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A Restricted Subset of Dendritic Cells Captures Airborne Antigens and Remains Able to Activate Specific T Cells Long after Antigen Exposure

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Summary

Mice sensitized for a Th2 response to *Leishmania* LACK antigen developed allergic airway inflammation upon exposure to LACK aerosol. Using multimers of I-A^d molecules bound to a LACK peptide as probes, we tracked the migration of LACK-specific Th2 cells to the airways. Elevated numbers of LACK-specific Th2 cells remained in the airways for 5 weeks after the last aerosol. Substantial numbers of DC presenting LACK peptides were found in the airways, but not in other compartments, for up to 8 weeks after antigen exposure. These LACK-presenting airway DC expressed CD11c and CD11b as well as high levels of surface molecules involved in uptake and costimulation. Taken together, our results may explain the chronic Th2 airway inflammation characteristic of allergic asthma.

Introduction

Asthma is a complex chronic inflammatory disease of the lung characterized by airflow obstruction, airway hyperresponsiveness, and airway inflammation (Wills-Karp, 1999). This inflammatory response is characterized by infiltration of the airway wall by mast cells, lymphocytes, and eosinophils, together with elevation of allergen-specific IgE. Past studies have established a critical role for Th2 cytokines such as IL-4, IL-5, and IL-13 in the disease process (Coyle et al., 1995; Foster et al., 1996; Grunig et al., 1998). Furthermore, abundant clinical and experimental evidence suggests that T helper cells initiate and maintain inflammation in allergic asthma (Cohn et al., 1997; Gavett et al., 1994; Robinson et al., 1992). Through the cytokines they secrete, Th2 lymphocytes drive the production of allergen-specific IgE and orchestrate the recruitment and activation of the primary effector cells, the mast cells, and eosinophils. Despite recent advances in understanding the pathology of asthma, little is known about the mechanisms leading to the chronicity of airway inflammation resulting in the disease.

Most studies of T cell responses to inhaled antigen describe the behavior of all CD4$^+$ or CD8$^+$ T cells in the lung, although most of these cells are not specific for the challenge antigen. Studies of antigen-specific T cells in asthma models have all used T cells from TCR-transgenic mice to obtain large numbers of T cells of a defined specificity (Cohn et al., 1997, 1998; Mathew et al., 2001; Tsitoura et al., 1999). This approach has been largely used to investigate the role of cytokines released by the Th2 cells; however, little is known about the fate of these cells in sensitized animals upon challenge with an inhaled allergen.

The development of peptide/MHC tetramers for identification and isolation of T cells of a single peptide specificity represents a major technical advance. The use of MHC class I tetramers has substantially advanced and altered our understanding of CD8$^+$ T cell responses to viral and bacterial infections (Busch et al., 1998; Doherty, 1998; Kim et al., 2000; White et al., 1999). More recently, tetramer reagents have been described for a few MHC class II-restricted T cell epitopes to track antigen-reactive CD4$^+$ T cells following an immunogenic challenge (Gütgemann et al., 1998; Liu et al., 2000; Malherbe et al., 2000; Rees et al., 1999). However, this strategy has not been adopted to visualize antigen-specific CD4$^+$ T cells in a mouse model of allergic airway inflammation.

Recently, Malherbe and his collaborators showed that multivalent peptide/MHC molecules that selectively bind to CD4$^+$ T cells reacting to the immunodominant *Leishmania* LACK antigen (Julia et al., 1996) could be used to accurately detect antigen-specific CD4$^+$ T cells in single-chain TCR-transgenic mice infected with *L. major* (Malherbe et al., 2000). The *Leishmania* LACK antigen has proven to be a particularly useful antigen with a single dominant I-A^d restricted epitope in BALB/c mice (Mougneau et al., 1995).

Here we describe a mouse model of allergic airway inflammation using LACK as the model allergen that permits us to track, enumerate, and purify allergen-responsive CD4$^+$ T cells in normal mice without the use of TCR-transgenic T cells.

Results

LACK-Sensitized BALB/c Mice Challenged with LACK Aerosols Exhibit the Hallmarks of Allergic Airway Inflammation

To demonstrate that *Leishmania* LACK protein could be used in a model of allergic airway inflammation, we immunized BALB/c mice i.p. at days 0 and 7 with 10 μg of LACK precipitated with 2 mg of alum. When splenocytes were harvested at day 14 and incubated in vitro with LACK peptide, they proliferated and produced high lev-

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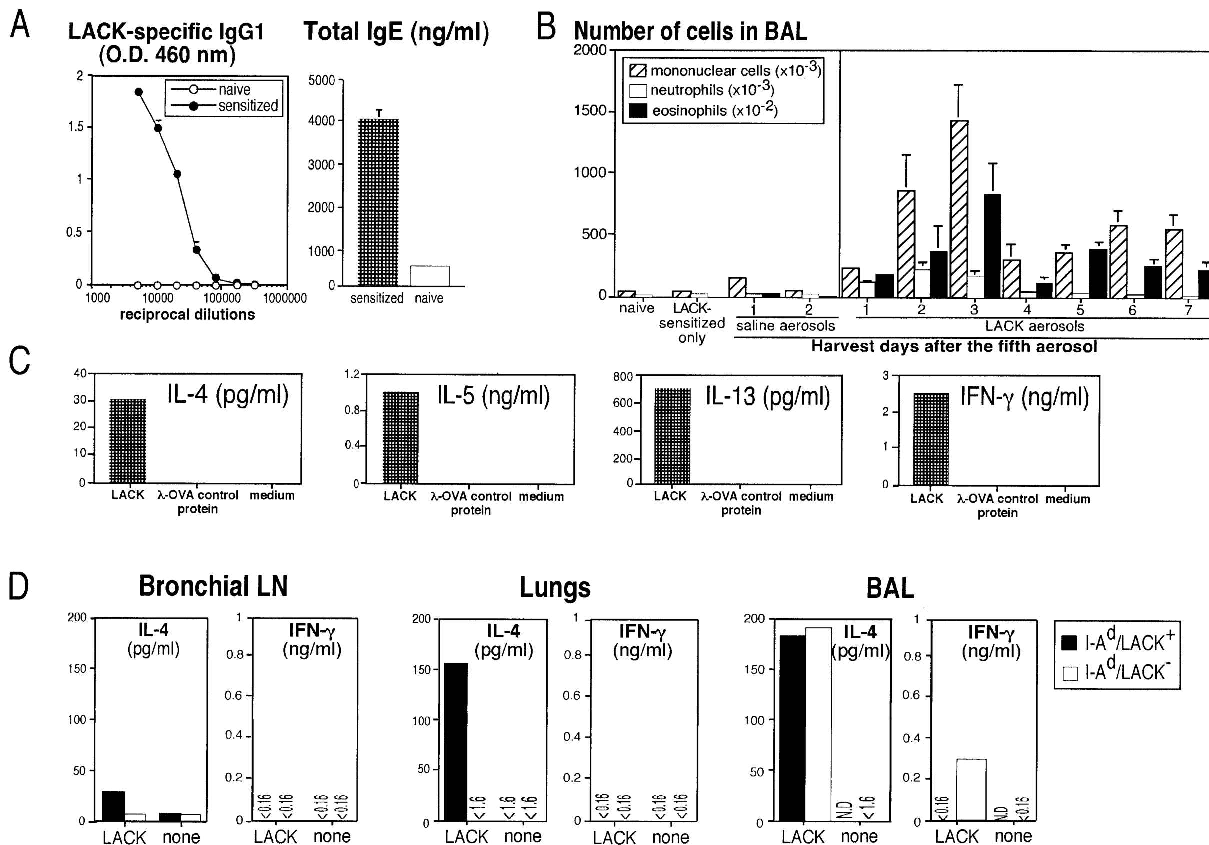

Figure 1. LACK Induces a Strong Th2 Response When Administered with Alum

BALB/c mice were immunized i.p. at days 0 and 7 with 10 μg LACK protein in 2 mg of alum. At day 14, mice were exposed to LACK or saline aerosol for 5 consecutive days.

(A) Serum was isolated at day 14. The presence of LACK-specific IgG1 and total IgE was measured by ELISA in the serum of sensitized and naive animals. Data represent the mean ± SEM of five mice.

(B) The cells from BAL of sensitized, challenged mice were collected at the indicated days after the last LACK or saline aerosol and counted. Cytospin preparations were stained with Wright Giemsa, allowing differentiation into mononuclear cells, neutrophils, and eosinophils by standard morphology. Data represent the mean ± SEM of five mice.

(C) 5×10^6 lung cells harvested 5 days after the last aerosol challenge were incubated with 0.25mg/ml of LACK or a recombinant control protein λ-OVA in 24-well plates. Cytokine contents in the supernatants were analyzed using ELISA after 72 hr of culture. Data show the responses of pooled cells from ten mice. One representative experiment (out of three) is shown.

(D) $CD4^+$ T cells were enriched from bronchial LN or from lavaged lung derived from a pool of 50 sensitized, challenged mice (2 or 4 days after the last aerosol, respectively). BAL cells were harvested 7 days after the last aerosol from a pool of 100 mice. 10^6 $CD4^+$ T cell preparations were incubated with I-A^d/LACK multimers coupled to protein A-Alexa^{488}, CyCR-anti-CD4 mAbs, and PE-anti-(B220, CD8, CD11b, Gr-1, and CD11c). After gating on $CD4^+$ PE^- T cells, I-A^d/LACK$^+$ and I-A^d/LACK$^-$ cells were sorted and 7500 cells were incubated with 8×10^5 irradiated APC in the presence of 25 μM LACK or in medium alone for 72 hr in 96 U-bottom plates. Supernatants were analyzed for IFN-γ and IL-4 contents using ELISA or a CT4S assay, respectively. Data show the results of one representative experiment out of three.

els of Th2 cytokines but no detectable IFN-γ (data not shown). LACK-sensitized mice contained high titers of LACK-specific IgG1 and IgE in their sera as compared to naive animals (Figure 1A), consistent with the strong Th2 polarization observed at the T cell level.

To induce allergic airway inflammation, sensitized mice were challenged with an aerosol of LACK protein or normal saline as a control on 5 consecutive days. The cellular content of the broncheoalveolar lavage (BAL) was analyzed on the indicated days after the fifth exposure (Figure 1B). BAL cells from naive and from sensitized, but not challenged, mice were used as controls. The number of mononuclear cells and eosinophils dramatically increased upon exposure to LACK challenges, peaking 3 days after the last aerosol and remaining elevated for 1 week after the last exposure. In LACK-sensitized mice challenged with saline for 5 days, the number of mononuclear cells was less elevated than in LACK-challenged mice 1 day later, and after 2 days the number had returned to the level of sensitized, nonchallenged controls.

To investigate the capacity of LACK-specific T cells to migrate in response to aerosol challenges, lung cells were isolated after the fifth challenge and stimulated in vitro with LACK protein, a recombinant control protein (λ-OVA), or no antigen (Figure 1C). A relatively polarized Th2 response including IL-4, IL-5, IL-13, and some IFN-γ was detected in response to LACK stimulation but not in the control cultures. LACK-specific T cells were purified by sorting of I-A^d/LACK multimer positive cells from

the bronchial LN, lung, and BAL of sensitized, challenged mice and restimulated in vitro with LACK protein. The I-A^d/LACK$^+$ cells produced high levels of the Th2-specific cytokines, IL-4 (Figure 1D), IL-5, and IL-13 (E.M.H. and V.J., unpublished data), but no IFN-γ (Figure 1D). Of the I-A^d/LACK$^-$ CD4$^+$ T cells, only those from the BAL responded to LACK protein, and they also produced a predominantly Th2 response (Figure 1D). Thus, BALB/c mice primed for a Th2 response to LACK developed strong Th2-mediated allergic airway inflammation when challenged with LACK aerosol.

Aerosol Challenge with LACK Increases LACK-Specific CD4$^+$ T Cells in the Lung

The ability to detect by flow cytometry antigen-specific CD4$^+$ T cells responding to an aerosol challenge afforded the opportunity to study the dynamics of a T cell response in different compartments of the lung. Sensitized mice were sacrificed on the indicated days after the fifth aerosol challenge, and cell preparations from spleen, bronchial LN, lavaged lung, and BAL cells recovered from the lavaged lungs were enriched for T cells and stained with I-A^d/LACK multimers (Figure 2). Cells from naive mice and from mice sensitized but not exposed to aerosol challenges were prepared as controls and similarly stained.

Five aerosol challenges with LACK protein produced sizeable increases in I-A^d/LACK$^+$ cells in the BAL, lung, and bronchial LN, but not in the spleen of LACK-sensitized mice (Figure 2). These increases were observed both as increases in the total numbers of I-A^d/LACK$^+$ cells in the organ and as the increased proportion of LACK$^+$ cells as a percentage of total CD4$^+$ T cells. In four independent experiments, we observed that, between days 2 and 7 after the last aerosol challenge in sensitized mice, up to 34-fold and 13-fold more I-A^d/LACK$^+$ cells were recovered from the BAL ($p < 0.001$) and lung ($p < 0.01$) of sensitized, challenged mice compared to sensitized, nonchallenged controls (Figure 2, right panels). The total numbers of CD4$^+$ T cells in the BAL and lung increased with similar kinetics but lesser magnitude ($p < 0.03$ and $p = 0.5$, respectively), accounting for the 3- to 5-fold increase in I-A^d/LACK$^+$ cells when expressed as a percentage of total CD4$^+$ T cells (Figure 2, left panels). In the BAL and lung, both the frequency and total numbers of I-A^d/LACK$^+$ cells began to decline after day 7. However, most clearly in the BAL, the total number of I-A^d/LACK$^+$ cells remained significantly elevated as compared to sensitized, nonchallenged controls ($p < 0.02$). Even after 5 weeks, the number of I-A^d/LACK$^+$ cells was 4-fold higher in the BAL and 2.5-fold higher in the lung than in sensitized controls. There was also a significant ($p < 0.05$) increase in the numbers of I-A^d/LACK$^+$ cells in the bronchial LN with a 9-fold increase on day 2, declining slowly thereafter. In the spleen, the frequency of I-A^d/LACK$^+$ cells doubled when mice were sensitized with LACK, but their proportion and number did not significantly increase ($p = 0.7$) after aerosol challenges with LACK. In conclusion, antigen-reactive T cells persist in the airways for long periods of time after low-dose aerosol exposures to the antigen.

LACK-Specific T Cells in the Lung and BAL Are Activated, Nonapoptotic Cells

The prolonged elevation of I-A^d/LACK$^+$ cells in the airways in the absence of a continued antigen challenge could have different implications depending on the functional status of these cells. To determine this, lavaged lung and BAL cells from the same mice were harvested 7 days after the last aerosol and stained with I-A^d/LACK multimers, anti-CD4 mAbs, annexin-V, and 7-ADD (Figure 3A). In the lung, approximatively one-third of the I-A^d/LACK$^+$ cells were either apoptotic (Annexin-V$^+$) or necrotic (annexin-V$^+$/7-ADD$^+$) as compared to fewer than 10% of the I-A^d/LACK$^-$ cells. This preferential death among the antigen-specific T cells was consistent with a waning immune response to LACK. The frequency of dying T cells in the BAL, however, was substantially lower and was the same for I-A^d/LACK$^+$ and I-A^d/LACK$^-$ cells—16% and 14%, respectively. This contrasts sharply with CD4$^+$ T cells in the BAL of sensitized, nonchallenged mice, in which 85% were dying cells (data not shown).

To distinguish whether the I-A^d/LACK$^+$ cells that persisted in the BAL were active or had matured into quiescent long-term memory cells, BAL cells harvested at various times after antigen aerosol challenge were stained with the I-A^d/LACK multimer and anti-CD69 mAbs at various times after antigen challenge. Days 1 and 35 after the last aerosol were selected as representative time points and are shown in Figure 3. In naive mice in which only few CD4$^+$ T cells were detectable and none stained with I-A^d/LACK multimers, 20% of the CD4$^+$ T cells were CD69$^+$. At both 1 and 35 days, the majority of the I-A^d/LACK$^+$ cells were positive for the activation marker CD69. This was considerably higher than the fraction of CD69$^+$ I-A^d/LACK$^-$ cells at either time point in the BAL of sensitized (data not shown) or naive mice (Figure 3B). Thus, the I-A^d/LACK$^+$ T cell population that remained significantly elevated in the BAL for at least 5 weeks after the last aerosol antigen exposure was a largely activated, nonapoptotic population capable of making a large effector cytokine response in vitro (Figure 1D).

LACK-Presenting APC Remain in the BAL 4 Weeks after the Last Aerosol

The persistence of elevated numbers of activated, nonapoptotic I-A^d/LACK$^+$ T cells in the airways may result from the persistence in this compartment of APC presenting the dominant LACK epitope. To test this hypothesis, we have assessed the presence of presented LACK peptide on APC harvested at various times after the last aerosol LACK challenge. The capacity of cell populations to retain and present LACK was assayed by the ability of cells to stimulate the production of IL-2 by a LACK-specific CD4$^+$ T cell hybridoma. Sensitized, challenged mice were sacrificed at different days after the last aerosol, and cell suspensions from their LN, lung, and BAL were irradiated and cultured with the LACK-specific T cell hybridoma LMR7.5 without the addition of LACK peptide in vitro. One day later, supernatants were harvested and their IL-2 content was measured by immunoassay (Figure 4A). APC isolated from the draining LN 1 day after the last aerosol stimulated the LMR7.5 T cell hybridoma poorly to secrete IL-2, and this produc-

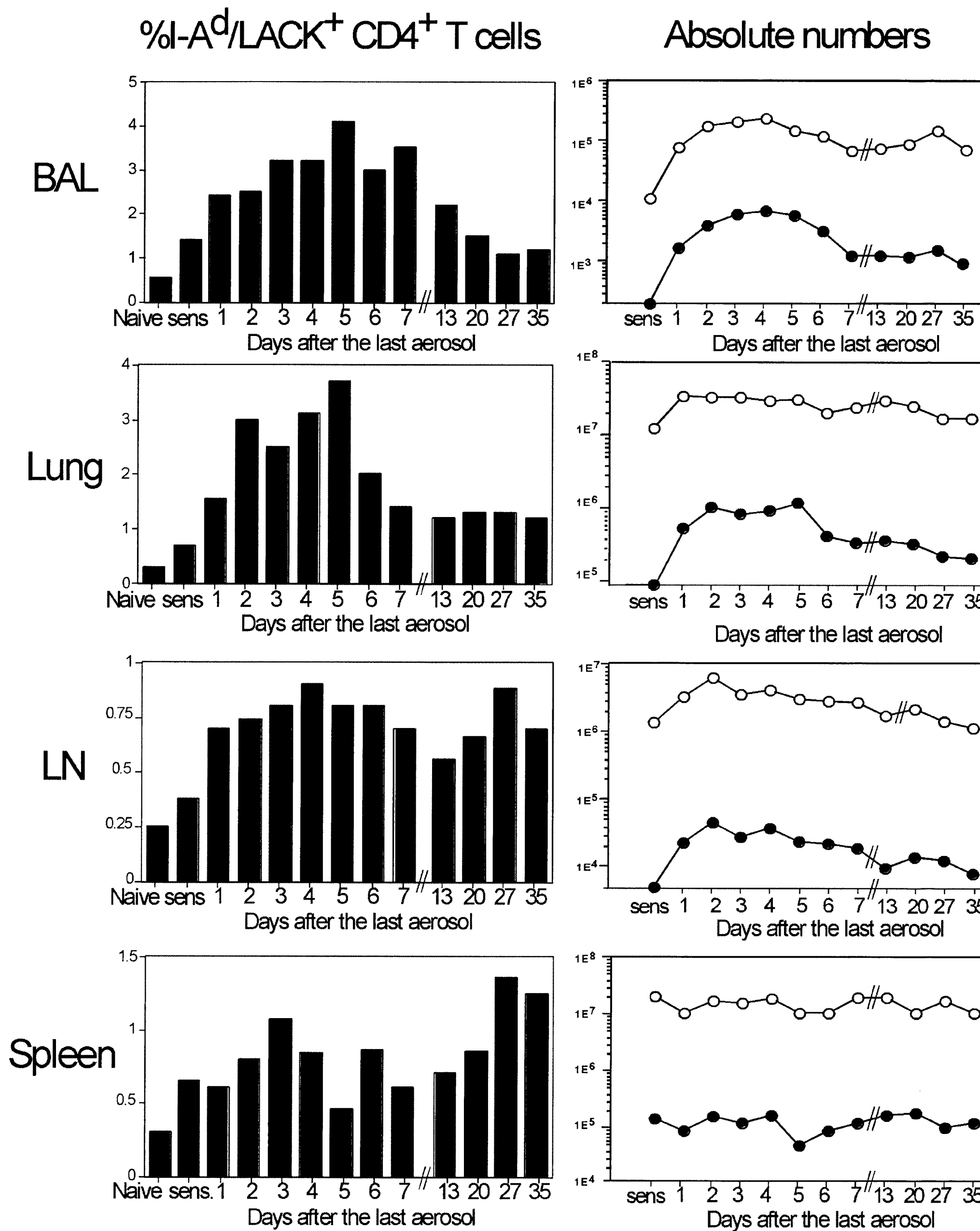

Figure 2. LACK-Specific T Cells Are Detected in Sensitized, Aerosolized Mice

BAL, lavaged lung, bronchial LN, and spleen cells from naive, sensitized, and sensitized, challenged mice were harvested at the indicated days after the last aerosol (10 mice per group, 15 mice in case of naive or sensitized only). 10^6 CD4$^+$ enriched T cell preparations from LN, lung, and spleen, and 10^6 total BAL cells were stained with I-A^d/LACK multimers coupled to protein A-Alexa^{488}, CyCR-anti-CD4, and a cocktail of PE-anti-(B220, CD8, CD11b, Gr-1, and CD11c) mAbs. The percentage (left panels) and the number of total CD4$^+$ (open dots) and I-A^d/LACK$^+$ CD4$^+$ T cells (filled dots, right panels) were calculated after gating on viable CD4$^+$ PE$^-$ cells. In the right panels, the data for naive mice were left out because of the scale. Numbers of I-A^d/LACK$^+$ T cells in BAL, lavaged lung, LN, and spleen of naive mice were 0.2×10^2, 3.2×10^4, 0.5×10^2, and 3.2×10^4, respectively. Numbers of CD4$^+$ T cells in BAL, lavaged lung, LN, and spleen of naive mice were 2.0×10^3, 1.1×10^7, 1.8×10^4, and 1.1×10^7, respectively. Data show the results of one representative experiment out of four.

tion ceased after day 2. APC isolated from the lavaged lungs, however, retained sufficient antigen to stimulate LMR7.5 up to 6 days after the last antigen challenge, although with rapidly declining efficiency. In striking contrast, APC activity remained quite high in the BAL for at least 35 days and was still detectable at the only later time point examined, day 57.

To account for differences in the number of I-A^d class II$^+$ cells per well that may account for the different APC activity (Figure 4A), lung, LN, and BAL cell suspensions

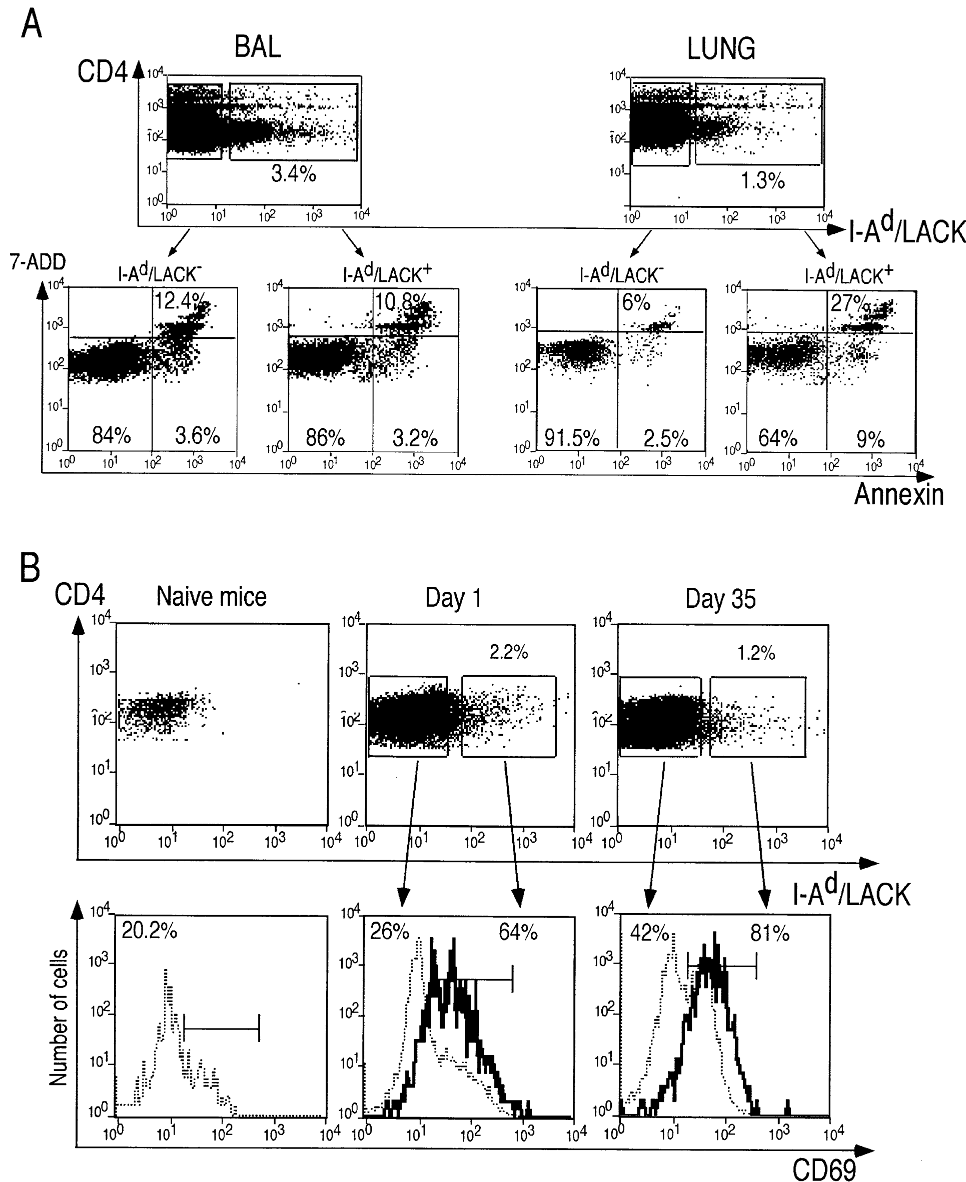

Figure 3. I-A^d/LACK$^+$ CD4$^+$ T Cells in the Airways Are Nonapoptotic Cells and Exhibit an Activated Phenotype

(A) One week after the last aerosol, CD4$^+$ preparations from lavaged lungs and BAL isolated from a pool of ten sensitized mice were incubated with I-A^d/LACK multimers coupled to protein A-Alexa^{488}, PE-anti-Annexin-V mAbs, and 7-ADD. I-A^d/LACK$^+$ and I-A^d/LACK$^-$ populations were analyzed for the expression of Annexin-V and 7-ADD. Data show the result of one representative experiment (out of two).

(B) BAL cells harvested from a pool of ten sensitized, challenged mice on days 1 and 35 after the last aerosol were incubated with I-A^d/LACK multimers coupled to protein A-Alexa^{488}, CyCR-anti-CD4, and PE-anti-CD69 mAbs. I-A^d/LACK$^+$ (thick-lined histograms) and I-A^d/LACK$^-$ (dotted-lined histograms) CD4$^+$ T cells were analyzed for CD69 expression. Data show the results of one representative experiment out of three.

were stained with anti-I-A^d mAbs, and the percentage and numbers of I-A^{d+} cells were calculated for all time points (Figure 4B). The normalized IL-2 secretion expressed as the amount of IL-2 induced per 10,000 I-A^{d+} cells is shown in Figure 4C. Despite variability in the frequency of I-A^{d+} cells, especially among the BAL samples, the results were qualitatively similar to the ones previously shown in Figure 4A. Thus, LN contained few LACK-presenting APC at any time point, and lungs contained LACK-presenting APC for only 1 week after the last aerosol, whereas MHC class II$^+$ cells from BAL retained the capacity to present LACK long after the last aerosol.

LACK Peptide Is Presented by the CD11c$^+$CD11b$^+$ DC Subset in BAL

To characterize the APC in BAL that presented the LACK$^{156-173}$ peptide in vivo, the BAL cells of sensitized mice 1 day after the last aerosol challenge were first analyzed by flow cytometry for surface markers expressed by B cells, myeloid, and DC lineages (Figure 5A). Thirty-nine percent of the cells were CD11c$^-$CD11b$^+$, 18% CD11c$^+$CD11b$^-$,

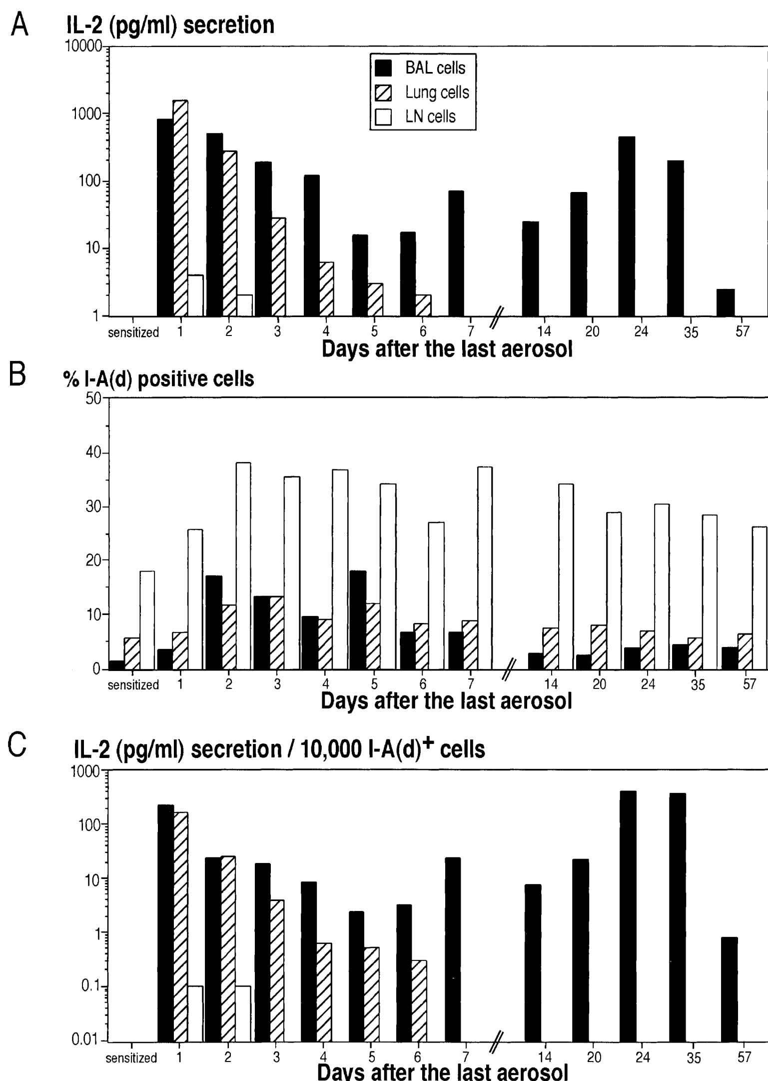

Figure 4. Antigen-Presenting Cells Loaded with LACK-Derived Peptides Are Detectable in BAL Long after the Last Aerosol

Cells were isolated from the BAL, lavaged lungs, and bronchial LN of sensitized and sensitized, challenged mice (ten mice per group) on various days after the last aerosol.

(A) Cells were irradiated and incubated with 10^5 cells of the LMR7.5 T cell hybridoma in 96 U-bottom plates for 24 hr. IL-2 content was analyzed by ELISA.

(B) 5×10^5 cells from BAL, lung, and bronchial LN were blocked with anti-FcγIII/II receptor mAbs and stained with PE-anti-CD4 and FITC-anti-I-A^d mAbs before being processed by flow cytometry. The percentage of I-A^{d+} cells is shown on various days after the last aerosol.

(C) The absolute numbers of I-A^{d+} cells were calculated from the percentage of I-A^{d+} cells found in (B), and using the absolute numbers, the IL-2 secretion by LMR7.5 was normalized to 10,000 class II$^+$ cells per well. Data show the results of one representative experiment out of three.

4.7% CD11c$^+$CD11b$^+$, and 4% B220$^+$CD11c$^-$CD11b$^-$. These four cell subsets were purified to 97% by cell sorting (Figure 5A), and the antigen-presenting capacity of each was tested by measuring stimulation of IL-2 secretion by the LACK-specific LMR7.5 T cell hybridoma (Figure 5B, left panel). Only the two subsets expressing the DC marker CD11c spontaneously presented LACK peptide to LMR7.5. Of these, the minor CD11c$^+$CD11b$^+$ subset was by far the most potent, giving effective presentation with as few as 2000 cells per well. In contrast, the CD11c$^+$CD11b$^-$ cells were about 100-fold less potent as inducers of T cell activation. The B220$^+$CD11c$^-$CD11b$^-$ and CD11c$^-$CD11b$^+$ subsets did not present appreciable levels of LACK peptide.

The difference in spontaneous APC activity among subsets was primarily an indication of the presence of processed and retained LACK rather than a difference in potential antigen-presenting activity. When the peptide was added in vitro, all cell subsets induced the activation of LMR7.5 (Figure 5B, right panel). CD11c$^+$CD11b$^+$ cells remained the most potent, requiring five to six times fewer cells than CD11c$^+$CD11b$^-$ cells to induce the same level of IL-2. B220$^+$CD11c$^-$CD11b$^-$ cells also became quite efficient APC when LACK peptide was added, and the CD11c$^-$CD11b$^+$ subset had a weak but significant APC activity, which could be accounted for by 1% contaminating DC. The relatively greater increase in APC activity with added peptide by the CD11c$^+$CD11b$^-$ and the B220$^+$CD11c$^-$CD11b$^-$ cells demonstrates that they were quite competent APC but retained antigen poorly in vivo. Thus, in vivo, despite the presence of multiple types of potential APC, only the restricted subset of DC expressing both CD11c and CD11b surface antigens was able to retain LACK after in vivo capture and present it to the T cells one day after antigen challenge.

The CD11c$^+$CD11b$^+$ DC in BAL Are Highly Activated APC

The different subsets of APC in the BAL of sensitized, challenged mice were further characterized by flow cytometry after staining with different mAbs directed to cell surface antigens (Figure 6). CD11c$^+$CD11b$^+$ cells expressed low levels of CD4 and CD8α but were positive

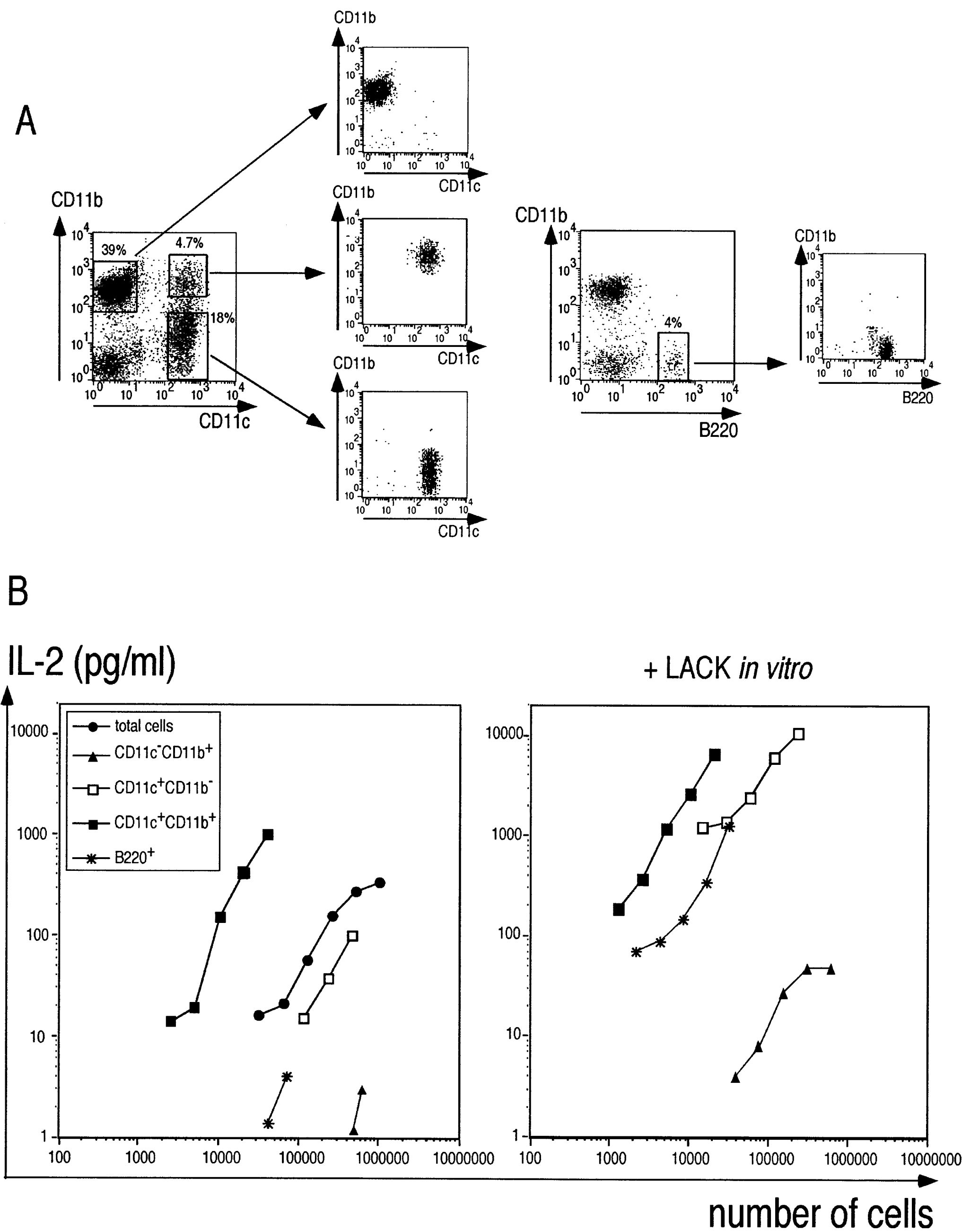

Figure 5. The CD11c⁺CD11b⁺ DC Subset Captured and Processed LACK In Vivo

(A) BAL cells were harvested from a pool of 100 sensitized mice 1 day after the last aerosol, and CD11c⁻, CD11b⁻, and CD11c/CD11b expressing cells were sorted. CD11c⁻CD11b⁺B220⁺ cells were sorted after gating on the lymphocyte population. Reanalysis of the purified population revealed a purity >97%.

(B) The purified APC were incubated with the LMR7.5 T cell hybridoma in the absence (left panel) or presence (right panel) of 5 μM LACK peptide. IL-2 contents of the supernatants were measured 24 hr later by ELISA. Data show the results of one representative experiment out of three.

for Ly6C, Ly6G (Gr-1), F4/80, and DEC-205 markers. These cells also expressed high levels of IL-3R, I-A^d, CD40, and CD80, but low levels of CD86. The CD11c⁺CD11b⁻ cells were low to negative for CD4 and CD8α and expressed lower levels of MHC class II, CD80, CD86, CD40, DEC-205, IL-3Rα, Ly6C, and Ly6G than the CD11c⁺CD11b⁺ population.

CD11c⁻CD11b⁺ cells stained brightly with anti-Ly6C and Ly6G mAbs but were low or negative for all the other markers tested, suggesting that they were granulocytes. To confirm this and to distinguish between eosinophils and neutrophils, we prepared cytospins with the purified CD11c⁻CD11b⁺ cells to analyze their morphology after Giemsa staining. Eosinophils represented 65% of this population, whereas the neutrophils constituted the remaining 35% (data not shown). All B220⁺CD11c⁻CD11b⁻ cells ex-

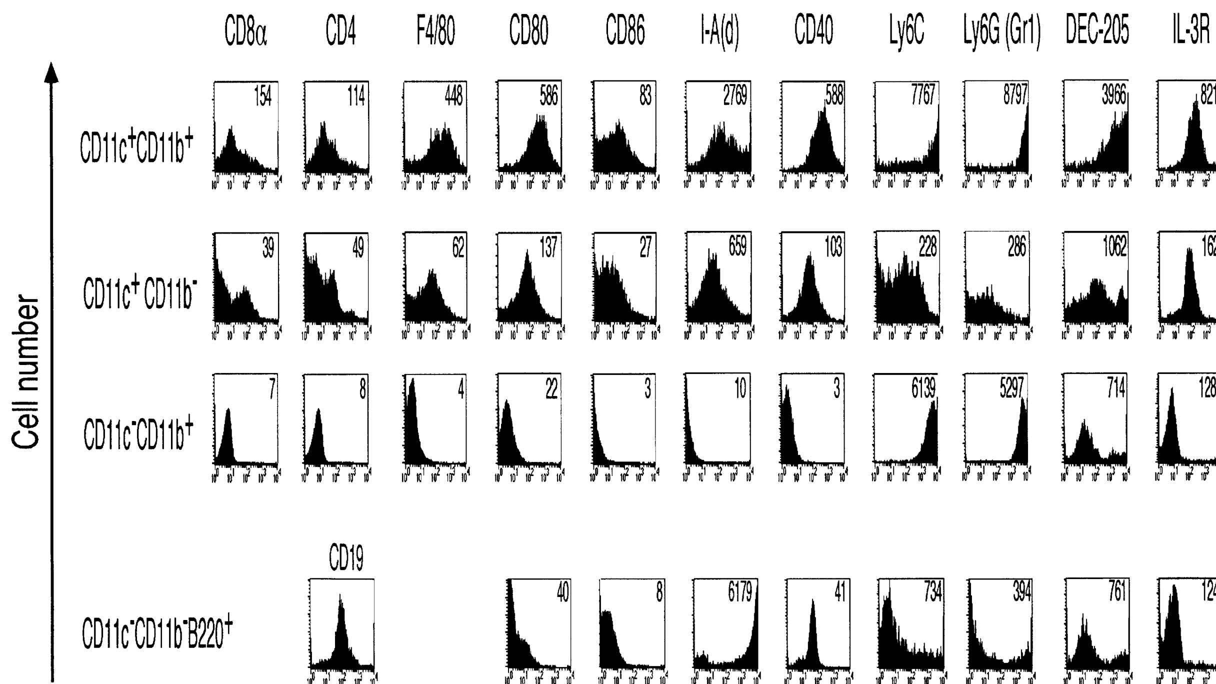

Figure 6. Flow Cytometry Analyses of Cell Surface Antigen Expression on APC Present in BAL 1 Day after the Last Aerosol

After gating on the indicated populations, each subpopulation was analyzed for expression of the indicated cell-surface markers. Representative FACS analyses are shown after gating on the indicated APC subpopulations. The mean fluorescence intensity is indicated in the right upper corner of each panel.

pressed the CD19 antigen, confirming that they were B-lymphocytes. They expressed very high levels of MHC class II molecules but low levels of CD40; were negative for CD80, CD86, and IL-3Rα; and had heterogenous staining for Ly6C, Ly6G, and DEC-205. Thus, the $CD11c^+CD11b^+$ DC were found to be the APC subset expressing the highest levels of MHC class II, DEC-205, and several costimulation molecules, all important for optimum uptake and/or presentation of antigen to T cells in vivo.

The $CD11c^+CD11b^+$ Cells Retain the Capacity to Activate Allergen-Specific T Cells Long after the Last Exposure

To determine the phenotype of the BAL cells that retained LACK peptide for many weeks after antigen exposure, APC subpopulations were isolated 3 weeks after the last aerosol from the BAL of sensitized, challenged mice and tested for their ability to stimulate the LACK-specific T cell hybridoma (Figure 7). BAL cells were stained with anti-CD11c and anti-CD11b mAbs, and subpopulations were isolated by cell sorting (Figure 7A) as described in Figure 5. Compared to the 1-day, post-challenge time point, the total BAL cell number was substantially reduced (data not shown), and the proportion of the $CD11c^-CD11b^+$ and $CD11c^+CD11b^+$ cells dropped to 2% and 1%, respectively, in contrast to the $CD11c^+CD11b^-$ cells, which increased to 40% (Figure 7A). Using BAL cells from 100 sensitized and aerosol-challenged mice, sufficient numbers were isolated to determine their ability to present LACK peptide spontaneously to LMR7.5. Three weeks after the last aerosol, only the $CD11c^+CD11b^+$ subset was able to spontaneously present antigen to the LACK-specific T cell hybridoma (Figure 7B, left panel). These cells constituted only 1% of total lung but were approximatively 100 times more effective at retaining and presenting the LACK antigen. No LACK presentation could be detected from the major APC subset, the $CD11c^+CD11b^-$ cells, or the $CD11c^-CD11b^+$ cells.

Exogenously added LACK peptide could be presented by the $CD11c^+CD11b^-$ but not by the $CD11c^-CD11b^+$ subset, similar to day 1 post-challenge BAL cells. Under these conditions, the $CD11c^+CD11b^+$ cells were again the most potent APC, as demonstrated by their ability to induce measurable IL-2 with as few as 100 cells per well. Thus, despite the presence of various APC in the BAL of sensitized, challenged mice, the $CD11c^+CD11b^+$ APC subset was the major source of presented antigen at early times after antigen challenge and was also the subset found to retain that capacity long after the last exposure to the antigen.

Discussion

$CD4^+$ Th2 cells are key initiators of the chronic lung inflammation responsible for atopic asthma, yet to date it has not been possible to directly track the migration and expansion of T cells specific for either a natural or experimental aeroallergen. This report describes a mouse model of allergic airway inflammation using the *Leishmania* LACK antigen as the experimental allergen in place of the frequently used protein OVA. BALB/c mice sensitized by i.p. injection of LACK protein plus

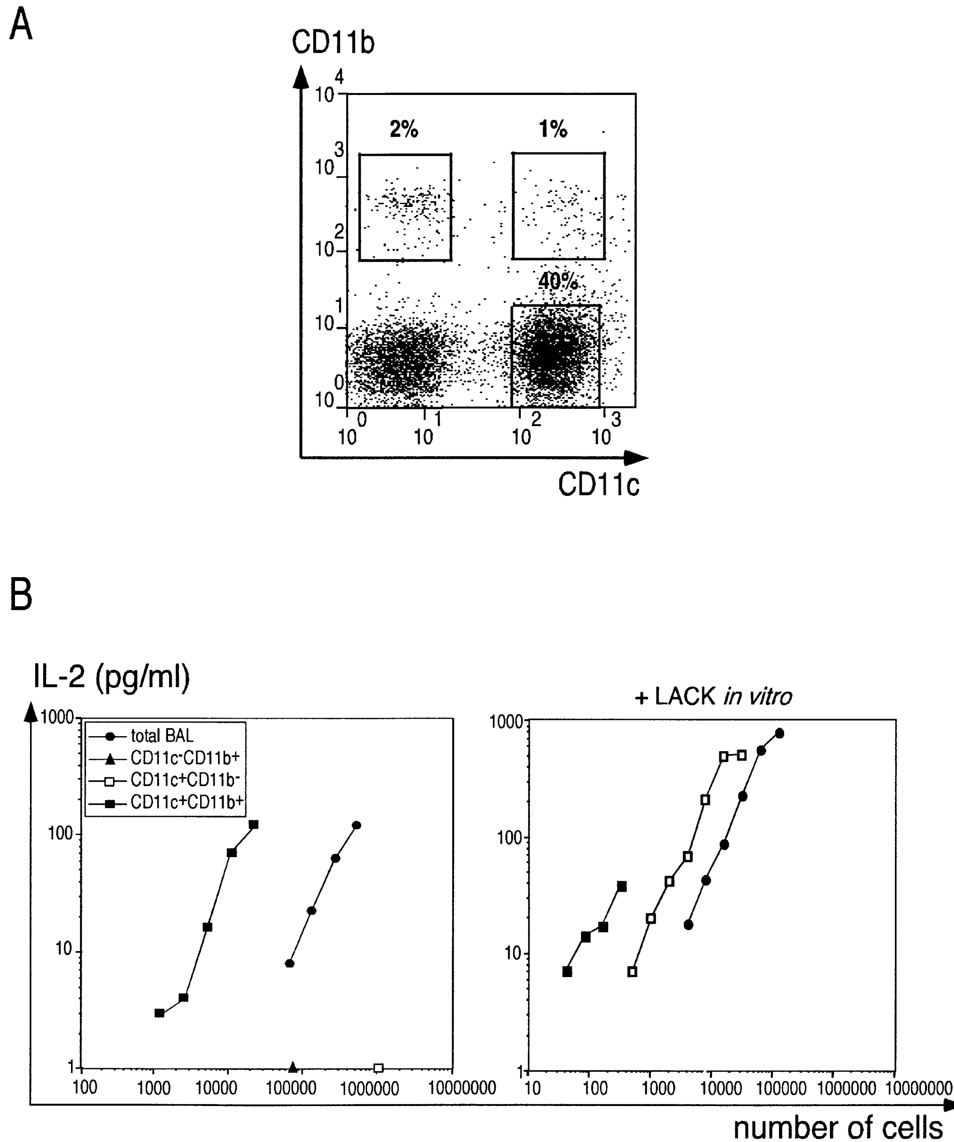

Figure 7. Late after the Last Exposure, the CD11c$^+$CD11b$^+$ Cells Still Present LACK-Derived Peptides

(A) BAL cells were harvested 3 weeks after the last aerosol challenge from a pool of 100 sensitized mice and stained with PE-anti-CD11c and APC-anti-CD11b. Subsequently, CD11c$^-$, CD11b$^-$, and CD11c/CD11b-expressing subpopulations were sorted.

(B) Purified APC were incubated with LMR7.5 T cell hybridoma in the absence (left panel) or presence (right panel) of 5 μM LACK peptide. IL-2 content was measured in the supernatant 24 hr later by ELISA. Data show the results of two similar experiments.

ALUM and challenged with low doses of LACK aerosols developed the expected, strong, polarized Th2 response with prominent lung eosinophilia and elevated IgE in vivo. We found that the LACK-specific T cells remained long after the last aerosol in the airways, while LACK-loaded DC expressing both the CD11c and CD11b surface antigens persisted a long time in the airways, retaining their antigen-presenting capacity over that time.

There are two important advantages in using LACK instead of OVA. LACK has been shown to have a single, highly dominant I-A^d-restricted epitope on peptide LACK$^{156-173}$ (Mougneau et al., 1995) that accounts for the majority of the CD4$^+$ T cell response to the whole protein. The second is that very specific and efficient I-A^d/LACK multimers have been developed that allow fluorescent staining and cell sorting of T cells specific for this epitope (Malherbe et al., 2000). Previous attempts to track antigen-specific T cells in vivo have either relied on functional assays to identify cells reacting to antigen or have used changes in total CD4$^+$ population as an approximation of the behavior of the antigen-specific T cells. TCR-transgenic T cells have been used to directly track antigen-specific T cells (Jenkins et al., 2001), yet the introduction of a large bolus of adoptively transferred T cells of a single specificity introduces a number of limitations and potential artifacts. Visualizing non-TCR-transgenic, antigen-specific T cells is now possible using soluble forms of the natural ligand, the peptide/MHC complex that is recognized by their TCR. This new technology has significantly changed our understanding

of the course of $CD8^+$ T cell responses to bacterial and viral antigens (Doherty, 1998). Here, using multivalent I-A^d MHC class II molecules linked to the dominant $LACK^{156-173}$ peptide, we have visualized, enumerated, and purified LACK-specific $CD4^+$ T cells to analyze effector function during a pulmonary immune response without the use of TCR-transgenic mice.

Sensitization of mice with two i.p. injections of LACK protein with alum adjuvant increased the number of I-A^d/$LACK^+$ T cells in the spleen, lung, bronchial LN, and BAL. The increase of I-A^d/$LACK^+$ T cells in the BAL and lung prior to any aerosol challenge suggested that some LACK-specific T cells had acquired a memory/effector phenotype allowing them to migrate to tissues not directly exposed to antigen (Reinhardt et al., 2001; Sallusto et al., 1999). When sensitized mice were challenged with LACK aerosol daily for 5 days, the percentage and number of I-A^d/$LACK^+$ cells increased dramatically in BAL, lung, and bronchial LN but not in the spleen, reaching nearly maximum numbers within 2 days and maintaining high levels through day 7 after the last aerosol. In the BAL and lung, I-A^d/$LACK^+$ T cells comprised up to 4%–5% of total $CD4^+$ T cells during this period. After 1 week, the numbers of I-A^d/$LACK^+$ T cells declined several-fold from peak numbers; however, as late as 35 days after the last aerosol exposure, I-A^d/$LACK^+$ cells remained elevated, as compared to sensitized mice, in the airway lumen. This prolonged elevation of antigen-specific or total $CD4^+$ T cells has not been reported in mouse models of airway inflammation, although Blyth et al. (1998) have reported prolonged elevation of lymphocyte infiltrates using a mouse model with OVA as the antigen. In man, however, chronic airway inflammation with $CD4^+$ T cells expressing Th2 cytokine mRNA has been shown to be a characteristic feature of both atopic and nonatopic asthma (Humbert et al., 1999; Robinson et al., 1992).

Highly purified I-A^d/$LACK^+$ T cells from all lung compartments produced high levels of the Th2 cytokines IL-4 (Figure 1D), IL-5, and IL-13 (E.M.H. and V.J., unpublished data), but no detectable IFN-γ when restimulated with LACK in vitro. In the LN and lung, virtually all of the response to the whole LACK protein was present in the I-A^d/$LACK^+$ subset, showing that responses to the $LACK^{156-173}$ epitope encompassed nearly all of the response to the intact protein. The reproducible observation that I-A^d/$LACK^-$ T cells purified from the BAL produced cytokines upon LACK stimulation may reflect the downregulation of TCR expression, as has previously been reported for BAL T cells (Yamaguchi et al., 1999). Alternatively, it could be due to T cell responses directed to minor LACK epitopes or cells with low affinity to the dominant LACK epitope.

The persistence in the airways of LACK-specific T cells with an activated phenotype 5 weeks after the last aerosol suggested the possibility of continued antigen stimulation at these sites. It is likely that long-lived antigen is retained in a processed or at least cell-bound form since inhaled protein antigens disappear from the lung quite rapidly in vivo (Seymour et al., 1998; Taylor and Bourne, 1985; Willoughby and Willoughby, 1977). To test this, we investigated whether APC present in the lung and airways retained $LACK^{156-173}$ on I-A^d that could be effectively presented. Antigen presentation by BAL APC proved remarkably long-lived, retaining significant, presented $LACK^{156-173}$ at day 57, the latest time tested. This contrasted sharply with APC in the lung parenchyma, which rapidly lost the ability to present $LACK^{156-173}$ during the first week after antigen exposure, and bronchial LN, which were nearly devoid of cells presenting $LACK^{156-173}$ even 1 day after aerosol exposure.

The long-lived, antigen-presenting capacity of BAL cells could be attributed entirely to a subset of DC with a distinct cell-surface phenotype. The $CD11c^+CD11b^+$ subset accounted for nearly all of the presented LACK at 1 day after aerosol challenge and all of the retained antigen at the latest time point analyzed, day 21. The survival of the $CD11c^+CD11b^+$ DC could be attributed to maturation stimuli and feedback signals from activated naive or memory T cells (Hermans et al., 2000). It is also possible that incoming migratory $CD11c^+CD11b^+$ cells phagocytose and process apoptotic $CD11c^+CD11b^+$ cells including their LACK content (Inaba et al., 1998). Alternatively, recent experiments suggest that preformed MHC/peptide fragments can be transferred from one DC to another (Knight et al., 1998; Smith and de St. Groth, 1999). We have performed experiments to estimate the turnover rate of the $CD11c^+CD11b^+$ in the BAL, using the technique of continuous bromodeoxyuridine labeling. Our preliminary results suggested a much longer life span for $CD11c^+CD11b^+$ DC population in the BAL as compared to LN DC. After 1 week of bromodeoxyuridine treatment, beginning 4 days after the last aerosol, only 8% of the $CD11c^+CD11b^+$ BAL DC population was labeled, compared to $>$70% of the LN DC in the same animals (data not shown).

The $CD11c^+CD11b^+$ subset was a minor subset of DC in normal mice (data not shown) but expanded considerably in lungs with an ongoing Th2 response. This subset was markedly reduced again by day 21, whereas the $CD11c^+CD11b^-$ increased to 40% at this time. $CD11c^+CD11b^-$ pulmonary DC have been previously shown to turn over rapidly (Holt et al., 1994) and to migrate readily into the lung-draining LN (Vermaelen et al., 2001; Xia et al., 1995).

In contrast to previous reports of antigen-presenting DC migrating from the airways to the bronchial LN after aerosol exposure to a protein antigen (Vermaelen et al., 2001; Xia et al., 1995), we found that the LN contained few LACK-loaded APC. In these reports, however, animals had not been sensitized prior to the aerosol exposure, and it is known that such primary aerosol exposure can lead to development of an abortive T cell response and IgE unresponsiveness (Sedgwick and Holt, 1985; Seymour et al., 1998). In contrast, aerosol exposure of LACK-sensitized mice rapidly produced a polarized Th2 memory response in the lung, which may account for the very different migration patterns of APC observed in our study. A second fundamental difference between the present experiments and the previous studies is the dose of antigen used in the pulmonary challenge. The dose of LACK protein delivered to the lower respiratory tract in each of the five exposures in the present experiments was estimated at 200–300 ng, whereas doses used for previous studies of lung APC migration were 700 μg (Vermaelen et al., 2001) or 100 μg (Xia et al., 1995), doses that far exceed any normally encountered airborne allergen and may result in unresponsiveness.

The CD11c$^+$CD11b$^+$ DC expressed high levels of several surface proteins important for APC function, including I-A^d, CD40, CD80, CD86, and DEC-205. Both the conventional CD11c$^+$CD11b$^-$ pulmonary DC and B cells could provide highly effective antigen presentation when pulsed with LACK$^{156-173}$ peptide in vitro. However, neither population appeared to retain LACK$^{156-173}$ efficiently after this in vivo low-dose aerosol challenge, which correlated with their lower expression of DEC-205.

It is difficult to relate the CD11c$^+$CD11b$^+$ cells found in the BAL of allergic mice to a previously described subset of DC since they expressed a combination of different markers thought to be characteristic of specific subsets. The CD11c$^+$CD11b$^+$ cells expressed DEC-205 found on Langerhans cells, and bone marrow-derived, thymic, and some splenic DC (Inaba et al., 1995; Kronin et al., 2000). They also expressed F4/80, which is thought to be characteristic of macrophages but was also recently reported to be on the surface of freshly isolated splenic DC (Leenen et al., 1998). In addition, they stained brightly for the granulocyte marker Ly6-G (Gr-1). This marker has recently been found on murine plasmocytoid DC, which express B220 but not CD11b and produce IFN-α (Nakano et al., 2001; Asselin-Paturel et al., 2001). A DC with very similar properties to the one we describe here has been reported as a major BAL population in mice overexpressing GM-CSF (Wang et al., 2000).

In conclusion, we have developed a model of allergic airway inflammation in which allergen-specific Th2 cells can be tracked and purified in non-TCR-transgenic mice. Using a sensitive T cell presentation assay, we have identified a unique subset of DC localized in the airways, which retained and presented the antigen for a prolonged period of time. These results suggest that the airways are a dynamic compartment where both antigen-presenting DC and memory/effector Th2 cells persist for long periods after exposure to the allergen. If these phenomena occur in man, they could account for the chronic infiltration of Th2 cells observed in the airways of people with atopic asthma.

Experimental Procedures

Mice and Induction of Allergic Airway Inflammation

Female BALB/cAnN mice were purchased from Taconic Farms, Inc. (Germantown, NY). All animals were raised under specific pathogen-free conditions at the DNAX Research Institute animal facility and used at 6 weeks of age. Sensitization was performed by two i.p. injections of 10 μg LACK protein precipitated in 2 mg of aluminum hydroxide (alum) at days 0 and 7. At day 14, sensitized mice were exposed to a LACK protein (1 mg/ml) aerosol challenge for 30 min on 5 consecutive days. Aerosolization was performed using a Passport aerosol compressor (Invacare Corporation, Elyria, OH) connected to a 6500 cm^3 box that served as the deposition chamber for the mice. Aerosols were given in groups of a maximum of 50 animals. Experiments were conducted following protocols approved by the DNAX animal care and use committee.

Reagents and Antibodies

LACK peptide (aa 161–173; SLEHPIVVSGSWD) was purchased from Bio-synthesis, Inc. (Lewisville, TX). LACK and λ-OVA recombinant proteins were produced in *E. coli* and purified as described (Mougneau et al., 1995). Soluble I-A^d/LACK dimers were produced and used as described (Malherbe et al., 2000). In brief, staining reagent was prepared by incubating 1.4 μl of Alexa^{488} coupled to protein A (Molecular Probes Inc., Eugene, OR) at the concentration of 0.5 mg/ml in PBS with 8 μg of I-A^d/LACK dimers for 30 min at room temperature (Malherbe et al., 2000). The following mAbs were used for stimulation, cell purification, or flow cytometry analysis: 145/2C11, anti-CD3; GK1.5, anti-CD4; 53-6.7, anti-CD8α; M1/70, anti-CD11b; RA3-6B2, anti-B220; AMS-32.1, anti-I-A^d; HL3, anti-CD11c; RB6-8C5, anti-Gr-1; 5B11, anti-IL3Rα; 16-10A1, anti-CD80; GL1, anti-CD86; HM40-3, anti-CD40; AL-21, anti-Ly6C; 1D3, anti-CD19; 2.4G2, anti-FcγIII/II receptor; and H1-2F3, anti-CD69. The coupled mAbs were purchased from BD Pharmingen (San Diego, CA), and the purified ones used for T cell depletion were prepared at DNAX. Cl:A3-1, FITC-labeled, anti-F4/80 and NLDC-145, anti-DEC-205 mAbs were purchased from Caltag (Burlingame, CA) and Serotec (Raleigh, NC), respectively.

In Vitro Restimulation of Lung and I-A^d/LACK$^+$ CD4$^+$ Cells

Lungs were lavaged, the associated bronchial LN were removed aseptically, and the lungs were forced through a sterile No. 100 steel mesh (Tylinter Inc., Mentor, OH) with the piston of a 10 ml plastic syringe. The cells were lysed with 3 ml of lysis buffer (Sigma Chemical Co., St Louis, MO) for 3 min and washed with Hanks BSS solution. 5 $\times$ 10^6 lung cells were stimulated in 24-well plates in culture medium containing 0.25 mg/ml of LACK protein. After sorting from lung, LN, or BAL, 7500 I-A^d/LACK$^+$ and 7500 I-A^d/LACK$^-$ CD4$^+$ cells were incubated with 10^6 irradiated syngenic splenocytes with or without LACK protein (0.25 mg/ml) in 96-well flat-bottom plates. The culture medium was RPMI 1640 (JRH Biosciences, Lenexa, KS) with 10% heat-inactivated FCS (Sigma Chemical Co., St Louis, MO), 0.05 mM 2-mercaptoethanol (Sigma), 2 mM L-glutamine (JRH Biosciences), and penicillin/streptomycin (GIBCO BRL, Gaithersburg, MD).

IgG1 and IgE Measurements

Mice were bled at day 14 and LACK-specific IgG1 were determined by ELISA as described (McSorley et al., 1997). Total IgE were quantitated by ELISA using rat anti-IgE mAbs EM95 as coating antibody and anti-IgE mAbs NIP-210E coupled to biotin as second antibody as described (Hurst et al., 2001).

Analysis of BAL Cells

After blood collection, a canula was placed into the trachea, and lung was washed four times with 1 ml of pyrogen-free saline warmed to 37°C. The cells were washed with PBS, resuspended in 200 μl, and counted using a Bürker-Türk chamber. For differential BAL cell counts, cytospin preparations were made and stained with Wright Giemsa. Per cytospin, 400 cells were counted and differentiated into mononuclear cells, neutrophils, and eosinophils by standard morphology.

Purification of Cells

For purification of CD4$^+$ T cells, LN and spleen cells were depleted of B220$^+$, Mac-1$^+$, CD8α^+, and Gr-1$^+$ by negative depletion using sheep anti-rat coated Dynabeads as described (Julia et al., 1996). Lavaged lung cells were enriched for CD4$^+$ T cells using a Percoll gradient. After lysing, the cells were washed and resuspended in 6 ml of 90% Percoll, and a 6 ml layer of 40% Percoll was added delicately on the top. After centrifugation at 1000 rpm for 30 min, the cells at the interface were collected.

Flow Cytometry Analysis

For stainings with I-A^d/LACK multimers, 10^6 CD4$^+$ T cell preparations from LN, spleen, lavaged lung, and BAL cells—all previously blocked with anti-FcR mAbs—were stained with 60 μg/ml of I-A^d/LACK multimers for 30 min at room temperature in PBS supplemented with 0.5% of BSA. Cells were washed and incubated with cychrome (CyCR)-anti-CD4 mAbs and with a cocktail of phycoerythrin (PE)-mAbs: anti-B220, -CD11c, -CD11b, -Gr-1, and -CD8α. Lymphocytes gated by forward- and side-scatter parameters were analyzed on a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA). For the purification of I-A^d/LACK$^+$ and I-A^d/LACK$^-$ cells, LN were harvested 2 days after the last aerosol from a pool of 50 sensitized mice. In brief, CD4$^+$ preparations were stained with I-A^d/LACK multimers, CyCR-anti-CD4 mAbs, and the cocktail of PE-mAbs described above

and sorted into I-A^d/LACK$^+$CD4$^+$PE$^-$ and I-A^d/LACK$^-$CD4$^+$PE$^-$ populations on a high-speed sorter VANTAGE SETLO$^+$ flow cytometer (Becton Dickinson). For the purification of I-A^d/LACK$^+$ and I-A^d/LACK$^-$ cells from the lung and BAL, cells harvested from a pool of 100 mice, 4 or 7 days after the last aerosol, respectively, were lysed, blocked, stained with CyCR-anti-CD4 mAbs, and sorted by CD4 expression. The CD4$^+$ were stained with I-A^d/LACK multimers and the cocktail of PE-mAbs and sorted into I-A^d/LACK$^+$CD4$^+$PE$^-$ and I-A^d/LACK$^-$CD4$^+$PE$^-$ populations. For sorting of APC, BAL cells were collected from 100 sensitized, challenged mice, blocked, and stained with PE-anti-CD11c, allophycocyanin (APC)-CD11b, and FITC-anti-B220 mAbs before being processed for flow cytometry. For apoptosis detection, CD4$^+$ T cells were first sorted from the BAL of sensitized mice (as described above) and stained with I-A^d/LACK multimers, PE-Annexin-V, and 7-ADD using the Annexin V-PE apoptosis detection kit provided by BD Pharmingen.

Cytokine Analysis

Supernatants were analyzed for IL-2, IL-5, IL-10, and IFN-γ content by ELISA as described (Seymour et al., 1998). IL-4 was detected by a bioassay using the IL-4-dependent CT.4S cell line (donated by Dr. William Paul, National Institutes of Health, Bethesda, MD) as described (Seymour et al., 1998). Supernatants were analyzed for IL-13 content by ELISA using the quantitative mouse IL-13 kit provided by R&D Systems, Inc. (Minneapolis, MN).

Detection of APC Presenting LACK

Cells from BAL, lavaged lung, and bronchial LN were resuspended at 8×10^6 cells/ml, and the indicated numbers of cells were added to a 96-well U-bottom plate. LMR7.5 T cell hybridoma were added to each well, and cellular supernatants were harvested 24 hr later and analyzed for IL-2 content by ELISA. In some experiments, LACK peptide was added in the wells at the concentration of 5 μM. Alternatively, lavaged lungs and bronchial LN were cut into small pieces, digested with collagenase (Gibco-BRL) at 37°C for 1 hr, and incubated with LMR7.5 as described above. This latter method demonstrated equivalent data.

Statistics

To evaluate the kinetic responses of I-A^d/LACK$^+$ and total CD4$^+$ T cells in the various organs as observed in four independent experiments, a nonparametric ANOVA (Kruskal-Wallis test) was applied. All tests were performed using the program Instat 3.00. A difference was considered significant when $p < 0.05$.

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