

Analysis of T-cell activation after bronchial allergen challenge in patients with atopic asthma

Maria L. Lara-Marquez, MD,^{a, e} Aaron Deykin, MD,^{a, e} Stephen Krinzman, MD,^{a, c, e} James Listman, MD,^{d, e} Elliot Israel, MD,^{a, e} Hongzhen He, MD,^{a, e} David C. Christiani, MD,^{c, e, f} David L. Perkins, MD, PhD,^{d, e} and Patricia W. Finn, MD^{a, b, e} Boston, Mass.

Background: T helper cells are a heterogeneous group of cells that have phenotypic and functional differences. Activated T helper cells have been found in peripheral blood after allergen challenge of subjects with atopic asthma, but the phenotypes of specific T helper subpopulation involved remains to be identified.

Objective: To characterize the T cell activation markers that may be regulated by allergens, we analyzed peripheral blood lymphocytes obtained before and after allergen challenge from subjects with atopic asthma.

Methods: We analyzed the distribution of the cell surface activation markers, interleukin 2 receptor (IL-2R) and major histocompatibility complex class II antigens (MHC II) among T helper subpopulations classified as naive (CD45RA) or memory (CD45RO) phenotypes. Nine adult subjects with atopic asthma underwent bronchoprovocative allergen inhalation and isocapnic cold air hyperventilation (ISH) challenge followed by serial spirometry. Peripheral blood mononuclear cells (PBMC) were isolated at baseline and 2 and 24 hours after challenge. Four-color flow cytometry was used to analyze the expression and distribution in vivo of IL-2R and MHC II activation markers on naive and memory T cell subsets after challenge.

Results: At 2 and 24 hours after allergen challenge, there was a significant increase in the CD45RO+IL-2R+ T helper cells compared with baseline (mean $\pm$ SE, baseline, 12.5% $\pm$ 1% versus 2 hours, 18.1% $\pm$ 1% and 24 hours, 17.8% $\pm$ 2%, $p < 0.025$). MHC II expression was not significantly increased after challenge on naive and memory T helper cells and coexpression of IL-2R and MHC II was only found in a small proportion of CD45RO+ T helper cells (2.7% $\pm$ 1%). No changes of IL-2R or MHC II expression on T helper subsets were observed after ISH challenge in the same patients. We also found that 31% to 46% of T helper cells coexpress CD45RA and CD45RO simultaneously, and upregulation of IL-2R and MHC II expression occurs only on those T helper cells that express CD45RO.

Conclusions: We have found that T helper cells express both CD45RA and CD45RO isoforms, which suggests the existence of a transitional phenotype among naive and memory T helper cells in peripheral blood. In subjects with atopic asthma, our in vivo analysis characterizes two populations of activated memory T helper cells based on the expression of IL-2R or MHC II surface molecules after allergen challenge. (J Allergy Clin Immunol 1998;101:699-708.)

Key words: Asthma, allergen challenge, T helper cells, CD45 R isoforms, Interleukin-2R, MHC class II

Asthma is a disease characterized by airway hyperresponsiveness and inflammation. Early reports of the presence of lymphocyte infiltration in bronchial autopsies from subjects with severe asthma¹ suggested the importance of the role of lymphocytes in the pathogenesis of asthma. Increasing evidence for T cell involvement in airway inflammation in asthma includes the presence of activated T cells in biopsies of subjects with mild stable asthma,² activated T helper cells in the peripheral blood of subjects with stable and severe asthma,³⁻⁵ and in bronchoalveolar lavage and peripheral blood after bronchial allergen challenge.^{6, 7} In addition, the data derived from animal models have provided further evidence for the involvement of T cells in antigen-induced airway hyperresponsiveness, a phenotype associated with asthma.⁸⁻¹¹

CD4+ T helper cells are a heterogeneous group of cells that can be divided into subpopulations on the basis of the expression of the cell surface markers. The expression of the CD45RA and CD45RO isoforms of the CD45 molecule, produced by alternative splicing,¹² define two subpopulations. Broadly, CD45RA+ T cells include the "naive" phenotype and CD45RO+ T cells include the "memory" phenotype.^{13, 14} Naive CD4+ T cells are essential for responses to new foreign antigens and have different antigen-presenting cell requirements than memory T cells.^{15, 16} Memory T cells have in vitro recall response to antigens,¹⁷ preferential locomotion in response to attractants and mitogens,¹⁸ and migrate toward inflammatory sites.¹⁹ Recent reports with human peripheral blood for in vitro studies suggest that a subset of CD45RA T helper cells provides helper function for IgE synthesis in synergy with the CD45RO T helper cells.^{20, 21} These data indicate an important functional

From the Respiratory Divisions, ^aBrigham and Women's and ^bBeth Israel Hospitals; the Pulmonary Division, ^cMassachusetts General Hospital; the Division of Nephrology, ^dBrigham and Women's Hospital; ^eHarvard Medical School; and ^fthe Department of Environmental Health of Harvard School of Public Health, Boston.

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Reprint requests: Patricia W. Finn, MD, Respiratory Division, Brigham and Women's Hospital, 75 Francis St., Boston, MA 02115.

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Abbreviations used

FCS:	Fetal calf serum
IL-2R:	IL-2 receptor
ISH:	Isocapnic cold air hyperventilation
MHC II:	Major histocompatibility complex class II antigens
PBMC:	Peripheral blood mononuclear cell
PBS:	Phosphate-buffered saline
VT:	Tidal volume

role of both CD45 subpopulations in the regulation of IgE synthesis.

Allergen challenge has been a useful model to assess mechanisms implicated in atopic asthma. Recent studies report increased numbers of activated CD4⁺ T helper cells expressing IL-2 receptor (IL-2R) in the peripheral blood and bronchoalveolar lavage after endobronchial segmental allergen challenge.⁷ However, the specific subpopulations of the CD4⁺ T cells involved in this allergic response have not yet been characterized. We previously found an increase in memory CD4⁺ T cells in a murine model of allergic airway hyperresponsiveness.¹⁰ For these human studies, we postulated that a specific phenotype of memory T cells would be identified among CD4⁺ T cells responding to allergen inhalation challenge. We analyzed the *in vivo* memory immune response to an inhaled allergen in subjects with atopic asthma. Specifically, we determined the expression of IL-2R, MHC class II on CD45RA⁺ and CD45RO⁺ T helper cells to characterize the subpopulations of CD4⁺ T helper cells implicated in an allergic immune response.

METHODS

Subjects

The study protocol was approved by the Brigham and Women's Hospital Human Research Committee, and written informed consent was obtained from each subject participating in the study.

Nine patients with atopic asthma were enrolled from the Asthma Research Center at the Brigham and Women's Hospital. The patients had mild asthma as defined by the National Asthma Education Program Expert Panel Report (1991); average age was 27.5 ± 8 years (four men, five women), and all had clinical history of allergy and with at least one positive skin test result from a panel of 14 environmental allergens (Table I). The patients were nonsmokers, had an FEV₁ greater than 70% of predicted, and were not taking any medications with the exception of β_2 agonists, which were withheld 8 hours before spirometry screening. Exclusion criteria included the use of inhaled or oral corticosteroid treatment within 3 months before the study, immunotherapy within the last 3 months, and concomitant systemic disease or pregnancy.

Study design

The study was designed to analyze the *in vivo* immune response of allergen-induced bronchoconstriction in subjects with atopic mild asthma by using bronchial allergen challenge with the specific allergen to which the patient had a positive skin response. An isocapnic cold air hyperventilation (ISH)

challenge was performed on a different day, separated by at least 7 days from the allergen challenge.

The study protocol consisted of three visits. On the first visit, an initial screening session included a medical history, a physical examination, and skin tests with 14 environmental allergens, described below. To establish a stable baseline for spirometry, repeated forced expiratory maneuvers were performed until three consecutive maneuvers with 5% variability between the highest and the lowest FEV₁ were achieved. The highest stable FEV₁ was recorded as the baseline. For the subsequent visits, baseline FEV₁ was required to be $\pm 10\%$ of baseline FEV₁ established during the first visit. On the second and third visits, either the bronchial allergen challenge or the ISH challenge was performed, with a desired end point of a fall of 20% or more of the baseline FEV₁. The order of the challenges was randomized, with a minimum of a 1-week interval between challenges. Both allergen and cold air challenges were performed during morning hours, with a starting time between 8:00 AM and noon. On the days of the allergen and cold air challenges, antihistamine medication was withheld 72 hours before the challenge. After the challenges, spirometry and hand-held peak flow determinations were made every 15 minutes for the first 2 hours and hourly thereafter to 8 hours. Follow-up spirometry was done at 24 hours.

Skin prick testing was performed with a panel of common aeroallergen extracts (glycerol diluent) of the New England area: maple, Birch white, oak, grass mix, ragweed g./*Ambrosia*, dust mix, *Dermatophagoides farinae*, *Dermatophagoides pteronyssinus*, *Alternaria tenuis*, *Aspergillus fumigatus*, cockroach, dog hair, cat hair (Fel d I) (Alk labs, Reed labs). Glycerol diluent and histamine were used as the negative and positive controls, respectively. Fifteen minutes after administration of the allergens, a positive skin response was read as a weal 3 mm above the negative control of the longest and orthogonal diameter.

Bronchial provocation test

Allergen challenge. On the day of the challenge, the patients had a baseline spirometry performed before challenge. Before proceeding with the challenge, the FEV₁ had to be $\pm 10\%$ of the baseline established during the first visit. For each patient, the allergen to be administered by aerosol was the allergen producing the largest weal on skin testing during the first visit. Ten serial threefold dilution of the allergen were made with aqueous solution of 0.9% NaCl, 0.03% albumin, and 0.4% phenol. A dose escalation skin test was performed with the 10 serial dilution. Dilution "0" was the equivalent or a twofold greater wt/vol concentration to the glycerol-based extract used during screening. After baseline spirometry, blood sample collection and an initial aerosolized diluent (aqueous solution of 0.9% NaCl, 0.03% albumin, 0.4% phenol) was administered to the patient in five vital capacity breaths with a 5-second breath hold through a DeVilbiss nebulizer attached to a 20 ψ compressed air source and a dosimeter. After 10 and 11 minutes, a forced expiratory maneuver was performed. Provided that there was not a greater than 10% decrease from the best of the three baseline FEV₁ maneuvers, subjects proceeded to the allergen challenge, inhaling five breaths of increasing concentration of antigen at 10-minute intervals. The initial allergen concentration was a twofold dilution weaker than that which elicited a weal (3 mm more than the diluent control during the dose escalation skin testing). Two FEV₁ maneuvers were done at each 10-minute interval, and the next highest dilution was administered in the same way until a 20% decrease of FEV₁

TABLE I. Characteristics of study subjects

Patient	Age (yrs)	Sex	FEV ₁ L*	% Predicted	Allergen
A1	44	F	2.16	88	Cat hair (Fel d I)
A2	28	F	3.29	83	Cat hair (Fel d I)
A3	19	M	4.34	96	<i>Alternaria</i>
A4	21	F	2.7	76	Cat hair (Fel d I)
A5	36	F	3.01	89	Timothy
A6	21	M	3.62	79	Cockroach
A7	29	M	3.5	84	Timothy
A8	19	M	4.09	85	Ragweed
A9	31	F	2.46	8	Cat hair (Fel d I)
Mean $\pm$ SD	27.5 $\pm$ 8		3.24 $\pm$ 0.7	84.4 $\pm$ 5.7	

*Baseline FEV₁.

(the better of two technically adequate maneuvers) occurred. If a 10% to 19% reduction in FEV₁ occurred, spirometry was repeated in 5 minutes before the administration of further antigen. Spirometry was then repeated each 15 minutes for the first 2 hours and hourly for the next 8 hours. Subjects returned to the hospital at 24 hours after bronchial challenge to provide FEV₁ measurements, to report nocturnal symptoms or need of rescue medication, and for blood sample collection.

ISH challenge. A baseline spirometry was performed before challenge. Before proceeding with the challenge, the FEV₁ had to be $\pm 10\%$ of the baseline established during the first visit. The patients breathed subfreezing dry air containing 5% CO₂ for 3-minute periods at increasing levels of minute ventilation. The initial minute ventilation was 20 breaths per minute at VT = FEV₁. Each escalation was 50% greater than the preceding level. FEV₁ determinations were made at 0.5, 1.5, and 3.5 minutes and every 2 minutes thereafter until there was a less than 1% drop (or an improvement) of the FEV₁. The best of two technically adequate maneuvers was recorded at each time point. Ventilation escalation continued until a nadir FEV₁ less than 80% of prechallenge or maximal voluntary ventilation was obtained. The subject repeated the forced expiratory maneuvers, and after 3.5 minutes, measurements were done each 15 minutes for 2 hours and then followed with same protocol as for the allergen challenge.

Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by Ficoll-Hypaque gradient.²² Briefly, 20 ml of whole blood was diluted 2:1 in phosphate-buffered saline (PBS) on a Ficoll-Hypaque gradient (Pharmacia) (density 1070 $\pm$ 0.001 gm/ml) and centrifuged at 1800 rpm for 30 minutes. The interface was recovered, washed twice, and resuspended in PBS with 3% fetal calf serum (FCS) (Life Technology).

Monoclonal antibodies

Monoclonal antibodies specific for CD3 (T cells), CD25 (IL-2R, alpha chain), CD45RA (leukocyte common antigen) (2H4), CD8 (cytotoxic-suppressor T cells), MHC class II (MHC II) monomorphic antigen for HLA-DR, HLA-DQ, and HLA-DP (I3) were obtained from Coulter Corporation. CD4+ (T helper) monoclonal antibodies was purified by a protein A column and eluted with 125 mmol/L acetate in distilled water. Purified monoclonal antibodies were brought to a final concentration of approximately 1 mg/ml and conjugated to Cy5 (80 nmol/L) (Biological Detection Systems) at room temperature for 30 minutes. Free dye was separated from Cy5-labeled

monoclonal antibodies by elution through a gel permeation column (G50 sephadex) with PBS and 0.02% NaN₃. Biotinylated anti-CD45RO (UCHL1) and biotinylated anti-CD25 were obtained from Dako Corporation. Streptavidin Red-613 was obtained from Life Technologies, Inc. Each antibody was titrated to determine the highest fluorescent signal. For CD3, CD4, CD8, CD45RA, and CD45RO, the isotope control was murine IgG₁ (Coulter) and for I3 and CD25, the isotype control was murine IgG_{2a} (Coulter). Isotype controls were used to establish background fluorescence for each fluorochrome.

Flow cytometry

For immunofluorescence staining, PBMCs were isolated as described above, incubated at a density of 5×10^5 cells in 50 μ l of PBS containing 3% FCS and 0.01% of NaN₃ (PBS-FCS), with saturating concentrations of fluorochrome-labeled monoclonal antibodies for 30 minutes at 4°C. The samples were washed twice with 1 ml of the same buffer and resuspended in 300 μ l of PBS-FCS. Samples analyzed with biotinylated antibodies required a second step and were incubated for additional 20 minutes in 50 μ l of PBS-FCS with saturating concentrations of streptavidin-Red 613 and washed twice and resuspended in 300 μ l of the same buffer. Samples were analyzed fresh without fixation on an Coulter Epics Elite fluorescence activation cell sorter with 488 (FITC, PE and Red 613) and 633 (Cy5) excitation wave lengths. Lymphocytes were gated according to size and granularity in a forward and side scatterplot. Fluorescence was detected at 525 (FITC), 590 (PE), 613 (R613), and 670 (Cy5) nm.

The CD3+, CD4+, and CD8+ cells were analyzed by scatter by gating on the lymphocyte population. The distribution of the other markers (IL-2R, CD45RA, CD45RO, MHC II) were analyzed for coexpression with CD4+ T cells in a two-parameter log scale histogram while gating on the CD4+ T cells to determine the percentage of activated T helper cells (CD4+IL-2R+ and CD4+MHC II+), naive (CD4+CD45RA+), and memory (CD4+CD45RO+) phenotypes. When analyzing CD45RO expression on CD4+ T cells, a "high" and a "low" expressing population was identified. CD45RO expression on CD4+ T cells was calculated on the basis of the "high" expressing population. Flow cytometry data were analyzed with the Listmode Coulter Elite software, as previously described.^{23,24} Graphics were prepared with WinMDI software (Joseph Trotter, Scripps Institute, La Jolla, Calif.).

White cell count

To calculate absolute values of the proportion of T cells and subpopulations, the absolute number of lymphocytes at baseline

TABLE II. Analysis of T lymphocyte subpopulations before and after bronchial allergen challenge (mean $\pm$ SD)

Sample	Lymphocytes*	CD3†	CD8†	CD4†	CD45RA‡	CD45RO‡	IL-2R‡	MHC II‡
Patients								
Baseline	1906 $\pm$ 619	72.3 $\pm$ 4.3	21.6 $\pm$ 7.7	49.9 $\pm$ 8.6	59.4 $\pm$ 11.8	34.6 $\pm$ 6.2	9.7 $\pm$ 2.2	7.9 $\pm$ 1.8
2 hours	1797 $\pm$ 358	74.9 $\pm$ 4.9	24.2 $\pm$ 7.7	48.1 $\pm$ 8.4	58.8 $\pm$ 11.5	34.3 $\pm$ 6.6	13.3 $\pm$ 1.8§	10.1 $\pm$ 2.6§
24 hours	2012 $\pm$ 349	74.4 $\pm$ 6.9	22.8 $\pm$ 8.3	47.7 $\pm$ 11.6	60.2 $\pm$ 12.4	36.4 $\pm$ 7.3	13 $\pm$ 2.8§	8 $\pm$ 2.6

*Absolute number of lymphocytes expressed as cells per milliliter.

†CD3, CD4, and CD8 T cells are expressed as percentage of total lymphocytes.

‡CD45RA, CD45RO, IL-2R, and MHC II are expressed as percentage of CD4+ T cells.

§ $p < 0.05$, comparison vs baseline.

|| $p < 0.05$, comparison vs baseline.

and each time point after challenge was derived from a complete and differential white cell count analysis by an automated cell counter (Clinical Laboratory, Brigham and Women's Hospital). Absolute numbers were obtained from eight of the nine subjects analyzed and are expressed as cells/mm³.

Statistical analysis

Paired data were analyzed with a paired *t* test or Mann-Whitney test. Unpaired data were analyzed with Student's *t* test or Wilcoxon U test. Sigma Stat statistical software (Jandel Scientific/SPSS Science) was used for all the statistical analyses.

RESULTS

Response to bronchoprovocation

Nine patients underwent challenge with inhaled allergen. The mean acute fall in FEV₁ was 25.4 $\pm$ 1.8. Six of these individuals also underwent ISH challenge, with a mean initial fall in FEV₁ of 22.7% $\pm$ 4.6%. There was no difference in the degree of bronchoconstriction produced in these six patients after ISH compared with the degree of bronchoconstriction produced in these individuals after allergen challenge (22.7% $\pm$ 4.6% vs 24.7% $\pm$ 2.2%, $p = 0.71$).

Analysis of T lymphocyte subpopulations

To characterize the T cell phenotype of subjects with atopic asthma after allergen challenge, PBMC were isolated and analyzed by flow cytometry at baseline and 2 and 24 hours after challenge. Two and 24 hours after allergen challenge, there were no significant differences in the percentage (Table II) and absolute number (not shown) of CD3+, CD8+, CD4+CD45RA+, CD4+CD45RO+ T cells relative to baseline. Of interest, we also analyzed atopic nonasthmatics ($n = 7$, data not shown) and found a lower percentage of CD4+CD45RA+ T cells (38.6 $\pm$ 9.5) but a higher percentage of CD4+CD45RO+ T cells (55.1 $\pm$ 10.3) compared with the atopic asthmatic subjects in this study (Table II). Our findings are consistent with other reports.^{20, 25} There were no other significant differences in comparison of baseline values for subjects with atopic asthma as compared with atopic subjects free from atopic asthma. We also observed that the CD4+CD45RA+ and CD4+CD45RO+ T cells were not modified in subjects with atopic asthma upon allergen challenge (Table II).

At 2 hours after allergen challenge, there was a significant increase in the proportion of CD4+ T cells expressing IL-2R (9.7 $\pm$ 2.2 vs 13.3 $\pm$ 1.8, $p < 0.01$) and MHC II (7.9 $\pm$ 1.8 vs 10.1 $\pm$ 2.6, $p < 0.05$) relative to baseline. At 24 hours after challenge, the proportion of CD4+ T cells expressing IL-2R remained elevated relative to baseline (9.7 $\pm$ 2.2 vs 13 $\pm$ 2.8), whereas CD4+MHC II+ T cells returned to baseline values (7.9 $\pm$ 1.8 vs 8 $\pm$ 2.6).

Comparison of T lymphocytes after allergen and cold air challenge

To evaluate whether the increased expression of the IL-2R and MHC II activation markers on CD4+ T cells was specific to allergen challenge, we also analyzed T lymphocytes after ISH challenge. After ISH challenge, there was no change in the expression of IL-2R (Fig. 1) or MHC II (not shown) on CD4+ T cells, in contrast to significant increases in these markers after allergen challenge. No significant changes were found on other T cell surface markers analyzed, including CD8, CD45RA, and CD45RO (data not shown). Thus in our allergen challenge protocol, T cell activation appears specific to bronchial allergen challenge and was not observed after ISH challenge.

Differential expression of IL-2R and MHC II among T helper subpopulations

To determine which CD4+ T cells express the IL-2R and MHC II activation markers after challenge, we extended our analysis to cell surface markers associated with naive (CD45RA) and memory (CD45RO) T cell phenotypes. This analysis was performed with flow cytometry with three- and four-color analysis.

We first analyzed the expression of IL-2R on subpopulations of CD4+ T helper cells. At baseline, IL-2R was expressed primarily on the CD45RO+ T helper cells (Fig. 2). After allergen challenge, there was a significant increase in the percentage of CD45RO+ T helper cells that expressed IL-2R+ at 2 hours (mean $\pm$ SE, 12.5% $\pm$ 1% vs 18.1% $\pm$ 1%, $p < 0.01$) and 24 hours (12.5% $\pm$ 1% vs 17.8% $\pm$ 2%, $p < 0.025$) relative to baseline. No significant changes in the IL-2R+CD45RA+ T helper cells were observed. Similar results were obtained after analyzing the changes in the absolute values of IL-2R+

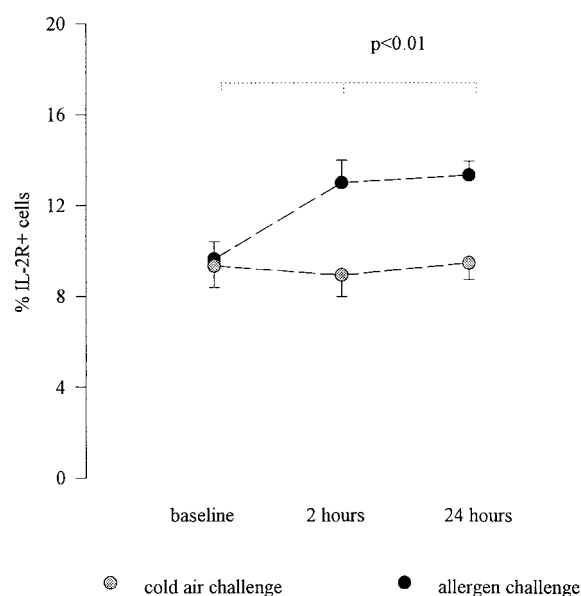


FIG. 1. Comparison of IL-2R expression after allergen challenge ($n = 9$) (black circles) and isocapnic cold air hyperventilation (ISH, cold air) challenge ($n = 6$) (gray circles). Shown is proportion of CD4+ T cells expressing IL-2R (y-axis) at baseline and 2 and 24 hours after challenge. Means $\pm$ SE are shown for each time point. The probability (p) value is for the comparison of 2 and 24 hours relative to baseline.

memory T helper cells at 2 hours (baseline, 78 ± 11 vs 105 ± 14 cells/mm³, $p < 0.05$) and at 24 hours (78 ± 11 vs 120 ± 20 cells/mm³, $p = 0.076$) after allergen challenge (not shown).

Similar to the analysis of IL-2R+ T helper cells at baseline, MHC class II was expressed primarily on the memory T helper cells (Fig. 3). However, at 2 hours there was only a trend toward an increased percentage of CD45RO+ T helper cells expressing MHC II+ (mean $\pm$ SE 10.6 ± 1.5 vs 12.7 ± 1.7 , $p = 0.066$), which returned to baseline at 24 hours (Fig. 3). No changes were observed in absolute values (not shown) of either the MHC II+ naive T helper cells or MHC II+ memory T helper cells relative to baseline. Taken together, our data show that IL-2R and MHC II are expressed primarily on the memory phenotype T helper cells (Figs. 2 and 3), and suggest different kinetics and cell phenotype expression of IL-2R and MHC II among CD45RO+ T helper cells.

Coexpression of IL-2R and MHC II on naive and memory T helper cells

To characterize the differences in the expression of IL-2R and MHC II observed on T helper cells after allergen challenge, we analyzed whether there was coexpression of IL-2R and MHC II among CD45RA+ and CD45RO+ T helper cells. We determined that IL-2R and MHC II molecules are only coexpressed on a small proportion of the memory T helper cells (Fig. 4) at the time points analyzed in this protocol (baseline, 2 hours, and 24

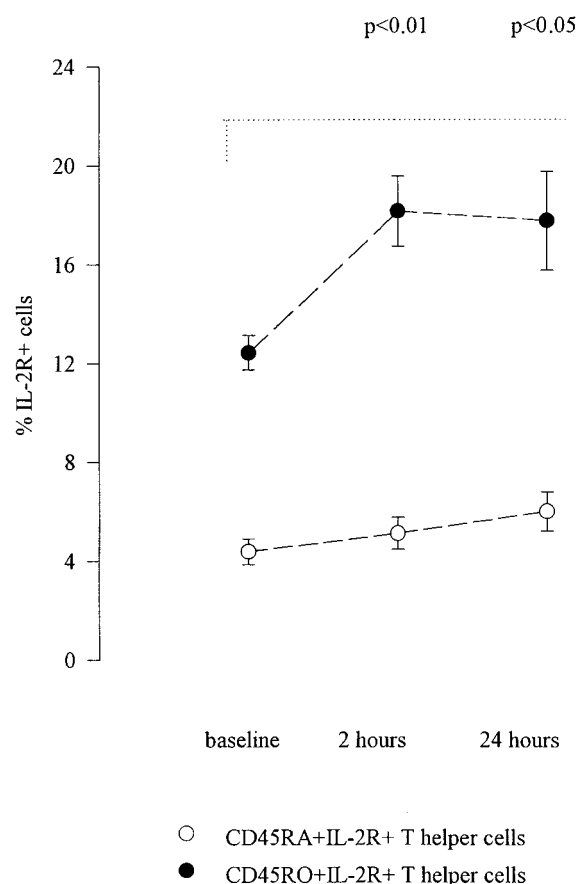


FIG. 2. Comparison of IL-2R expression on naive and memory T cells after allergen challenge. Shown is mean $\pm$ SE of percentage of CD4+CD45RO+ T cells that are IL-2R+ (y-axis, open circles) and percentage of CD4+CD45RO+ T cells that are IL-2R+ (y-axis, filled circles) at baseline and 2 hours and 24 hours after challenge ($n = 9$). The probability (p) value is for comparison of postchallenge values versus baseline of respective subpopulation.

hours). Although no significant changes were observed in terms of absolute values (mean $\pm$ SE, baseline, 20 ± 2 ; 2 hours, 22 ± 4 ; 24 hours, 19 ± 6 cells/mm³), there was a discrete elevation of the proportion of CD45RO+ T helper cells coexpressing IL-2R and MHC II at 2 hours (2.9 ± 1 vs 4.2 ± 1.5 , $p < 0.05$), which returned to baseline at 24 hours after challenge. Thus the increased expression of IL-2R molecules appears primarily on the IL-2R+ MHC II-memory T helpers.

CD45RA and CD45RO distribution among activated T helper cells after allergen challenge

Because the IL-2R and MHC II molecules are expressed primarily on the memory T cell phenotype (CD4+CD45RO+) that is preferentially activated in response to allergen challenge (Figs. 1 and 2), further analysis of the CD45 isoforms was undertaken (Table III and Fig. 5). The goal of this simultaneous analysis of the CD45 isoforms was to identify the kinetics of coexpression of these isoforms in response to allergen exposure

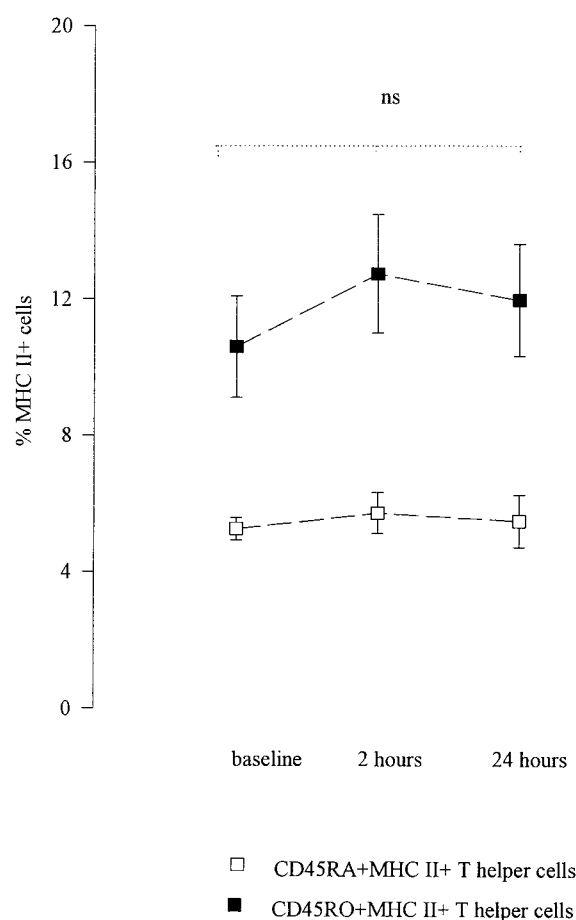


FIG. 3. Comparison of MHC II surface molecule expression on naive and memory T cells after allergen challenge. Shown is mean $\pm$ SE of percentage of CD4+CD45RA+ T cells that are MHC II+ (y-axis, open circles) and percentage of CD4+CD45RO+ T cells that are MHC II+ (y-axis, filled circles) at baseline and 2 hours and 24 hours after challenge ($n = 9$). The probability (p) value is for comparison of postchallenge value versus baseline of respective population.

and to address whether we might identify a transition between the naive phenotype to the memory phenotype. For these analyses, we indicate the proportion of CD4+ T cells that express exclusively each marker alone (CD45RA+RO-, CD45RA-RO+) and the cells that coexpress both isoforms (CD45RA+CD45RO+) (Table III). We found that the CD4+ T cells coexpress both isoforms to a significant extent at baseline, supporting the existence of a transitional phenotype in peripheral blood. Although the proportion of each population of the T helper cells does not change significantly after challenge, the CD4+ memory T cells tend to increase by 24 hours after challenge.

By using four-color flow cytometry analysis, CD45RA+ and CD45RO+ T helper cells were further characterized, focusing on the presence of either IL-2R or MHC II surface molecules at baseline and 2 and 24 hours after allergen challenge. A representative two-

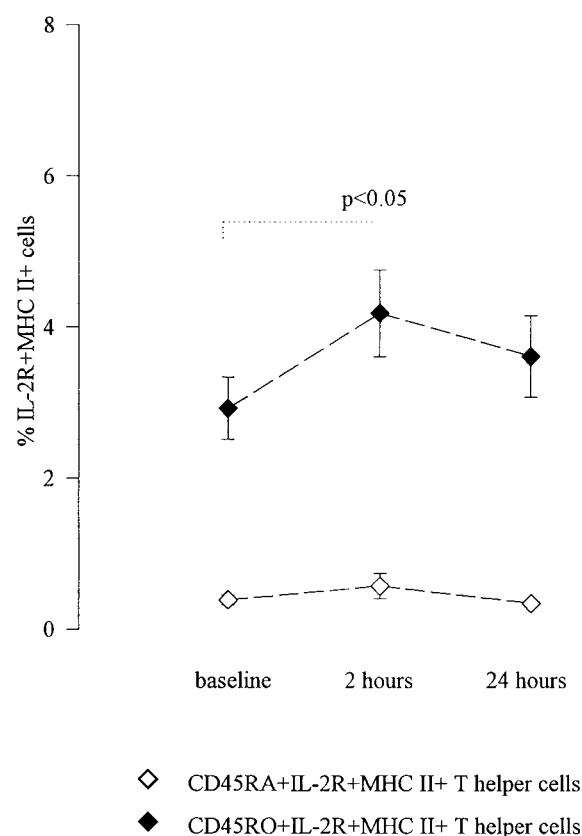


FIG. 4. Comparison of IL-2R and MHC II expression on naive and memory T cells after allergen challenge. Shown is mean $\pm$ SE of percentage of CD4+CD45RA+ T cells that are IL-2R+MHC II+ (y-axis, open circles) and percentage of CD4+CD45RO+ T cells that are IL-2R+MHC II+ (y-axis, filled circles) at baseline and 2 hours and 24 hours after challenge ($n = 6$). The probability (p) value is for comparison of postchallenge values versus baseline of respective subpopulation.

parameter histogram is shown (Fig. 5, *a* through *c*). The distribution of CD45RA and CD45RO surface molecules was analyzed on the CD4+ cells. We determined the proportion of CD4+ T helper cells that were CD45RA+RO-, CD45RA-RO+, or the cells that coexpress both isoforms CD45RA+CD45RO+ (upper right quadrant), which constitute the transitional cells (Fig. 5, *a*). Furthermore, after gating on the CD4+IL-2R+ T cells or on the CD4+MHC II+ T cells, we found the following: at 2 hours, the proportion of CD4+IL-2R+ T helper cells that were CD45RA-RO+ phenotype changed from 58% to 61%, and the proportion of CD4+MHC II+ T helper cells that were of the CD45RA-RO+ phenotype changed from 45% to 52% compared with baseline. At 24 hours, the same trends were observed, with an increase to 91% of the CD4+IL-2R+ T helper cells that were CD45RA-RO+ phenotype and an increase to 60% of the CD4+MHC II+ T helper cells that were CD45RA-RO+ phenotype when compared with baseline (Fig. 5, *b* and *c*, respectively). Together, these data suggest that an *in vivo* allergen-

TABLE III. Distribution of CD45RA and CD45RO surface molecules on T helper cells

Patients	CD45RA+RO-*		CD45RA+RO+*		CD45RA-RO+*	
	%	Absolute value†	%	Absolute value†	%	Absolute value†
Baseline						
A6	24.2	266	31.9	351	43.8	482
A7	36.9	312	31	265	32.1	272
A9	17	168	46	454	37	365
Mean ± SD	26 ± 8	249 ± 60	38.9 ± 6.1	385 ± 85	37.6 ± 5	373 ± 85
2 Hours						
A6	33.1	245	24.1	179	42.6	315
A7	36	352	27	264	37	362
A9	21	216	42	431	37	380
Mean ± SD	30 ± 6.4	271 ± 58	31 ± 7.8	291 ± 105	38.9 ± 2.6	352 ± 27
24 Hours						
A6	25.7	179	26.8	186	47.5	518
A7	29	348	31	372	40	480
A9	14	133	42	398	44	417
Mean ± SD	22.9 ± 6.4	220 ± 92	33.3 ± 6.4	319 ± 94	43.8 ± 3.1	472 ± 42

*Percentage of CD4+ T cells.

†Absolute values expressed as cells per milliliter.

driven T cell response is characterized by an increase in the memory T helper cells and a decrease in the naive and transitional T helper cells among activated T helper cells.

DISCUSSION

T cell activation has been implicated as an important event in the induction of the manifestations of asthma, including airway hyperresponsiveness in animal models.⁸⁻¹¹ In studies of human beings with asthma, activated T cells correlate with bronchoconstriction induced by allergen challenge.^{7, 26, 27} However, the specific subsets and kinetics of T cell activation in response to allergen challenge remain to be determined. Using multiple color flow cytometry, we examined the kinetics of the expression of IL-2R, MHC II, CD45RA, and CD45RO surface molecules on the CD4+ T cells to characterize the cell subpopulations involved in the in vivo memory allergen-driven immune response to aerosolized antigen bronchoprovocation.

IL-2R and MHC II are activation markers that are acutely upregulated on activated T cells. In human beings with asthma, IL-2R is elevated both in the peripheral blood and bronchoalveolar lavage, and increased IL-2R expression may correlate with disease severity.^{3, 4, 6, 28} T lymphocytes expressing IL-2R are also found in the lungs of other immunologically mediated diseases, including sarcoidosis.²⁹ Increased MHC class II expression on human T cells has also been found in allergic inflammation, including cutaneous models.³⁰ After bronchial allergen challenge, we found differences in the kinetics of cell expression of these markers. The expression of IL-2R marker was increased as early as 2 hours after challenge on T helper cells and remained elevated at 24 hours. In contrast, the expression of MHC class II antigens showed only a discrete increase at 2 hours, which returned to baseline by 24 hours. Import-

tantly, after challenge, the upregulation of both IL-2R and MHC II occurred on the CD45RO T cell subset, which has been correlated with memory T cell responses.^{18, 31, 32} These results suggest that the patients with atopic asthma in our study had a population of allergen-specific memory T cells that were activated by bronchial allergen challenge. Because we did not detect naive T cells that had been activated during the early phase of the allergen specific response, we suggest that the activation and effector functions of the T cell memory subset may provide targets for potential therapeutic interventions.

Using four color flow cytometry to analyze the simultaneous expression of IL-2R and MHC class II on the CD4+CD45RO and CD4+CD45RA T cell subsets, we demonstrated that most activated T cells expressed either IL-2R or MHC II molecules but not both together. Only a small percentage expressed both IL-2R and MHC II and were only detected on the CD45RO+ T helper cells (Fig. 4). Although a discrete increase was observed in the percentage of cells expressing both markers (IL-2R and MHC II) 2 hours after challenge, there was no change in absolute numbers of these group of cells. At 2 and 24 hours after challenge, there was a significant increase in the percentages (Fig. 2) and absolute values of IL-2R+ and a trend toward increase in MHC II+ memory T helper cells (Fig. 3). These results suggest that T cell activation may progress in two pathways involving the upregulation of either IL-2R or MHC II expression. Alternatively, this differential expression of IL-2R and MHC II among memory T helper cells after allergen challenge could represent different stages of activation of the same cell. In both instances, there may be differential effector and regulatory roles for T cells that express either IL-2R or MHC II in the modulation of a self-limited allergen response as in our allergen challenge protocol. By 24 hours, eight of nine

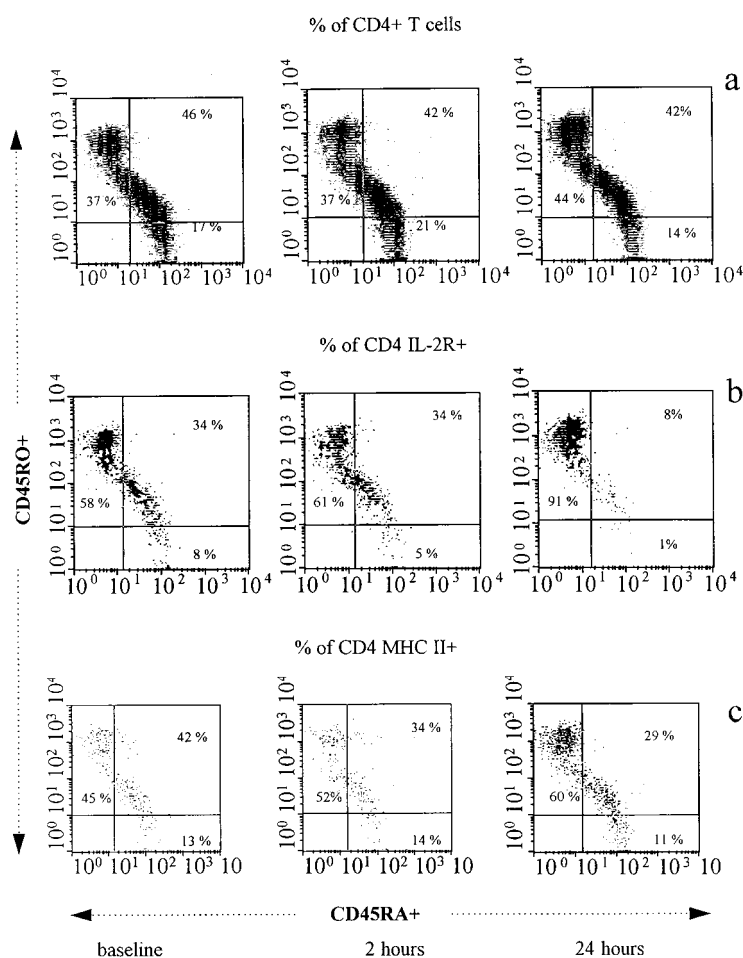


FIG. 5. Analysis of CD45RA and CD45RO isoforms on T helper cells after allergen challenge. PMBCs were obtained from patient (A9) at baseline and 2 hours and 24 hours after challenge and analyzed by flow cytometry. Distribution of CD45RA and CD45RO surface molecules were analyzed on CD4+ cells (a). The CD45RA+ T helper cells (x-axis) and the CD45RO+ T helper cells (y-axis) are shown on three sets of histograms. After gating on CD4+ IL-2R+ (b) or CD4+ MHC II+ (c) T cells, we show distribution of CD45RA and CD45RO surface markers on CD4+ IL-2R+ cells (b) and CD4+ MHC II+ (c) T cells. Quadrants are set on the basis of negative controls as described in Methods section. Analysis of lymphocytes at baseline and 2 hours and 24 hours is indicated by first, second, and third histograms in each panel. The CD45RA+RO- T helper cells are represented on lower right quadrant, CD45RA-RO+ T helper cells in upper left quadrant, and double-positive transitional CD45RA+CD45RO+ T helper cells are represented in upper right quadrant.

patients had spontaneously returned to baseline FEV₁ values, underscoring that the initial allergic response had been limited by endogenous regulatory immune mechanisms. We speculate that IL-2R+ T helper cells may have a role amplifying the allergen-specific response, possibly favoring clonal expansion of allergen specific T helper cells. Conversely, MHC II+ T cells may limit the immune response because they can present degraded antigens but, in the absence of accessory molecules they behave as “fail-safe APC”,³³ contributing to the termination of the immune response. Of interest, MHC class II expression may differ in cell type expression and the regulatory elements between humans and mice, indicating potential important functional differences.³⁴ It will be important to determine the signals that promote the differential activation of IL-2R versus MHC class II

expressing T cells and the functional role of these two phenotypes in the pathogenesis of atopic asthma.

CD45 is a tyrosine phosphatase that is expressed in different isoforms from differential RNA splicing.¹² Expression of the CD45RA isoform has been correlated with “naive” T cells, whereas CD45RO has been correlated with “memory” T cells.^{13, 14, 18, 31} Our results show that approximately 31% to 46% of all peripheral T cells simultaneously express both markers (Fig. 5). Previous studies have shown that long-term T cell clones and up to 30% of the cells in adult blood express both CD45 isoforms, with a proportion of these cells expressing IL-2R.³⁵⁻³⁷ Another report showed that approximately 50% of newborn infant CD4+ T cells and, similar to our findings, approximately 40% of adult T cells express both CD45 isoforms.³⁸ The functional significance of

cells expressing both isoforms is unknown. One possibility is that they are transitional cells progressing from a naive to a memory phenotype or from resting to recently primed cells. In vitro studies have established that the transition from CD45RA to CD45RO is accompanied by cell activation and proliferation.³⁹ Others suggest that in the newborn, the CD4+ naive phenotype expressing IL-2R may be an important transitional population into the memory phenotype.⁴⁰ These observations are in agreement with our in vivo results, which clearly show that IL-2R or MHC class II is upregulated only on the CD4+ T cells expressing CD45RO molecules whether or not they coexpress the CD45RA molecule (Fig. 5). Also, we have extended the phenotypic characterization of cells positive for both CD45 isoforms by analyzing the coexpression of IL-2R and MHC II. Importantly, we characterized these T cells after the in vivo stimulation of subjects with atopic asthma.

The correlation between peripheral blood lymphocytes and bronchoalveolar lavage T cells is controversial. Some studies have reported correlation between PBL and BAL CD4+ T cells^{7, 28}; however, other groups have not detected significant correlations.^{6, 26, 41} Our results demonstrate that upregulation of activation markers can be detected on memory T cells in peripheral blood as early as 2 hours after allergen challenge. Of note, we have observed changes in IL-2R and MHC II in the periphery at this relatively early time point. Although detection of this specific marker panel at 2 hours has not previously been reported for in vivo allergen challenge, there is evidence to indicate that the changes in activation markers may be specific to the allergen response. First, in these subjects, a nonspecific response (ISH) did not increase expression of IL-2R or MHC II. Second, although we have not measured the numbers of allergen-specific T cells, which may be low but nevertheless potent, we have measured changes in activated T cells by sensitive flow cytometry techniques. Thus we were able to detect the small but significant proportion of naive or memory T helper cells that were expressing IL-2R or MHC II. Third, other human models have found increased activated markers at time points earlier than 24 hours.⁷ Last, using a murine model of superantigen induced T cell activation, we have found that IL-2R expression on peripheral T cells is upregulated within 2 to 24 hours, similar to our findings in this analysis (data not shown). Obtaining peripheral blood T cells is less invasive than bronchoalveolar lavage lymphocytes, and detection of activated memory T cells in response to bronchial allergen challenge may be used to better understand the mechanisms related to the pathogenesis of the allergic response. We have found that T helper cells express both CD45RA and CD45RO isoforms, suggesting the existence of a transitional phenotype among naive and memory T helper cells in peripheral blood. Using four-color flow cytometry analysis, we have identified the IL-2R+ memory T helper cells as the primary population involved in the in vivo allergen response in subjects with atopic asthma.

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