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Airborne lipid antigens mobilize resident intravascular NKT cells to induce allergic airway inflammation

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Airborne exposure to microbial cell wall lipids such as lipopolysaccharide triggers innate immune responses that regulate susceptibility to allergic airway inflammation. α-Glycosylceramides represent another widespread class of microbial lipids that directly stimulate innate-like, IL-4– and IL-13–producing, CD1d-restricted NKT cells. In this study, we demonstrate that NKT cells constitutively accumulate and reside in the microvasculature of the mouse lung. After a single airborne exposure to lipid antigen, they promptly extravasate to orchestrate the formation of peribronchiolar and interstitial lymphohistiocytic granulomas containing numerous eosinophils. Concomitant airborne exposure to ovalbumin (OVA) induces the priming of OVA-specific Th2 cells and IgE antibodies by the same dendritic cell coexpressing CD1d and MHC class II. Although NKT cell activation remains confined to the lipid-exposed lung and draining lymph nodes, Th2 cells recirculate and seed the lung of a parabiotic partner, conferring susceptibility to OVA challenge months after the initial exposure, in a manner independent of NKT cells and CD1d. Thus, transient recruitment and activation of lung-resident intravascular NKT cells can trigger long-term susceptibility to allergic airway inflammation.

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Abbreviations used: α-GalCer, α-galactosylceramide; BAL, bronchoalveolar lavage; EdU, 5-ethynyl-2'deoxyuridine; MCh, methacholine.

Airborne exposure to microbial products is linked to asthma in complex ways (Williams et al., 2005). Although heavy exposure early in life seems to be inversely correlated with the development of allergy, most aeroallergens are contaminated with microbial products such as LPS that can promote allergic airway inflammation in experimental systems. The importance of lipids is further highlighted by the strikingly high frequency of lipid-binding proteins among major allergens. For example, the common dust mite allergen Der p 2, which is structurally and functionally homologous to MD-2, can bind a range of lipids, including LPS, to activate the TLR4 pathway and render mice susceptible to allergic airway inflammation (Trompette et al., 2009).

In signaling through TLR4, LPS generally promotes Th1 responses through the induction of IL-12. Specific connections between microbial lipids and the induction of Th2 cytokines that are the hallmark of allergic airway inflammation have not been conclusively established. Gram-negative bacteria that lack the LPS synthesis pathway, particularly those belonging to the genus *Sphingomonas*, are ubiquitous bacterial types found in soil and aqueous environments and are particularly abundant in aerosols (Fahlgren et al., 2010). *Sphingomonas* characteristically uses α-glycuronosylceramides instead of LPS in the outer leaflet of the cell wall outer membrane (Kawahara et al., 2000). These glycolipids evade the TLR pathway and have attracted much attention because of their ability to bind CD1d and directly activate the semi-invariant TCR of NKT cells, an innate-like lineage of effector-type T cells that explosively release IL-4 and IL-13 in both mice and humans (Kinjo et al., 2005; Mattner et al., 2005; Sriram et al., 2005). Likewise, a recent study suggested that house dust extracts could contain

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uncharacterized lipids that directly activated NKT cells independently of TLR signaling (Wingender et al., 2011).

Several observations suggest that NKT cell activation during exposure to protein antigen can direct Th2 responses to this antigen. Mice immunized systemically (Singh et al., 1999) or intranasally (Kim et al., 2004) with a mixture of OVA and the NKT ligand α-galactosylceramide (α-GalCer), a synthetic analogue of the bacterial α-glycuronosylceramides, developed a Th2 response to OVA, as expected from the abundant release of IL-4 by NKT cells and their ability to interact with the same DCs that prime adaptive T cells (Fujii et al., 2007). In a nasal immunization model, *Sphingomonas* lipids alone or their synthetic analogue α-GalCer was sufficient to induce lung eosinophilic infiltration and airway hyperreactivity (Meyer et al., 2006), whereas co-administration of OVA created a state of susceptibility to allergic airway inflammation upon later intranasal exposure to OVA alone (Kim et al., 2004).

The anatomical location of NKT cells in the lung and the cellular mechanisms underlying their lipid-mediated activation and subsequent promotion of allergic responses have not been fully elucidated. In this study, we demonstrated that NKT cells naturally accumulated as long-term residents of the lung microvascular network in the resting state. Upon a single intratracheal exposure to their lipid ligands, intravascular NKT cells rapidly extravasated to peribronchiolar and interstitial spaces to orchestrate the formation of lymphohistiocytic granulomas with numerous eosinophils. Concomitant exposure to OVA through the same intratracheal route resulted in the priming of OVA-specific Th2 cells in the mediastinal LN and the production of IgE in a manner dependent on the coexpression of CD1d and MHC class II by CD11c$^+$ DCs but not B cells. Although NKT cell activation and their cytokine secretion remained localized to the sensitized lung and its draining LNs, OVA-specific Th2 cells recirculated and could seed the unsensitized lung of a parabiotic partner. These Th2 cells conferred susceptibility to airborne OVA challenge months after the initial exposure and in a manner independent of NKT cells and CD1d. Thus, these experiments demonstrate that resident intravascular NKT cells are rapidly mobilized by exposure to airborne microbial lipids and can respond locally and transiently in the lung to induce long-lasting systemic allergic sensitization to associated protein antigens.

RESULTS

NKT cells accumulate in the lung microvasculature and rapidly extravasate upon airborne exposure to lipid

NKT cells are markedly overrepresented in the healthy lung as they represent up to 5–10% of the T cells recovered from this organ, compared with a frequency of 0.2% in peripheral circulating blood. Pulmonary blood is enriched in NKT cells, suggesting that, as in the liver, a fraction of NKT cells reside within the intravascular compartment (Thomas et al., 2011). To identify their precise anatomical distribution, we used mice in which NKT cells are selectively GFP labeled through a knockin of *egfp* into the *cxcr6* gene (Geissmann et al., 2005) and crossed them to Vα14-Jα18 TCR transgenic mice. Multiple GFP$^+$ cells were observed, mostly within small blood vessels delineated by in vivo staining with DyLight592–tomato lectin, with a broad distribution in peribronchiolar and interalveolar capillaries (Fig. 1 A). These GFP$^+$ cells accumulated in the extravascular compartment after airborne exposure to

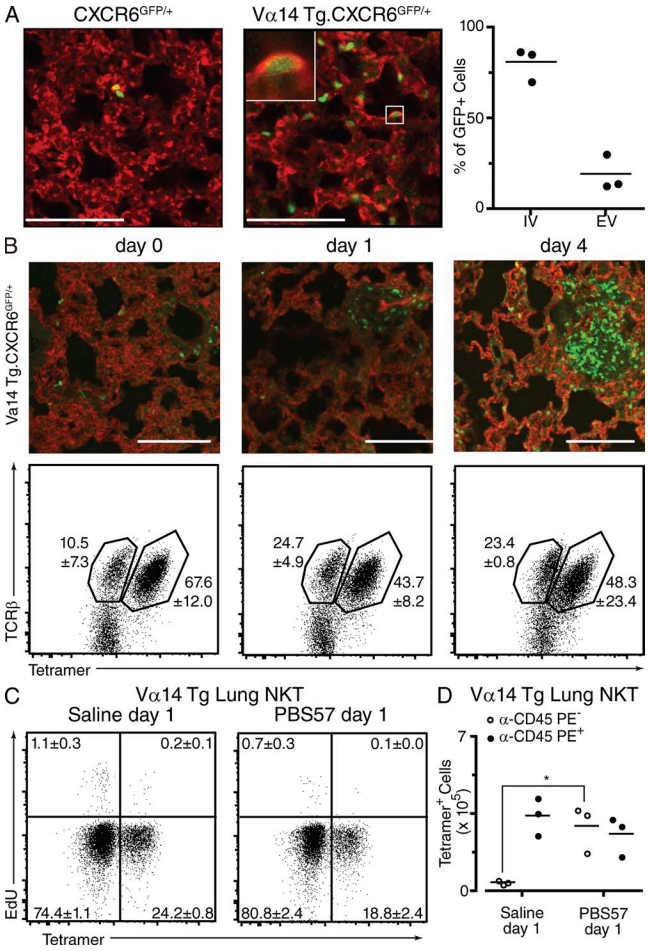

Figure 1. Intravascular location of lung Vα14 NKT cells.
(A, left) Two-photon fluorescence microscopy of lung harvested from CXCR6$^{GFP/+}$ mice and Vα14tg CXCR6$^{GFP/+}$ mice (as indicated) injected with DyLight592–tomato lectin to visualize blood vessel endothelia. The inset demonstrates the intravascular location of a GFP$^+$ cell (boxed area). (right) The frequency of intravascular (IV) and extravascular (EV) NKT cells was measured in three independent Vα14tg CXCR6$^{GFP/+}$ samples counting at least 100 cells per lung. A GFP$^+$ cell was scored as intravascular if it appeared wholly within the structure of a blood vessel within the 50-µm depth of examination. (B, top) Images of rehydrated 10-µm frozen sections of Vα14 transgenic CXCR6$^{GFP/+}$ lungs at days 0, 1, and 4 after airway administration of 100 ng of the NKT ligand PBS57. (bottom) Characterization of GFP$^+$ cells by TCR-β and CD1d–α-GalCer tetramer staining. Numbers indicate mean percentage and SEM in each gate (n = 3/group). (C) EdU incorporation (3 h after i.v. injection) by Vα14tg lung lymphocytes stained with tetramers (n = 3/group). (D) Anti–CD45-PE label acquisition by CD1d–α-GalCer tetramer-gated lymphocytes obtained from lungs harvested 2 min after i.v. injection of the antibody in Vα14tg mice exposed to airborne saline or PBS57, as indicated (n = 3/group). (A and D) Horizontal bars indicate the mean. *, $P < 0.05$. Bars, 100 µm.

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0.1 μg of the α-GalCer analogue PBS57 (Liu et al., 2006) and included between 43 and 67% of CD1d–α-GalCer$^+$ NKT cells at different time points before and after exposure to lipid, attesting to their NKT lineage origin (Fig. 1 B). At 24 h after exposure, many GFP$^+$ cells were already found inside the pulmonary tissue (Fig. 1 B). This accumulation was not caused by local expansion because they did not incorporate 5-ethynyl-2'deoxyuridine (EdU) after 3 h (Fig. 1 C), suggesting rapid mobilization and invasion of the pulmonary tissue. To quantitatively follow the extravasation of NKT cells into the lung tissue, we briefly exposed them to anti–CD45-PE antibodies injected i.v. (Pereira et al., 2009). 2 min after injection, the lungs were perfused through the right ventricle with PBS (until they turned white) to flush out both free antibody and nonadherent red and white blood cells. Lymphocytes were then extracted from the lung tissue and analyzed by flow cytometry. This procedure identifies lymphocytes tightly adhering to the endothelial lining of the vessels because they are brightly labeled by the anti–CD45-PE antibody but remain in the lung despite the perfusion. At 24 h after lipid exposure, we observed a 7.4-fold increase in the absolute number of CD45-PE–negative NKT cells and a corresponding decrease in CD45-PE–positive NKT cells, without overall changes in total NKT cell numbers, confirming NKT cell extravasation and pulmonary invasion (Fig. 1 D).

In contrast, although airway exposure to a high dose of 1 μg of the microbial TLR ligand LPS also induced the extravasation of NKT cells, little NKT cell expansion or cytokine production could be detected (Fig. S1). Thus, direct NKT cell activation by agonist TCR ligands was required for maximal recruitment and for cytokine production and expansion.

Airborne NKT ligand triggers NKT cell activation and the recruitment of allergic granulomas

0.1 μg of the α-GalCer analogue PBS57 was administered intratracheally to follow the local consequences on NKT cell activation in BALB/c mice over time. Within 24 h, a cellular infiltration was noted in the peribronchiolar and interstitial space, which led to the formation of well-defined granulomas made of monohistiocytic and giant cells, lymphocytes, and eosinophils by day 4 (Fig. 2 A). These allergic granulomas were highly reminiscent of the lesions observed in human extrinsic allergic alveolitis, an acute condition induced by exposure to organic dusts and commonly reported as farmer's or bird fancier's lung disease, where microbial products are believed to play a key role in allergic sensitization to associated protein antigens (Patel et al., 2001). To follow NKT cell accumulation in the extravascular compartment, mice were exposed to intravascular anti–CD45-PE labeling for 2 min, as described in the previous section. Whereas 17.8 ± 4.4% of lung NKT cells were protected from labeling in the absence of exposure to PBS57, consistent with their predominant intravascular location, 38.3 ± 5.7% were no longer stained 24 h after exposure, confirming their extravasation into the lung tissue, and this proportion rose to 71.7 ± 13% at day 4 (Fig. 2 B) and 89.8 ± 3% at day 7 (not depicted). In parallel, the relative and absolute numbers of lung NKT cells were greatly increased at day 4 (Fig. 2 C). Granulomas peaked in size by day 7 with several of them including typical giant cells, which are thought to result from the fusion of histiocytic cells, whereas the lung recovered a largely normal aspect by day 14 (Fig. S2). The nature of this granulomatous infiltration was also assessed by flow cytometry of cells extracted from the lungs, confirming the extensive eosinophilic and NKT cell infiltration (Fig. 2 C).

The local activation of NKT cells to release cytokines was directly demonstrated by intracellular staining for IL-4 and IFN-γ ex vivo (without restimulation or Golgi blocking in vitro) after cell extraction from the lung tissue at different time points. Release of both cytokines was observed at 12 h and peaked at 24 h (Fig. 3 A). Expression of CD25 and further up-regulation of CD69

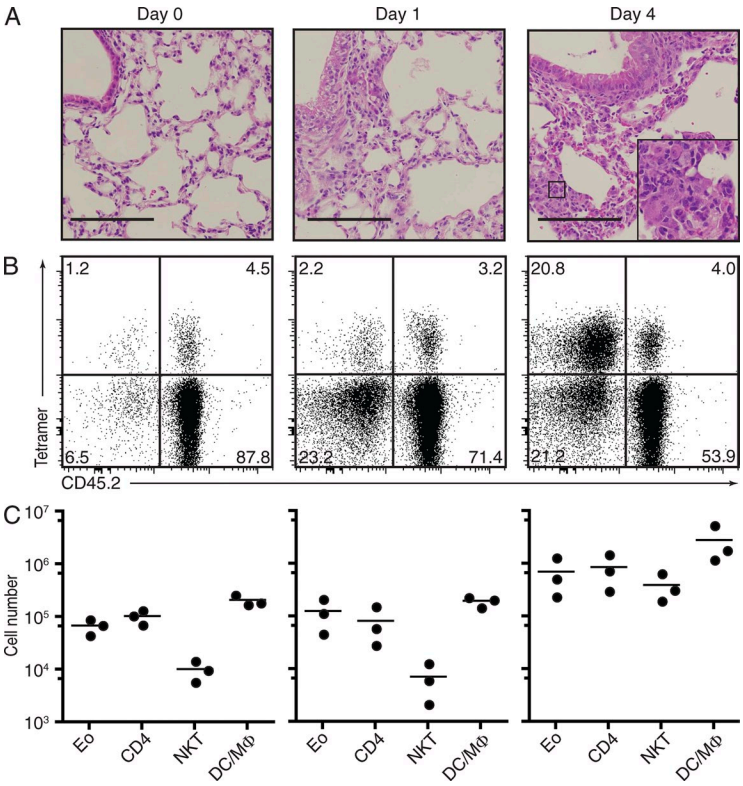

Figure 2. Extravasation of intravascular NKT cells upon airborne exposure to their lipid antigen. BALB/c mice received one intratracheal instillation of 100 ng PBS57 at day 0. (A) H&E staining of lungs (inset shows granuloma, day 4), representative of three mice/group. Bars, 200 μm. (B) Anti–CD45-PE label acquisition by B220$^-$TCR-β$^+$ gated lymphocytes obtained from lungs harvested 2 min after i.v. injection of the antibody (n = 3 mice/group). (C) Absolute numbers of eosinophils (Eo), CD4$^+$ T cells (CD4), NKT cells (NKT), and DCs/macrophages (DC/MΦ) harvested from the lungs at the corresponding time points. All experiments are representative of three mice per group. Horizontal bars indicate the mean.

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followed the same kinetics in the lung, as expected, but also in the mediastinal LNs, suggesting rapid activation of NKT cells in the draining LNs as well (Fig. 3 A). In contrast, splenic NKT cells failed to exhibit cytokine production or up-regulation of CD25 or CD69, further confirming the loco-regional nature of the immunological reaction to airborne NKT lipid antigen. Experiments of EdU incorporation by NKT cells also demonstrated that DNA synthesis began after 24 h and peaked at 36 h with >30% EdU^+ cells in the lung and mediastinal LNs, whereas the spleen showed only modest incorporation (Fig. 3 A, right column).

Lipid APCs

To identify the cell types involved in lipid antigen presentation in vivo, we purified DCs and macrophages from the lung, mediastinal LNs, and spleen 24 h after airway exposure to the NKT ligand and used them to stimulate the NKT hybridoma DN32.D3 in vitro (Fig. 3 B). In the lung, both cell types reached near maximal presentation level at 24 h. In mediastinal LNs, presentation was delayed by 24 h, which is consistent with migration of pulmonary DCs or migration of lipid and its capture by LN DCs. In contrast, splenic and inguinal LN DCs failed to stimulate NKT cells at 24 and 48 h, in agreement with the observation that NKT cells were locally rather than systemically activated.

Early chemokines drive the extravasation of NKT cells

A survey of chemokine messenger RNAs induced in the lung 18 h after exposure to airborne NKT ligand identified multiple candidates involved in their extravasation (Fig. 4 A). Furthermore, experiments in CD1d-deficient mice demonstrated that chemokine induction was strictly dependent on CD1d expression, ruling out a direct proinflammatory effect of the lipid on epithelial cells for example. An exception was CXCL2 (Fig. 4 A, #22), which was induced in response to saline as well. Known NKT attractants such as CCL17 (TARC), CXCL9 (MIG), and CXCL13 (BLC; Johnston et al., 2003; Thomas et al., 2003; Semmling et al., 2010) were rapidly up-regulated in total lung proteins (Fig. 4 B). Furthermore, airborne exposure to CCL17 mobilized intravascular NKT cells into the lung parenchyma, as judged by the intravascular anti–CD45-PE assay (Fig. 4 C).

NKT-induced allergic airway inflammation to OVA

Next, we co-administered the model antigen OVA intratracheally along with the NKT lipid antigen. After a single intratracheal exposure to the mixture of 0.1 µg PBS57 and 10 µg OVA at day 0, mice were rested for 2 wk before a series of four intratracheal challenges with 10 µg OVA alone at days 14, 15, 17, and 18. As demonstrated by the examination of cells recovered from bronchoalveolar lavage (BAL), a massive eosinophilia developed in the airways after the OVA challenges (Fig. 5 A). Increased airway reactivity to methacholine (MCh; Fig. 5 B) and

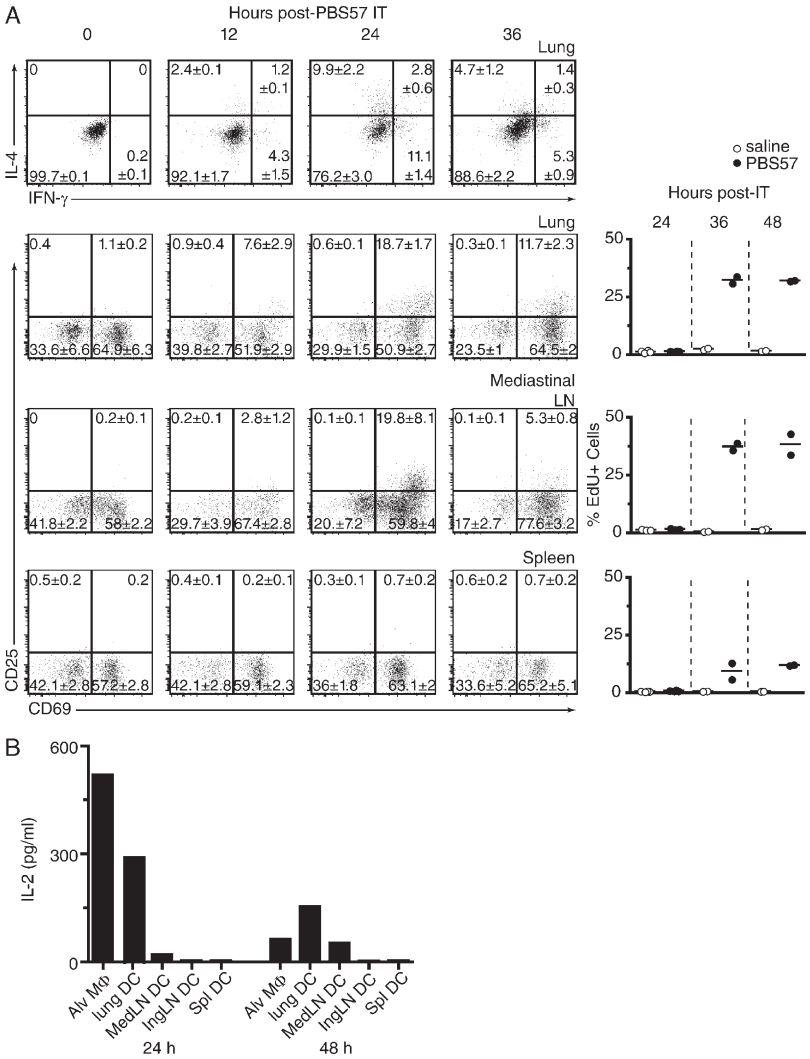

Figure 3. Local activation of lung NKT cells after airborne exposure to lipid ligand. (A, top row) Intracellular IL-4 and IFN-γ staining of NKT cells ($B220^-$, TCR-β^+, CD1d-tetramer$^+$) in the lung of BALB/c mice at the indicated times after intratracheal (IT) instillation of 100 ng PBS57. Quadrant numbers represent percent mean and SEM of three mice per group. (bottom rows) CD25 and CD69 expression by tetramer$^+$ NKT cells at the indicated times in the lung, mediastinal LNs, and spleen. The right column shows the percentage of EdU^+ NKT cells at the indicated times after intratracheal instillation of PBS57 or saline in the corresponding tissues. All of the mice in the EdU incorporation experiment also received 10 µg OVA intratracheally. Horizontal bars indicate the mean. (B) Lung macrophages and DCs from lung, mediastinal (Med) and inguinal (Ing) LNs, and spleen (Spl) were FACS sorted 24 and 48 h after intratracheal instillation of 100 ng PBS57 and cultured with the PBS57-specific NKT cell hybridoma DN32.D3 for 1 d before assessing IL-2 released in supernatants. Data are representative of two independent experiments.

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a typical presentation of allergic airway inflammation (Fig. 5 C) with peribronchiolar eosinophilic and lymphocytic infiltrates and mucus hypersecretion were observed at day 20. Note that mice sensitized with OVA alone did not develop allergic airway inflammation or airway hyperreactivity and that the *Sphingomonas* lipid GSL-1, an agonist ligand of mouse and human NKT cell, had equivalent effects to PBS57 (Fig. S3). Notably, allergic airway inflammation was totally absent in CD1d$^{-/-}$ mice but was unaltered in MyD88$^{-/-}$ mice (Fig. S4), directly demonstrating that the adjuvant effect was exclusively provided by the interaction between lipid and NKT cells.

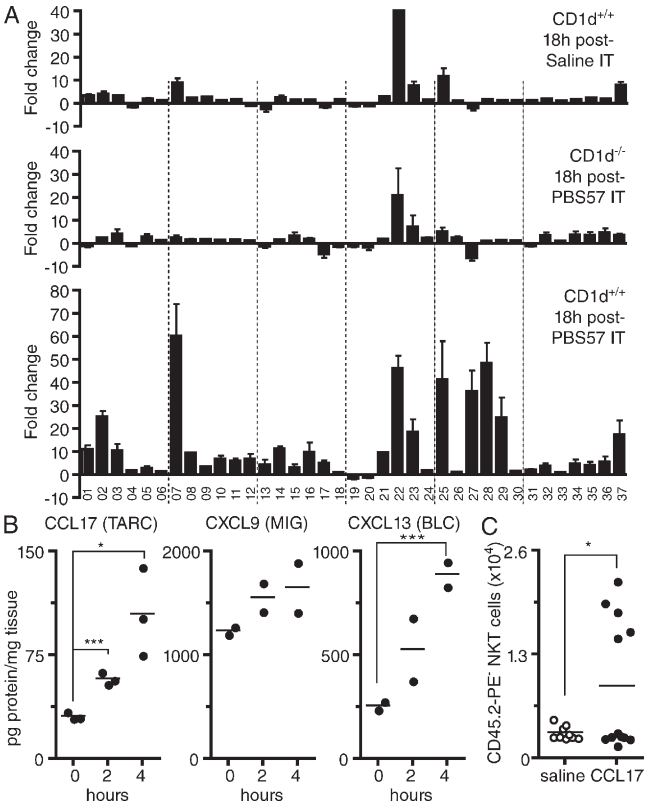

Figure 4. Local chemokines induce NKT cell extravasation. (A) Chemokine messenger RNA expression profile of total lungs from WT or CD1d$^{-/-}$ mice 18 h after airway exposure to saline or PBS57 as indicated. Data were normalized to control unmanipulated WT mice, with bars showing the mean ± SEM of two independent experiments. Shown are *ccl1* (01), *ccl2* (02), *ccl3* (03), *ccl4* (04), *ccl5* (05), *ccl6* (06), *ccl7* (07), *ccl8* (08), *ccl9* (09), *ccl11* (10), *ccl12* (11), *ccl17* (12), *ccl19* (13), *ccl20* (14), *ccl21* (15), *ccl22* (16), *ccl24* (17), *ccl25* (18), *ccl27a* (19), *ccl28* (20), *cxcl1* (21), *cxcl2* (22), *cxcl3* (23), *pf4/cxcl4* (24), *cxcl5* (25), *ppbp/cxcl7* (26), *cxcl9* (27), *cxcl10* (28), *cxcl11* (29), *cxcl12* (30), *cxcl13* (31), *cxcl14* (32), *cxcl15* (33), *cxcl16* (34), *cxcl17* (35), *cx3cl1* (36), and *xcl1* (37). IT, intratracheal. (B) Time course of protein expression of CCL17 (TARC), CXCL9 (MIG), and CXCL13 (BLC) in total lung lysates after intratracheal exposure to PBS57. Data are representative of two separate experiments with two to three individual mice analyzed at each time point. (C) NKT cell extravasation 48 h after intratracheal administration of 100 ng CCL17 was measured by the absolute number of lung tetramer+ cells that were protected from a 2-min intravascular anti–CD45-PE labeling. EdU incorporation after a 3-h pulse was <1% in all groups (not depicted). (B and C) Horizontal bars indicate the mean. *, $P < 0.05$; ***, $P < 0.001$.

Surprisingly, despite the absence of NKT ligand during the OVA challenge, a massive increase of NKT cell numbers was observed as well (Fig. 5 A). Unlike the earlier NKT cell expansion observed at the sensitization phase, this late increase was independent of CD1d presentation as it could not be blocked with anti-CD1d antibodies (Fig. S5), suggesting a cytokine rather than TCR-driven increase or accumulation. Importantly, when the challenges were delayed and administered 6 wk rather than 3 wk after sensitization, the increase in NKT cells was reduced by 22-fold on average, despite equivalent counts of eosinophils and CD4 T cells (Fig. S6). Thus, the NKT cell increase observed during early OVA challenge might involve residual NKT cells that were previously activated by their lipid ligand and had not yet been cleared from the lung.

OVA-specific T cells recovered from the mediastinal LNs demonstrated an exclusive Th2 cytokine profile by secreting IL-4, IL-5, and IL-13 and no IFN-γ in response to OVA in vitro, which is consistent with a polarization of the response (Fig. 6 A). A similar polarization was evidenced in the antibody response to OVA, which consisted mainly of IgE and IgG1 (Fig. 6 B). This polarized cytokine response was also indicated by direct cytokine protein analysis in the lavage samples obtained after OVA challenge and contrasted with the mixed response observed during the sensitization phase (Fig. S7 A). As expected, airway inflammation was reduced in mice lacking IL-4 or IL-4Rα, but it was not modified in mice lacking IFN-γ or IL-10 (Fig. S7 B), suggesting that the latter cytokines did not play a major role in regulating the Th2 outcome of the response.

Resident versus recirculating cells in NKT cell–mediated allergic airway inflammation

The aforementioned experiments suggested that the local activation of NKT cells provided the adjuvant effect and the cytokines required for priming OVA-specific Th2 cells and IgE antibodies. However, NKT cell invasion and granuloma formation are likely to induce broader changes in the bronchopulmonary microenvironment as well, which may be important for the development of susceptibility to allergic airway inflammation. To further dissect the role of local and recirculating effector mechanisms in the pathogenesis of allergic airway inflammation, we generated parabiotic pairs using B6 mice with different CD45 alleles (Fig. 7 A). One parabiont was sensitized intratracheally with OVA + PBS57 while both parabiotic partners received intratracheal OVA-only challenges starting at day 15. Notably, both mice developed comparable accumulation of eosinophils. NKT cells only expanded in the sensitized mouse, further demonstrating that their prior activation by lipid antigen was a prerequisite for local expansion upon OVA challenge but that this expansion per se was not required for allergic inflammation. Interestingly, whereas each lung contained similar proportions of CD45.1 and CD45.2 CD4 T cells, consistent with the recirculation properties of T cells, NKT cells exhibited a

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marked bias for their original host, highlighting their resident properties (Fig. 7 B). Thus, NKT cells remained resident in their lung of origin and mainly acted as a local adjuvant system for priming a systemic recirculating effector response, including OVA-specific Th2 cells and anti-OVA–specific IgE-producing B cells, which in turn was sufficient to transfer susceptibility to allergic airway inflammation.

Dual expression of CD1d and MHC class II by DCs required for allergic airway inflammation

Using newly developed CD1d1$^{fl/fl}$ mice crossed to CD11c-, Lyz2-, and CD19-Cre–deleter strains (Fig. S8), we investigated the role of different APCs. CD1d expression by DCs was specifically required for the induction of allergic inflammation to OVA, as shown by the abrogation of allergic inflammation, including eosinophilic infiltration and anti-OVA IgE and IgG1, in *Cd11c-Cd1d*$^{\Delta/\Delta}$ mice and its persistence in *Lyz2-Cd1d*$^{\Delta/\Delta}$ mice (Fig. 8, A and B). *Cd19-Cd1d*$^{\Delta/\Delta}$ mice lacking CD1d on B cells showed intact allergic inflammation, indicating that CD1d presentation of lipid antigen did not play an important or nonredundant function in this process (Fig. 8 C). Surprisingly, the serum levels of anti-OVA IgG1 and IgE antibodies in *Cd19-Cd1d*$^{\Delta/\Delta}$ mice were comparable with controls, indicating that cognate interactions between NKT and B cells were not required for anti-OVA antibody production. Therefore, it is likely that although NKT cells enabled the DC-mediated priming of OVA-specific MHC class II–restricted CD4$^+$ Th2 cells, the latter provided most of the help to OVA-specific B cells.

This scenario would predict that CD1d and MHC class II must be present on the same DC to induce allergic inflammation to its full extent. Indeed, in lethally irradiated CD1d$^{-/-}$ hosts reconstituted with a 1:1 mixture of MHC II$^{-/-}$ and CD1d$^{-/-}$ bone marrow cells, allergic inflammation was significantly impaired, as judged by the extent of eosinophilic infiltration (Fig. 9 A) and anti-OVA IgE antibody secretion (Fig. 9 B), compared with mice reconstituted with mixtures of WT and either MHC II$^{-/-}$ or CD1d$^{-/-}$ bone marrow cells.

DISCUSSION

Although CD1d-restricted Vα14 NKT cells have long been known to accumulate in the healthy lung, our experiments demonstrated that they mainly resided in the intravascular space rather than in the pulmonary tissue itself and, paradoxically, that they failed to recirculate. Strikingly, these intravascular NKT cells were rapidly mobilized after exposure to airborne lipid antigen and they responded by extravasation, the secretion of multiple cytokines and chemokines, and the recruitment of allergic inflammatory granulomas. In the process, NKT cells activated DCs and enabled them to stimulate and differentiate CD4 Th2 cells specific for protein antigens associated with the airborne lipid. We proved the requirement for a three-cell NKT–DC–CD4 interaction and the predominant role of antigen-specific CD4 T cells over NKT cells in helping B cells produce IgE and IgG1 antibodies to the protein antigen. These events ultimately created a state of allergic susceptibility whereby new challenge with protein antigen alone rapidly induced allergic airway inflammation independently of further NKT cell involvement.

These findings have several important implications for our understanding of the biology of NKT cells and for their potential involvement in various forms of allergic pulmonary reactions ranging from extrinsic allergic alveolitis to asthma. The intravascular location of NKT cells was previously established

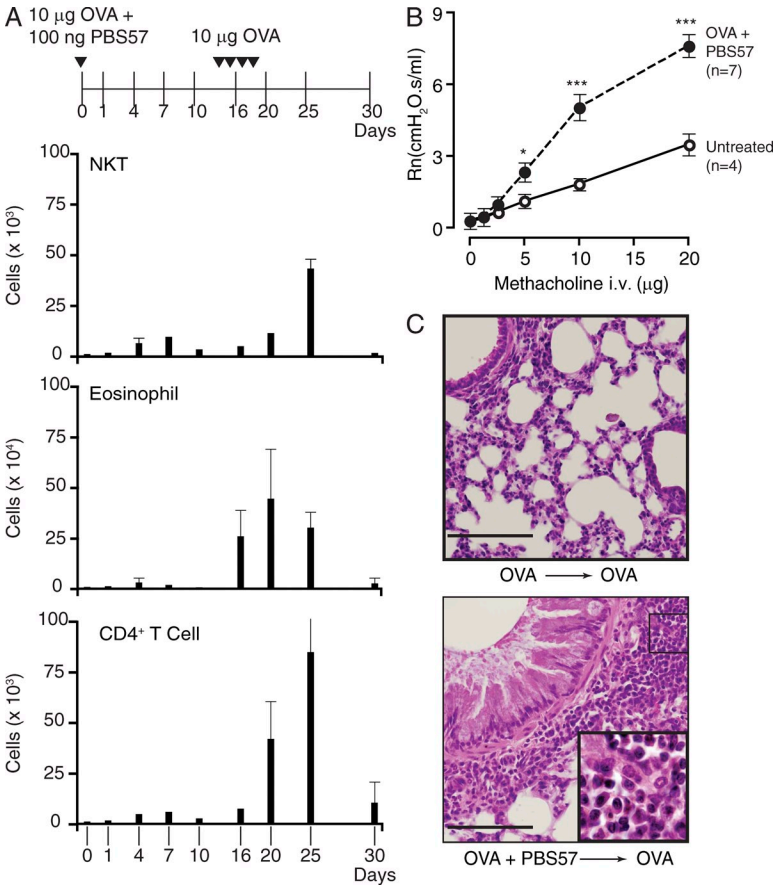

Figure 5. Airborne exposure to PBS57 + OVA renders mice susceptible to asthma-like response upon OVA-only challenge. (A) Mice received 10 μg OVA along with 100 ng PBS57 intratracheally at day 0 and were challenged intratracheally with 10 μg OVA at days 14, 15, 17, and 18. Absolute numbers of CD1d–α-GalCer$^+$ NKT cells, eosinophils, and CD4$^+$ T cells in the BAL at the indicated days are shown as mean ± SD of three mice per group. (B) Airway hyperresponsiveness in response to MCh assessed at day 20 (n = 4–7/group). Error bars represent SEM. *, $P < 0.05$; ***, $P < 0.001$. (C) Representative H&E micrographs of lungs from mice sensitized with OVA alone or with OVA + PBS57 at day 0 and challenged with OVA alone before harvesting at day 20. Note the mucus hypersecretion and the lymphocytic and eosinophilic infiltration (inset) in mice sensitized with PBS57. Micrographs are representative of four mice per group. Bars, 100 μm.

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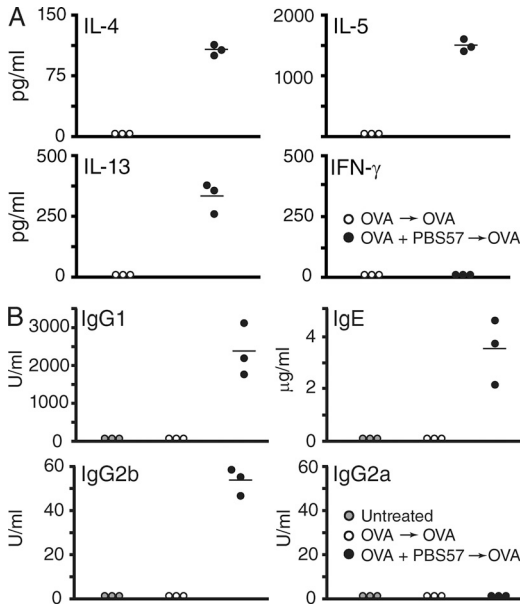

Figure 6. Th2 polarization of OVA-specific T cells in mediastinal LNs. (A) Cytokine release by LN cells extracted at day 20 and cultured with 50 μg/ml OVA for 3 d. Circles represent individual mice. (B) Serum isotypes of anti-OVA antibodies at day 20 in mice untreated or exposed to OVA and PBS57 as indicated. Data are representative of two independent experiments, each with three mice per group. (A and B) Horizontal bars indicate the mean.

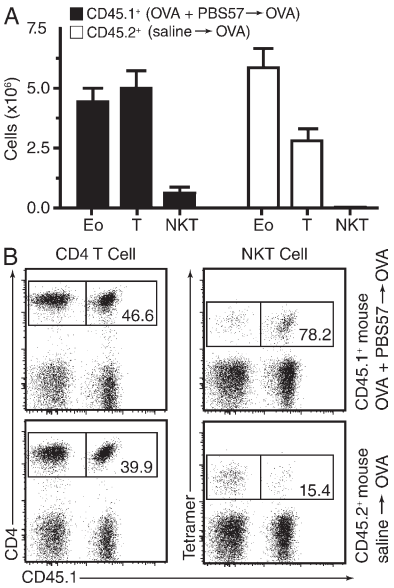

Figure 7. Airborne priming induces recirculating effectors of allergic airway inflammation. (A) The CD45.1+ member of a parabiotic pair was sensitized with OVA + PBS57, whereas its CD45.2+ partner received saline at day 0. Both mice were then challenged with OVA alone at days 14, 15, 17, and 18, and lungs were harvested at day 20 to measure absolute numbers of eosinophils (Eo), T cells, and NKT cells. Error bars represent SEM. (B) Origin of the CD4 and NKT cells recovered from the lungs of each parabiont at day 20. Data are representative of three parabiotic pairs.

in the liver, where live imaging demonstrated a crawling behavior on the luminal surface of endothelial cells (Geissmann et al., 2005; Lee et al., 2010). In the context of infection with a blood-borne pathogen such as *Borrelia*, liver NKT cells interacted with Kupffer cells before the formation of intrahepatic granulomas (Lee et al., 2010). In our study, the synthetic NKT cell antigen was administered intratracheally and was readily picked up by both macrophages and DCs. Although the role of lung DCs and their coexpression of CD1d and MHC class II molecules appeared to be dominant for the priming of OVA-specific allergic responses, DCs were not absolutely required for the initial extravasation of lung NKT cells upon intratracheal exposure to their lipid ligand (unpublished data). It is likely that various CD1d-expressing cell types can pick up and present the lipid ligand to NKT cells in a redundant manner. The inhaled lipid antigen might travel all the way to the vascular endothelium before activating NKT cells, or alternatively, a small proportion of NKT cells constitutively present within the lung tissue might be first activated and induce the selective extravasation of their intravascular counterparts within 24 h through the release of chemokines such as CCL17 directly or via activated APCs, as recently suggested (Semmling et al., 2010).

It is interesting in that regard that most other T cells retrieved from the lung of healthy mice were effector-type cells and also seemed to be inside the microvascular bed, as judged by

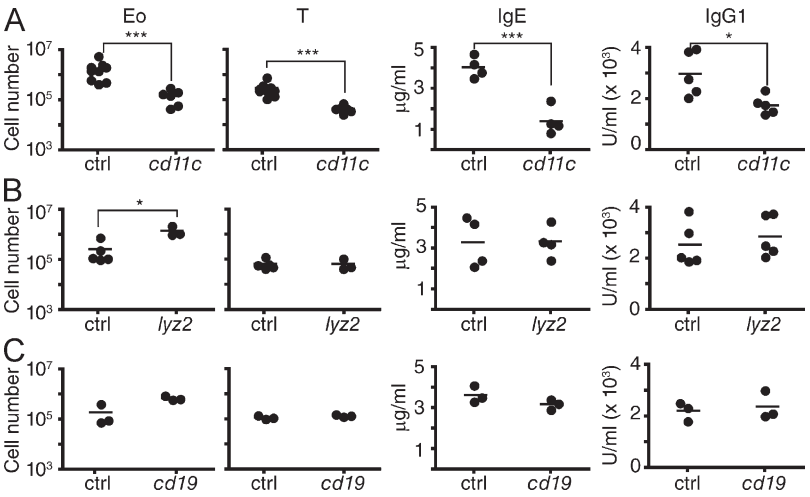

Figure 8. Allergic airway inflammation in CD1d1$^{fl/fl}$ mice. (A–C) Cell counts of BAL eosinophils (Eo) and T cells and serum levels of anti-OVA IgE and IgG1 at day 20 in Cd11c-Cd1d$^{\Delta/\Delta}$ (A), Lyz2-Cd1d$^{\Delta/\Delta}$ (B), or Cd19-Cd1d$^{\Delta/\Delta}$ (C) mice sensitized with OVA + PBS57 at day 0 and challenged with OVA on days 14, 15, 17, and 18. Controls were littermates expressing the Cre transgene and CD1d1$^{fl/+}$ or expressing CD1d1$^{fl/fl}$ in the absence of the Cre transgene. Circles represent individual mice. Horizontal bars indicate the mean. *, $P < 0.05$; ***, $P < 0.001$.

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their intravascular staining with anti–CD45-PE. As these cells were obtained after perfusion of the lung, they likely represented transiently or permanently adhering populations. Some of these effector type T cells also extravasated within the first few days after airborne administration of the NKT ligand but in much smaller relative proportion than NKT cells. Interestingly, a recent study has documented the presence of very large populations of effector-type T cells in the noninflamed lung of humans (Purwar et al., 2011). These findings suggest that both adaptive and innate-like effector T cells, possibly enriched for specificity against pathogens or allergens commonly encountered in these tissues, such as influenza virus in the lung, may reside within intravascular compartments, ready to invade the tissue to provide rapid host defense.

The requirement for coexpression of CD1d and MHC class II on DCs suggests that a three-cell interaction involving NKT cells, DCs, and OVA-specific T cells must occur to enable the full-blown allergic response after OVA challenge. Such a requirement may play out at multiple levels (Moody, 2006; Bendelac et al., 2007; Fujii et al., 2007). Resting or immature DCs effectively present lipid to NKT cells, independently of TLR signaling. In return, NKT cells activate DCs through CD40L–CD40 interactions and enhance their capacity to activate OVA peptide–specific CD4 T cells (Fujii et al., 2004). DCs activated by NKT cells secrete a distinct set of chemokines that attract CD8 T cells and likely CD4 cells as well (Semmling et al., 2010). DCs conditioned by Th2 cytokines may also promote the differentiation of OVA-specific CD4 T cells along the Th2 pathway. Indeed, the Th2 differentiation of OVA-specific CD4 T cells is a prominent feature of this model. Notably, whereas lung NKT cells produced both Th1 and Th2 cytokines, they drove an OVA-specific CD4 T cell response that was exclusively of the Th2 phenotype. This is not the case in other immune responses where NKT cells were suggested to be involved, such as *Borrelia* infection in the liver (Kinjo et al., 2006) or influenza infection in the lung (De Santo et al., 2008), suggesting that additional elements, including signaling through TLRs for production of IL-12 and activation of NK cells may tilt the balance in the opposite, Th1 direction.

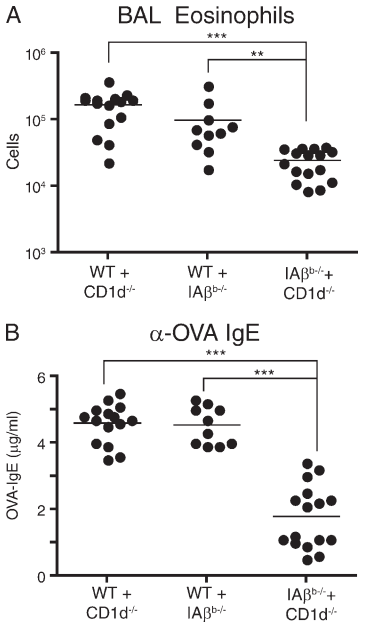

Figure 9. Mixed bone marrow chimeras demonstrate a requirement for joint expression of CD1d and MHC class II by APCs. (A and B) BAL eosinophil counts (A) and anti-OVA IgE serum levels (B) in individual CD1d$^{-/-}$ mice reconstituted with 1:1 mixtures of bone marrows as indicated. Data are a compilation of three separate experiments in which reconstitution by CD45.1$^+$ and CD45.2$^+$ bone marrow–derived cells was 38.8 ± 4.7% and 61.2 ± 4.7%, respectively. Horizontal bars indicate the mean. **, $P < 0.01$; ***, $P < 0.001$.

Another key component of the adaptive immune response to allergens is the production of IgE antibodies. Although it has been shown that NKT cells could directly help B cells produce isotype switched antibodies in vivo and in vitro (Galli et al., 2003; Lang et al., 2008; Leadbetter et al., 2008; Mattner et al., 2008), the secretion of anti-OVA IgE and IgG1 antibodies in our model could occur in the absence of CD1d expression by B cells, likely through cognate interactions with OVA-specific CD4 T cells. Similar findings were reported in other systems (Tonti et al., 2009), suggesting that different helper pathways are available. Thus, B cell help coming from either CD4 helper T cells or NKT cells may be redundant, or alternatively, one pathway may predominate depending on the form and strength of antigens.

The model of allergic airway inflammation examined in this study differs fundamentally from the traditional OVA-alum model in which sensitization is induced by intraperitoneal injection, and in the absence of defined NKT cell antigen, a direct involvement of NKT cells remains controversial (Akbari et al., 2003; Lisbonne et al., 2003; Das et al., 2006). Our model is potentially relevant to several forms of human diseases caused by pulmonary hypersensitivity. First, some of the most abundant gram-negative bacteria on earth do not express LPS in their cell wall, and the best characterized alternatives to LPS are the α-glycuronosylceramides used by members of the widespread *Sphingomonas* genus, which function as NKT cell agonists (Long et al., 2007; Kinjo et al., 2008). Because *Sphingomonas* is ubiquitously found in soil and aqueous environments and is particularly abundant in airborne particles (Fahlgren et al., 2010), pulmonary exposure to NKT agonist ligands is likely to occur repeatedly in some environments. Notably, NKT agonists can exert biological effects at very low doses. Although in this model we used a dose of 100 ng to obtain consistent results across the entire lung, we have also observed reliable allergic inflammation after a single exposure to 10 ng of lipid (unpublished data). Therefore, it seems likely that repeated exposure to natural aerosols containing even very low amounts of microbial lipids might have significant pathological implications in some individuals. In addition, phospholipids from pollens were reported to bind CD1d and activate human Vα24 NKT cells (Agea et al., 2005). Finally, it is

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striking that a large fraction of allergens, including aeroallergens, are lipid-binding or lipid transfer proteins (Thomas et al., 2005) and that some of them such as Der p 2 can signal through the TLR pathway and promote allergic airway inflammation (Trompette et al., 2009). Repeated exposure to trace amounts of these airborne lipids and lipid transfer proteins, alone or in combination, might represent a key environmental component of asthma susceptibility in humans. In that respect, a recent study of house dust extracts suggested the presence of lipid ligands that could directly activate NKT cells (Wingender et al., 2011).

It is noteworthy that the histopathological pattern associated with NKT ligand inhalation, with its diffuse interstitial granulomas made of monohistiocytes, lymphocytes, and eosinophils, resembled extrinsic allergic alveolitis or hypersensitivity pneumonitis, a human condition observed in subjects exposed to organic dusts loaded with microbial products (Patel et al., 2001). So-called farmer's or bird fancier's lung are occupational diseases with symptoms beginning a few hours after exposure to moldy hay in a barn or to bird droppings, for example. These acute diseases can sometimes evolve toward chronic lung fibrosis, perhaps induced by IL-13. The patients may also develop allergy to associated protein antigens, such as avian proteins, similar to the sensitization to OVA in our experimental system, suggesting that NKT cells could be involved in a spectrum of pulmonary hypersensitivity reactions.

Interestingly, whereas NKT cells recovered from the lung or the BAL were markedly increased after exposure to airborne lipid at the sensitization phase of our experimental model, they did not increase upon a delayed (>6 wk) challenge to OVA alone, despite intense allergic airway inflammation. This may be relevant to the controversial reports regarding the presence of NKT cells in the BAL of humans with asthma (Akbari et al., 2006; Thomas et al., 2010). Indeed, a striking conclusion emerging from our study is that pulmonary NKT cells may act locally and transiently to induce long-term allergy to protein antigens. When reexposure to protein allergen occurred late after the sensitizing event, there was no obvious marker of the involvement of NKT cells.

In summary, this study establishes the unique topography and biology of lung NKT cells and their ability to induce a spectrum of pulmonary hypersensitivity reactions ranging from extrinsic allergic alveolitis to asthma. These results and a recent study suggesting the presence of NKT ligands in house dust extracts (Wingender et al., 2011) emphasize the need to consider NKT cells and their airborne ligands as potential contributors, along with other known factors, to allergic pulmonary diseases in humans. In addition, this study helped characterize a robust and simple experimental model of allergic airway inflammation exclusively based on airborne exposure to allergens, which stands in contrast with the broadly used experimental systems based on intraperitoneal immunization with alum-adsorbed antigen. Models involving unique components of the local innate immune system of the lung (Barrett and Austen, 2009) should yield novel insights into the immunopathology of allergic pulmonary inflammation.

MATERIALS AND METHODS

Mice. WT C57BL/6J, CXCR6-EGFP knockin (B6.129P2-Cxcr6tm1Litt/J), CD1d$^{-/-}$ (B6.129S6-Cd1d1/Cd1d2tm1Spb/J), and CD45.1 congenic mice (B6.SJL-Ptprca Pep3b/BoyJ), all on the C57BL/6 background, were purchased from The Jackson Laboratory. CD45.1 congenic I-A$\beta^{b-/-}$ mice (B6.SJL(129)-Ptprca/BoyAiTac H2-Ab1tm1Gru N7+N6) on the C57BL/6 background were purchased from Taconic. WT BALB/cJ, IL-4$^{-/-}$ (BALB/c-Il4tm2Nnt/J), IL-4R$\alpha^{-/-}$ (BALB/c-Il4ratm1Sz/J), IL-10$^{-/-}$ (C.129P2(B6)-Il10tm1Cgn/J), IFN-$\gamma^{-/-}$ (C.129S7(B6)-Ifngtm1Ts/J), and CD1d$^{-/-}$ (C.129S2-Cd1tm1Gru/J), all on the BALB/c background, were purchased from The Jackson Laboratory. BALB/c.MyD88$^{-/-}$ mice were obtained from A. Chong (University of Chicago, Chicago, IL). Jα18$^{-/-}$ mice on the B6 and BALB/c backgrounds were obtained from M. Taniguchi (RIKEN Research Center, Yokohama, Japan; Cui et al., 1997). Vα14-Jα18 transgenic mice in the C57BL/6 background were generated as previously described (Griewank et al., 2007). Age- and sex-matched animals were used between 5 and 12 wk of age. For experiments involving knockout mice, controls were heterozygous littermates.

The C57BL/6.CD1d1$^{fl/fl}$ strain carries loxP sites flanking exons 2 and 6 of the C57BL/6 Cd1d1 allele. These mice were crossed with CD19-cre (B6.129P2I-Cd19tm1(cre)Cgn/J), CD11c-cre (C57BL/6J-Tg(Itgax-cre,-EGFP) 4097Ach/J), or LysM-cre mice (B6.129P2-Lyz2tm1(cre)Ifo/J), all purchased from The Jackson Laboratory. All mice were raised in a specific pathogen-free environment at the University of Chicago, and experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee.

Intratracheal administration of lipids and proteins. Mice were anesthetized with 1.5 mg ketamine and 150 mg xylazine and received 10 mg OVA (grade V; Sigma-Aldrich), 100 ng of the synthetic NKT lipid agonist PBS57 (Liu et al., 2006), or a mixture of both in a final volume of 100 ml PBS, administered in two intratracheal aspirations of 50 ml each separated by 15 min on day 0. For LPS experiments, mice received 1 μg ultrapure LPS (*Escherichia coli* O111: B4; InvivoGen) in a final volume of 100 μl PBS, administered in two intratracheal aspirations of 50 μl each separated by 15 min. For chemokine administration experiments, mice received 100 ng of carrier-free recombinant murine CCL17 (<1 endotoxin unit/μg; R&D Systems) in a final volume of 100 μl PBS, administered in two intratracheal aspirations of 50 μl each separated by 15 min.

Allergic airway inflammation model. To induce allergic inflammation to OVA, mice were anesthetized and sensitized by intratracheal administration of 10 mg OVA and 100 ng of the synthetic NKT lipid agonist PBS57 as described in the previous section and then challenged on days 14, 15, 17, and 18 with OVA alone intratracheally. Mice were killed on day 20 for analysis.

Airway hyperresponsiveness experiments. Mice were anesthetized with 2.5 mg ketamine and 250 μg xylazine. After tracheostomy, the trachea was cannulated with a blunt 18-gauge metal needle, and the mouse was ventilated with a computer-controlled small-animal ventilator (flexiVent; SCIREQ) using tidal volume 1 of 10 ml/kg and a respiratory frequency of 150 breaths/min. Positive end-expiratory pressure of 2 cm H_2O was applied throughout. An external jugular vein was isolated for i.v. infusion of MCh. To obtain a dose–response curve, a bolus of MCh was injected starting at a dose of 1.25 μg (solution at 125 μg/ml, with volume bolus of 10, 20, 40, 80, and 160 μl). After each dose was administrated, the expiratory path was obstructed for 15 s to produce a deep inflation after which exhalation was immediately allowed. Ventilation was continued for 2 min between consecutive MCh doses. Airway responsiveness was quantified as Newtonian resistance at the highest dose of MCh given.

Parabiotic mice. Mice were anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine. A linear incision was made from the scapulae to the lower abdomen on opposing sides of each member of the pair. Animals were placed side by side, and skin edges were sewn together. Mice were rested a minimum of 10 d before experiments.

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Preparation of lung tissue for cellular analysis. Cell suspensions of lung tissue were prepared by first perfusing lungs with ice-cold PBS in situ. Lung tissue was then diced and incubated in 5 ml RPMI 60 medium with 0.1% DNase I (fraction IX; Sigma-Aldrich) and 1.6 mg/ml collagenase P (Roche) in a 37°C shaking incubator for 30 min. Cell suspensions were then strained through a nylon mesh cell strainer (Thermo Fischer Scientific) and washed with 25 ml RPMI with 5% FCS. Cells were centrifuged once at 300 *g* for 5 min with no brake. Cell pellets were resuspended in 5 ml of 44% Percoll (Sigma-Aldrich), underlaid with 3 ml of 66% Percoll, and centrifuged for 20 minutes at 30,000 *g* with no brake. Leukocytes were recovered from the interface of the gradient layers and washed.

Preparation of tissues for microscopic analysis. For histology, lungs were perfused through a needle inserted in the right ventricle with cold PBS in situ before removal and fixation in 4% paraformaldehyde (histological grade; Thermo Fischer Scientific) under a vacuum overnight and then transferred to PBS for 24 h at 4°C. Lobes were sectioned sagittally, embedded in paraffin, and cut into 5-µm sections before staining with hematoxylin and eosin (H&E). For experiments of $CXCR6^{GFP/+}$ mice, lungs were prepared as in the same way, but fixation was in 2% buffered paraformaldehyde made from 16% EM grade paraformaldehyde (Electron Microscopy Sciences). Lungs of $CXCR6^{GFP/+}$ mice were examined as whole tissues in some experiments using two-photon imaging. In other experiments, they were embedded in OCT (Tissue-Tek), cut onto 5–15-µm sections, allowed to air dry, and rehydrated just before analysis.

Imaging. To visualize blood vessels, anesthetized mice were i.v. injected with 100 µg of Dylight594-labeled *Lycopersicon esculentum* (tomato) lectin (Vector Laboratories) in a 200-µl volume. After 5 min, mice were killed, and tissues were prepared as described in the previous section. Whole lungs were placed on a dish with PBS and visualized using an SP5 II microscope (Leica). Histology micrographs were taken with the FSX-100 microscope camera system (Olympus). Data were analyzed using ImageJ software (National Institutes of Health).

Cytokine analysis. BAL fluid and supernatants from cultured cells were assayed for cytokines using Mouse Grp I Cytokine 23-Plex Panel (Bio-Rad Laboratories) and the BioPlex analyzer (Bio-Rad Laboratories) or using the Cytometric Bead Array mouse Th1/Th2/Th17 cytokine kit (BD) and the LSR II flow cytometer (BD) according to the manufacturer's instructions. Data were analyzed using corresponding commercial software.

Chemokine analysis. Perfused lungs were snap-frozen in liquid nitrogen and homogenized in 1 ml PBS with 1× Protease Inhibitor Cocktail and 1 µM EDTA (Thermo Fisher Scientific). Total protein was quantitated using the Protein Assay (Bio-Rad Laboratories) according to the manufacturer's instructions. Chemokines were quantitated by incubating protein homogenates on the Quantibody Mouse Chemokine Array 1 (RayBiotech) according to the manufacturer's instructions. Data extraction was performed using a GenePix 4000B Microarray scanner and GenePix Pro software (Molecular Devices). CCL17 was also independently measured using the mouse CCL17 DuoSet ELISA kit (R&D Systems) according to the manufacturer's instructions. Total RNA was isolated from snap-frozen perfused lungs as described above, and expression levels were measured using mouse genome 430 2.0 array analyzed with GeneChip analysis software (Affymetrix).

Quantitative real-time PCR. Total RNA was isolated from the perfused lungs that were then snap-frozen in liquid nitrogen and homogenized in a 1-ml volume of TRIZOL (Invitrogen). RNA was isolated using the RNeasy mini kit (QIAGEN) according to the manufacturer's instructions and then reverse transcribed with random primers using the AffinityScript qPCR cDNA Synthesis kit (Agilent Technologies). Quantitative real-time PCR was performed on an Mx3005p using Brilliant SYBR green qPCR Master Mix (Agilent Technologies), and data were analyzed using qPCR software (Agilent Technologies). Primers used were IL-4–F (5′-TCATCGGCATTTTGAACGAG-3′), IL-4–R (5′-CGTTTGGCACATCCATCTCC-3′), IL-13–F (5′-GAGCAACATCACACAAGACCAGA-3′), IL-13–R (5′-GGCCAGGTCCACACTCCATA-3′), IFN-γ–F (5′-AACGCTACACACTGCATCTTGG-3′), and IFN-γ–R (5′-GCCGTGGCAGTAACAGCC-3′).

Serum antibodies. Serum was prepared from blood samples using yellow cap serum separator tubes (BD). Total and specific antibody levels were determined by means of sandwich ELISA as previously described (Ganeshan et al., 2009). For anti-OVA IgE ELISAs, a reference monoclonal anti-OVA IgE was provided by P. Bryce (Northwestern University, Chicago, IL).

Cell culture. Culture medium consisted of RPMI 1640 (Cellgro) supplemented with 10% FCS (Bio-West), 50 µM β-mercaptoethanol, 100 U/ml penicillin–100 µg/ml streptomycin (Sigma-Aldrich), and 25 µg/ml gentamicin (Invitrogen). Cells were cultured at 37°C, 5% CO_2. For OVA stimulation of mediastinal LN cells, isolated mediastinal LNs were adjusted to a final concentration of 2×10^5 cells/well in a round-bottom 96-well plate and were cultured with or without 50 µg/ml OVA for 3 d in 200 µl of culture medium.

Flow cytometry. CD1d-PBS57 tetramers were prepared as previously described (Benlagha et al., 2000). Fluorochrome-labeled monoclonal antibodies (clone indicated in parentheses) against mouse B220 (RA3-6B2), CCR3 (83101), CD1d (1B1), CD3ε (17A2), CD4 (GK1.5 or L3T4), CD8α (53–6.7), CD11b (M170), CD11c (HL3), CD19 (1D3), CD45.1 (A20), CD45.2 (104), F4/80 (BM8), GR-1 (RB6-8C5), IA/IE (2G9), IL-4 (11B11), IFN-γ (XMG1.2), NK1.1 (PK136), and TCR-β (H57-597) were purchased from eBioscience, BD, R&D Systems, or BioLegend. For intracellular flow cytometry, cells were first stained for extracellular antigens and then fixed, permeabilized, and stained with fluorescently labeled anticytokine antibodies for 30 min using the Cytofix/Cytoperm kit (BD).

EdU incorporation experiments. 1 mg EdU (Invitrogen) was injected i.v. 3 h before analysis. Cells were fixed and stained with a Pacific blue–labeled Click-iT anti-EdU kit (Invitrogen) according to the manufacturer's instructions.

In vivo tracking of lipid APCs. Mice were given 100 ng PBS57 or saline via intratracheal aspiration at day 0. At 24 and 48 h, lungs were prepared as described in Preparation of lung tissue for cellular analysis. Spleens were injected with 1 ml of 1 mg/ml collagenase P (Thermo Fischer Scientific) in HBSS, minced, and mixed with an additional 1 ml of 1 mg/ml collagenase P and incubated in 5% CO_2 at 37°C for 20 min before isolation of APCs. Alveolar macrophages were sorted as green autofluorescent$^+$CD11c$^+$CD19$^-$CD3$^-$ cells, whereas DCs were sorted as green autofluorescent$^-$CD11c$^+$CD19$^-$CD3$^-$ cells. The NKT hybridoma DN32.D3 was added at 5×10^4 cells/well and incubated overnight with 5×10^4 splenic DCs, inguinal LN DCs, mediastinal DCs, lung DCs, or alveolar macrophages. The 24-h supernatants were assayed for IL-2 using an ELISA kit (R&D Systems).

Generation of mixed bone marrow chimeras. 6–8-wk-old B6.CD1d$^{-/-}$ mice were subjected to 9.5 Gy irradiation with a gamma cell 40 irradiator equipped with a cesium source. 3–6 h later, irradiated mice were i.v. injected with a total of 2×10^6 bone marrow cells obtained from femurs. Mice were rested for at least 6 wk after irradiation before experiments.

In vivo treatment with anti-CD1d antibodies. Mice were injected with 100 µg anti-CD1d antibody 20H2 (rat IgG1; Roark et al., 1998) or isotype control at days −1.5 and 1.5 (sensitization) or days 12.5 and 15.5 (challenge).

Data analysis. Results are expressed as the means ± SEMs. Statistical significance was determined using the unpaired Student's *t* test, except for the MCh response (two-way analysis of variance) and the NKT cell extravasation assay in response to CCL17 (Fisher's exact test, two tailed), using Prism Software 4.0 c for Macintosh (GraphPad Software).

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Online supplemental material. Fig. S1 shows that LPS mobilizes but does not activate lung intravascular NKT cells. Fig. S2 details the histological lesions of extrinsic allergic alveolitis induced by intratracheal administration of PBS57. Fig. S3 shows that microbial GSL-1 and the analogue PBS57 induce a similar degree of allergic airway inflammation. Fig. S4 shows that allergic airway inflammation is abolished in CD1d-deficient mice but preserved in MyD88-deficient mice. Fig. S5 shows that treatment with anti-CD1d impairs allergic airway inflammation when the antibodies are injected before sensitization but not before challenge. Fig. S6 shows that delayed OVA challenge at day 40 induces little NKT cell expansion compared with challenge at day 20. Fig. S7 shows that the mixed cytokine burst detected in the BAL after sensitization resolves into a polarized Th2 response after challenge. Fig. S8 shows the cell type–specific CD1d depletion achieved in CD1d1$^{fl/fl}$ mice crossed to Cre deleter strains. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20110522/DC1.

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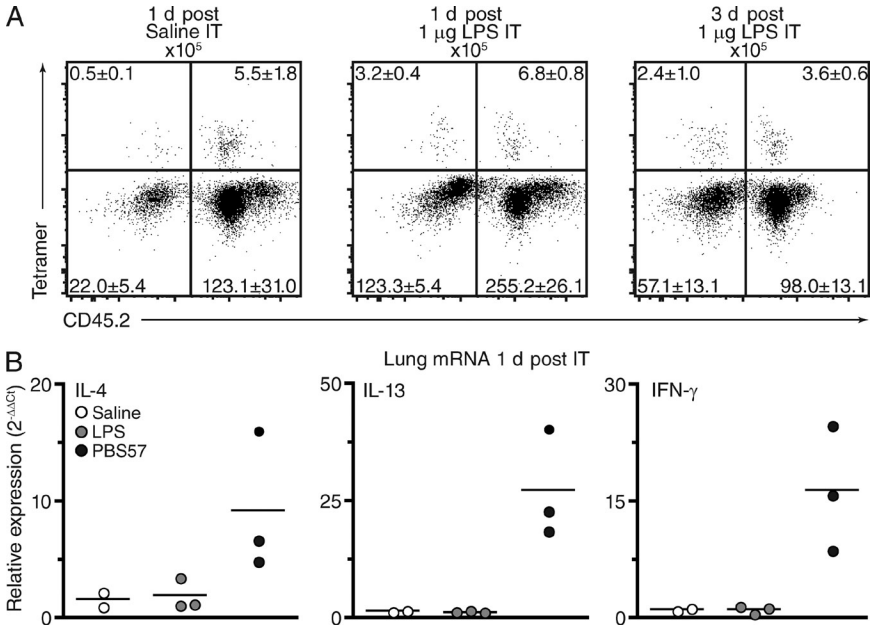

Figure S1. Comparison of 1 µg LPS and 100 ng PBS57 administered intratracheally. (A) High doses of LPS induce NKT cell extravasation measured by the intravascular anti–CD45-PE labeling assay. Numbers indicate mean percentage and SEM of three mice per group. (B) IL-4, IL-13, and IFN-γ messenger RNA in lung tissue 24 h after intratracheal (IT) saline, LPS, or PBS57 as indicated. Horizontal bars indicate the mean.

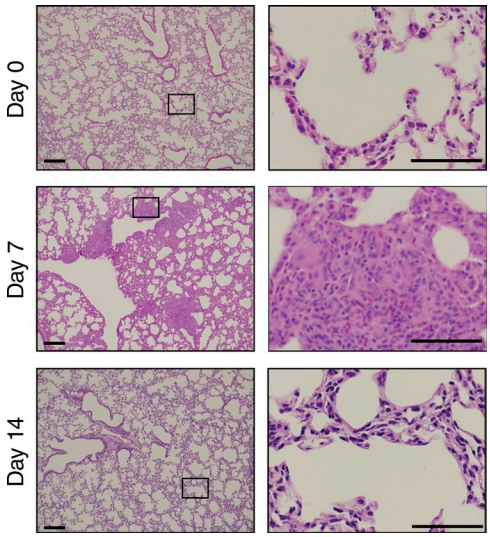

Figure S2. Extrinsic allergic alveolitis induced by PBS57. H&E staining of lung sections at days 0, 7, and 14 after intratracheal administration of 100 ng PBS57. Right column pictures are higher magnification images of the areas boxed in the left column. Data are representative of three lungs/group. Bars, 200 µm.

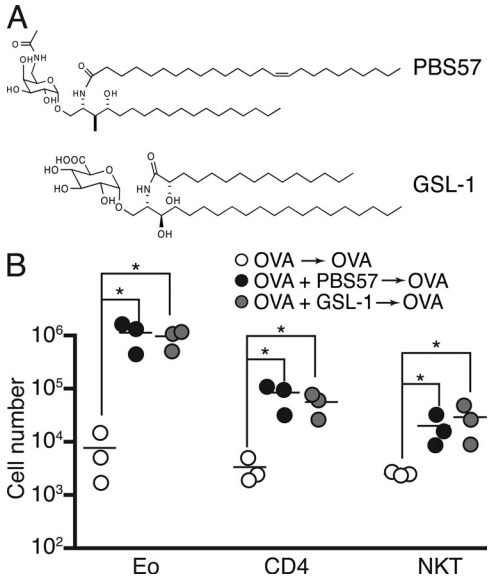

Figure S3. Microbial GSL-1 and the synthetic analogue PBS57 induce similar allergic airway inflammation. (A) Chemical structures of PBS57 and GSL-1. (B) Mice sensitized with OVA, OVA + 100 ng PBS57, or OVA + 100 ng GSL-1 as indicated were challenged with OVA alone. BAL cell counts of eosinophils (Eo), CD4 lymphocytes, and NKT cells at day 20 are shown for individual mice. Horizontal bars indicate the mean. *, P < 0.05.

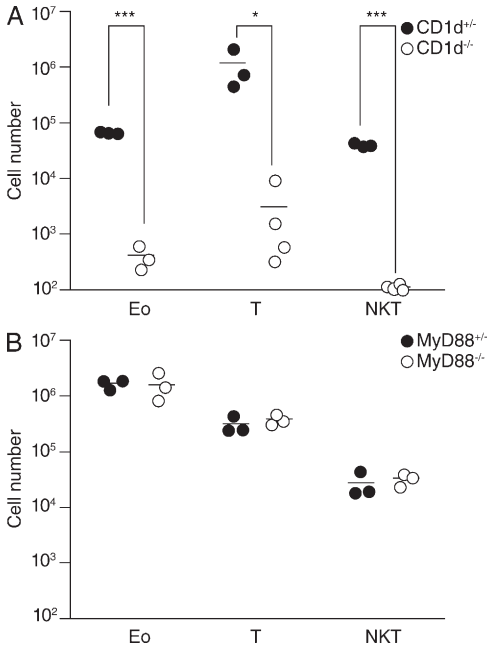

Figure S4. Allergic airway inflammation is CD1d dependent and MyD88 independent. (A) CD1d$^{+/-}$ and CD1d$^{-/-}$ littermates were sensitized with OVA + PBS57 at day 0 and challenged with OVA as described in Allergic airway inflammation model. Shown are BAL eosinophils (Eo), T cells, and NKT cells at day 20. (B) Similar experiment comparing MyD88$^{+/-}$ and MyD88$^{-/-}$ littermates. (A and B) Horizontal bars indicate the mean. *, P < 0.05; ***, P < 0.001.

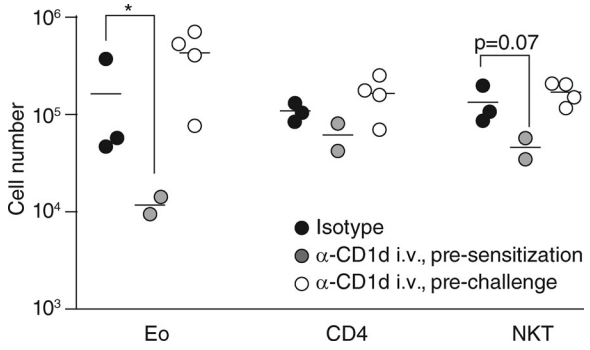

Figure S5. Effect of anti-CD1d blocking on allergic airway inflammation. Mice i.v. injected with 100 µg anti-CD1d antibody 20H2 either presensitization or prechallenge or with isotype control, as indicated, were assessed for allergic airway inflammation by counting eosinophils (Eo), CD4 T cells, and NKT cells in BAL. Horizontal bars indicate the mean. *, P < 0.05.

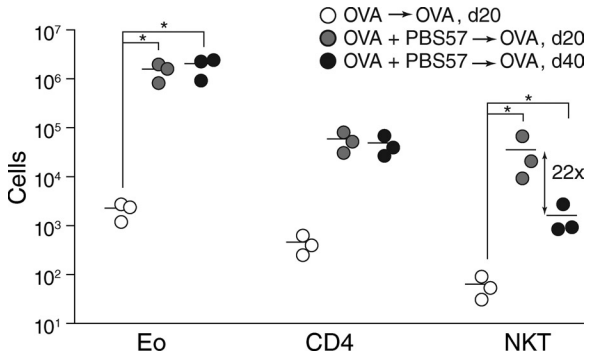

Figure S6. Decreased NKT cell expansion upon delayed OVA challenge. Mice received the indicated intratracheal sensitization and challenge before counting BAL eosinophils (Eo) and CD4 and NKT cells. Horizontal bars indicate the mean. *, P < 0.05.

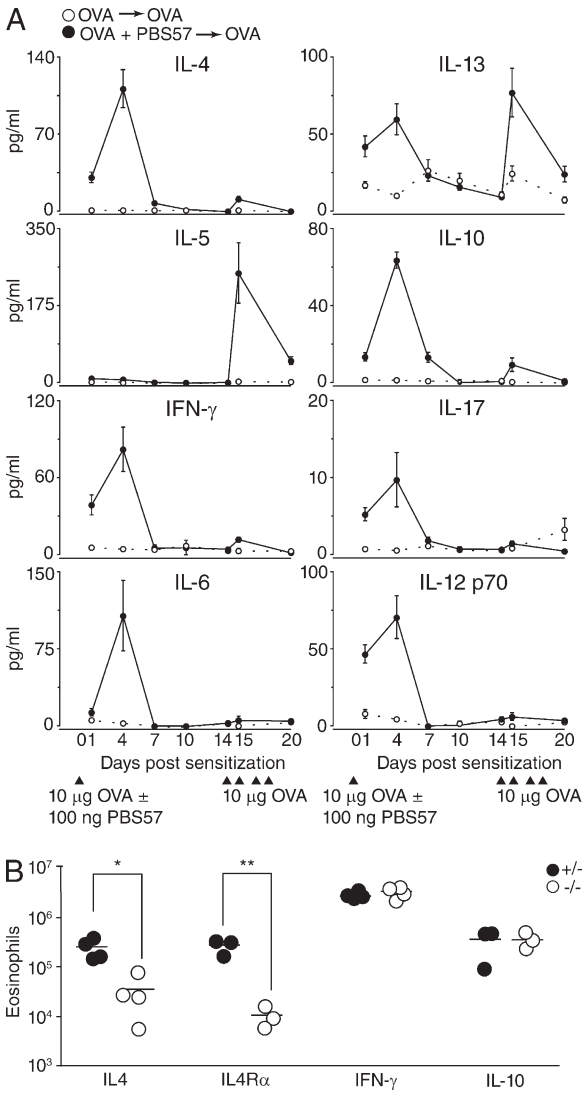

Figure S7. Mixed cytokines burst after sensitization resolves into a polarized Th2 response after challenge. (A) Quantification (mean ± SEM of three mice/group) of cytokines in BAL obtained at the indicated days. (B) Mice lacking IL-4 or IL-4Rα but not IFN-γ or IL-10 have impaired allergic airway inflammation assessed by BAL eosinophil counts at day 20. Horizontal bars indicate the mean. *, $P < 0.05$; **, $P < 0.01$.

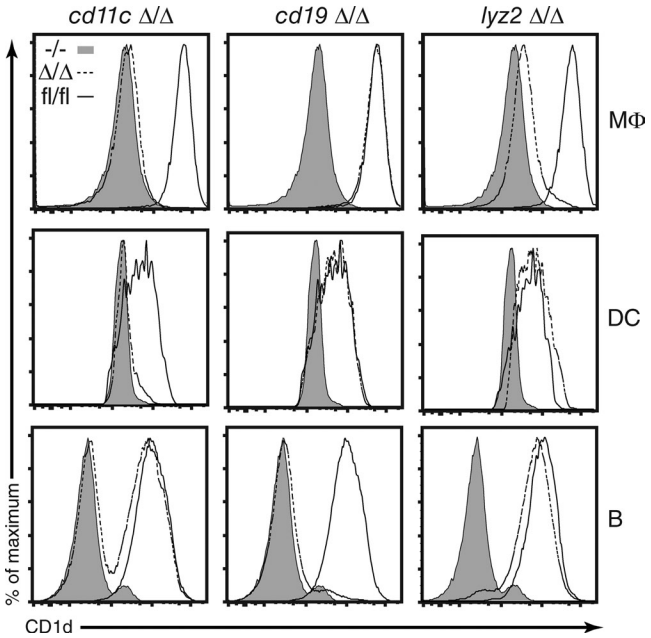

Figure S8. Cell type–specific CD1d depletion in CD1d1$^{fl/fl}$ mice. Representative histograms of CD1d expression in lung macrophages (MΦ), DCs, and B cells of CD1d1$^{fl/fl}$ mice (fl/fl), CD1d1$^{fl/fl}$ mice crossed to indicated Cre deleter strains (Δ/Δ), and CD1d$^{-/-}$ mice (−/−). Note that both macrophages and DCs lost CD1d expression in cd11c Δ/Δ mice and that a fraction of B cells lost CD1d expression in cd11c Δ/Δ mice. Data are representative of four mice/group.