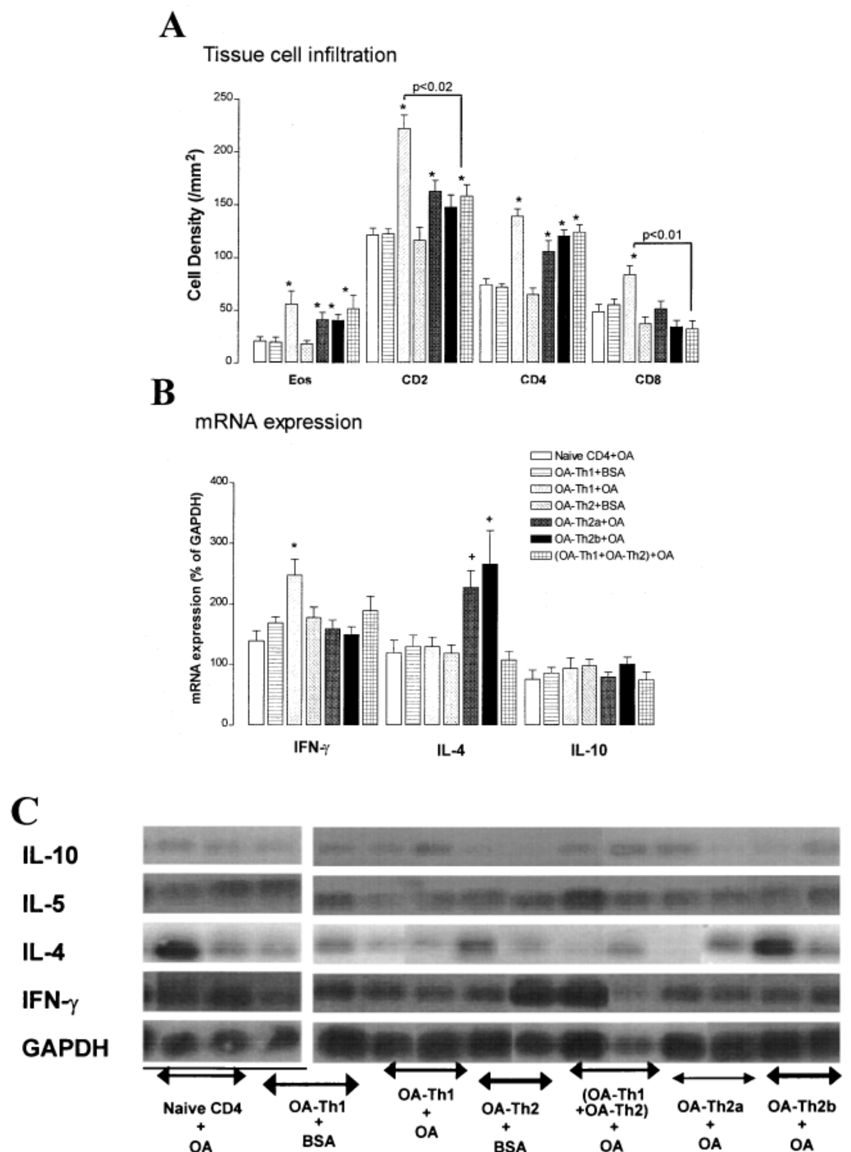

FIGURE 3. Airway mucosal cell infiltrates and cytokine mRNA expression in lungs of recipients of T cell subsets. *A,* Density of eosinophils (eos) and $CD2^+$, $CD4^+$, and $CD8^+$ T cells in airway tissue (mean ± SEM) for seven different groups of rats that received adoptive transfer of various T cell lines and were exposed to allergens. The groups are similar to those shown in Fig. 1, with similar legends. Adoptive transfer of OVA-specific Th1 cells caused an increase in eosinophils and T cell subtypes in recipient airways following exposure to OVA. Transfer of Th2 cells induced a similar degree of tissue eosinophilia that was not inhibited by simultaneous transfer of Th1 cells. Th2 cell transfer also caused an increase in $CD4^+$ T cells, but not of $CD8^+$ T cells, and combined transfer of Th1 and Th2 cells did not attenuate the $CD4^+$ T cell increase. $*$, $p < 0.05$ compared with negative control recipient naive $CD4^+$ T cells. *B,* IFN-γ, IL-4, and IL-10 mRNA expression was measured by RT-PCR and Southern blotting and expressed as percentage of GAPDH mRNA expression in lung tissue for the seven experimental groups of rats. Recipients of Th1 cells showed a significant increase in IFN-γ, but not of Il-4 and IL-10 mRNA while recipients of Th2 cells showed an increase in IL-4 mRNA. However, those receiving a combination of Th1 and Th2 cells showed no increase in IL-4 mRNA, indicating that Th1 cells suppressed the induction of IL-4 mRNA expression by Th2 cells. $*$, $p < 0.05$ and $**$, $p < 0.01$ compared with negative control recipient naive $CD4^+$ T cells. *C,* Representative hybridized dot blots of PCR products for IL-10, IL-5, IL-4, IFN-γ, and GAPDH cDNA in recipient rat lungs following adoptive transfer of Th1 and Th2 cells.

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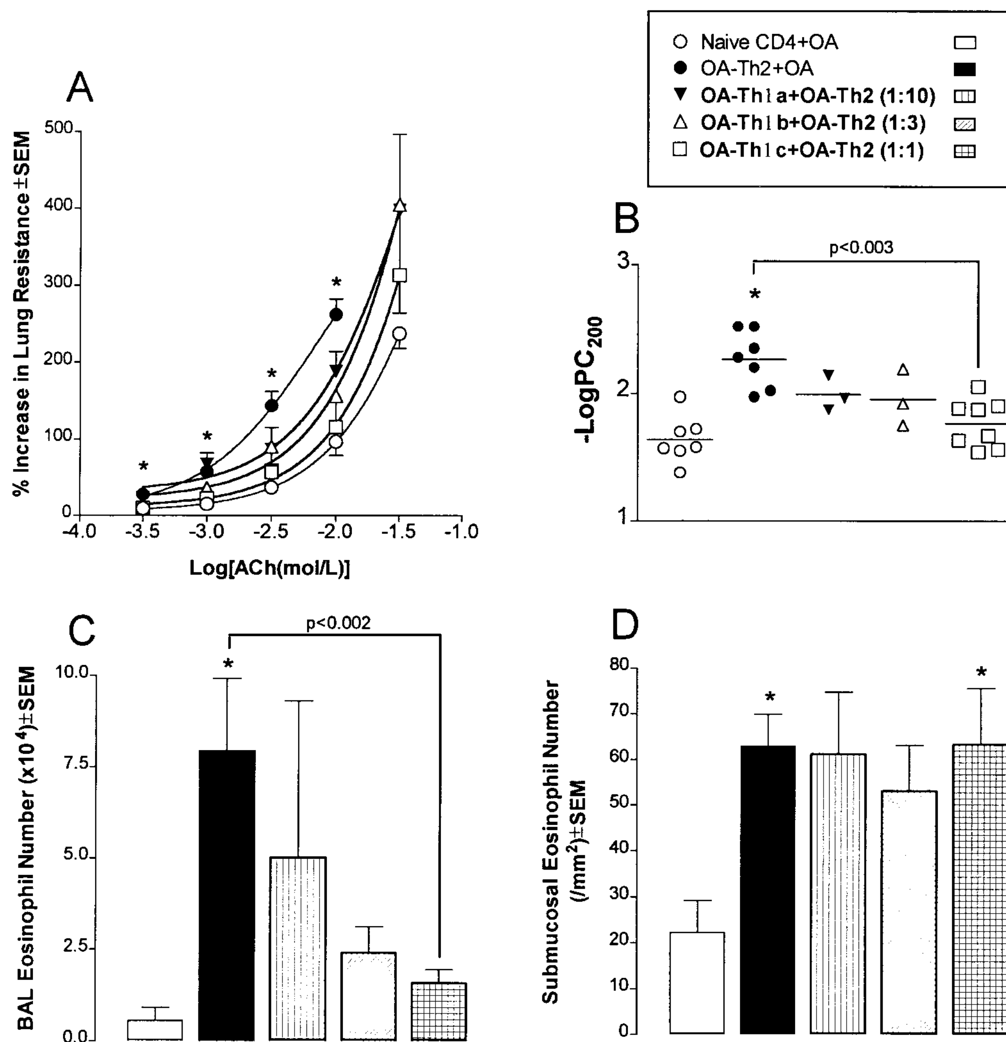

FIGURE 4. Number of Th1 cells inhibiting Th2-dependent BHR and BAL eosinophilia. *A,* To determine the number of Th1 cells required to suppress Th2 cell-mediated events in allergen-challenged airways, the mean percent increase in lung resistance to increasing concentrations of ACh was measured in five different groups of rats. Number of rats per experiment as indicated: ○, naive CD4 + OVA; negative control recipients of 10×10^6 naive $CD4^+$ cells, then OVA airway challenged, $n = 7$; ●, OA-Th2 + OVA: positive control recipients of 10×10^6 OVA-specific Th2 cells, then OVA challenged, $n = 7$; ▼, (OVA-Th1a + OVA-Th2) + OVA: recipients of mixed 1×10^6 OVA-specific Th1 cells and 10×10^6 OVA-specific Th2 cells, then OVA challenged, $n = 3$; △, (OVA-Th1b + OVA-Th2) + OVA: recipients of mixed 3×10^6 OVA-specific Th1 cells and 10×10^6 OVA-specific Th2 cells, then OVA challenged, $n = 3$; □, (OVA-Th1c + OA-Th2) + OA: recipients of mixed 10×10^6 OVA-specific Th1 cells and 10×10^6 OVA-specific Th2 cells, then OVA challenged, $n = 8$. Notably, transfer of 1, 3, or 10×10^6 Th-1 cells suppressed 10×10^6 Th2 cell-transferred BHR in a cell number-dependent manner, as indicated by the rightward shift of the ACh concentration-response curves. *B,* Mean $-\log PC_{200}$ is shown for the five groups of rats detailed in *A*. All Th1 cell doses significantly suppressed Th2 cell-induced BHR, while transfer of the maximal number (10×10^6) of Th1 cells showed a nearly complete inhibition. *C,* Mean ± SEM of eosinophils in BAL fluid in the five groups of rats. Transfer of OVA-specific Th1 cells, along with OVA-specific Th2 cells dose-dependently suppressed the influx of eosinophils in BAL fluid, although the effect of 1 or 3×10^6 Th1 cells was not statistically significant. *, $p < 0.05$ as compared with group (naive CD4 + OA). *D,* Tissue eosinophils. Mean ± SEM of eosinophils in airway tissue in the five groups of rats. Transfer of OVA-specific Th1 cells along with OVA-specific Th2 cells did not inhibit tissue eosinophilia. *, $p < 0.05$ as compared with group (naive CD4 + OVA).

BSA via the airways, then the OVA-specific Th2-dependent increase in BHR and BAL eosinophils was reversed significantly. These results suggested that the BSA-specific Th1 cells down-regulated Th2 asthmatic responses by an allergen-specific mechanism, although the resulting final mechanisms through IFN-γ may be nonspecific (Fig. 5, *A–C*). By contrast, there was persistent increased eosinophilia in the airway submucosa (Fig. 5*D*). Similar to OVA-specific Th1 cells, the BSA-specific Th1 cells given alone were unable to increase BHR and BAL eosinophils following airway challenge with BSA, the specific allergen for these T cells (Fig. 2).

Anti-IFN-γ reversed Th1 inhibition of Th2-dependent BHR and BAL eosinophilia

To test whether the prototypic Th1 cytokine IFN-γ was involved in suppression of BHR and BAL eosinophilia by allergen-specific local airway stimulation of Th1 cells, we systemically treated recipients of Th1 plus Th2 cells with anti-IFN-γ mAb, compared with an isotype control. Administration of anti-IFN-γ to recipients before transfer of mixed OVA-specific Th1 and Th2 cells partly and significantly reversed the Th1 inhibition of Th2-induced BHR (Fig. 6, *A* and *B*), but the Th-1 inhibition of BAL eosinophilia did

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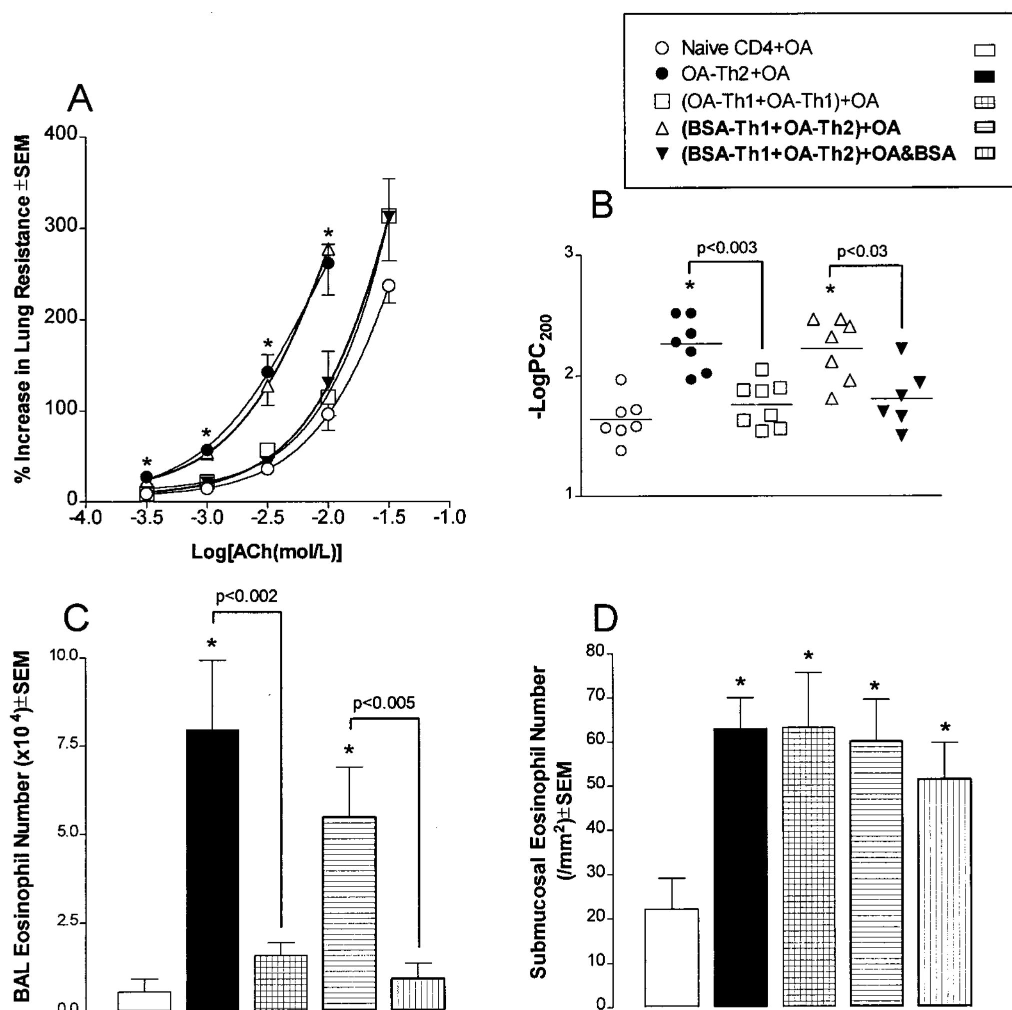

FIGURE 5. Allergen specificity of Th1 inhibition of Th2-dependent BHR and BAL eosinophilia. To determine the possible allergen-specificity of Th1 suppression of Th2 cell effects, heterologous allergen-specific BSA-sensitized Th1 cells were transferred with OVA-specific Th2 cells, and airways were challenged with OVA alone or with a mixture of OVA and BSA. The mean percent increase in R_L to increasing concentrations of ACh was determined in five different groups. Number of rats per experiment as indicated: ○, naive CD4 + OVA: negative control recipients of 10×10^6 naive $CD4^+$ cells. then OVA airway challenged, $n = 7$; ●, OVA-Th2 + OVA: positive control recipients of 10×10^6 OVA-specific Th2 cells, then OVA challenged, $n = 7$; □, (OVA-Th1c + OVA-Th2) + OVA: recipients of mixed 10×10^6 OVA-specific Th1 cells and 10×10^6 OVA-specific Th2 cells, then OVA challenged, $n = 8$; △, (BSA-Th1 + OA-Th2) + OA: recipients of mixed 10×10^6 BSA-specific Th1 cells and 10×10^6 OVA-specific Th2 cells, then OVA challenged, $n = 7$; ▼, (BSA-Th1 + OVA-Th2) + OA and BSA: recipients of mixed 10×10^6 BSA-sensitized Th1 cells and 10×10^6 OVA-specific Th2 cells, then airway aerosol challenged with combined OVA + BSA, $n = 6$. Notably, the BSA-specific Th1 cells transferred with OVA-specific Th2 cells had no effect on OVA-specific Th2 cell-transferred BHR in rats exposed to OVA aerosol alone (△), but significantly reversed Th2-induced BHR in rats that were challenged with both OVA and BSA (▼). *B,* Mean $-\log PC_{200}$ for the five groups of rats detailed in *A*. BSA-specific Th1 cells exerted a suppressive effect on BHR transferred by OVA-specific Th2 cells, only when rats were exposed to both BSA and OVA aerosol (▼). *C,* Mean ± SEM of eosinophils in BAL fluid in the five groups of rats. Transfer of BSA-specific Th1 cells along with OVA-specific Th2 cells suppressed the influx of eosinophils in BAL fluid, only in rats challenged with BSA and OVA, but not OVA aerosol alone (first and second bars from the right). *, $p < 0.03$ compared with groups: naive CD4 + OVA, (OVA-Th1c + OVA-Th2) + OVA, and (BSA-Th1 + OVA-Th2) + OVA and BSA. Data shown as mean ± SEM. *D,* Tissue eosinophils. Mean ± SEM of eosinophils in airway tissue in the five groups of rats. There was no suppression of airway eosinophilia induced by Th2 cells with the transfer of BSA-specific Th1 cells and exposure to BSA. *, $p < 0.05$ as compared with group (naive CD4 + OVA).

not reach significance (Fig. 6*C*). However, anti-IFN-γ significantly increased mucosal eosinophil counts (Fig. 6*D*). In contrast, BHR and eosinophil responses of rats treated with an appropriate isotype control were not affected (Fig. 6, *A* and *B*).

Because of the small amounts of IFN-γ produced by the Th2 cell lines, it is possible that the findings in the adoptive recipients of Th2 cells were affected by the IFN-γ, or that IFN-γ may influence results of transfers with the mixed Th1 and Th2 cells or of mixed Th1 and Th2 cells in recipients treated with anti-IFN-γ. Therefore, the effect of anti-IFN-γ on adoptive transfers of Th1 and Th2 cells alone was examined. Anti-IFN-γ had no effects on bronchial responsiveness measured after transfer of Th2 cells alone. Thus, $-\log PC_{200}$ was 1.94 ± 0.18 after Th2 cell transfer and isotype IgG control administration ($n = 5$) and was 1.90 ± 0.09 after Th2 cell transfer with anti-IFN-γ Ab ($n = 5$). BAL eosinophil counts with Th2 cell transfer increased to $10.0 \times 10^4 \pm$ 2.1 eosinophils, with no change after treatment with anti-IFN-γ ($13.0 \times 10^4 \pm 2.5$). With Th1 cell transfer, $-\log PC_{200}$ was 1.45 ± 0.09 with isotype IgG control ($n = 5$) and 1.87 ± 0.21 after anti-IFN-γ Ab ($n = 5$), indicating a nonsignificant increase on bronchial responsiveness. BAL eosinophils were not increased after Th1 cell transfer ($1.8 \times 10^4 \pm 0.21$) or after Th1 cell transfer

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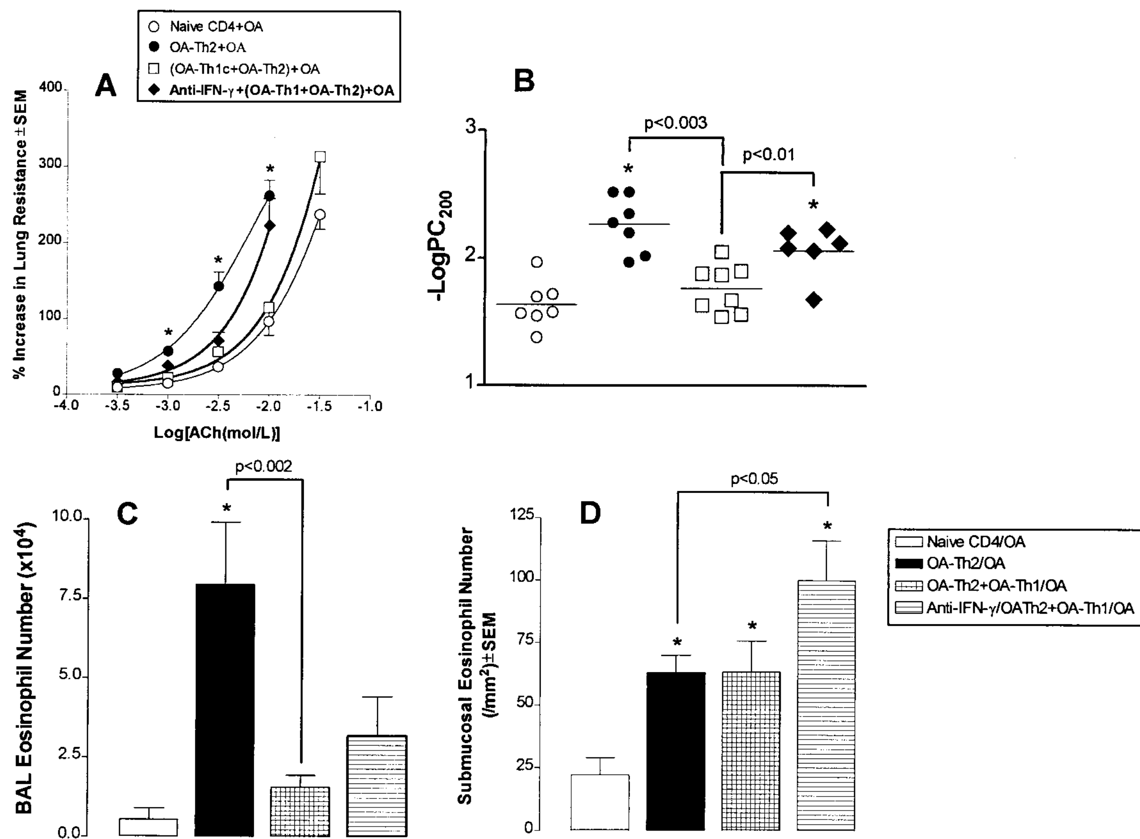

FIGURE 6. Anti-IFN-γ reverses Th1 inhibition of Th2-dependent BHR and BAL eosinophilia. *A,* To determine the possible role of IFN-γ in Th1 suppression of Th2 airway effects, recipients of combined Th1 and Th2 transfers were treated with anti-IFN-γ mAb, and the mean percent increase in R_L to increasing concentrations of ACh was measured in four different groups of rats. Number of rats per experiment as indicated: ○, naive CD4 + OVA: negative control recipients of 10×10^6 naive $CD4^+$ cells, then OVA aerosol challenged, $n = 7$; ●, OVA-Th2 + OVA: positive control recipients of 10×10^6 OVA-specific Th2 cells, then OVA challenged, $n = 7$; □, (OVA-Th1 + OVA-Th2) + OA: recipients treated with isotype control and transferred with mixed 10×10^6 OVA-specific Th1 cells and 10×10^6 OVA-specific Th2 cells, then OVA challenged, $n = 8$; ◆, anti-IFN-γ + (OVA-Th1 + OVA-Th2) + OVA: recipients treated with anti-IFN-γ Ab (DB1, 0.3 mg/rat) and transferred with mixed 10×10^6 OA-specific Th2 and OVA-sensitized Th1 cells, then OVA challenged, $n = 6$. Notably, the anti-IFN-γ treatment significantly attenuated the suppressive effect of Th1 cells on the BHR transferred by Th2 cells (◆). *B,* Mean $-\log PC_{200}$ for the four groups of rats as detailed in *A*. Anti-IFN-γ Ab treatment partially, but significantly, reversed Th1 cells suppression of Th2-induced BHR (◆). *, $p < 0.03$ for responses of negative control group (naive CD4 + OA) compared with the OVA-Th2 + OVA group, or the anti-IFN-γ + (OVA-Th1 + OVA-Th2) + OVA group. *C,* Mean ± SEM of eosinophils in BAL fluid. Anti-IFN-γ Ab treatment did not significantly reverse the Th-1 induced suppression of Th2 effects. *, $p < 0.002$ comparing the effect of combined Th1 and Th2 cells against Th2 cells alone. *D,* Mean ± SEM of eosinophils in airway mucosal tissues in the four groups of rats. Anti-IFN-γ Ab treatment caused a significant increase in tissue eosinophils in recipients of Th1 and Th2 cell transfer compared with Th2 cells alone. *, $p < 0.05$ compared with the effect of naive $CD4^+$ T cells.

with anti-IFN-γ treatment ($1.6 \times 10^4 \pm 0.51$). There was significantly greater bronchial responsiveness after Th2 transfer ($-\log PC_{200} = 1.94 \pm 0.18$) compared with that after Th1 transfer ($-\log PC_{200} = 1.45 \pm 0.09$; $p < 0.05$).

Transferred Th1 and Th2 cells in recipient lungs

DAPI-labeled cells were easily identified under fluorescent microscopy at 12 h after adoptive transfer, particularly in rats that received OVA-specific Th1 and Th2 cells and exposed to OVA. Endogenous cells showed no blue autofluorescence, apart from the airway epithelial cells, and no DAPI-positive nuclei were observed in the control sections. However, the DAPI-labeled Th1 and Th2 cells could be localized to the airway subepithelial mucosa and also in the alveolar walls (Table I). After OVA-Th1 cell transfer, there were 64 ± 4.3 and 28.5 ± 7.4 DAPI-positive cells/mm² in the airway epithelium and submucosa and in the lung parenchyma, respectively. For OVA-Th2 transfer, the corresponding values were 120 ± 14 and 41.1 ± 9.9. In sections of lungs obtained from rats receiving Th1 and Th2 cells and sacrificed at 24 h after OVA exposure, DAPI-positive nuclei were still present within the bronchial wall and lung parenchyma. These indicate that transferred Th1 or Th2 cells reached the airways and lungs.

Discussion

Adoptively transferred allergen-specific Th1 cells inhibited the asthma effector function of Th2 cells by mechanisms that partly involved local production of IFN-γ in a Brown Norway rat model of asthma. Inhibition of the Th2 asthma-effector responses by regulatory Th1 cells has been sought in other systems and is a mechanism postulated for the efficacy of various immunotherapy procedures in allergic diseases. However, previous studies in mice have failed to directly demonstrate Th1 suppression of Th2 effector mechanisms in asthma (36–38). In our study, transferred Th2 cells probably arrived and functioned in airways of rats undergoing Th1 suppression of Th2-dependent BHR, BAL eosinophilia, and diminished IL-4 mRNA expression following local airway challenge

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Table I. *DAPI-positive cells in lungs from rats receiving Th1 or Th2 cells and exposed to OVA*[a]

	Airway Epithelium and Submucosa (/mm^2)	Lung Parenchyma (/mm^2)
OVA-Th1 + OVA exposed (n = 3)	64 ± 4.3	28.5 ± 7.4
OVA-Th2 + OVA exposed (n = 3)	120 ± 14	41.1 ± 9.9

[a] Data are shown as mean ± SEM.

with specific allergen. Thus, as demonstrated by the tracking experiments, Th2 cells reached the airways and lungs and were activated. Airway Ag challenge may have caused expression of their gene program for production of Th2 cytokines, but when Th1 cells were administered and reached the airways, the Th2 cells were suppressed and could not lead to IL-4 expression, BHR, and BAL eosinophilia.

In other studies, Th2-induced asthma was modulated by administration of Th1-favoring agents at the induction phase for developing immune responses that lead to the generation of Th2 cells (14, 16, 18). In contrast, we have uniquely demonstrated Th1 cell down-regulation of Th2-mediated asthma at the efferent or elicitation phase of the responses when Th2 cells are fully developed and capable of promoting asthma. This is an effect probably more appropriately applied to asthmatic patients who have already developed asthma effector Th2 cells when seen by physicians. Therefore, our experimental maneuvers are more relevant to clinical asthmatics with pre-existing asthma effector Th2 cells. In contrast, previous studies employed Th1-promoting agents at the time of immunization, such as IL-12 (16, 17), IL-12 and IL-18 (18), or infections with mycobacteria (19) or *Listeria* (20), to cause subsequent impaired development of Th2 responsiveness. In contrast, our data pertain not to inhibiting the induction of Th2 asthma-promoting cells, but to modulating the effector functions of fully developed Th2 cells in the airways, as encountered in established asthma patients.

In contrast to our successful demonstration of Th1 cell inhibition of Th2 cell-dependent asthma, a recent study in mice employing Th1 and Th2 lines that expressed monoclonal TCR specific for a relevant peptide of OVA, which is the same allergen that we employed, failed to find Th1 inhibition of elicited Th2 asthma (37). It is difficult to know the exact reasons for the difference, but this highlights the usefulness of examining different systems before making firm conclusions about immunoregulatory mechanisms. One difference may be that our experiments were performed in the Brown Norway rat, which seems to favor Th2 responses and thus has been used successfully in a variety of other Th2 systems (22–24). Many features of Th2 asthma occur in this rat model (4–6, 25), including adoptive transfer of BHR and airway eosinophilia by CD4$^+$ T cells (4, 6, 7) and by allergen-specific Th2 lines, as shown in this study. As a Th2 predominant model, this system may be more optimal for demonstrating Th1 down-regulation of Th2 cells in asthma. Thus, Th2 responses in this model may be more susceptible to suppression because they are subject to less endogenous Th1 suppression. In a previous study, we found that exogenous IFN-γ administration in actively sensitized rats inhibited BHR and BAL eosinophilia, but we induced only a small augmentation of BHR with no effect on BAL eosinophilia by treatment with anti-IFN-γ Ab (39). In the current study, anti-IFN-γ Ab also did not modulate Th2-induced BHR or BAL eosinophilia that was induced by the transfer of Th2 cell lines alone, followed by airway Ag challenge. Thus, the potentially very small amount of IFN-γ production by the Th2 lines had no significant effect in our system. These findings are consistent with our formulation that Brown Norway rats have a predominant Th2 asthma with little endogenous Th1 down-regulation. An important overriding issue concerns which responses in various experimental systems are akin to those in humans. In this case, the ability to down-regulate Th2 asthma effector cell responses by specific Th1 cells is desirable and should be sought and eventually applied to human asthma.

This is the first in vivo study in rats, of which we are aware and certainly in a model of asthma, that employed deviating cytokine culture conditions to generate allergen-specific Th1 and Th2 lines. These T cell lines were polyclonal in OVA specificity compared with the monoclonal anti-OVA T cell lines that were employed in similar murine studies that failed to show Th1-induced down-regulation of BHR (37) or of airway inflammation (38). Thus, differences in T cell specificity could have influenced results, since some monoclonal TCR clonotypes may be more able to promote Th2 airway inflammatory responses, whereas others of similar peptide/MHC specificity, but of different clonotype, and present in polyclonal T cells we employed, could be more involved in down-regulating responses (40, 41). Thus, when TCR of both Th1 and Th2 cells are identical, there would be no opportunity for anticlonotypic down- regulation to operate (42).

It was important to demonstrate whether the OVA-specific Th1 suppressive cells were acting in an allergen- or Ag-specific fashion, since these cultured lines were producing a skewed IFN-γ dominant response in vitro before transfer, and might have continued to do so in vivo, and thus inhibited the Th2 asthma-promoting cells by a nonspecific effect, although still partly IFN-γ dependent. Against such nonspecificity was the finding that similarly derived Th1 cells of a completely different specificity to BSA, when added to OVA-specific asthma-promoting Th2 cells, were not inhibitory when airways were challenged with OVA alone. In contrast, when these recipients of the combined BSA-specific Th1 cells plus OVA-specific Th2 cells were airway challenged with an aerosol mixture of OVA and BSA, then BHR and BAL eosinophils were inhibited. This demonstrated that the allergen- or Ag-cognate specificity of the down-regulatory Th1 TCRs probably led to activation in vivo via host APC surface complexes of BSA peptides and MHC class II molecules. This triggered the BSA-specific Th1 line, resulting in local production of inhibitory Th1 cytokines, including IFN-γ. It is important to note that both OVA and BSA can act as allergens in this system to elicit asthma in actively sensitized rats or in recipients of specific Th2 cells eliciting asthma. In contrast, Th1 cells specific for both allergens do not mediate BHR, but when the allergen-specific Th1 population is mixed with OVA-specific Th2 cells there is suppression of asthma, only when the Th1 cell specificity also is triggered. The findings that the control BSA-specific Th1 cells inhibiting Th2 asthma due to the OVA allergen-specific Th2 cells suggest that Th1 cells of a variety of specificities may be able to inhibit asthma if the cognate specificity of their TCRs can be triggered to secrete inhibitory cytokines. This raises the possibility that immunity could be raised and then possibly stimulated by irrelevant or innocuous Ags in asthmatic patients to provide a useful strategy to control Th2 asthma.

The mechanisms by which Th1 cells down-regulate Th2 cell-dependent processes that locally effect the asthmatic increases in BHR and BAL eosinophils are unclear. Understanding how Th1 cells down-regulate asthma would be aided by knowing exactly how Th2 cells lead to asthma. There may be a sequential local airway Th2 asthma-promoting cascade reaction in which Th2 cells initially are recruited out of the vessels and into the lung tissues, thus allowing initial Th2 activation via allergen peptide-MHC

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class II complexes on local APCs, leading to local production of Th2 cytokines, like IL-4, IL-5 (2, 43, 44), IL-9 (45), and IL-13 (46, 47). These cytokines are important in recruiting and then activating bone marrow-derived circulating eosinophils, leading to increased BHR. It has been hypothesized that the activated eosinophils damage airways through cytotoxic mechanisms, thereby removing normal protective mechanisms and producing BHR (48, 49). Alternatively, eosinophils may not always be required for BHR (50, 51) and perhaps Th2 cells and cytokines may effect changes on airway smooth muscle cells directly (52). In our experiments, the fact that the added allergen-stimulated Th1 cells simultaneously suppressed BHR, IL-4 expression, and BAL eosinophilia is consistent with an action of Th1 cells on the Th2 asthma effector cascade formulated above.

Our results suggest that Th1 cells were also recruited into the airways and were also activated by allergen peptide/MHC class II on local APCs, but in contrast produced IFN-γ, since anti-IFN-γ treatment significantly reversed the suppression of Th2 asthmatic responses. However, the incompleteness of inhibition by anti-IFN-γ treatment suggests that other cytokines, such as IL-12 or even IL-18, that also could be derived via Th1 effects may also be involved in down-regulation of asthma. It is possible that the point in the Th2 asthma effector cascade that this IFN-γ inhibition acted was through suppression of mRNA synthesis of key Th2 cytokines, such as IL-4, as was found. In addition, Th1 cells may have inhibited eosinophil activation or protected bronchial smooth muscle cells from eosinophil cytotoxic proteins (48), or blocked direct effects of Th2 cells or cytokines on airway smooth muscle cells. An interesting finding was that Th1 lines suppressed elevated BHR and BAL eosinophils, while bronchial mucosal eosinophilia was not affected. Also, Th1 cells themselves induced eosinophil infiltrates in airway mucosae but not in BAL eosinophilia; Th1 cells also did not increase BHR. Together, these findings associate BAL eosinophil responses, but not airway mucosal eosinophilia, with increased BHR, and suggests that the down-regulatory Th1 cells acted on distal events in the Th2 asthma cascade, perhaps at the epithelium, necessary to generate BAL eosinophils and possibly also on smooth muscle to inhibit BHR, rather than the down-regulatory Th1 cells acting more proximally on inflammatory events in the submucosa. The relationship between BAL eosinophil numbers and BHR is not straightforward, since the anti-IFN-γ Ab caused a partial reversal of BHR that was not accompanied by a change in BAL eosinophils, although in this case tissue eosinophil numbers increased significantly. It is likely that the activation status of the eosinophils is also important for the genesis of BHR.

Th1 inhibition of the tissue eosinophils, but not the BAL eosinophils, has been reported in other rodent asthma models, especially in mice (36, 37). In contrast to our findings in rats that Th1 cells caused no changes in BAL cell composition, but increased mucosal eosinophils and T cells, studies in mice showed that Th1 cells induced a very prominent neutrophilia in BAL fluid (44). The reason for these differences is not known but could be due to the stronger and broader inhibitory effects of the polyclonal, allergen-specific Th1 cells we used in the Brown Norway rat system, in contrast to the TCR-transgenic Th1 and Th2 cells used in the mice studies.

In conclusion, our study demonstrates that two different allergen-specific Th1 lines repeatedly and specifically suppress asthma-promoting Th2 lines. These findings confirm the effectiveness and potential clinical usefulness of promoting desirable Th1 responses to optimally down-regulate the allergic asthmatic response. One such approach is based on using DNA vaccines that encode the genes for allergens, in addition to incorporating additional DNA sequences such as certain CpG motifs (14), which may lead to eventual immunity to the DNA-encoded allergen that is skewed toward Th1 protective responses. Our demonstration of the protective effects of Th1 responses in suppressing Th2 asthma effector T cells suggests that such Th1-promoting approaches should be pursued clinically.

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