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Long-Lived Th2 Memory in Experimental Allergic Asthma[1]

Nazanin Mojtabavi,* Gerhard Dekan,† Georg Stingl,* and Michelle M. Epstein[2]*

Although life-long immunity against pathogens is beneficial, immunological memory responses directed against allergens are potentially harmful. Because there is a paucity of information about Th2 memory cells in allergic disease, we established a model of allergic asthma in BALB/c mice to explore the generation and maintenance of Th2 memory. We induced disease without the use of adjuvants, thus avoiding Ag depots, and found that unlike allergic asthma in mice immunized with adjuvant, immunizing with soluble and aerosol OVA resulted in pathological lung lesions resembling human disease. To test memory responses we allowed mice with acute disease to recover and then re-exposed them to aerosol OVA a second time. Over 400 days later these mice developed OVA-dependent eosinophilic lung inflammation, airway hyperresponsiveness, mucus hypersecretion, and IgE. Over 1 year after recuperating from acute disease, mice had persistent lymphocytic lung infiltrates, Ag-specific production of IL-4 and IL-5 from spleen and lung cells in vitro, and elevated IgG1. Moreover, when recuperated mice were briefly aerosol challenged, we detected early expression of Th2 cytokine RNA in lungs. Taken together, these data demonstrate the presence of long-lived Th2 memory cells in spleen and lungs involved in the generation of allergic asthma upon Ag re-exposure. ***The Journal of Immunology*****, 2002, 169: 4788–4796.**

Allergic asthma is a syndrome initiated by a specific immune response to a myriad of allergens and is defined by intermittent and reversible airway obstruction, hyperreactivity, and lung inflammation. High serum IgE and an intense inflammatory infiltration particularly rich in eosinophils, mast cells, and neutrophils are involved in the pathogenesis of the disease. The cells that initiate the allergen-specific response and generate cytokines involved in the production of IgE and the recruitment, activation, and survival of the infiltrating cells are CD4 Th2 lymphocytes (Th2) (1). The pathogenic role of Th2 cells is further underscored by their ability to adoptively transfer allergic asthma to naive mice (2–4) and by the findings that elimination of Th2 cells or Th2 cytokines prevents induction and abolishes allergic asthma in mouse models (5). Clinically, patients have intermittent and recurrent episodes of asthma associated with allergen exposure. Such allergen-specific relapses support the idea that immunological Th2 memory plays a crucial role. While cells of the innate immune system and vasoactive and proinflammatory molecules contribute to disease pathogenesis, adaptive immunity is required for the maintenance of responses initiating disease relapses.

Immunological memory is characterized by a more effective response to repeated Ag exposure (reviewed in Refs. 6–8). Although this is a mechanism important for protecting the host against pathogens, it is not obvious what an effective immune response is to an allergen that subsequently leads to asthma. However, long-lived, vigorous immune responses against allergens can be potentially life threatening, and remarkably little is known about CD4 Th2 memory cells in allergic diseases.

Immunological memory has been classically studied in animals using replicating micro-organisms such as viruses, Ag coinjected with adjuvant, and Ag-specific TCR transgenic mice (9). However, the study of memory has been difficult for many reasons. In a normal immune response there is a low precursor frequency of memory cells often below the level of detection. Additionally, it is difficult to study the survival of memory cells in the absence of Ag due to immunization protocols including adjuvants, which leave large Ag depots, or replicating organisms, which also result in a constant and available Ag load. To circumvent these problems, many laboratories use adoptive transfers into syngeneic recipients (10), but there may be transferred Ag, and this may not reflect the immune response under normal circumstances. Studies exploiting the large precursor frequency in TCR transgenic mice do not take into account the lack of nonspecific bystander cells that may play a role in disease. However, adoptive transfer of TCR transgenic T cells provides an opportunity to have a sufficient number of Ag-specific cells within the context of a heterogeneous repertoire. We sought to induce experimental allergic asthma in mice by immunizing with soluble and aerosolized Ag and to subsequently explore the generation and maintenance of Th2 immunological memory upon rechallenge in the same host. The advantages of this approach are that we avoid Ag depots and adoptive transfers and can evaluateTh2 immunological memory by disease and by characterizing Th2 immune responses in lymphoid organs and the respiratory tract.

Materials and Methods

Mice

Six- to 8-wk-old female BALB/c mice maintained in the VIRCC facility and provided OVA-free food and water ad libitum were used in all experiments. All experimental protocols complied with the requirements of the Animal Care Committee of the University of Vienna Medical School.

Immunization

To establish acute allergic asthma, we immunized large groups of mice i.p. with 10 μg of OVA (grade V; Sigma-Aldrich, St. Louis, MO) in 200 μl of PBS or with PBS on days 0 and 21. One week later, on days 28 and 29, mice were nebulized with 1% OVA in PBS or with PBS alone using an

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ultrasonic nebulizer for 60 min twice daily. Groups of mice following acute disease induction are referred to as OVA-OVA ($OVA_{ip}OVA_{aerosol}$), OVA-PBS ($OVA_{ip}PBS_{aerosol}$), and PBS-PBS ($PBS_{ip}PBS_{aerosol}$). To determine whether immunological memory was generated and maintained, we allowed mice with acute disease to recover, and then challenged them a second time with aerosolized 1% OVA or PBS for 60 min twice daily on 2 consecutive days. Memory and control groups are referred to as OVA-OVA-OVA ($OVA_{ip}OVA_{aerosol}OVA_{aerosol}$), OVA-OVA-PBS ($OVA_{ip}OVA_{aerosol}$ $PBS_{aerosol}$), OVA-PBS-OVA ($OVA_{ip}PBS_{aerosol}OVA_{aerosol}$), and PBS-PBS-PBS ($PBS_{ip}$ $PBS_{aerosol}PBS_{aerosol}$). More than six experimental groups were immunized at different time points over 4 years. Evaluation for disease was performed in two or three separate experiments on days 31, 60, 90, 120, 143, 170, 220, 260, and 433. Mice at later time points (days 675, 708, and 807) were used for investigating lung T cells and Ig. Evaluations for disease on indicated days are representative. See Table I for protocol details.

Lung inflammation and mucus production

Forty-eight hours following the last aerosol challenge, mice were evaluated for inflammation and mucus hypersecretion. The tracheas of lethally anesthetized mice were cannulated and lavaged with 1 ml of PBS. Total leukocytes in the bronchoalveolar lavage fluid (BAL)[3] were counted with a hemocytometer, and cytospin slides were prepared and stained with May-Grünwald-Giemsa to determine the cell differential. Following BAL, tracheas were perfused with PBS and then with 4% formalin. Paraffin-embedded lung sections of 4 μm were stained with H&E for morphological evaluation and with periodic acid-Schiff (PAS) for mucopolysaccharide staining, which reflects mucus production. Stained lung sections were evaluated in all experiments.

Airway responsiveness

Twenty-four hours following the last aerosol challenge, mice were evaluated for airway hyperresponsiveness (AHR). We measured AHR in conscious, unrestrained mice by whole-body plethysmography (Buxco Electronics, Sharon, CT) using a method previously reported (11). Briefly, mice were placed into the main chamber, and pressure differences between the main and a reference chamber were recorded. Readings were taken at 5-min intervals at baseline and then with nebulized PBS and titrated doses of methacholine (Sigma-Aldrich) for 5 min each. Pressure readings were averaged for each nebulization dose in a progressive fashion. Results are expressed as a calculated, dimensionless value termed the enhanced pause (Penh) that correlates with pulmonary resistance and airway responsiveness.

Serum Ig

Sera for the measurement of OVA-specific Ig were obtained at 48 h and in some experiments at 96 h after the last aerosol challenge. For the measurement of OVA-specific IgG1, ELISA plates were coated with OVA at 10 μg/ml overnight at 4°C. The plates were washed and blocked with 2% BSA/0.05% Tween 20 for 2 h at 37°C. Titrated sera were incubated for 2 h at room temperature. After washing, biotinylated anti-IgG1 detection mAb (BD PharMingen, San Diego, CA) was added, and plates were incubated for 1 h. Europium (Eu^{3+})-streptavidin (Delfia; Wallac, Turku, Finland) was added to each well after the plates were washed. Enhancement solution (100 μl) was added (Delfia), and Eu^{3+} release was measured by fluorometry at 340 nm excitation and 614 nm emission. The assay used to measure OVA-specific IgE was the same as that described above, except that the plates were coated with anti-mouse IgE (LO-ME-3; Serotec, Oxford, U.K.) and detected with biotinylated OVA.

Cytokine analysis

Pooled spleen or mediastinal lymph node cells from mice 48 h after the last aerosol challenge were incubated at 5×10^5/well with 1000 μg/ml OVA, Con A/IL-2, or medium alone (IMDM with 10% FCS and additives) for 48 h on anti-IL-4- and anti-IL-5-coated Immunospot ELISPOT plates (Cellular Technology, Cleveland, OH). The mAb pairs used for capture/biotinylated detection were as follows: IL-4, BVD4-1D11/BVD6-24G2; IL-5, TRFK-4/TRFK-5 (BD PharMingen). For color development, we added streptavidin POD (Roche, Mannheim, Germany), diaminobenzidene/$NiCl_2$ substrate (Sigma-Aldrich), and then water. Spots were then counted per well and subtracted from wells containing medium alone. For spleen cell cytokine analysis, cells (5×10^5/well) were incubated with OVA (1 mg/ml) or medium alone for 96 h. Cell supernatants were tested for IL-4 and IL-5 using ELISAs (Endogen, Woburn, MA) according to the manufacturer's instructions.

To prepare lung cells for cytokine analysis, lungs were cut into small pieces and were then incubated at 37°C for 1 h with 150 U/ml collagenase D (Sigma-Aldrich) and 50 U/ml DNase (Sigma-Aldrich). Tissue fragments were gently homogenized with a glass homogenizer (Kontes, Vineland, NJ). The resulting cell suspension was flushed through 70-μm pore size mesh filter and centrifuged over a Lympholyte-M (Cedarlane Laboratories, Hornby, Ontario, Canada) gradient. To further purify $CD4^+$ T cells from lung cells, we incubated total lung cells with $CD4^+$ Dynabeads (Dynal, Oslo, Norway) for 20 min at 4°C. The cell suspension was placed on the Dynal Magnetic Particle Concentrator and washed with medium. Beads were removed by incubating the cell suspension with DETACHaBEAD for 45 min at 4°C, followed by bead removal using the Dynal Magnetic Particle Concentrator. Lung cells were incubated with equal numbers of irradiated T depleted spleen APC (5×10^5/well) in the presence of graded doses of OVA in IMDM with 10% FCS and additives or in medium alone on ELISPOT plates for 48 h (as above). Purified populations of lung CD4 cells (2×10^5/well) were incubated with equal numbers of irradiated T cell-depleted spleen APC in the presence of graded doses of OVA in IMDM with 10% FCS and additives or in medium alone in 96-well microtiter plates for 96 h. Supernatants were frozen at −20°C until use. Supernatants were tested for IL-4 and IL-5 levels using ELISA assays (Endogen, Woburn, MA) following the manufacturer's instructions.

Lungs removed 3 h after a 1-h secondary aerosol challenge in mice on day 475 for IFN-γ, TNF-α, IL-2, IL-4, IL-5, IL-6, IL-10, and IL-13 RNA expression were immediately frozen in liquid nitrogen. Total RNA from lungs was extracted with TRIzol reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions. Total RNA (20 μg) was incubated with RQ1 DNase buffer, DTT, RNasin RNase inhibitor, and RQ1 RNase-free DNase (Promega, Madison, WI) for 25 min at 37°C. RNA was further purified by phenol/chloroform/isoamyl (25/24/1) extraction. First-strand cDNA was synthesized from all the digested RNA with avian myeloblastosis virus reverse transcriptase (Roche) in a final volume of 50 μl. cDNA was diluted to a total volume of 500 μl with Advantage buffer

Table I. *Immunization protocol*

Intervention	Day	Evaluation
Sensitization OVA with 10 μg or PBS i.p.	0 21	
Primary aerosol challenge with OVA or PBS, 1 h, twice daily	28 29	
	30	AHR
	31	Inflammation, mucus, Ig
Secondary aerosol challenge with OVA or PBS, 1 h, twice daily	87 88	
	89	AHR
	90	Inflammation, mucus, Ig
	92	Ig
Secondary aerosol challenge with OVA or PBS, 1 h, twice daily	257 258	
	259	AHR
	260	Inflammation, mucus, Ig
	262	Ig
Secondary aerosol challenge with OVA or PBS, 1 h, twice daily	430 431	
	432	AHR
	433	Inflammation, mucus, Ig
	435	Ig
Secondary aerosol challenge with OVA or PBS, 1 h, twice daily	492 493	
	495	Lymph node and spleen cytokine assay

[3] Abbreviations used in this paper: BAL, bronchoalveolar lavage fluid; AHR, airway hyperresponsiveness; HPRT, hypoxanthine-guanine phosphoribosyltransferase; PAS, periodic acid-Schiff; Penh, enhanced pause; TBC, total BAL cell counts.

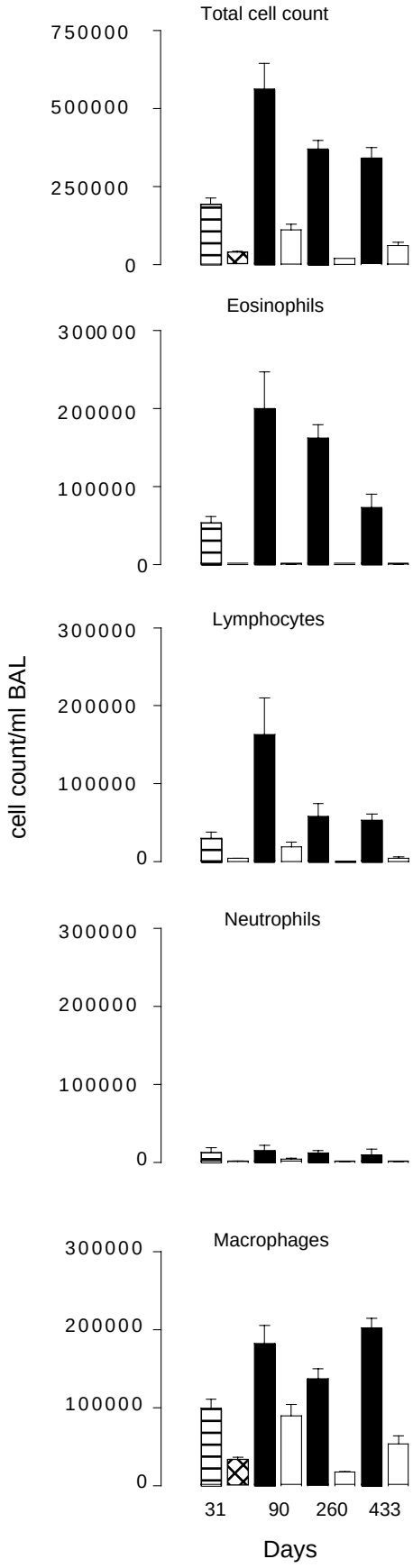

FIGURE 1. Inflammatory infiltrates in BAL of mice with acute disease and following secondary aerosol challenge. Female BALB/c mice were immunized with 10 μg OVA on days 0 and 21, and 7 days later were challenged with 1% OVA aerosol for 1 h, twice daily on 2 consecutive days. Groups of mice (n = 3–5) were rechallenged with OVA aerosol at one time point following primary disease induction and were evaluated for memory recall responses. BAL taken 48 h after the last aerosol was stained, and cells were enumerated. Total cell counts and number of individual leukocytes per milliliter of BAL. Data are from three experiments and are expressed as the mean ± SEM. Groups of mice are represented as OVA-OVA (▤), OVA-PBS (▨), OVA-OVA-OVA (■), and OVA-OVA-PBS (□).

(Clontech, Palo Alto, CA) and was frozen at −20°C until use. Amplification protocols for cytokines consisted of 35 repetitive cycles of denaturing at 95°C (0.5 min), annealing at 60−64°C (0.5 min), and extension at 72°C (1.30 min). cDNA (5 μl) was added to a reaction mixture containing Advantage polymerase according to the manufacturer's protocol. Primers for the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT) were used as internal controls. The PCR primers used to detect all cytokines and HPRT were purchased from Clontech. Amplified cDNA was separated by agarose gel electrophoresis and visualized with ethidium bromide.

FACS analysis

Total lung cells were stained with mAb directed against CD4 (GK1.5-FITC), CD8 (53-6.7-PE), B cells (B220-PE), and MHC class II (M5/114-FITC). Purified CD4 T cells were stained with anti-CD4-PE and counterstained with mAb directed against CD45RB (16A-FITC), CD44 (Pgp-1-FITC), CD62L (Mel-14-FITC), and CD25 (7D4-FITC). All mAb were purchased from BD PharMingen.

Statistical analysis

Groups were compared by unpaired Student's t test. Values of $p < 0.05$ were considered significant. For AHR, mean Penh values at each dose for each group were summed, and then the sums were compared using unpaired Student's t test. All data are presented as the mean ± SEM.

Results

Evaluation of lung inflammation

Inflammation of the respiratory tract in acute allergic asthma is characterized by Ag-specific eosinophilic inflammatory infiltrates. To test whether mice developed allergic lung inflammation, we evaluated mice by BAL and H&E-stained sections 48 h following the last aerosol challenge. We assessed airway inflammation using BAL cell counts and leukocyte differentials. Total BAL cell counts (TBC) indicates the intensity of airway inflammation. Upon the induction of acute allergic asthma, we observed a 20-fold Ag-specific increase in TBC in OVA-OVA compared with OVA-PBS mice on day 31 (Fig. 1). However, when we rechallenged mice a second time with aerosol-OVA, airway inflammation was higher than during acute disease ($p < 0.05$), illustrating Ag-specific memory responses.

To evaluate the composition of the BAL infiltrates, we analyzed the cell counts of individual cell types (Fig. 1). We observed a consistent, statistically significant, OVA-specific increase in absolute counts per milliliter of BAL for each cell type ($p < 0.05$). Airway inflammatory infiltrates in OVA-OVA-OVA mice consisted predominantly of eosinophils and lymphocytes, but neutrophils and macrophages were also increased compared with OVA-OVA-PBS control mice ($p < 0.05$). Although in the absence of further Ag exposure eosinophils disappeared within 30 days, OVA-OVA-PBS mice on day 90 had increased numbers of BAL neutrophils, lymphocytes, and macrophages, suggesting that mice had not yet remitted from acute airway inflammation. Aerosol challenge with BSA, an irrelevant Ag, did not induce inflammation (data not shown). Taken together, these data show an OVA-specific Th2-type inflammatory response in the airways >400 days after disease induction.

Further evaluation of lung inflammation in H&E-stained sections demonstrated that OVA challenge-induced inflammation that

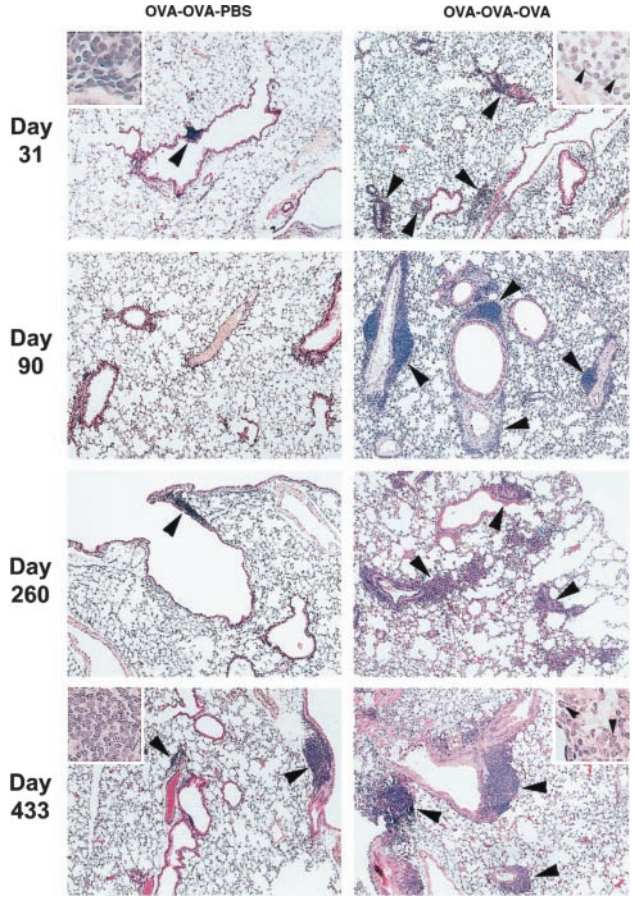

FIGURE 2. Eosinophilic inflammatory infiltrates in the lungs of mice with acute disease and following secondary aerosol challenge. Female BALB/c mice were immunized as previously indicated. Lungs were removed and perfused with PBS and formalin following lavage. These are representative photomicrographs of paraffin-embedded, H&E-stained lung sections taken from mice on the indicated days. Arrowheads in the lower power field (×100 objective lens) indicate foci of inflammation. High power field (×400) insets illustrate eosinophils in OVA-OVA-OVA mice and persistent infiltrates containing lymphocytes, plasma cells, and macrophages in OVA-OVA-PBS mice.

was similar to human asthma. We observed both peribronchial and perivascular infiltrates containing ~30% eosinophils, numerous lymphocytes, macrophages, scattered neutrophils, and few plasma cells in tight foci in the periphery of lungs from OVA-OVA mice during acute disease on day 31 (Fig. 2). Lung tissue sections from OVA-OVA-OVA mice during relapses revealed ~30–50% eosinophils, with many macrophages, lymphocytes, and plasma cells within the infiltrates up until day 433. In the absence of further Ag aerosol exposure, infiltrating eosinophils disappeared within 30 days of acute disease (data not shown). However, lungs from recuperated OVA-OVA-PBS mice contained focal peribronchial and perivascular lymphocytic infiltrates for >1 year after acute disease. These infiltrates were not present in PBS-PBS-PBS or naive age-matched control mice (data not shown). These results demonstrate eosinophilic lung inflammation closely resembling human lesions, memory responses >400 days after acute disease, and persistent lung inflammatory infiltrates in the absence of Ag re-exposure.

Evaluation of mucus production

A characteristic feature of allergic asthma is excessive production of viscous mucus. To test Ag-specific mucus hypersecretion, we stained paraffin-embedded lung sections with PAS. Upon OVA aerosol challenge a large percentage of goblet cells produced mucus in the respiratory mucosa of the large airways compared with a very low level in naive, PBS-PBS-PBS, and OVA-OVA-PBS mice (Fig. 3). Peak mucus hypersecretion appeared on day 90, as evaluated by high numbers of mucus-producing cells, goblet cell hyperplasia, and mucus within the airways. Mucus hypersecretion did not follow BSA aerosolization and remitted 3 wk after the last Ag aerosol exposure (data not shown). These results demonstrate Ag-dependent mucus hypersecretion in response to aerosol-OVA.

Evaluation of methacholine-induced AHR

Wheezing, difficulty breathing, and coughing are cardinal features of asthma and are a consequence of airway obstruction and AHR. To determine whether mice developed Ag-specific AHR, we tested the response of mice to a methacholine aerosol challenge. We nebulized mice with methacholine and measured airway resistance using total body plethysmography 24 h after the last OVA aerosol challenge. OVA aerosol was necessary for the induction of AHR in response to graded doses of nebulized methacholine at all time points tested (Fig. 4). Naive, OVA-PBS, OVA-OVA-PBS, PBS-PBS, and PBS-PBS-PBS mice also respond to methacholine, but the responses were significantly less than those of OVA-OVA and OVA-OVA-OVA mice. Experiments were internally controlled

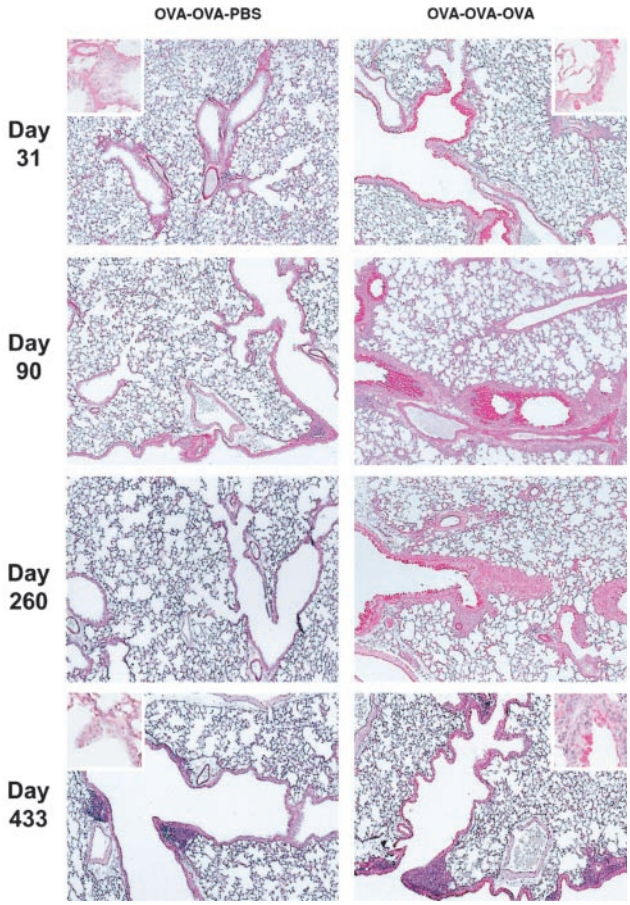

FIGURE 3. Mucus hypersecretion in mice with acute disease and following secondary aerosol challenge. Representative photomicrographs of paraffin-embedded, PAS-stained lung sections at low (×100) and high (×400) power fields for OVA-OVA-OVA and OVA-OVA-PBS mice on the indicated days. Intense fuchsia staining of mucopolysaccharide within goblet cells of the respiratory epithelium and the airway lumen demonstrates mucus production.

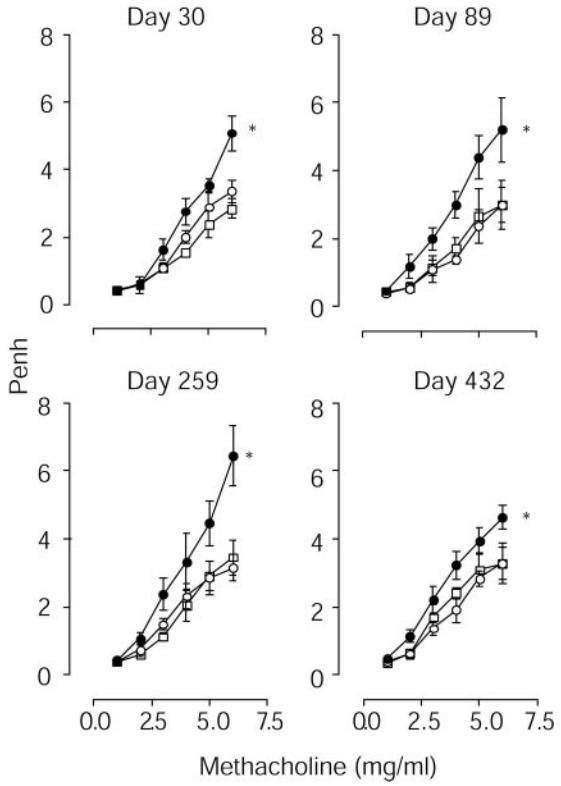

FIGURE 4. AHR in mice with acute disease and following secondary aerosol challenge. Mice were nebulized with titrated doses of methacholine and were tested for AHR by whole-body plethysmography 24 h after the last aerosol. On day 30 the groups are represented as OVA-OVA (●), OVA-PBS (○), and PBS-PBS (□); on days 89, 259, and 432 the groups are represented as OVA-OVA-OVA (●), OVA-OVA-PBS (○), and PBS-PBS-PBS (□). Data are expressed as the mean Penh ± SEM ($n = 4–6$) and are representative of three independent experiments. Statistical significance (*) is considered at $p < 0.05$ for OVA-OVA and OVA-OVA-OVA compared with OVA-PBS and OVA-OVA-PBS groups of mice, respectively.

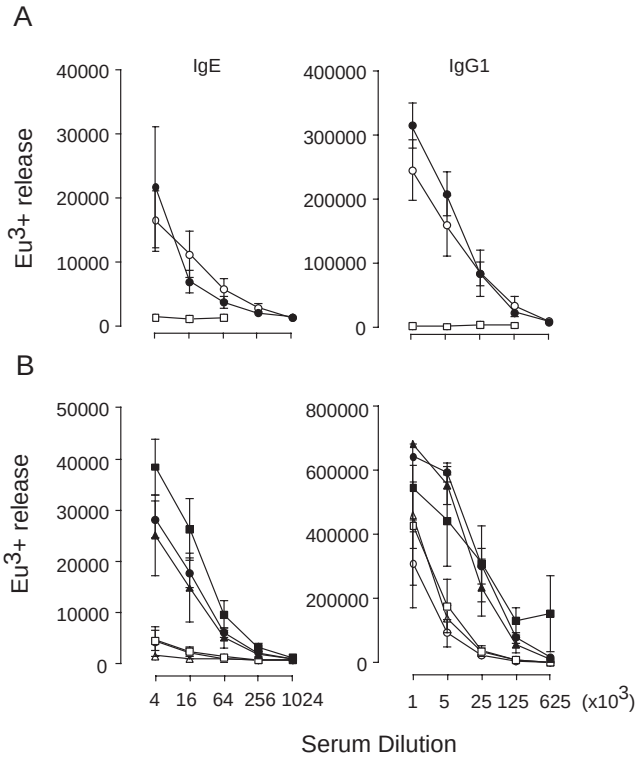

FIGURE 5. Serum OVA-specific IgE and IgG1 in mice with acute disease and following secondary aerosol challenge. Sera were tested 48 h after challenge for OVA-OVA (●), OVA-PBS (○), and PBS-PBS (□) mice on day 31 (*A*), and 96 h following secondary aerosol challenge on days 92 (□, OVA-OVA-PBS; ■, OVA-OVA-OVA), 262 (○, OVA-OVA-PBS; ●, OVA-OVA-OVA), and 435 (△, OVA-OVA-PBS; ▲, OVA-OVA-OVA) (*B*). PBS-immunized mice have no detectable Ig at all indicated days. Data shown are from three independent experiments. These data were generated from testing sera from different time points in the same assay. Each line represents the mean ± SEM Eu^{3+} release for each serum dilution for each group of mice ($n = 4–6$).

and were performed on different days; thus, relative differences between groups were not assessed.

Serum Ig determination

To test whether Th2-type Abs were produced in acute disease and whether they were boosted following aerosol rechallenge, we measured serum OVA-specific IgG1 and IgE at 48 and/or 96 h after aerosol challenge. We observed that serum OVA-specific IgG1 and IgE were elevated on day 31, 48 h after the last aerosol challenge, in mice immunized with i.p. OVA (OVA-OVA and OVA-PBS) regardless of aerosol challenge compared with PBS-PBS mice ($p < 0.05$, Fig. 5*A*), indicating that systemic immunization was effective at generating Ab. We then tested serum OVA-specific IgG1 and IgE at 48 h after secondary aerosol challenge and observed that Ab levels were not significantly different for OVA-OVA-OVA and OVA-OVA-PBS mice (data not shown), suggesting that aerosol challenge did not boost Ab responses. However, we reasoned that aerosol challenge might boost Ab production later than 48 h. When we tested sera 96 h after challenge with OVA aerosol, both OVA-specific IgG1 and IgE were significantly elevated compared with levels in the PBS aerosol controls ($p < 0.05$; Fig. 5*B*), indicating that aerosol OVA boosted Ab production. BAL Ab levels mirrored serum levels for both isotypes at all time points (data not shown). OVA-specific IgG2a was consistently low compared with Th2-type isotypes (data not shown). IgG1, but not IgE, titers were persistently elevated in OVA-OVA-PBS mice, indicating the maintenance of IgG1 production regardless of Ag re-exposure. In one experiment OVA-specific IgG1 levels continued to be elevated at 675 days (data not shown). Remarkably, serum and BAL IgE 48 h after secondary aerosol challenge were undetectable at the later time points, demonstrating that serum IgE levels do not correlate with the onset of OVA-specific inflammation, AHR, and mucus hypersecretion (data not shown). Taken together, these data show that Th2 Ig isotype production increases following aerosol Ag exposure long after initial Ag priming and long-lived production of IgG1 in recuperated mice.

Memory responses in OVA-sensitized mice following delayed aerosol challenge

To prove that memory responses that induce disease depend upon aerosol lung challenge, we tested the OVA-PBS-OVA groups for recall disease and cytokine production. Animals sensitized with OVA i.p., nebulized on days 28 and 29 with PBS, and subsequently nebulized with OVA (OVA-PBS-OVA) developed less BAL and parenchymal inflammation than OVA-OVA-OVA mice on days 60 and 90 and had no inflammation after day 120 (data not shown). This indicates that lung challenge is required <4 mo after sensitization for lung memory responses. A representative experiment from day 260 demonstrated diminished BAL infiltrates upon OVA aerosol challenge in OVA-PBS-OVA mice compared with OVA-OVA-OVA mice (Fig. 6*A*). Nebulized methacholine did not

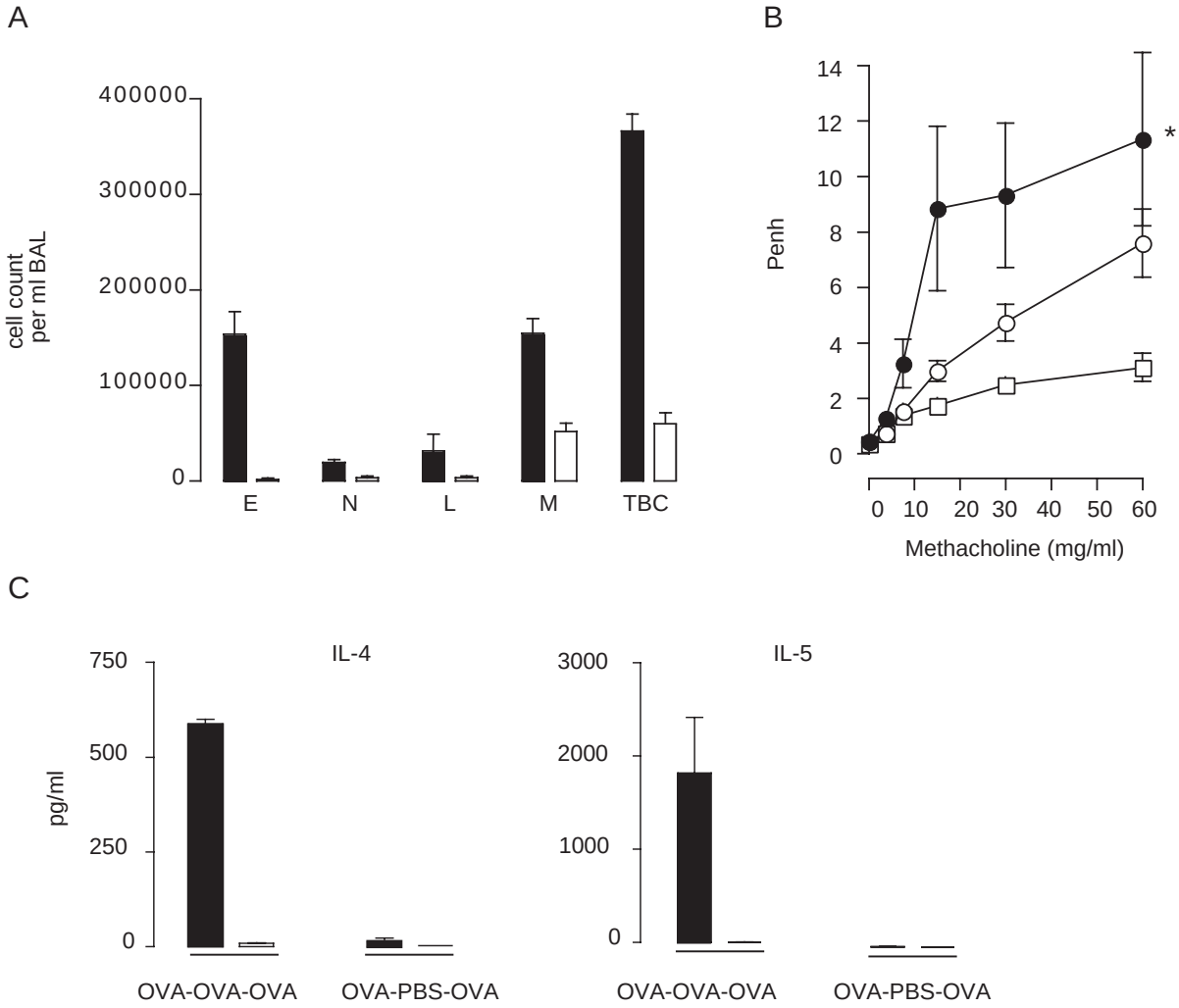

FIGURE 6. Memory responses in sensitized mice following delayed OVA aerosol challenge. Female BALB/c mice were immunized with 10 μg OVA on days 0 and 21, and 7 days later were challenged with 1% OVA aerosol or PBS for 1 h, twice daily on 2 consecutive days. Groups of mice (n = 3–5) were rechallenged with OVA aerosol on days 257 and 258 and evaluated for memory recall responses. *A*, BAL taken on day 260, 48 h after the last aerosol was stained and cells were enumerated. Total cell counts per milliliter of BAL and number of individual leukocytes in BAL. Data from two experiments are presented and are expressed as the mean ± SEM. Groups of mice are represented as OVA-OVA-OVA (■) and OVA-PBS-OVA (□). E, eosinophils; N, neutrophils; L, lymphocytes; M, macrophages. *B*, On day 259 OVA-OVA-OVA (●), OVA-PBS-OVA (○), and PBS-PBS-PBS (□) mice were nebulized with titrated doses of methacholine and tested for AHR by whole-body plethysmography. Data are expressed as the mean Penh ± SEM (n = 4–6) and are representative of three independent experiments. Statistical significance (∗) is considered at $p < 0.05$ for OVA-OVA-OVA compared with PBS-PBS-PBS groups of mice. *C*, Pooled (5×10^5 cells/well) spleen cells from OVA-OVA-OVA (■) and OVA-PBS-OVA (□) mice (n = 5) on day 260 were incubated with OVA (1 mg/ml) for 96 h. Cell supernatants were tested for IL-4 and IL-5 concentrations by ELISA. Data are presented as the average of triplicate determinations ± SEM. All cells responded to Con A/IL-2. These data are representative of eight separate experiments.

induce AHR in OVA-PBS-OVA mice compared with OVA-OVA-OVA mice (Fig. 6*B*). After 120 days mucus hypersecretion and inflammation, as evaluated by histology, were absent in OVA-PBS-OVA mice, and by day 90 serum OVA-specific IgE was not detected (data not shown). Additionally, spleen cell production of IL-4 and IL-5 diminished rapidly and was not detected on day 260 in OVA-PBS-OVA compared with OVA-OVA-OVA mice (Fig. 6*C*), indicating that aerosol allergen challenge plays a role in maintaining spleen Th2 memory. Taken together, these data demonstrate that aerosol allergen challenge soon after sensitization is important for the maintenance of Th2 memory responses.

Spleen and lymph node Th2 memory

To test for the presence of Ag-specific Th2 cells in lymphoid tissue, we tested spleen and draining mediastinal lymph node cytokine secretion from OVA-OVA-OVA and OVA-OVA-PBS mice. In vitro stimulation with OVA induced Ag-specific IL-4 and IL-5 from OVA-OVA-OVA and OVA-OVA-PBS spleen cells (Fig. 7). These data indicate the persistence of Th2 memory cells in spleen and an in vivo aerosol Ag memory response. In contrast, we detected OVA-specific IL-4 and IL-5 secretion from OVA-OVA-OVA, but not OVA-OVA-PBS, draining lung lymph nodes (Fig. 7), suggesting that long-lived Th2 cells are not present in draining mediastinal lymph nodes. Taken together, these data provide evidence for long-lived Th2 memory cells in mice long recovered from acute disease in spleen, but not in draining lymph nodes unless mice receive in vivo aerosol Ag.

Local respiratory tract Th2 memory

Mice recuperated from acute allergic asthma had persistent lymphocytic lung infiltrates as seen in H&E-stained lung sections from

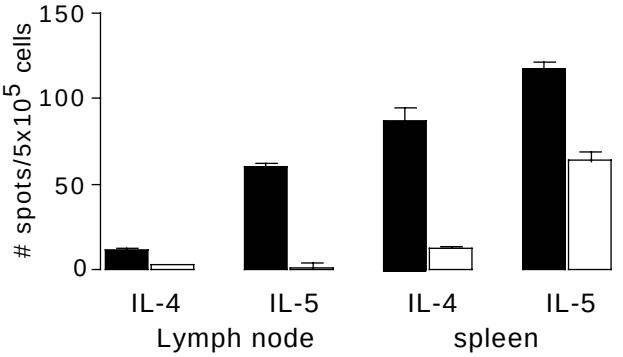

FIGURE 7. Spleen and thoracic lymph node cell production of IL-4 and IL-5 upon stimulation with OVA in vitro. Pooled (5×10^5 cells/well) spleen cells or mediastinal lymph node cells from OVA-OVA-PBS (■), and OVA-OVA-OVA (□) mice ($n = 5$) on day 495, 48 h after the last aerosol challenge, were incubated with OVA (1 mg/ml). Cells were cultured on IL-4 and IL-5 mAb-bound ELISPOT plates for 48 h. Addition of detecting mAb and color development resulted in black spots, which were enumerated. Data are presented as the difference between the numbers of spots per 5×10^5 cells/well from wells cultured with OVA (1 mg/ml) and medium alone and are the average of triplicate determinations ± SEM. All cells responded to Con A/IL-2. These data are representative of four experiments.

OVA-OVA-PBS mice (Fig. 2). To determine whether these infiltrates contained Ag-specific memory Th2 cells, we stained the total lung cell suspensions and observed that the population contained 25% $CD4^+$, 40% $B220^+$, 45% MHC class II^+, and no $CD8^+$ cells (Fig. 8*A*). To demonstrate the phenotype of CD4 T cells present in the lungs, we purified $CD4^+$ cells from total lung cells. A >90% enriched population of lung CD4 T cells expressed high CD44 and low CD45RB, CD62L, and CD25, indicating a memory cell phenotype (Fig. 8*A*). Additionally, we stained frozen lung sections with anti-CD4 and observed many CD4 T lymphocytes within the infiltrates in OVA-OVA-PBS mice, with little difference between OVA-OVA-PBS and OVA-OVA-OVA stained sections (data not shown).

To explore the Ag specificity of persistent lung CD4 T cells, we stimulated isolated lung cells and purified CD4 T cells from recovered mice with APC and OVA in vitro. Total lung cells and CD4 T cells from OVA-OVA-PBS, but not from PBS-PBS-PBS, mice produced OVA-specific IL-4 and IL-5 (Fig. 8, *B* and *C*), indicating the presence of long-lived, Ag-specific memory CD4 T cells in the lungs of mice recuperated from acute allergic asthma. To further investigate the Ag specificity of persistent lung memory cells, we evaluated in vivo Ag responsiveness by measuring total lung cytokine RNA expression 3 h after a 1-h aerosol challenge from recovered mice. We reasoned that early evaluation after a brief aerosol exposure was necessary to avoid the contribution of immigrant systemic memory cells, particularly from the spleen. While IFN-γ and TNF-α remained at background levels, a 1-h OVA aerosol challenge induced increases in RNA expression of IL-2, IL-4, IL-5, IL-6, IL-10, and IL-13 on day 475 (Fig. 8*D*). Taken together, these data demonstrate that Ag-specific Th2 memory cells persist in the lungs of mice >1 year after recuperating from acute disease and suggest that they play a role in the initiation of allergic asthma relapses.

Discussion

Here we show that mice immunized with soluble and aerosolized Ag develop allergic asthma with both peribronchial and perivascular, focal, and peripheral lung inflammatory infiltrates that closely resemble human lesions. These infiltrates persist in the lungs of mice recovered from acute disease in the absence of further Ag exposure. However, upon Ag stimulation, lung memory CD4 T cells act rapidly

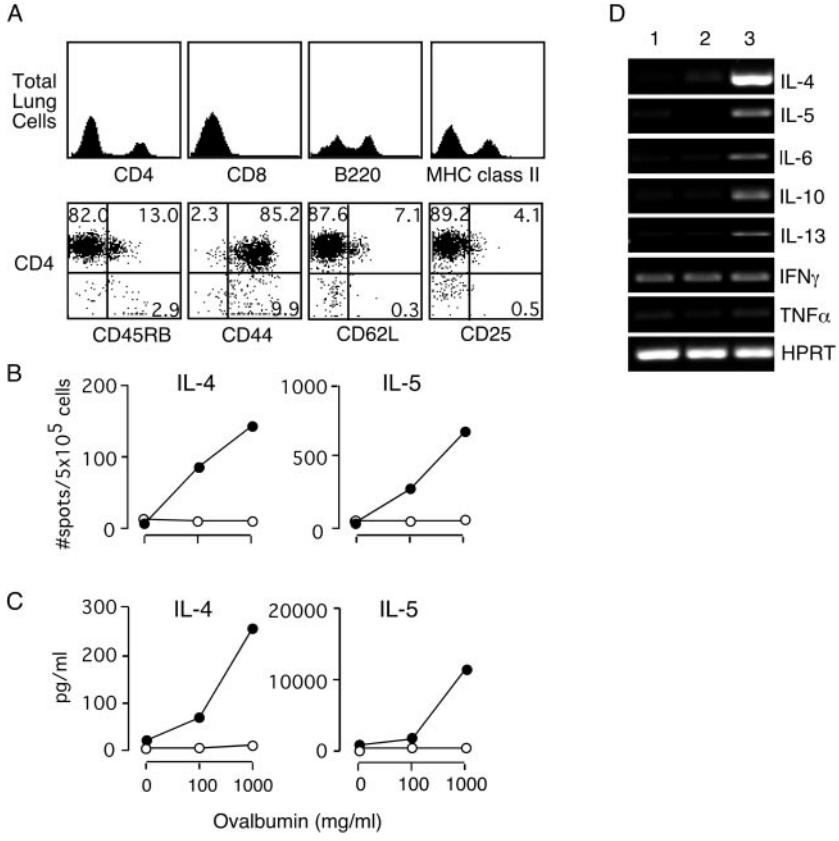

FIGURE 8. Lung memory cells in OVA-OVA-PBS mice. *A*, FACS analysis of total lung cells from OVA-OVA-PBS mice ($n = 5$) from day 708 stained with anti-CD4, -CD8, -B220, and -MHC class II mAbs (*top panel*) and purified CD4 cells from OVA-OVA-PBS mice ($n = 12$) on day 807 stained with mAb directed against CD45RB, CD44, CD62L, and CD25. *B*, Pooled (5×10^5 cells/well) total lung cells from OVA-OVA-PBS, and PBS-PBS-PBS mice ($n = 5$) on day 436 were incubated in triplicate wells with equal numbers of irradiated APC and graded doses of OVA. Cells were cultured on IL-4 and IL-5 mAb-bound ELISPOT plates for 48 h. Addition of detecting mAb and color development resulted in black spots, which were enumerated. Data are presented as the difference between the numbers of spots per 5×10^5 cells/well with OVA and medium alone and are the average of triplicate determinations ± SEM. All cells responded to Con A/IL-2. *C*, Pooled (2×10^5 cells/well) purified CD4 T cells from OVA-OVA-PBS and PBS-PBS-PBS mice ($n = 12$) on day 807 were incubated in triplicate wells with equal numbers of irradiated APC and graded doses of OVA for 96 h. Cell supernatants were tested for IL-4 and IL-5 concentrations by ELISA. Data are presented as the average of triplicate determinations ± SEM. All cells responded to Con A/IL-2. *D*, Lung RNA expression of IFN-γ, TNF-α, IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, and HPRT from mice ($n = 3$–4) 3 h following a 1-h aerosol challenge with PBS or OVA on day 475. *Lane 1*, PBS-PBS-PBS; *lane 2*, OVA-OVA-PBS; *lane 3*, OVA-OVA-OVA. These data are representative of three experiments.

to produce Th2 cytokines, resulting in disease relapses >1 year after the last Ag exposure. In addition, persisting Ag-specific cells in the spleen and OVA-specific IgG1 production demonstrate long-lived systemic memory. Thus, local and systemic Th2 memory are generated and maintained in experimental allergic asthma.

Long-lived Th2 memory cells persist in the spleen and lungs of mice long recovered from acute allergic asthma. They have a memory phenotype and respond in vitro by secreting IL-4 and IL-5 upon Ag stimulation. Additionally, they respond rapidly to in vivo aerosol challenge, as detected by the expression of Th2 cytokine RNA in the lungs as early as 3 h. This suggests rapid activation of lung Th2 cells, which then initiates the subsequent cascade of events leading to disease. Moreover, our observation that OVA-PBS-OVA mice did not sustain memory responses in the absence of early aeroallergen encounter illustrates that rapid immune responses depend on previous lung encounter with allergen, which results in sentinel tissue-specific memory cells. The evidence that nonlymphoid tissue harbors resting memory cells that upon activation become effector-memory cells that are capable of initiating allergic asthma is supported by an emerging paradigm suggesting that memory T cells in nonlymphoid tissues contribute to systemic immunity (12). In several studies functionally distinct memory T cell subsets were distinguished by surface receptors such as CCR7, CD62L, and CD45RB; effector function; and capacity to home to lymphoid organs and sites of tissue inflammation (12–17). Furthermore, memory CD8 T cells have been found in the lungs of mice recovered from viral diseases (18–22). Taken together, these data implicate a role for tissue-specific memory T cells.

Although memory Th2 cells were present in the spleen and lungs of recovered mice, we were unable to detect OVA-specific IL-4 and IL-5 in draining lung lymph nodes unless the mice were OVA aerosol challenged. This may be due to the sensitivity of our assay or the absence of memory Th2 cells. We speculate that memory cells are not present in the draining lymph nodes during remission, and that following aerosol challenge Th2 cells migrate to draining lymph nodes. Data demonstrating that memory cells may not be in peripheral lymph nodes (23) and that memory, naive, and effector cells have distinct circulation patterns (24–26) support this hypothesis. Moreover, in a recent study draining lymph node and nonlymphoid tissue T cells were distinguished based on their response to Ag (27). Interestingly, they found that Ag-specific T cells in the lungs did not clonally expand, but produced effector cytokines, while those in draining lymph nodes did the opposite (27). Similarly, cells from resting lungs (OVA-OVA-PBS mice) produced effector cytokines, although draining lung lymph node cells did not. However, upon aerosol exposure Th2 cells were present in the draining lymph nodes. In addition, the numbers of CD4 T cells in lung infiltrates in OVA-OVA-PBS and OVA-OVA-OVA groups were not different. Thus, it is possible that distinct subsets of nonlymphoid/lung and lymphoid memory T cells stimulated upon Ag challenge develop into memory effector cells capable of either producing Th2 cytokines or proliferating, respectively.

A fundamental question regarding the maintenance of memory T cells that remains unanswered is whether persisting Ag is a requirement. Many experiments focusing on this question have resulted in disparate results, particularly when addressing the mechanisms underlying CD4 vs CD8 T cell memory (28–38), and little is known about the role of persisting Ag in the maintenance of Th2 memory cells, especially during allergic disease. It has been shown that persistent Ag is necessary for Th cell survival (10, 38). Ag epitopes in the form of immune complexes reside in vivo on the surface of follicular dendritic cells and are thought to be a reservoir of Ag for circulating memory T cells (39). Often the study of immunological memory is hindered by the presence of Ag depots when animals are immunized with Ag precipitated in alum, emulsified in Freund's adjuvant, and replicating micro-organisms. Although we avoided Ag deposits in this model, we cannot exclude the role of Ag persistence in the maintenance of memory.

T cell memory has also been shown to depend on B cells and Ig (39, 40). While serum and BAL OVA-specific IgE were boosted after OVA aerosol challenge, they were not detectable in OVA-OVA-PBS mice after 143 days. However, elevated levels of serum and BAL OVA-specific IgG1 persisted up until day 675 in OVA-OVA-PBS mice without further Ag challenge (data not shown). While this demonstrates that an allergic response can occur with low levels of serum and BAL IgE, it does not exclude a role for IgE during disease relapses or in the maintenance of Th2 cell memory, because IgE may be tissue bound. Alternatively, IgG1 may play an important role in the maintenance of memory Th2 cells.

The administration of soluble OVA, followed by aerosol challenge, resulted in focal areas of eosinophilic inflammation that tended to be near the periphery of the lung rather than centrally located and to resemble human lesions (41, 42). This pattern of inflammatory cell infiltration differs from well-established protocols inducing disease in <2 wk using OVA precipitated in alum. Immunization with 25 μg of OVA precipitated in alum on days 0 and 5, followed by aerosol OVA 1 wk later, leads to eosinophilic inflammation characterized by diffuse, centrally located lesions (43). The mechanism underlying the differences remains unknown.

Although persistent lymphocytic infiltrates remained in the lungs of recuperated OVA-OVA-PBS mice, other physiologic changes, including, mucus hypersecretion, eosinophilia, and AHR, were not apparent. Patients with seasonal or intermittent allergic asthma are asymptomatic unless exposed to their inciting allergen. However, asymptomatic asthmatics may exhibit methacholine-induced AHR, which has been correlated with persistent subclinical inflammation (44–46). We observed that methacholine-induced AHR was transient and was evident in OVA-OVA-OVA mice for up to 5 days after OVA aerosol challenge (data not shown). It is not clear why AHR is short-lived in mice.

In summary, we established a model of experimental allergic asthma that more closely resembles human asthma and demonstrated the maintenance of long-lived Th2 memory cells in spleen and lungs. Whether the long-lived local and systemic memory we observed depends on Ag persistence remains to be tested. However, the immunization protocol we used reduces continuous Ag exposure in the form of Ag depots. This model offers an opportunity to further study peripheral and systemic Th2 immunological memory and may assist in the development of novel strategies for the treatment of allergic asthma.

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