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Research Article

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Nonatopic Asthma: In Vivo Airway Hyperreactivity Adoptively Transferred to Naive Mice by THY-1$^+$ and B220$^+$ Antigen-specific Cells That Lack Surface Expression of CD3

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Abstract

To investigate the cellular immune events contributing to airway hyperreactivity (AHR), we studied an in vivo mouse model induced by the hapten picryl (trinitrophenyl) chloride (PCl). Mice were immunized by cutaneous contact sensitization with PCl and airway challenged subsequently with picryl sulfonic acid (PSA) antigen (Ag). Increased airway resistance was produced late (24 h) after Ag challenge, disappeared by 48 h, and was associated with no decrease in diffusion capacity. AHR could be produced in PCl immune/PSA challenged mice on day 7 or even, with challenge, as early as 1 d after contact sensitization, after adoptive transfer of immune cells lacking CD3$^+$ contact sensitivity effector T cells, or after transfer of Ag-specific lymphoid cells depleted of conventional T lymphocytes with surface determinants for CD3, CD4, CD8, TCR-β, or TCR-δ molecules. Further experiments showed that development of AHR depended upon transfer of immune cells expressing surface membrane Thy-1 and B220 (CD45RA) determinants. We concluded that a novel population of Ag-specific lymphoid cells with a defined surface phenotype (Thy-1$^+$, CD3$^-$, CD4$^-$, CD8$^-$, TCR-αβ$^-$, TCR-γδ$^-$, and CD45RA$^+$) is required in a mouse model for the development of AHR. (*J. Clin. Invest.* 1997. 100:629–638.) Key words: asthma • T cell • airway hyperreactivity • immunity • CD45

Introduction

The considerable evidence suggesting a role for T cells in the pathogenesis of human asthma includes: (*a*) the presence of activated T cells [expressing IL-2R(CD25) and MHC class II] in the blood of asthmatics with acute exacerbations (1); (*b*) expression of Th2-like cytokine IL-4, and IL-5 mRNA in upper airway biopsies harvested from patients with asthma (2); and (*c*) presence of T cells and cytokine protein indicative of Th2

cell origin in asthmatic bronchoalveolar lavage fluid (3–5). Equivalent definitive findings have as yet not been made in the study of patients with nonatopic asthma syndromes such as intrinsic asthma, hapten-induced asthma induced by toluene diisocyanate, or chronic bronchitis, although some data suggest parallels (6, 7). However, the precise mechanism by which T cells or subsets mediate the functional changes of the asthmatic state has not been determined.

A hapten-induced murine model of bronchial immune inflammation produced in picryl chloride (PCl)[1] contact sensitized mice (8) was studied histologically and found to be characterized by peribronchial accumulation of mononuclear cells that could not be produced in athymic nude mice or in normal animals treated with serotonin antagonists (9–11). This suggested that the bronchial inflammatory response depended upon the presence of sensitized T cells and was mediated in part by serotonin. Using ex vivo techniques in studying isolated trachea from immunized and challenged mice, significant airway hyperreactivity (AHR) to nonspecific bronchoconstrictor agents was shown that could not be demonstrated in similarly immunized and challenged nude mice (10, 11).

Here we describe further work with the PCl murine model and show that AHR previously demonstrated in isolated trachea ex vivo can also be shown in vivo in anesthetized intact mice by measuring pulmonary function using forced oscillations and body plethysmography. The duration of this in vivo AHR persists for many days after antigen (Ag) challenge, recapitulating in mice an important clinical aspect of human asthma (12). In addition, we show that early after immunization, at a time thought to be too early for the induction, activation, and recirculation of conventional T cells, in fact, as soon as only 1 d after sensitization, subsequently challenged animals manifest in vivo AHR that also could be transferred adoptively to naive animals with 1-d immune lymphoid cells. Adoptive cell transfer experiments showed that a subpopulation of lymphoid cells, specifically cells that carry surface determinants for Thy-1 and B220 (CD45RA), but that did not express surface CD3, CD4, CD8, TCR-β, and TCR-δ molecules, was necessary for the Ag-specific transfer of AHR.

Methods

Mice. Female BALB/c mice were purchased from Jackson Laboratory (Bar Harbor, ME). 8–12-wk-old, 22–25 g mice were rested for at

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1. *Abbreviations used in this paper:* Ag, antigen; AHR, airway hyperreactivity; Con A, concanavalin A; CS, contact sensitivity; DLCO, diffusion capacity of the lungs; OX, oxazolone; PCl, picryl chloride; PSA, picryl sulfonic acid (trinitro benzene sulfonic acid); Rrs, respiratory system resistance; RT, reverse transcription; TNP, trinitrophenyl.

least 1 wk before use. Mice were housed in filter frame cages changed by personnel wearing gowns, gloves, and masks, and were fed autoclaved food and sterile water.

Reagents. PCl (trinitrophenyl [TNP] chloride; Chemotronix, Swannonoa, NC) was stored desiccated in the dark. PSA (picryl [trinitrobenzene] sulfonic acid, an antigenically equivalent, water-soluble analogue of PCl) (Eastman Kodak Co., Rochester, NY) was used for airway challenge at a concentration of 0.6% in PBS, neutralized to pH 7.2 with Na_2CO_3 before use. Non–cross reactive hapten oxazolone (OX) (Gallard Schlesinger Inds., Inc., Carle Place, NY) was used as a control for PCl. Rabbit complement (Pel-Freeze Biologicals, Brown Deer, WI) and immunomagnetic dyanal beads (BioMag; Becton Dickinson, Bedford, MA) were used for cell lysis and cell separation experiments, respectively. Magnetic activated cell sorting was performed using a SuperMACS® system (Miltenyi Biotech Inc., Auburn, CA). FACS® reagents included FITC-labeled anti–murine Thy-1.2, Red 670–labeled anti–murine CD3 and PE-conjugated anti–murine B220 antibodies (PharMingen, San Diego, CA), and propidium iodide (P4170; Sigma Chemical Co., St. Louis, MO). cDNA primers for murine CD3 and β-actin for use in reverse transcription (RT)–PCR were synthesized at the Boyer Center for Molecular Medicine (Yale University, New Haven, CT). Mouse ascites fluid containing monoclonal mouse IgE anti-TNP was the kind gift of Dr. F.-T. Liu (Scripps Research Institute, La Jolla, CA) (13).

Sensitization and challenge. Mice were contact sensitized epicutaneously with 200 μl 0.5% solution PCl or with 0.5% solution OX, both in absolute alcohol, to the shaved abdomen and four paws on day 0. On day 1 or day 7, the airways were challenged by intranasal, inhaled administration of 50 μl 0.6% PSA solution under light ether anesthesia, and pulmonary function testing was performed at various intervals afterward. In other experiments, mice were injected intravenously with monoclonal IgE anti-TNP (14) on day 0, PSA airway-challenged on day 1, and pulmonary function tested subsequently.

Histological analysis. At various intervals after challenge, mice were anesthetized intramuscularly with 50 μl of a cocktail of ketamine, xylazine, and atropine (3:3:1 by vol). Lungs were perfused by injection of the right ventricle with cold PBS, cannulated via the trachea with PE-50 polyethylene tubing, and inflated with either 10% buffered formalin for routine histology and processed, or for immunochemistry with 33% OCT and frozen by submersion in liquid nitrogen–cooled methylbutane. Sections of formalin-fixed paraffin-embedded tissue, stained with hematoxylin and eosin, were evaluated for cellular infiltration. For immunohistochemistry, frozen sections of lung tissue were incubated with mAbs to CD3, CD4, CD8, and B220 as previously described (15) using alkaline phosphatase—anti–alkaline phosphatase substrate system (Vector Laboratories, Inc., Burlingame, CA) and fast red (Sigma Chemical Co.).

Pulmonary function testing. Mice were anesthetized with 60–90 mg/kg i.p. sodium pentobarbital (Nembutal, NDC 0074-3778-05; Abbott Laboratories Diagnostic Division, Chicago, IL), and a tracheal cannula was inserted (18-gauge Teflon catheter cut to 2 cm with Luer Plug, 2N112; Baxter Healthcare Corp., Deerfield, IL). Three vital capacity breaths (1.0 ml) were given by a syringe connected to the tracheal cannula to eliminate areas of atelectasis.

Respiratory system impedance (Zrs) was measured by discrete frequency (4–40 Hz in 11 equal logarithmic steps) sinusoidal forced oscillations (amplitude of 1 ml/s ±20% at each frequency) superimposed on spontaneous tidal breathing as previously described (16, 17). The respiratory system resistance (Rrs) was defined as the mean of the real component of the impedance throughout the frequency range. Measurements were independent of the breathing pattern of the animal, and the resistance of the tracheal cannula was accurately eliminated.

AHR was determined by methacholine challenge using an ultrasonic nebulizer (Portasonic model 8600; DeVilbiss Healthcare Inc., Sommerset, PA) connected to a rodent ventilator (model 683; Harvard Apparatus, Inc., South Natick, MA). Methacholine (Sigma Chemical Co.) in PBS was administered serially by nebulization, be-

ginning at a concentration of 0.001 mg/ml delivered through the tracheal cannula in 20 1-ml breaths at a rate of 60 breaths/min. 1 min after each challenge, pulmonary function testing was performed. The concentration of methacholine producing an increase in Rrs of 100% above baseline was extrapolated from the linear portion of the curves for individual mice using linear regression analysis, and was expressed as the mean $-\log_{10}$ PC 100±SE for each group of mice. Thus, multiple 10-fold increases in reactivity to methacholine were reflected by each unitary increase in value on the log scale. In other experiments, the concentration of methacholine that first produced an increase of Rrs of 100% or more above baseline Rrs was determined and expressed on a $-\log_{10}$ scale.

Pulmonary function was also performed using a second technique. Mice were anesthetized, tracheostomized, and actively ventilated with tidal volumes of 0.4 ml at a rate of 150/min. Baseline Rrs and respiratory system dynamic compliance (C_{dyn}) were determined by plethysmography, and serial methacholine challenge was performed. Actively ventilated mice studied in this fashion require higher doses of inhaled methacholine to constrict the airway, and therefore AHR was expressed on a $\log_{10}$ scale as we have previously described (15, 18).

Determination of diffusion capacity. Diffusion capacity of the lungs (DLCO) was measured by a carbon monoxide single breath method, as previously described (17), using a gas chromatograph (model 0111, Carle AGC series 100; Carle Chromatography, Loveland, CO).

mAb and cell transfers. Hybridomas used to produce mAb included: TIB 146 [clone RA3-3A1/6.1 rat IgM anti–murine B220 (CD45RA)] and TIB 164 (clone 14.8, rat IgG anti–murine CD45RA) from the American Type Culture Collection (Rockville, MD). mAb produced by clone RA3-3A1/6.1 was shown to detect a surface Ag on mature B cells and bone marrow precursor cells, as well as a putative Ag-specific delayed-type hypersensitivity–initiating Thy-1+ lymphocyte (19–21). mAb produced by clone 14.8 detects an Ag expressed on B cells as well as a subpopulation of peripheral T lymphocytes of the Th2 phenotype (22). Other hybridomas included: TIB 107 (clone 30-H12, rat IgG2b anti–murine Thy-1.2), TIB 207 (clone GK1.5, rat IgG2b mAb anti–murine CD4), TIB 211 (clone 3.155, rat IgM anti–murine CD8) (23) (ATCC), clone C363.69B (rat IgG anti–murine CD3ε), the kind gift of K. Bottomly (Yale University) (24), clone H57-597 (hamster IgG anti–murine TCRβ) (25), the kind gift of R. Kubo (Cytel Corp., San Diego, CA), and clone UC7-13D5 (hamster IgG anti–murine TCRδ) (26), the kind gift of J. Bluestone (University of Chicago). All hybridomas were expanded in culture media (DMEM 5% FCS, 10 mmol penicillin and streptomycin). Supernatants were sterile filtered and stored at 4°C for use in depletion experiments at concentrations of ∼ 20 ml/10^8 lymphoid cells, determined in previous adoptive cell transfer experiments to be required to completely deplete cell transfers of initiator, effector, and regulatory cells of in vivo, T cell–dependent, PCl cutaneous contact sensitivity responses in mice (14, 23). In all experiments, control cells were incubated with similar volumes of culture media alone before treatment with complement (C′) or before incubation with immunomagnetic beads.

Cell depletion using mAb and C′. Immunized mice were killed by cervical dislocation on day 1 or day 7 after contact sensitization (with either PCl, OX, or sham sensitized). Lymphoid cells from axillary and inguinal lymph nodes and spleen, or from lymph nodes alone, were harvested, and single-cell suspensions were prepared, cells counted, and viability assessed. Cells were then incubated at 4°C for 45 min with mAbs, followed by extensive washing in cold PBS, and resuspended to a concentration of 10^7 cells/ml.

mAb-treated cells were then incubated with C′ at a concentration of 5 ml of 1:75 dilution/10^8 cells for 45 min at 37°C, followed by extensive washing with cold PBS as described previously (14, 21, 23). Cells were pelleted, resuspended in PBS, and injected intravenously at 2×10^8 cells/recipient. Controls received similar numbers of cells incubated with culture media alone and treated with similar quantities of C′.

Magnetic bead cell separation. For magnetic bead separation, cells incubated with mAb or media were washed in cold PBS, pelleted, resuspended in 2% FCS to 10^7/ml and then incubated for 30 min on ice with magnetic beads (Becton Dickinson) coupled to hamster mAb directed against rat IgG (for use with cells incubated with clone 14.8) or against rat IgM (to select B220$^+$ cells), and positively (adherent) or negatively (nonadherent) selected cells were isolated by magnetic separation.

In other experiments, we used SuperMACS® to obtain highly purified populations of CD3-depleted cells. PCl immune lymph node cells were incubated with biotinylated mAb to CD3, or with culture media alone for 30 min at 4°C. Then cells were washed with cold PBS, blocked with Fc blocker (clone 2.4G2; Sigma Chemical Co.), incubated with strepavidin microbeads for 30 min at 4°C, and then passed twice through a SuperMACS® system equipped with a cell separation column with a capacity of 10^9 cells. Depleted cells were harvested as the eluate after the second pass. Adherent cells in the column were washed with cold PBS containing 2% BSA while in the presence of the magnetic field. The column was then removed from the magnetic field, and adherent CD3+ cells were harvested by extensive washing with PBS/FCS. Cells were counted and viability assessed. All of these methods provided cells that were > 95–98% viable and were subsequently transferred intravenously or in some experiments intraperitoneally at a ratio, unless otherwise indicated, of one donor per one recipient into naive animals.

FACS® analysis and propidium iodide staining. We performed parallel experiments with mAb to Thy-1 to determine the efficacy of cell depletion using in vitro techniques. After mAb and C' treatment with Thy-1, immune cells were washed and resuspended in 2% FCS in PBS. Cells were incubated with FITC-labeled anti–Thy-1.2 at 4°C for 20 min, then washed twice. FACS® analysis of lymphocyte gated cells (by forward and side scatter) was then performed using FACScan II®. Separate aliquots of cells were incubated with propidium iodide (0.01% in PBS), a vital stain with a defined fluorescent intensity, used to quantify viability of cells determined by analysis of FL2 channel generated histograms.

In vitro cell proliferation and thymidine incorporation. To assure that mAbs did not result in ex vivo cell activation, cell proliferation studies were undertaken. 6-d PCl immune cells were treated with mAb used for cell depletion experiments with or without the addition of C' as described above. Cells were then cultured in DMEM 5% FCS for 24 h at 37°C (mimicking the time lag between adoptive transfer and lung challenge), then treated with varying doses of concanavalin A (Con A) (0, 1, and 2 μg/ml), and subsequently pulsed with thymidine for an additional 24 h. Thymidine incorporation was determined and compared to immune cells incubated with culture media alone (no mAb), which otherwise were similarly treated both with and without C'.

RT-PCR analysis of CD3 mRNA. To assess the efficiency of CD3 cell depletion, we employed RT-PCR, using a modification of a method previously described, to quantify CD3$^+$ cells in murine tissue (27, 28). 6-d PCl immune cells were treated ex vivo with either culture media alone, mAb to CD3 alone, mAb to CD3 followed by C', or treated with C' alone. Total cellular RNA was isolated by chloroform/ethanol extraction method (28). 2.5 μg of RNA was reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (Stratagene, La Jolla, CA) and specific cDNA primers (27) for murine CD3 (both with and without RT, to exclude DNA contamination) and murine β-actin in order to assess RNA loading of lanes. Subsequently, the reverse transcriptase was inactivated by heating and PCR carried out as described previously (27, 28).

Statistical analysis. Statistical analysis by the Statistics Department of Yale University indicated normal distribution of all data, therefore two-tailed, unpaired Student's *t* test was used to compare groups within experiments, unless otherwise indicated. The Bonferroni correction was applied to experiments where multiple comparisons were made. The *P* values reported were considered significant if ≤ 0.05.

Results

Late increases in Rrs are produced in immunized and challenged mice 24 h after airway challenge, and resolve by 48 h after challenge. Mice were immunized with PCl on day 0 and their airways challenged with PSA on day 7. Baseline Rrs measurements were performed at various intervals after challenge and compared to nonimmune animals that were airway challenged with PSA (Fig. 1). Differences in baseline resistance for PCl immunized and PSA challenged groups in comparison to control groups for the 24 h time point were significant, but for each time point 48 h and more (not shown) after challenge were not significant.

Increased Rrs and DLCO. Groups of mice were either contact sensitized with PCl or not and then airway challenged on day 7 with PSA. Lung diffusion measurements were obtained at various intervals after challenge. No significant changes in DLCO were appreciated when comparing nonimmune to immune mice early (2 h) after challenge (11.6±0.6 vs. 10.1±0.8 ml/min/mmHg, respectively; $P > 0.05$), or at the time of maximum increase in respiratory resistance, at 24 h after challenge (11.9±0.9 vs. 12.7±0.2; $P > 0.1$). DLCO values of unchallenged female mice of similar size ranged from 11 to 12 μl/min/mmHg. DLCO appeared to increase in immune mice by 24 h compared to 2 h after challenge (12.7±0.2 vs. 10.1±0.8 μl/min/mmHg; $P < 0.01$), which was not observed in nonimmune controls ($P > 0.37$).

Lung compliance measurements (C_{dyn}) were made at the time of Rrs calculations, including the 24 h time point, and did not change significantly (not shown). These findings defined the physiologic abnormality as bronchoconstriction of the cen-
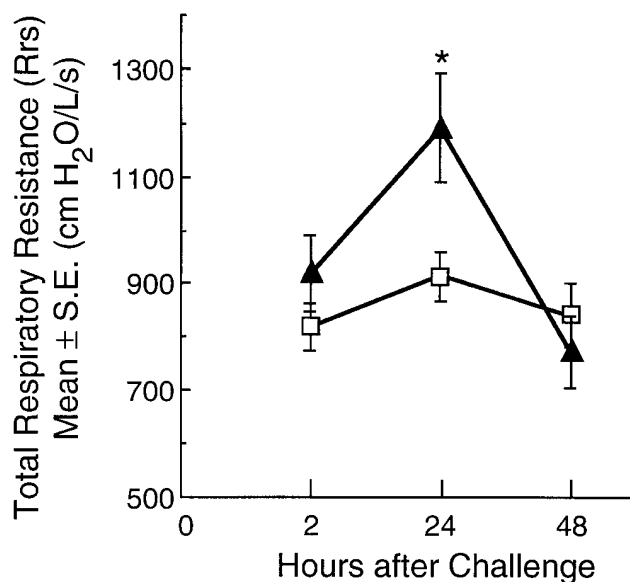

Figure 1. Late phase airway resistance in PCl contact sensitized mice peaks 24 h after airway challenge with PSA. 8–12-wk-old female BALB/c mice were contact sensitized on day 0 with 0.5% PCl or not sensitized. On day 7, mice were airway challenged with 0.6% PSA. Pulmonary function testing was performed at various intervals after challenge. Rrs was significantly increased 24 h after airway challenge (*$P < 0.05$) compared to controls. Differences in Rrs at 2 and 48 h compared to nonsensitized mice were not significant. n = 5–8 mice/group. □, Nonimmune and challenged. ▲, Immune and challenged.
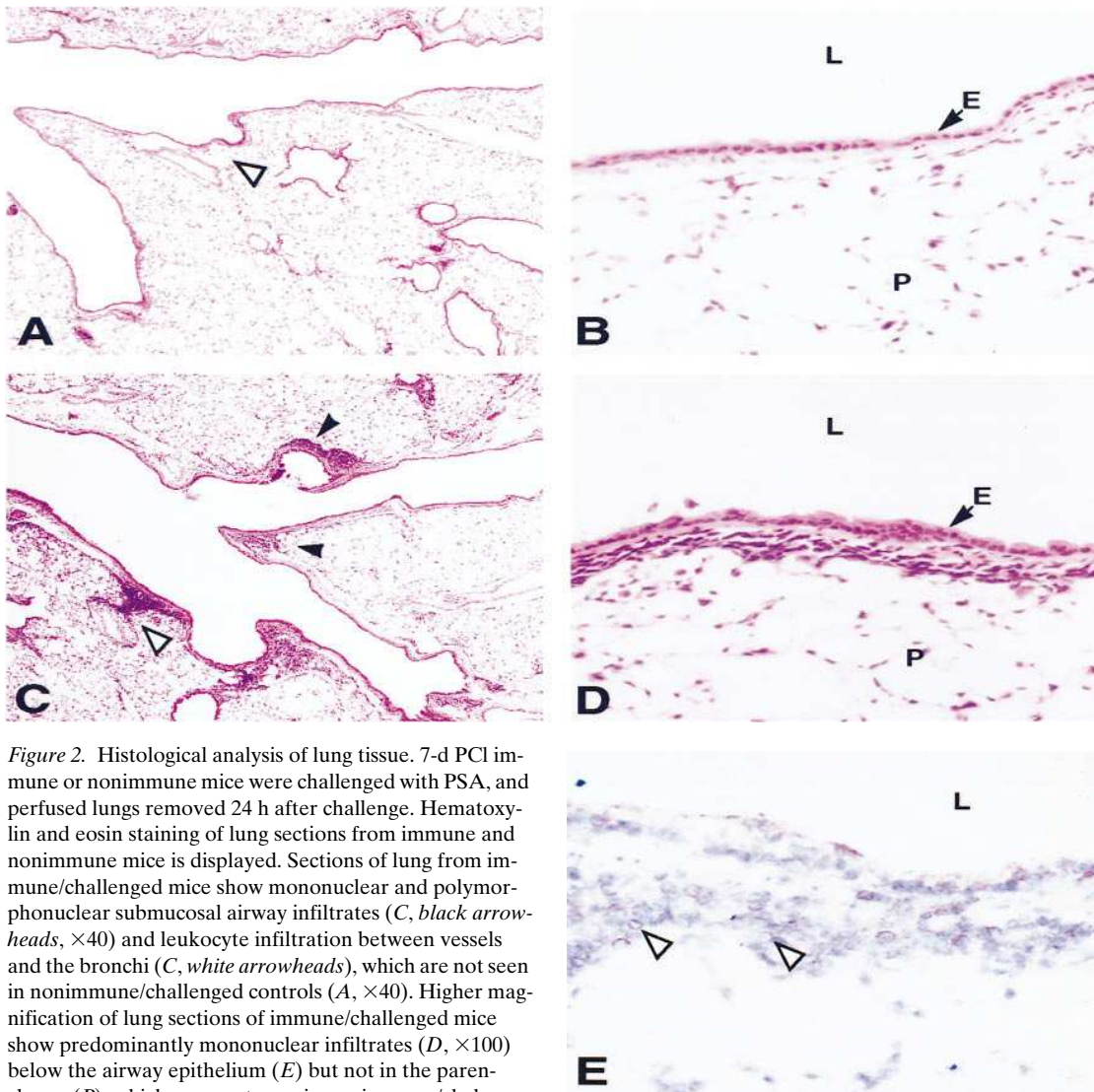
Figure 2. Histological analysis of lung tissue. 7-d PCl immune or nonimmune mice were challenged with PSA, and perfused lungs removed 24 h after challenge. Hematoxylin and eosin staining of lung sections from immune and nonimmune mice is displayed. Sections of lung from immune/challenged mice show mononuclear and polymorphonuclear submucosal airway infiltrates (*C, black arrowheads*, ×40) and leukocyte infiltration between vessels and the bronchi (*C, white arrowheads*), which are not seen in nonimmune/challenged controls (*A*, ×40). Higher magnification of lung sections of immune/challenged mice show predominantly mononuclear infiltrates (*D*, ×100) below the airway epithelium (*E*) but not in the parenchyma (*P*), which were not seen in nonimmune/challenged mice (*B*, ×100). Immunohistochemistry showed these infiltrates to be composed predominantly of CD3+ cells (*E, white arrowheads*, ×400). *L*, lumen.

tral airways, a pattern that has been described previously in bronchoconstrictor responses in mice (29). We concluded that the immune inflammatory responses produced in this system did not lead to physiologically significant alterations of lung parenchyma, and that the increased Rrs 24 h after challenge reflected alterations in airway resistance.

Lung histology is characterized by the presence of peribronchial infiltrates of T cells. Cellular infiltration of peribronchial areas of the lung was assessed at the time of maximal induced airway resistance at 24 h. Representative tissue sections of lungs of nonimmune and PCl immune mice, both taken 24 h after airway challenge with PSA, are displayed in Fig. 2. In comparison to nonimmune and challenged mice (Fig. 2, *A* and *B*), 7-d PCl immune and PSA challenged mice (Fig. 2, *C* and *D*) showed a subtle but unequivocal submucosal infiltration (*black arrowheads*, Fig. 2 *C*) composed of mononuclear cells, a few polymorphonuclear leukocytes, and virtually no eosinophils, which were not seen in lungs of nonimmune mice that

were similarly airway challenged (*white arrowheads*, Fig. 2 *A*). Immunohistochemical analysis showed the mononuclear cells to be CD3+ (*white arrowheads*, Fig. 2 *E*), and CD4+, with fewer CD8+ (not shown). Approximately 80% of the mononuclear cells were CD3+. Of these cells, the ratio of CD4+ to CD8+ cells was ∼ 2:1. Despite transfers of AHR by B220+ cells (see Figs. 4, 8, and 10), very rare B220+ cells were identified in these airway infiltrates. The infiltrates were maximal at 24 h after challenge and were not observed at time points > 48 h after challenge. In contrast to the airways, no significant infiltration of the lung parenchyma was observed at any time point. We concluded that airway resistance changes at 24 h, were associated with a submucosal inflammatory infiltrate dominated by T cells.

In vivo AHR can be produced after 7-d sensitization, peaks between 48 and 72 h after airway challenge, and persists for up to 8 d after challenge. The time course of in vivo AHR to methacholine challenge was studied in mice immunized with
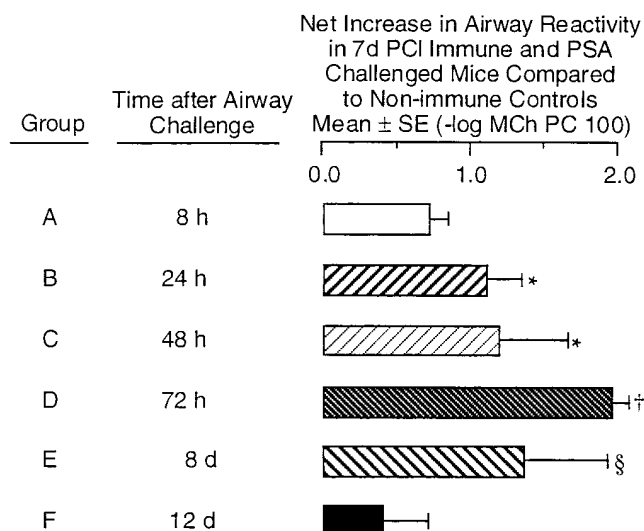
Net Increase in Airway Reactivity
in 7d PCl Immune and PSA
Challenged Mice Compared
to Non-immune Controls
Mean ± SE (-log MCh PC 100)

Group	Time after Airway Challenge	
		0.0 1.0 2.0
A	8 h	⊏══════⊣
B	24 h	▨▨▨▨▨▨⊣*
C	48 h	▨▨▨▨▨▨⊣*
D	72 h	▨▨▨▨▨▨▨▨▨⊣†
E	8 d	▨▨▨▨▨▨▨⊣§
F	12 d	■■■⊣

Figure 3. Time course of AHR induced by immune responses to PCl. Mice were contact sensitized with PCl or OX, or were not sensitized. All mice were challenged on day 7, and pulmonary function testing and methacholine challenge were determined at various intervals thereafter. In comparison to control mice, airway reactivity to methacholine increased significantly by 24 and 48 h (*$P < 0.01$) after airway challenge, peaked at 72 h (†$P < 0.005$), was still significantly increased 8 d after challenge (§$P < 0.025$), and resolved by 12 d after challenge. $n = 4$–10 mice/group.

PCl and airway challenged with PSA. As seen in Fig. 3, AHR was significantly increased ($P \leq 0.05$) 24 h after airway challenge (*Group B*), increased further at 48 h, and peaked 72 h after challenge, reaching a level ∼ 100-fold greater (2 logs) than that of time-matched nonimmune and challenged groups (*Groups C* and *D*, respectively). Airway reactivity of mice sensitized with the distinct and non–cross-reactive immunogen, OX, followed by PSA challenge, did not differ from nonimmune and challenged groups, or from nonimmune mice that were not challenged. Thus the immunization process itself, or the challenge procedure alone, or OX immunization followed by challenge with PSA, all failed to lead to increased AHR, which was produced only in those groups of mice that were immunized with PCl and challenged with the matched Ag PSA. AHR remained significantly increased up to 8 d after Ag challenge of immunized and PSA challenged mice (despite lack of histological evidence of airway infiltration) (*Group E*), and decreased to baseline levels by day 12 after challenge (*Group F*).

Airway hyperresponsiveness can be transferred to naive animals with immune cells harvested 7 d after immunization. Adoptive cell transfer experiments were performed to determine the importance of cell subsets in the induction of AHR. Mice were contact sensitized with PCl on day 0. Lymphoid cells (lymph nodes and spleen) were harvested 7 d later. Single cell suspensions were generated and injected intravenously into naive mice that were PSA airway challenged 1 d later. AHR was evaluated 48 h after challenge. As seen in Fig. 4, transfer of 7-d immune cells (*Group B*) led to a significant increase of airway reactivity, measured 48 h after challenge, in transfer recipients.

Depletion of Thy-1⁺, CD45RA⁺ cells abrogated adoptive cell transfer of AHR, but depletion of CD3⁺, TCR-β⁺, and TCR-δ⁺ cells did not. Adoptive transfers of 7-d PCl immune cells were then performed after ex vivo cell depletion using mAb and C′. In assays of contact sensitivity in vivo, we previously showed complete functional depletion of cell transfers of initiator, effector, and regulatory cell subsets using these antibodies (14, 21, 23). Depletion of cells expressing CD3, the T cell receptor beta chain, or the delta chain of the γδ T cell receptor, did not significantly alter the ability of 7-d immune cells to transfer AHR. In contrast, treatment of immune cells with mAbs to Thy-1 or B220 (anti-CD45RA) and C′ significantly depleted the transfer of AHR by 7-d immune cells (Fig. 4, *Groups F* and *G*). We concluded that the transfer of AHR depended on a particular population of immune cells that lacked characteristic determinants found on T cells.

Assessment of cell subset depletion. To assess the degree of depletion of 7-d immune lymphoid cells produced by mAbs against Thy-1.2, we performed FACS® analysis and propidium iodide staining described in Methods. Results showed > 90% depletion of Thy-1.2⁺ cells after treatment with mAb and C′ compared to immune cells that were treated with culture supernatant and C′ alone. Thus, Thy-1.2 depletion in vitro coincided with depletion of the ability to transfer AHR in vivo.

B220⁺ cells appeared to transfer AHR. Therefore, to exclude the possibility that the anti-B220 antibodies used stimulated the immune cells transferred, thymidine incorporation was used to assess effects of mAb to CD45RA (B220) on lymphoid cell transfers. 7-d immune cells were treated ex vivo with mAb RA3-3A1/6.1 and C′, and then assessed for thymidine incorporation and compared to immune cells treated with C′ alone. Results showed that mAb to B220 did not directly stimulate proliferation (B220-treated cells: 445±77 cpm vs. non-
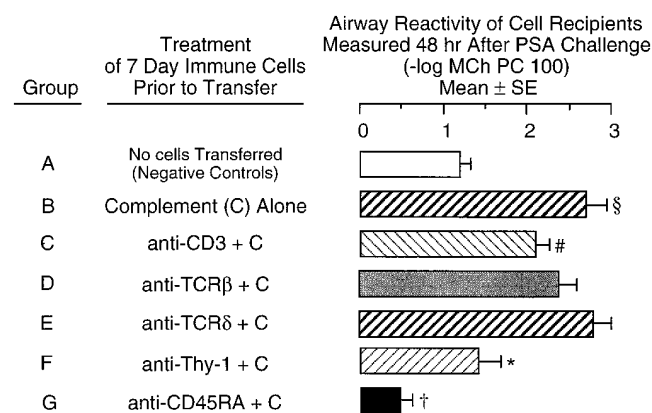
Airway Reactivity of Cell Recipients
Measured 48 hr After PSA Challenge
(-log MCh PC 100)
Mean ± SE

Group	Treatment of 7 Day Immune Cells Prior to Transfer	
		0 1 2 3
A	No cells Transferred (Negative Controls)	⊏═⊣
B	Complement (C) Alone	▨▨▨▨▨▨▨▨⊣§
C	anti-CD3 + C	▨▨▨▨▨▨⊣#
D	anti-TCRβ + C	▨▨▨▨▨▨▨▨⊣
E	anti-TCRδ + C	▨▨▨▨▨▨▨▨⊣
F	anti-Thy-1 + C	▨▨▨⊣*
G	anti-CD45RA + C	■■⊣†

Figure 4. Adoptive transfer of AHR by 7-d PCl immune cells: effect of mAb plus C′ depletion of specific cell subsets. BALB/c mice were contact sensitized with PCl on day 0. Immune cells were harvested on day 7. Cells were then depleted of CD3⁺, TCR-β⁺, TCR-δ⁺, Thy-1⁺, or CD45RA⁺ cells (*Groups C–G*, respectively), or not depleted (*Group B*) by mAb (or culture media alone, *Group B*) and rabbit complement. 2×10^8 cells (1:1, donor/recipient) were then transferred iv to naive mice which were airway challenged with PSA 1 d later. Pulmonary function was determined 48 h after challenge and compared to mice that received PBS alone (*Group A*). Results shown represent pooled data from two experiments. $n = 6$–9 mice/group. *$P < 0.01$, †$P < 0.005$ vs. *B*; §$P < 0.0005$ vs. *A*; #$P = $ NS vs. *B*.
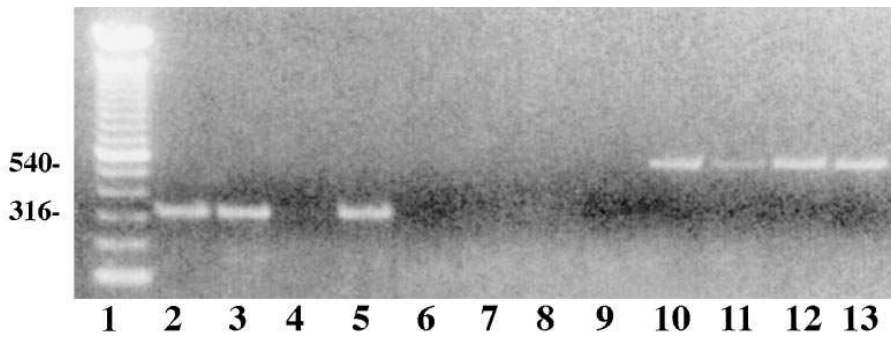
Figure 5. RT-PCR for CD3δ. 7-d PCl immune cells were treated with culture media alone (control), mAb to CD3 alone, mAb to CD3 followed by C′, or with C′ alone. Total cellular RNA was isolated, reverse transcribed, and cDNA product was amplified using PCR and primers for murine CD3 (lanes *2–9*) and for murine β-actin (lanes *10–13*). Lane *1*, kB ladder showing expected size for cDNA for CD3 (316 kb) and β-actin (540 kb). Lane *2*, 7-d immune cells treated with culture media alone. Lane *3*, 7-d immune cells treated with mAb to CD3 alone. Lane *4*, 7-d immune cells treated with mAb to CD3 followed by C′ treatment. Lane *5*, 7-d immune cells treated with C′ alone. Lanes *6–9*, same as lanes *2–5*, without the addition of reverse transcriptase, showing no DNA contamination. Lanes *10–13*, same sequence as lanes *2–5*, using primers for murine β-actin.

treated cells: 339±75 cpm, $P > 0.375$) and also led to no significant change in the proliferative response to Con A (for immune cells that were B220- and C′-treated vs. immune cells C′-treated alone, respectively: 1 mg/ml Con A–stimulation indices: 508±20 vs. 390±27, $P > 0.375$; 2 mg/ml Con A: 632±13 vs. 661±26, $P > 0.375$). We concluded that mAb to B220 (CD45RA) at the doses used did not stimulate immune cells ex vivo, nor result in any comitogenic effect on transferred T cells.

CD3 depletion did not deplete the ability to transfer AHR in vivo in this model. Therefore, to confirm CD3 depletion, we used RT-PCR with primers for CD3δ mRNA and β-actin to control for RNA loading, as described previously, to determine T cell presence in murine tissues (27, 28). Results displayed in Fig. 5 showed complete depletion of CD3 mRNA (no signal) of anti-CD3– and complement-treated PCl immune cells (Fig. 5, lane *4*) when compared to culture media–treated immune cells alone (Fig. 5, lane *2*), immune cells treated with anti-CD3 alone (Fig. 5, lane *3*), or immune cells treated with C′ alone (Fig. 5, lane *5*). DNA contamination was excluded by the absence of signal in the absence of reverse transcriptase (Fig. 5, lanes *6–9*). RNA loading, assessed by amplification using β-actin–specific primers (lanes *10–13*), confirmed that CD3 mRNA was completely depleted using anti-CD3 followed by C′ (lane *4*, compared to lanes *2* and *5*), despite a similar level of β-actin message (lane *12*, compared to lanes *10* and *13*).

We concluded that antibodies to Thy 1.2 and CD3 gave high degrees of depletion of targeted cell subsets. Thy 1.2–depleted lymphoid cells failed to transfer AHR, whereas immune cells highly depleted of CD3 cells were still capable of transfer of AHR. Taken together these data show that the transfer of AHR in this model required the presence of Thy-1^{+} and B220^{+} cells that lack surface expression of CD3.

Depletion of both CD4 and CD8 positive cells, required for transfer of T cell–mediated contact sensitivity, fails to affect transfer of AHR. To reconfirm that conventional T cells were not required for the adoptive cell transfer of AHR to naive recipients, we performed additional T cell depletion experiments using mAbs against CD4 and CD8 surface determinants, which are present on virtually all CD3^{+} cells, and assayed for the successful adoptive transfer of contact sensitivity responses and AHR in these mice. Donor mice were contact sensitized to either PCl or OX on day 0. On day 7, lymphoid cells were harvested and transferred directly intravenously to naive animals

or after depletion of CD4^{+} and CD8^{+} cells. On day 8, all groups were airway challenged with PSA and on day 9 ear challenged with PCl. Pulmonary function was performed on day 10 (48 h after airway challenge), immediately after which ear thickness was determined for contact sensitivity (CS) responses (24 h after ear challenge). Pulmonary function and ear swelling responses were compared to control mice which were recipients of OX immune lymphoid cell transfers that were similarly challenged, to test for Ag specificity.

Results presented in Fig. 6 show that transfer of AHR was Ag-specific (*Group A* vs. *Group B*). Furthermore, although CS ear swelling responses were abolished by mAb and C′ depletion using anti-CD4 and anti-CD8 antibodies (indicating a complete functional depletion of CD4/CD8 T cells that mediate CS), AHR transferred with the same cell population was not affected (Fig. 6, *Group C*). We concluded that T cells with surface determinants for CD4 or CD8 were not required for the Ag-specific transfer of AHR.
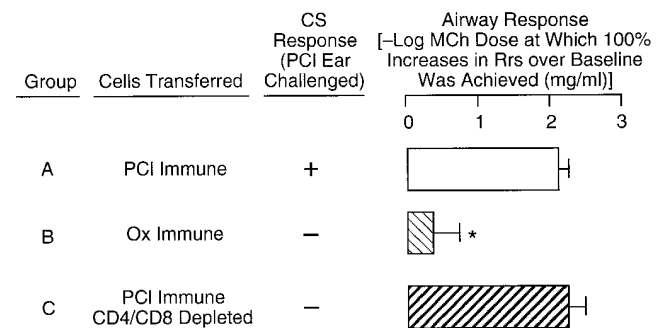

Figure 6. Depletion of CD4^{+} and CD8^{+} T cell subsets abrogates contact sensitivity without affecting the Ag-specific cell transfer of AHR. Mice were contact sensitized with PCl or OX on day 0. On day 7, immune cells were harvested, either not depleted (*Groups A and B*) or depleted of CD4 and CD8 cells (*Group C*), before transfer to naive recipients which were airway challenged (day 8) to induce AHR and also were ear challenged to elicit CS 2 d later (day 9). Airway reactivity to methacholine was determined 48 h after PSA airway challenge on day 10, and ears were measured immediately afterward. Airway responses: *$P < 0.005$ *A* vs. *B* and *B* vs. *C*; $P = NS$ for comparison, *A* vs. *C*. CS ear swelling responses: *A*: 2.3 U; *B*: 0.7 U; *C*: 0 U. Background swelling of mice that received no cells and were challenged alone: 0.7 U. $P < 0.05$, *A* vs. *B* and *A* vs. *C*.

PCl immune cells, completely depleted of CD3$^+$ cells, transfer AHR. 7-d PCl immune lymph node cells were separated into CD3$^+$ and CD3$^-$ populations using SuperMACS®. Aliquots of immune CD3$^+$ and CD3$^-$ harvested cell populations were then incubated with FITC anti-CD3 or anti-B220, and FACS® was performed to quantitate depletion. PCl immune lymph node cells were 61% CD3$^+$ and 26% B220$^+$ before the column. After the column, depleted cells were shown to be 0% CD3$^+$ and 92% B220$^+$. Positively selected cells were 80% CD3$^+$ and < 10% B220$^+$. CD3$^-$ cells that were also Thy-1$^+$ represented 2.3% of the transferred population. Then, equal amounts of positively or negatively selected cells (10^7 cells in 0.5 ml PBS) were transferred to naive recipients and compared to mice that received PBS alone.

1 d later, all mice were airway challenged with PSA, and pulmonary function was assessed 48 h later in actively ventilated mice using body plethysmography as described in Methods. In a representative experiment shown in Fig. 7, PCl immune CD3$^+$ cells failed to transfer AHR to naive recipients (Fig. 7, *Group B*), whereas CD3$^-$ cells (100% depleted of CD3$^+$ T cells) successfully transferred AHR (Fig. 7, *Group C*). These results were in agreement with antibody and C′ depletions (Fig. 3), and taken together confirmed that the adoptive transfer of AHR in this model required immune Thy-1$^+$, B220$^+$ (CD45RA) cells that lack surface expression of CD3.

Adoptive cell transfer of AHR by immunomagnetic bead enrichment for B220$^+$ cells. Positive selection of immune lymphocytes was performed using immunomagnetic dyanal bead selection. Axillary and inguinal lymph nodes (no spleen) were harvested 7 d after contact sensitization with PCl. As shown in Fig. 8, positive selection of B220$^+$ cells (using the mAb produced by clone RA3-3A1/6.1, *Group C*) transferred AHR (*Groups B* and *C*, $P < 0.01$ vs. *Group A*), whereas the cells remaining after this positive selection (B220-ve, *Group D*) were incapable of transfer ($P < 0.05$ vs. *Group C*, and $P = NS$ vs. *Group A*). Thus, these immunomagnetic bead cell separation experiments confirmed multiple prior mAb + C′ experiments, showing that a population of lymphocytes with surface determinants for B220 was necessary for the adoptive transfer of AHR.
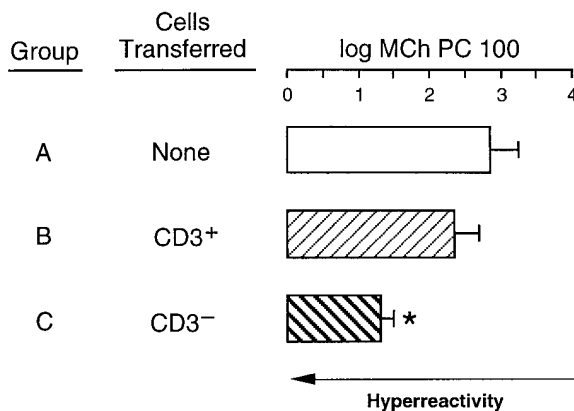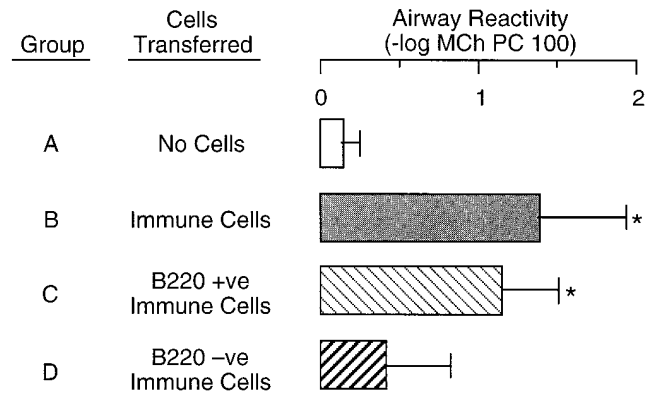
Figure 8. Immunomagnetic bead fractionation and transfer of B220$^+$ (CD45RA$^+$) cells induces AHR. Mice were contact sensitized with PCl and immune cells harvested on day 7. Cells were then either transferred without depletion (*Group B*) or enriched in or depleted of CD45RA$^+$ cells by immunomagnetic bead selection using mAb RA3-3A1/6.1 anti-CD45RA, anti-B220 (*Groups C* and *D*, respectively). Immune cells (*Group B*), B220$^+$ cells (*Group C*), and the cells remaining after harvest of B220$^+$ cells (B220-ve cells, *Group D*) were transferred to naive mice which were subsequently airway challenged 1 d later. Pulmonary function and methacholine challenge were performed 48 h after airway challenge. $n = 3$–5 mice/group. *$P \leq 0.05$ for comparisons, *B* vs. *A* and *B* vs *D*. $P = NS$ for comparisons, *A* vs. *D* and *B* vs. *C*.

FACS® analysis of Thy-1.2$^+$, B220$^+$, CD3$^-$ lymphoid cells. To determine some preliminary morphologic characteristics of PCl immune Thy-1$^+$, B220$^+$, CD3$^-$ cells, we performed parallel experiments whereby pooled lymph node cells from 7-d PCl immune BALB/c mice were triply stained with monoclonal antibodies (PharMingen) that included FITC-labeled anti–Thy-1.2, PE-labeled anti-B220, and Red 670–labeled anti-CD3. Analysis of all (ungated) cells confirmed our previous calculations (shown above), that ∼ 2% of the total harvested PCl immune lymphoid cell population was simultaneously Thy-1$^+$, B220$^+$, and CD3$^-$. Forward and side scatter analysis showed that, compared to conventional CD3$^+$ cells simultaneously present in these lymph node preparations, the Thy-1$^+$, B220$^+$, CD3$^-$ cells were characterized by similar low values for side scatter (i.e., were lymphocytes) and somewhat higher forward scatter, suggesting that these cells are larger lymphocytes, with a mean diameter of ∼ 9 μm compared to 6 μm for conventional CD3$^+$ lymphocytes. We concluded that the Thy-1$^+$, B220$^+$, CD3$^-$ cells in the PCl immune lymphoid cell preparations transferring AHR were probably large lymphocytes.

Ag-specific AHR is induced as early as 1 d after sensitization. To evaluate the effect of a shorter immunization on generation of Ag-specific AHR, mice were contact sensitized and then just 1 d later were airway challenged with PSA. Pulmonary function was tested 48 h after challenge. As can be seen in Fig. 9, challenge alone (*Group B*), or sensitization without challenge (*Group C*), led to no increase in AHR above that of unmanipulated mice (*Group A*). However, mice that were both PCl immunized and PSA challenged (*Group D*) manifested significant AHR (more than an eightfold increase), even after this short 1-d immunization protocol ($P > 0.005$). This contrasted with the 30–100-fold increases in AHR pro-

Figure 7. CD3 depleted cells transfer AHR. 7-d PCl immune lymphoid cells were depleted of or enriched in CD3 cells by MACS® and then transferred to naive mice which were PSA airway challenged 1 d later. Pulmonary function of actively ventilated mice was performed 48 h later. *$P < 0.025$, *A* vs. *C*; $P < 0.005$, *B* vs. *C*. $n = 3$–4 mice/group.

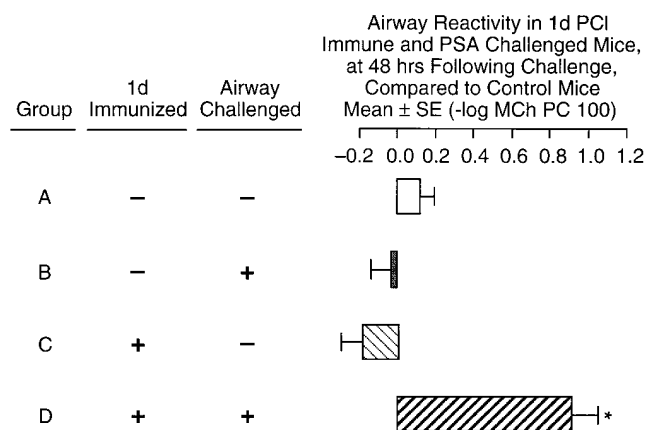| Group	1d Immunized	Airway Challenged	Airway Reactivity in 1d PCl Immune and PSA Challenged Mice, at 48 hrs Following Challenge, Compared to Control Mice Mean ± SE (-log MCh PC 100)
			−0.2 0.0 0.2 0.4 0.6 0.8 1.0 1.2
A	−	−	
B	−	+	
C	+	−	
D	+	+	*

Figure 9. AHR is produced in PCl contact sensitized and PSA airway challenged BALB/c mice as early as 1 d after sensitization. Mice were contact sensitized with PCl on day 0 and airway challenged with PSA on day 1. Pulmonary function testing and determination of airway reactivity to methacholine was performed 48 h after airway challenge (*Group D*) and compared to that of groups of control mice (*Groups A–C*). $n = 5$ mice/group. *$P < 0.005$ compared to all control groups.

duced in challenged animals 7 d after immunization. Interestingly, the elicitation of AHR after challenge of mice only 1 d after immunization was not associated with changes in Rrs or any significant cellular infiltration of the airways typical of mice challenged 7 d after immunization.

AHR can be transferred by 1-d immune Thy-1$^+$, CD45RA$^+$, CD3$^−$ cells. Lymphoid cells (lymph nodes and spleen) were harvested 1 d after PCl immunization and transferred to naive animals that were airway challenged 1 d later. AHR was evaluated 48 h after challenge. As seen in Fig. 10, transfer of 1-d immune cells (*Group B*) led to significant increases of airway reactivity (*Group A* vs. *B*). Treatment of 1-d immune cells with mAb to CD3 + C′ failed to deplete transfer (Fig. 10, *Group B* vs. *E*), whereas treatment with mAbs to Thy-1 and B220 completely abrogated the cell transfer (*Groups C* and *D*, respectively, vs. *Group B*). We concluded that the cell responsible for the transfer of AHR in this mouse model both early (1 d) and late (7 d) after immunization, was Thy-1$^+$, CD3$^−$, and B220$^+$ (CD45RA$^+$).

Repeat experiments were performed using anti-B220 and culture supernatant from another mAb directed to CD45RA, clone 14.8. The results showed that 1-d immune lymphocytes transferred AHR (−log PC 100 of immune cell transfer: 3.1±0.1 vs. nonimmune mice similarly challenged: 0.9±0.03 $P \leq 0.0005$), whereas transfer was depleted by mAb and C′ using two different antibodies to deplete cells expressing CD45RA (−log$_{10}$ PC 100 for B220-depleted mice: 0.9±0.04; mAb 14.8–depleted mice: 0.8±0.06; $P \leq 0.0005$ for either depleted group vs. immune cell transfer). Thus, both antibodies were similarly able to deplete the transfer of AHR by 1-d PCl immune cells. This confirmed, with two different mAbs, the importance of CD45RA$^+$ cells in transfer of airway reactivity, and that both 1-d and 7-d airway responses were dependent upon immune cells expressing CD45RA.

Sensitization with anti-TNP IgE mAb and airway challenge with PSA fails to lead to AHR, except at extremely high, nonphysiologic doses. To test whether AHR in this model was

mediated by Ag-specific IgE, naive mice received intravenously either PBS alone or anti-TNP IgE at doses of 2, 20, and 200 μg/mouse. The 20 μg dose was shown previously to produce macroscopically detectable early and late phase ear swelling after local skin challenge with PCl in passive serum transfer of cutaneous anaphylaxis (30). 1 d after injection of IgE, animals were airway challenged with PSA, and AHR was assessed 48 h later. The results show (Fig. 11) that AHR could only be demonstrated for the highest dose of IgE (*Group D*, 200 μg), a quantity very unlikely to be generated in active sensitization protocols at day 7, and especially unlikely by day 1 after immunization, or within 1 d of the transfer of immune lymphoid cells in adoptive transfer experiments, and therefore nonphysiologic. We concluded that IgE, potentially generated in an atopic immune response after PCl immunization, was not sufficient for the induction of AHR in this mouse model.

Discussion

Studies of asthmatic bronchoalveolar lavage fluid, autopsies of patients dying of status asthmaticus, and bronchial biopsies from asthmatics confirm the presence of neutrophils, lymphocytes, and eosinophils in the asthmatic airway (31–34). Cellular activation also appears to be associated with the airway inflammation. Infiltrating eosinophils express EG2, a marker indicative of degranulation, mast cells appear to be present in increased numbers and are degranulated, and T lymphocytes express surface activation markers like CD25 and MHC class II (35, 36).

In this study, we focused on the possible involvement of T cells in producing the altered airway reactivity typical of human asthma in an animal model of AHR, where bronchial inflammation was shown previously to be T cell dependent (10). We proposed to test the hypothesis that T cells independently
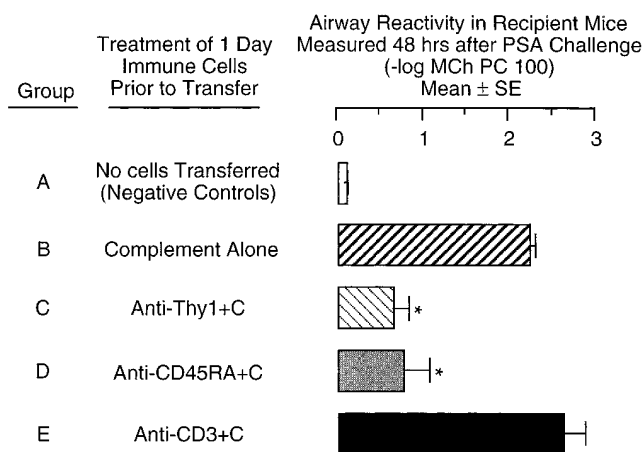
Group	Treatment of 1 Day Immune Cells Prior to Transfer	Airway Reactivity in Recipient Mice Measured 48 hrs after PSA Challenge (-log MCh PC 100) Mean ± SE
		0 1 2 3
A	No cells Transferred (Negative Controls)	
B	Complement Alone	
C	Anti-Thy1+C	*
D	Anti-CD45RA+C	*
E	Anti-CD3+C	

Figure 10. Adoptive transfer of AHR is mediated by 1-d PCl immune cells: effect of mAb and complement depletion of specific cell subsets. Mice were contact sensitized with PCl on day 0. On day 1, lymphoid cells were harvested and either treated with complement alone (*Group B*), or were incubated with mAb to Thy-1 (*Group C*), B220 (*Group D*), or CD3 (*Group E*) and later treated with C′ as described in Methods. After extensive washing, cells were transferred intravenously to naive mice, and pulmonary function was performed 48 h after PSA airway challenge. $n = 4$–5 mice/group. *$P < 0.005$ vs. *B*, P = NS, *B* vs. *E*.

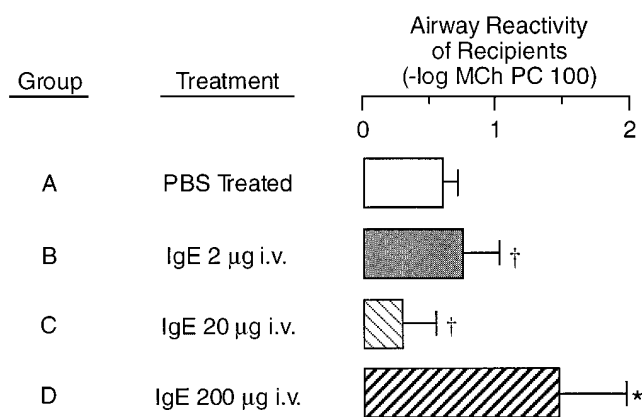| Group	Treatment	Airway Reactivity of Recipients (-log MCh PC 100)
		0 1 2
A	PBS Treated	
B	IgE 2 μg i.v.	†
C	IgE 20 μg i.v.	†
D	IgE 200 μg i.v.	*

Figure 11. Effect of passive transfer of TNP-specific IgE on induction of AHR. Groups of mice received various doses of monospecific IgE or PBS intravenously on day 0. 1 d later, mice were airway challenged with PSA, and pulmonary function was performed 48 h later. $n = 4–5$ mice/group. $*P < 0.05$ for comparison, *A* vs. *D*; $^\dagger P$ = NS, *B* vs. *C*. P = NS, *B* and *C* vs. *A*.

mediate the development of AHR. Surprisingly, our findings indicated that conventional mature T lymphocytes that express surface CD3, T cell receptor $\alpha\beta$ or $\gamma\delta$, or coreceptor molecules CD4 or CD8, do not appear to be required for the successful adoptive transfer of AHR in this model. Instead, a subset of Thy-1$^+$ cells with surface determinants for B220 (CD45RA) was required. These data suggest that conventional T cells found to infiltrate asthmatic airways may not directly and independently mediate all the airway alterations that define asthma.

In vivo cell-mediated immune responses are complicated processes requiring the participation of a variety of cell types. Functionally distinct cell populations, such as mast cells, platelets, $\gamma\delta$ T cells, and suppressor cells have been shown to play an important role in the immunoregulation of in vivo cell-mediated immune responses like CS (14, 23, 37). For example, it was shown that two different lymphocyte subsets are required to elicit CS responses (38). The first, an early-acting Ag-specific cell was shown to mediate the vascular events and tissue swelling that develops early (2–4 h) after Ag challenge in CS responses in the skin of mice. This Ag-specific cell could be generated within 1 d of immunization, and appeared to be required for local recruitment of second-acting conventional $\alpha\beta$ T cells which mediated classical CS inflammatory cell recruitment occurring late (24–48 h) after Ag challenge. It was shown later that the cell responsible for early events characteristically expressed B220, the surface marker later recognized as the high molecular weight isoform of CD45 (CD45RA), but was not present on the late acting, conventional delayed-type hypersensitivity effector T lymphocyte.

In this study, we show for the first time that Thy-1$^+$ and B220$^+$ lymphoid cells lacking CD3 appear to be responsible for the Ag-specific initiation of AHR in vivo in a mouse model. AHR could be produced after Ag challenge as early as 1 d after immunization (8-fold above that of similarly challenged, nonimmune mice), and increased to maximal levels (30–100-fold) by day 7 after sensitization. In multiple experiments, depletion of adoptive cell transfer populations using

mAb to Thy-1 and B220 (CD45RA) resulted in complete abrogation of AHR after transfer of either 1-d immune or 7-d immune cells, thereby confirming the crucial requirement for this lymphoid cell in AHR generated in this model.

The mechanism of Ag specificity of a Thy-1$^+$, CD3$^-$, B220$^+$ cell population and how it mediates the development of AHR are not known. Thy-1 is expressed by T cells as well as by other cells, including hemopoietic precursor cells (39, 40), bone marrow stromal cells (41), neural cells (42), and some B cells (39–41, 43). CD45 is expressed exclusively on leukocytes and hematopoietic progenitor cells (44). Thus it is possible that Ag specificity of the Thy-1$^+$, CD3$^-$, B220$^+$ cells is the result of other membrane receptors such as those associated with natural killer cells (45), or immunoglobulin (non-IgE), or an altered form of the T cell receptor that is not amenable to depletion using the mAbs described above, or by a previously unrecognized family of Ag receptors conferring specificity. How such an Ag-specific cell induces AHR will be studied in the future.

In summary, we show that altered pulmonary physiology induced by contact immunization and airway challenge with a derivative of PCl is characterized by late phase airway obstruction, submucosal airway infiltration dominated by T cells but not eosinophils, and prolonged AHR to methacholine. Adoptive transfer experiments showed that a particular population of Ag-specific lymphoid cells expressing surface Thy-1 and B220 (CD45RA) but lacking CD3, CD4, CD8, or T cell receptors $\alpha\beta$ and $\gamma\delta$ are required for elicitation of this hapten-induced AHR. Moreover, this in vivo AHR occurred in immunized and challenged mice as early as 1 d after immunization, and failed to develop after transfer of specific monoclonal IgE antibody, all suggesting nonatopic mechanisms.

If Thy-1$^+$, CD3$^-$, B220$^+$ cells also prove to occur in nonatopic, human hapten–induced asthma such as toluene diisocyanate, in other nonatopic intrinsic asthma syndromes, or even in relatively more common allergen-driven atopic asthma, then developing treatments aimed at such a population of cells may be more effective in attenuating airway edema (46), cellular infiltration, and bronchial hyperreactivity associated with these disorders than strategies focused on the conventional T cells shown to infiltrate asthmatic airways (47).

Acknowledgments

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