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# Invariant NKT cells are required for airway inflammation induced by environmental antigens

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**Invariant NKT cells (*i*NKT cells) are a unique subset of T lymphocytes that rapidly carry out effector functions. In this study, we report that a majority of sterile house dust extracts (HDEs) tested contained antigens capable of activating mouse and human *i*NKT cells. HDEs had adjuvant-like properties in an ovalbumin (OVA)-induced asthma model, which were dependent on Vα14*i* NKT cells, as vaccinated animals deficient for *i*NKT cells displayed significantly attenuated immune responses and airway inflammation. Furthermore, the administration of HDEs together with OVA mutually augmented the synthesis of cytokines by Vα14*i* NKT cells and by conventional CD4⁺ T cells in the lung, demonstrating a profound immune response synergy for both Th2 cytokines and IL-17A. These data demonstrate that *i*NKT cell antigens are far more widely dispersed in the environment than previously anticipated. Furthermore, as the antigenic activity in different houses varied greatly, they further suggest that *i*NKT cell responses to ambient antigens, particular to certain environments, might promote sensitization to conventional respiratory allergens.**

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Abbreviations used: α-GalCer, α-galactosylceramide; BALF, bronchoalveolar lavage fluid; BLN, bronchial LN; BM-DC, BM-derived DC; HDE, house dust extract; i.n., intranasal(ly); *i*NKT cell, invariant NKT cell; TLR, toll-like receptor; TLR-L, TLR ligand.

Recent increases in the prevalence of asthma and other allergic diseases have prompted investigators to consider the role of the environment in the genesis of atopy (von Mutius, 2009; Horner, 2010). Modern public health practices have eliminated many of the microbial threats for humans. The hygiene hypothesis proposes that higher allergic disease prevalence rates in developed countries are an unintended consequence of this deficiency in the timing, quantity, and quality of immune provocation by microbes (Holt and van den Biggelaar, 2010; Horner, 2010; von Mutius, 2010).

Several investigators have shown that endotoxin is ubiquitously distributed (Gereda et al., 2001; Gehring et al., 2002; Rabinovitch et al., 2005; Dassonville et al., 2008), and several have reported that infants raised in homes with high endotoxin levels were less likely to develop allergic stigmata, although others have been unable to confirm this association (Gereda et al., 2000; Braun-Fahrländer et al., 2002; Tse and Horner, 2008). Apart from endotoxin, homes contain a variety of other microbial products that are potent stimulants of the innate immune system (Roy et al., 2003; van Strien et al., 2004). In this vein, we have previously reported that house dust extracts (HDEs) contain ligands that activate DCs by toll-like receptor 2 (TLR2)–, TLR4-, and TLR9-dependent pathways (Boasen et al., 2005; Batzer et al., 2007). We have further established that HDEs have the potential to function as Th2 adjuvants in mice receiving intranasal (i.n.) OVA vaccinations (Ng et al., 2006; Lee et al., 2011).

NKT cells with an invariant TCR-α chain (invariant NKT cells [*i*NKT cells]) recognize glycolipids presented by CD1d, an MHC class I–like antigen-presenting molecule, and their rapid and explosive production of cytokines is more similar to innate immune cells than to the responses of adaptive immune cells (Kronenberg, 2005; Tupin et al., 2007). The first and most potent

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*i*NKT cell antigen to be identified was α-galactosylceramide (α-GalCer), a synthetic version of a glycolipid originally isolated from a marine sponge (Bendelac et al., 1997). Subsequently, *i*NKT cell antigens have been found in only two bacteria, *Sphingomonas* species, which based on the chemical similarity are the likely source of the original sponge antigen, and *Borrelia burgdorferi*, which is the causative agent of Lyme disease (Kinjo et al., 2006). However, the distribution and prevalence of *i*NKT cell antigens in the microbiota and in the wider environment, and whether the presence of these antigens influences asthma or autoimmunity, remain to be determined. *i*NKT cells have been implicated in directing many types of immune responses in mice, including several asthma models (Lisbonne et al., 2003; Bilenki et al., 2004; Pichavant et al., 2008; Kim et al., 2009) and a model for chronic obstructive pulmonary disease initiated by virus infection (Kim et al., 2008). Furthermore, airway challenge with glycolipid antigens induced pathological features of asthma in MHC class II–deficient mice that lack conventional $CD4^+$ T cells (Meyer et al., 2006). Vα24*i* NKT cells also have been implicated in the pathogenesis of asthma in humans (Akbari et al., 2006), although controversy remains about the role and especially about the magnitude of their increased frequency within the lungs of asthmatics (Hachem et al., 2005; Pham-Thi et al., 2006; Michel et al., 2007; Thomas et al., 2010). Given these considerations, the current series of investigations assessed the distribution and prevalence of *i*NKT cell antigens in indoor environments by analyzing HDEs from different homes and determined whether *i*NKT cells contribute to the adjuvant activities of these HDEs in a mouse airway inflammation model.

## RESULTS

### HDEs contain antigens for Vα14*i* NKT cells

To address whether HDEs contain *i*NKT cell antigens, we tested their ability to stimulate mouse Vα14*i* NKT cell hybridomas in a cell-free, CD1d-dependent, antigen presentation assay. Hybridoma DN3A4-1.2 utilizes the Vβ8.2 chain, expressed by ~55% of mouse *i*NKT cells (Bendelac et al., 1997; Benlagha et al., 2000; Matsuda et al., 2000). Seven out of eight HDEs stimulated the release of IL-2 by DN3A4-1.2 hybridoma cells when added to a plate coated with soluble mouse CD1d (Fig. 1 A). Seven out of eight HDEs also induced IL-2 release by 2H4 hybridoma cells (Fig. 1 B), which express Vβ7, used by ~14% of primary Vα14*i* NKT cells (Bendelac et al., 1997; Benlagha et al., 2000; Matsuda et al., 2000). In contrast, only four out of seven HDEs activated DN3A4-1.4 hybridoma cells (Fig. 1 C), which express the Vβ10 chain, but primary Vα14*i* NKT cells rarely express this Vβ (Bendelac et al., 1997; Benlagha et al., 2000; Matsuda et al., 2000). Furthermore, DN3A4-1.4 hybridoma cells are less capable of recognizing α-GalCer variants with substitutions at the 3″ and 4″ positions of the galactose sugar compared with their Vβ8.2- and Vβ7-expressing counterparts (Raju et al., 2009). We have previously reported that HDEs contain several TLR ligands (TLR-Ls; Boasen et al., 2005; Batzer et al., 2007). However, as seen in Fig. 1, the Vα14*i* NKT cell hybridomas did not respond to TLR2 or TLR4 ligands. Moreover, these hybridomas were nonresponsive to HDEs, unless plates were precoated with soluble CD1d (unpublished data). Additionally, both the type II NKT cell hybridoma 24 (CD1d restricted without the Vα14*i* TCR) and the OVA-specific non-NKT cell hybridoma B3Z did not respond to HDEs in the CD1d-coated plate assay (unpublished data). Collectively, these findings indicate that HDEs induced CD1d- and TCR-dependent IL-2 production by several Vα14*i* NKT cell hybridomas. Moreover, variable responsiveness of the hybridomas toward the eight HDEs indicates that the particular Vβ gene can influence the response, as has been observed with model antigens (Scott-Browne et al., 2007; Pellicci et al., 2009; Raju et al., 2009). Finally, this variable response pattern suggests that different HDEs may contain different antigens with varying affinities for the TCRs expressed by these *i*NKT cell hybridomas.

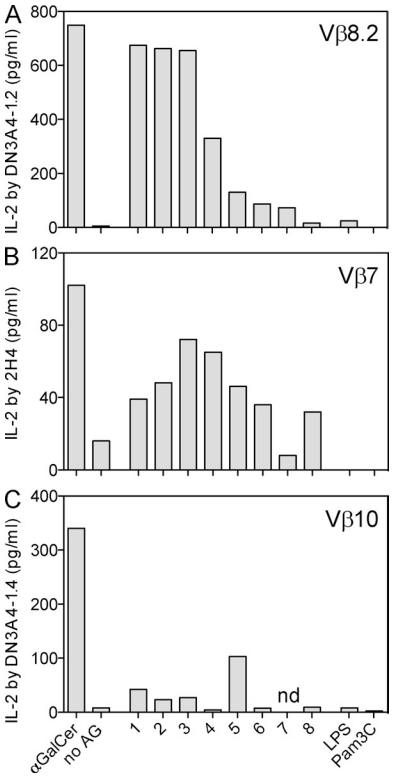


**Figure 1. HDEs contain antigens for Vα14*i* NKT cells.** (A–C) Microtiter wells coated with CD1d monomers were left untreated (no AG) or were incubated with α-GalCer or the indicated (x axis) HDEs for 18–22 h. Wells were washed and incubated with $5 \times 10^4$ of the indicated *i*NKT cell hybridomas (y axis) for 22–24 h. The Vβ usage of each hybridoma is indicated in the top right of each graph. The IL-2 concentration in the supernatant was determined by ELISA. The depicted values represent the mean of the observed ELISA replicates. Background values have been subtracted. None of the *i*NKT cell hybridomas responded to HDEs in the absence of CD1d (not depicted). Representative data of at least two experiments are shown. nd, not determined.

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### HDEs stimulate human *i*NKT cell lines

We next determined whether human Vα24*i* NKT cell lines expanded from PBMC-recognized antigens within HDEs. Indeed, 8 of the 12 HDEs tested induced cytokine production by a human *i*NKT cell line (Fig. 2 A). Similar results were obtained with a second Vα24*i* NKT cell line (unpublished data). Moreover, of the 12 tested HDEs, 9 induced similar responses by human and mouse *i*NKT cells (Fig. 2, A and B). This high degree of concordance between HDE-induced responses by the human *i*NKT cell line and mouse *i*NKT cell hybridoma DN3A4-1.2 (Vα14/Vβ8.2) suggests that they were responding to the same antigen or antigens. Consistent with this view, human Vβ11, the most common TCR-β expressed by human Vα14*i* NKT cells, is an orthologue of mouse Vβ8.2 (Bendelac et al., 1997; Kronenberg, 2005).

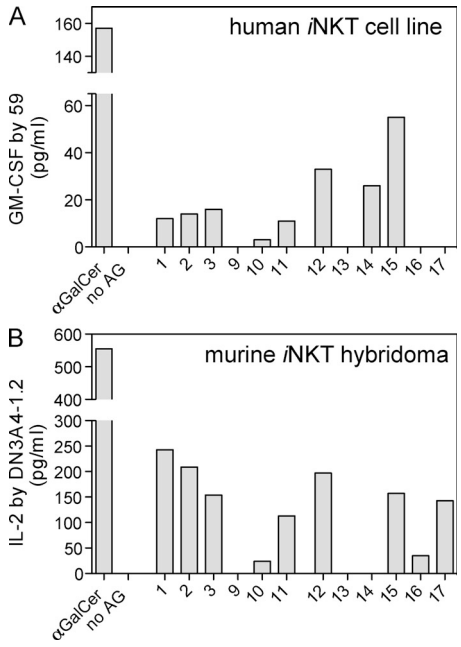


**Figure 2. HDEs stimulate human *i*NKT cell lines.** (A) Human PBMCs were left untreated (no AG) or were incubated with α-GalCer or the indicated (x axis) HDEs. After 20 h, cells were washed, and $2 \times 10^5$ PBMCs were incubated with $4 \times 10^4$ *i*NKT cells of the human *i*NKT cell line 59 for 72 h. The GM-CSF concentration in the supernatant was determined by ELISA. Similar results were obtained with the human *i*NKT cell line 2225 (not depicted). (B) Cell culture plates, coated with CD1d monomers, were left untreated (no AG) or were incubated with α-GalCer or the indicated (x axis) HDEs. After 20 h, plates were washed and incubated with $5 \times 10^4$ of the mouse *i*NKT cell hybridoma DN3A4-1.2 for 22 h. The IL-2 concentration in the supernatant was determined by ELISA. The depicted values represent the mean of the observed ELISA replicates. Background values have been subtracted. Representative data of at least two experiments are shown. Note that samples 1–3 are the same as those tested with DN3A4-1.2 in Fig. 1 A, whereas samples 9–17 were obtained from different houses from those analyzed in Fig. 1.

### HDE-loaded BM-derived DCs (BM-DCs) stimulate mouse *i*NKT cells in vivo

To determine whether HDEs could activate mouse Vα14*i* NKT cells in vivo, we immunized mice with BM-DCs pulsed with an HDE standard prepared from pooled samples found to have high levels of Vα14*i* NKT cell antigen by the APC-free hybridoma assay presented in Fig. 1 (hereafter HDE standard; see Materials and methods). As Vα14*i* NKT cells also can be activated by cytokines from APCs stimulated by TLR-L, in a CD1d-independent manner, we used antigen-pulsed BM-DCs derived from MyD88 and Trif double-deficient animals (BM-DC$^{DKO}$), which cannot respond to any known TLR-L (Hoebe et al., 2003). BM-DC$^{DKO}$ pulsed with α-GalCer was used as a positive control for these experiments. BM-DC$^{DKO}$ pulsed with LPS provided a negative control because these BM-DC$^{DKO}$ should not be able to provide the required cytokines for Vα14*i* NKT cell activation in the absence of exogenous antigen. 14–16 h after the injection of BM-DC$^{DKO}$, *i*NKT cells from recipient mice were analyzed. Challenge with BM-DC$^{DKO}$ loaded with α-GalCer (BM-DC$^{DKO}$/α-GalCer) led to a significant reduction in the frequency of Vα14*i* NKT cells (Fig. 3 A) and to the up-regulation of CD69 on the *i*NKT cells that could still be detected (Fig. 3, B and C), which are both indicators of *i*NKT cell activation. In contrast, BM-DC$^{DKO}$ loaded with LPS had, as expected, no effect on either parameter (Fig. 3, A–C). Compared with challenge with BM-DC$^{DKO}$/α-GalCer, injection of BM-DC$^{DKO}$ loaded with HDE (BM-DC$^{DKO}$/HDE) had a smaller but nonetheless significant effect on Vα14*i* NKT cell recovery (Fig. 3 A), and these *i*NKT cells displayed increased expression of CD69 (Fig. 3, B and C). Furthermore, BM-DC$^{DKO}$/HDE challenge induced the production of IFN-γ (Fig. 3 D) and IL-4 (Fig. 3 E) by *i*NKT cells. In previous experiments, although IFN-γ synthesis could be elicited from Vα14*i* NKT cells activated by self-antigen or solely by cytokines, IL-4 was correlated with foreign antigen activation (Nagarajan and Kronenberg, 2007; Tupin et al., 2008).

To address the CD1d dependency of *i*NKT cell activation by BM-DC$^{DKO}$/α-GalCer and BM-DC$^{DKO}$/HDE in vivo, we repeated the aforementioned experiments in the presence of blocking α-CD1d antibodies. This blockade significantly reduced the activation of *i*NKT cells by either α-GalCer or HDE, as measured by up-regulation of CD69 and cytokine production (Fig. 3, F and G). Importantly, the α-CD1d antibody–mediated impairment of *i*NKT cell activation by BM-DC$^{DKO}$/HDE was for all parameters and in all experiments comparable with or more pronounced than the reduction observed with the stimulation by BM-DC$^{DKO}$/α-GalCer. Collectively, these data suggest an exogenous antigen-dependent activation of Vα14*i* NKT cells in vivo by BM-DC$^{DKO}$ pulsed with HDEs.

NK cells can be activated downstream of Vα14*i* NKT cell activation, although after exposure to HDEs, it is possible that NK cell activation occurs independently of Vα14*i* NKT cells. Therefore, to assess the impact of HDE-mediated *i*NKT cell activation on NK cells, we also analyzed CD69 expression on these cells. After BM-DC$^{DKO}$/HDE injection, a clear increase

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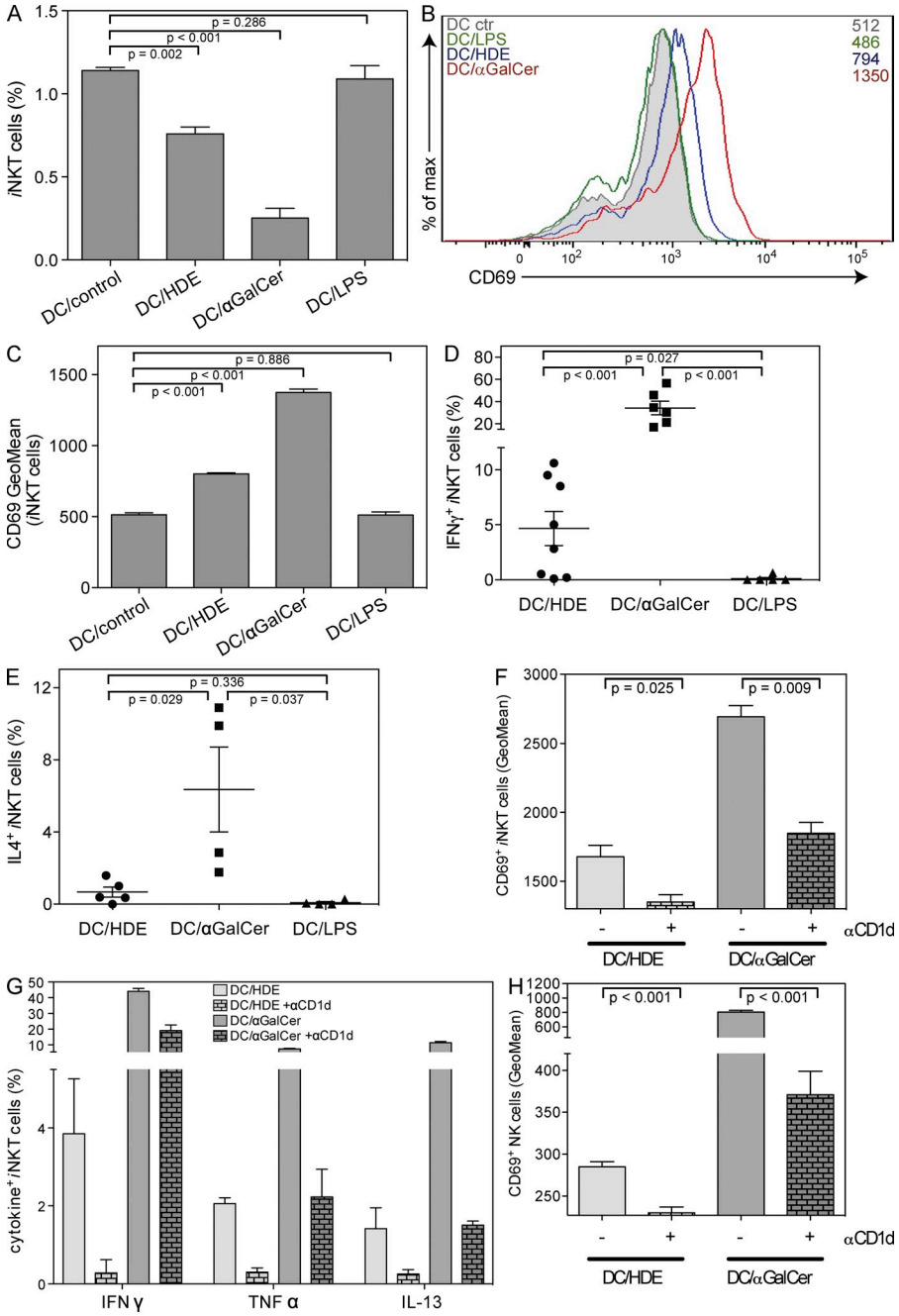


**Figure 3. HDE-loaded BM-DCs stimulate mouse *i*NKT cells in vivo.** DCs were generated with GM-CSF from the BM of MyD88 and Trif double-deficient animals (BM-DC$^{DKO}$; DC in the graphs). BM-DC$^{DKO}$ were washed and incubated with the indicated substances (50 µl/ml HDE, 150 ng/ml LPS, or 50 ng/ml α-GalCer) for 5 h, and 5 × 10$^5$ BM-DC$^{DKO}$ were injected i.v. into C57BL/6 mice. 12–15 h later, splenic *i*NKT cells were analyzed. (A) Relative proportion of *i*NKT cells in the spleen. (B and C) Representative expression of CD69 by splenic *i*NKT cells from single mice (B) and a summary of data from at least three mice per group from a representative experiment (C). The numbers in the histogram denote the geometric mean values of CD69 on *i*NKT cells. (A–C) Representative data from one of four independent experiments, with three to four mice per group, are shown. (D and E) IFN-γ (D) and IL-4 expression (E) by splenic *i*NKT cells after challenge with the indicated BM-DC$^{DKO}$. Background values have been subtracted. The graphs in D and E summarize data from three and two independent experiments, respectively, with two to four mice per group. (F–H) Mice were injected with 200 µg α-CD1d antibody (1B1) together with BM-DC$^{DKO}$ loaded with the indicated antigens. Up-regulation of CD69 on *i*NKT cells (F) or NK cells (H) and production of the indicated cytokines by *i*NKT cells (G) are depicted. The difference in cytokines production (G) with and without α-CD1d antibody treatment was statistically significant for all cytokines and BM-DC$^{DKO}$ challenges. Representative data from one of two independent experiments, with three to five mice per group, are shown. Error bars indicate mean ± SEM.

in CD69 expression by NK cells was observed (Fig. S1). To test whether other innate immune sensors beside TLRs could induce such activation of NK cells independent of Vα14*i* NKT cell activity, we injected the BM-DC$^{DKO}$/HDE into Jα18$^{-/-}$ mice, which lack Vα14*i* NKT cells (Cui et al., 1997), or into CD1d$^{-/-}$ mice. Only a small proportion of NK cells from Jα18$^{-/-}$ mice responded by up-regulating their expression of CD69 (Fig. S1), and similar results were obtained in CD1d$^{-/-}$ recipients (not depicted). Consistent with these findings, the NK cell trans-activation was dependent on *i*NKT cell recognition of CD1d as blocking with an α-CD1d antibody abrogated NK cell CD69 up-regulation (Fig. 3 H). These data indicated that the NK cell activation after BM-DC$^{DKO}$/HDE delivery was mainly Vα14*i* NKT cell driven.

### Vα14*i* NKT cells contribute to the adjuvant effect of HDE

HDEs have previously been found to be potent Th2-biasing mucosal adjuvants in a mouse asthma model (Boasen et al., 2005; Ng et al., 2006; Batzer et al., 2007; Lee et al., 2011). Therefore, we determined whether Vα14*i* NKT cells helped mediate the Th2 adjuvant activity of HDEs. To this end, BALB/c mice and Jα18$^{-/-}$ mice were i.n. vaccinated three times with chicken OVA alone or together with HDE standard. Sensitized mice were then airway challenged twice with OVA alone, and the parameters of OVA-specific immunity and airway inflammation were analyzed 24 h after the last challenge (experimental outline in Fig. 4 A). Wild-type

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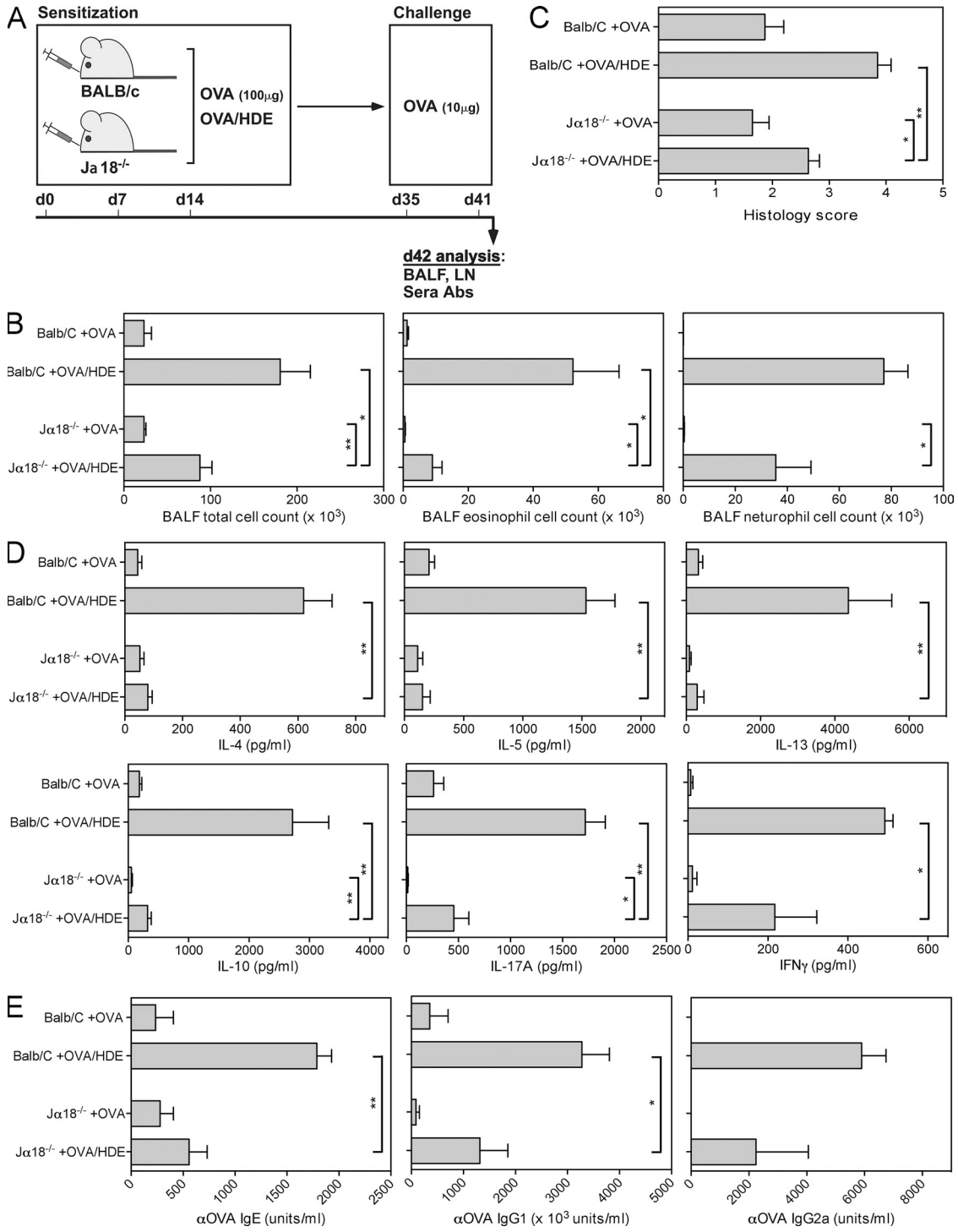


**Figure 4. Vα14*i* NKT cells contribute to the adjuvant effect of HDE during allergen-induced airway inflammation.** (A) Experimental outline: BALB/c mice and Jα18-deficient mice on the BALB/c background (Jα18$^{-/-}$) were sensitized three times, once per week (days 0, 7, and 14) with chicken OVA alone (100 µg/mouse) or together with HDE standard (20 µl/mouse). On days 35 and 41, the mice were challenged with OVA alone (10 µg/mouse) and analyzed 24 h after the last challenge. Abs, antibodies. (B) Total BALF cell count (left) and relative percentages of eosinophils (middle) and neutrophils (right) within BALF from the indicated mice. (C) Histological scores from the lungs of the indicated mice. (D) BLN-derived lymphocytes from the indicated mice were restimulated with 50 µg/ml OVA, and the concentrations of the indicated cytokines in the supernatant were measured by ELISA. (E) OVA-specific antibodies in the sera of the indicated mice including IgE (left), IgG1 (middle), or IgG2a (right). Representative data from one of two independent experiments, with three to four mice per group, are shown. P-values for Jα18$^{-/-}$ + OVA/HDE vs. Jα18$^{-/-}$ + OVA and for Jα18$^{-/-}$ + OVA/HDE vs. BALB/c + OVA/HDE are indicated, where the difference is statistically significant (P, * < 0.05; P, ** < 0.01). Error bars indicate mean ± SEM.

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and Jα18$^{-/-}$ mice vaccinated with OVA alone displayed little evidence of pulmonary inflammation upon airway allergen challenge (Fig. 4, B and C; and Fig. S2), and their OVA-specific immune responses were similar (Fig. 4, D and E). In comparison with wild-type mice i.n. vaccinated with OVA alone, mice vaccinated with OVA and HDE standard (OVA/HDE) developed significantly stronger airway inflammation and immune responses (Fig. 4, B–E). Consistent with a previous study, the HDE standard acted as a Th2-biasing mucosal adjuvant in this asthma model (Boasen et al., 2005) as OVA/HDE immunized wild-type mice developed eosinophil-rich inflammatory responses after airway allergen challenge and robust OVA-specific Th2 cytokine and IgE responses (Fig. 4, B–E). However, other immune parameters were also increased, including IFN-γ and IL-17A synthesis by OVA-stimulated bronchial LN (BLN) cells (Fig. 4 D). Finally, although the HDE standard retained some adjuvant activity in Jα18$^{-/-}$ mice (Fig. 4, B–E), immune responses to i.n. OVA/HDE vaccination, as well as airway inflammation after OVA challenge, were markedly attenuated compared with those of wild-type mice. (Fig. 4, B–E). Collectively, these observations indicate that Vα14*i* NKT cells played a major role in mediating the adjuvant activities of the HDE standard in this mouse model of allergen-induced airway inflammation.

### Synergistic activation of lung Vα14*i* NKT and CD4 T cells

Having established an important role for Vα14*i* NKT cells in mediating the adjuvant activity of the HDE standard, we tested their short-term response to i.n. HDE challenge. Bronchoalveolar lavage fluid (BALF) and lung lymphocytes were analyzed 24 h after a single challenge with HDE, but no significant differences in Vα14*i* NKT cell numbers, phenotype, or cytokine production in the lung or BLNs were detected (Fig. 5 A and not depicted). However, in the BALF, the total number of Vα14*i* NKT cells and their expression of CD69 increased significantly 24 h after a single HDE challenge, although cytokine synthesis did not increase (Fig. 5 A and not depicted).

Given that we observed only limited Vα14*i* NKT cell activation and no activation of conventional T cells after a single airway exposure to HDE, we next considered whether these cellular responses would be more pronounced at the end of the sensitization phase of experiments described in Fig. 4 A. Therefore, mice were administered reagents on three occasions, at weekly intervals. However, rather than allowing the adaptive response to develop further, mice were sacrificed on day 15, 1 d after the third vaccination with OVA, HDE, or OVA together with HDE. Whereas the phenotype of lung Vα14*i* NKT cells from mice vaccinated with OVA alone did not differ from control mice, we observed clear signs of Vα14*i* NKT cell activation in the lungs of mice treated either with HDE or OVA/HDE (Fig. 5 B). In particular, CD69 was upregulated and CD122 was down-regulated on Vα14*i* NKT cells (Fig. 5 B). Furthermore, we detected a higher percentage of CD4-negative Vα14*i* NKT cells in the lungs of mice vaccinated with HDE, and this finding was even more pronounced after OVA/HDE vaccination (Fig. 5 B and Fig. S3 A), suggesting either the selective recruitment and/or expansion of this subset or the down-regulation of CD4 expression by Vα14*i* NKT cells. Importantly, by intracellular cytokine staining, we could detect significantly more cytokine-producing lung Vα14*i* NKT cells after challenge with OVA/HDE than with OVA or HDE alone (Fig. 5 C), and this was true not only for Th2 cytokines but also for Th1 cytokines and IL-17A. These observations suggest that the concurrent adaptive immune response against OVA enhanced cytokine production by activated Vα14*i* NKT cells. The CD4-negative subset of Vα14*i* NKT cells produced most of the cytokines, a trend which was especially prominent for IL-17A (Fig. 5 D and Fig. S3 B).

Without additional ex vivo stimulation, we could also detect cytokine production by a subset of CD44$^{high}$ memory CD4 T cells isolated from the lungs of vaccinated mice, and it was significantly higher in mice receiving OVA and HDE than in those receiving OVA or HDE alone (Fig. 5, E and F). The CD44$^{high}$ population of conventional TCR-β$^{+}$ T cells outnumbered Vα14*i* NKT cells by ~10:1 in the lung (unpublished data). Given the similarly elevated percentages of cytokine-producing cells within this population (Fig. 5 F), we conclude that most of the cytokine-producing TCR-β$^{+}$ T cells detected after challenge with OVA/HDE were conventional CD4 T cells. Importantly, cytokine production by CD4$^{+}$ CD44$^{high}$ T cells was almost completely abrogated in Jα18$^{-/-}$ mice (Fig. 5, E and F), indicating a crucial role for Vα14*i* NKT cells in the induction of the conventional CD4$^{+}$ T cell response in the lung. Collectively, these observations demonstrate synergy between Vα14*i* NKT cell and conventional CD4 T cell responses in mice i.n. vaccinated with OVA and HDE.

## DISCUSSION

We have shown that a majority of HDEs tested contain antigens capable of activating mouse Vα14*i* NKT cell hybridomas and human Vα24*i* NKT cell lines. Stimulation of mouse Vα14*i* NKT cell hybridomas was shown to be CD1d dependent and not dependent on TLR agonists present in HDEs. Activation of Vα14*i* NKT cells also was seen in vivo after injection of BM-DCs pulsed with an HDE standard containing high levels of activity. Furthermore, in a mouse model of airway inflammation, the HDE standard had adjuvant activity that was largely Vα14*i* NKT cell dependent. Additionally, although i.n. delivery of OVA or HDE standard alone led to some degree of lung CD4 cell and Vα14*i* NKT cell activation, injection of these reagents together had synergistic effects on cytokine production by both cell types.

For several years, the only antigens identified that activated *i*NKT cells included α-GalCer and its derivatives, but the distribution of *i*NKT cell antigens in the microbiota, and more generally in the environment, remains incompletely characterized. Although the finding of *i*NKT cell antigens in *Sphingomonas* suggested that antigens could be found in seawater and soil, in this study, we have made the surprising finding that a significant amount of antigenic activity appears to be present in many households as well. An unresolved issue is the origin of the *i*NKT cell antigens detected in HDEs.

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Although house dust mites have been shown to stimulate the innate and adaptive immune systems, in preliminary experiments, we found that whole body extracts of *Dermatophagoides farinae* or *Dermatophagoides pteronyssinus* did not stimulate Vα14*i* NKT cell hybridomas (unpublished data), suggesting that house dust mites are not an important source of *i*NKT

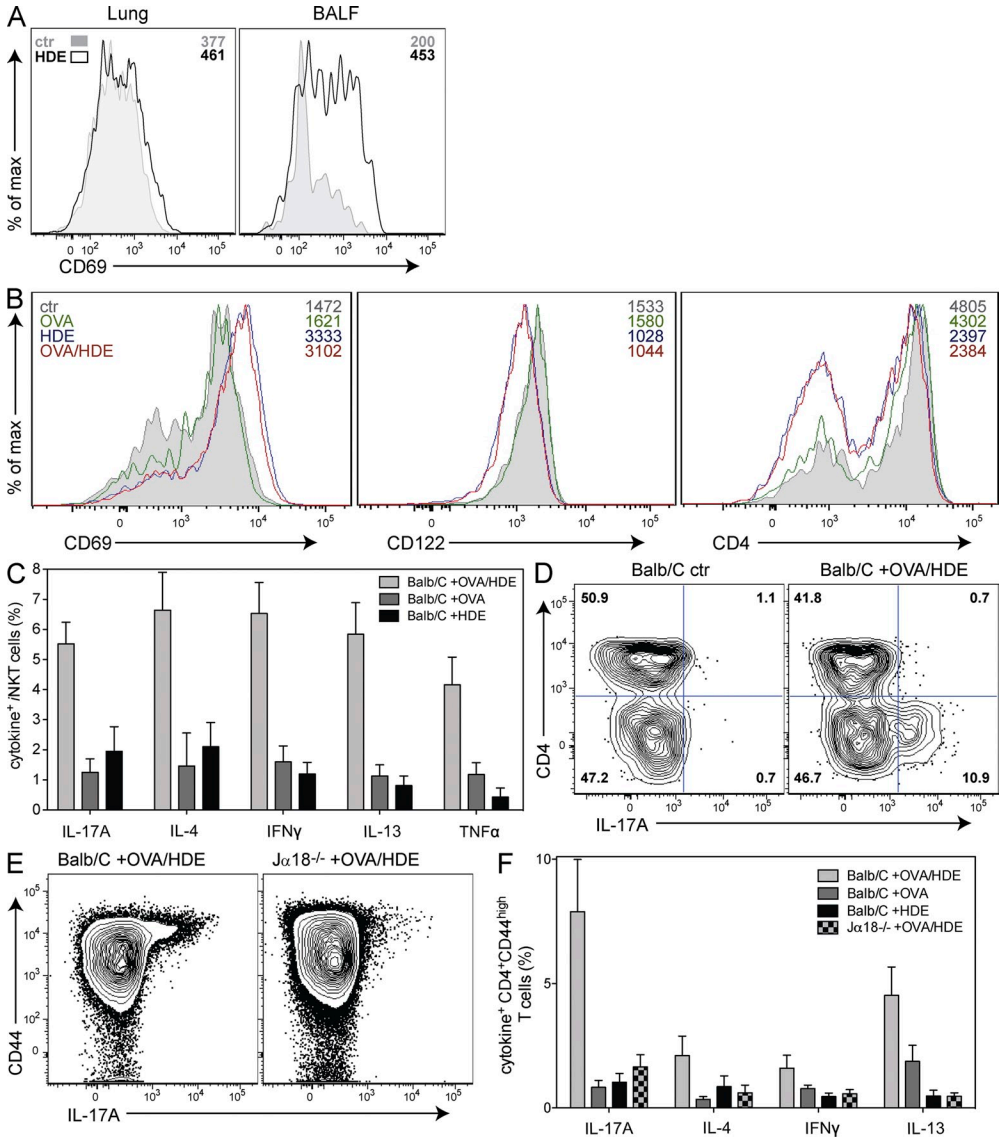


**Figure 5. Synergistic activation of lung Vα14*i* NKT cells and CD4 T cells.** (A) BALB/c mice were challenged once with HDE, and the surface expression of CD69 on *i*NKT cells from lung and BALF was determined 18 h later. The numbers in the histograms denote the geometric mean values of CD69 on *i*NKT cells. Light gray, closed histogram: BALB/c control; solid line, open histogram: BALB/c challenged with HDE. (B–F) BALB/c mice and Jα18$^{-/-}$ on the BALB/c background were vaccinated three times, once per week (days 0, 7, and 14) with chicken OVA alone (100 µg/mouse), HDE alone, or with OVA together with HDE and analyzed on day 15. Representative data of two or three independent experiments, with two to five mice per group, are shown. (B) Surface expression of CD69, CD122, and CD4 on lung *i*NKT cells from the indicated groups. The numbers in the histograms denote the geometric mean values of the indicated surface molecules on *i*NKT cells. (C) Cytokine production of lung *i*NKT cells ex vivo after the indicated treatments. Lymphocytes were kept 2 h in the presence of degranulation inhibitors to accumulate intracellular cytokines without further stimulation. The graph summarizes data from two or three independent experiments, with three to four mice per group. All cytokine values obtained from the OVA/HDE group were significantly different ($P < 0.05$) from the values obtained with the other treatments. (D) IL-17A production of lung CD4$^+$ and CD4$^-$ *i*NKT cells from control mice (BALB/c ctr) or from mice challenged with OVA/HDE. (E) CD44 expression and IL-17A production of lung conventional CD4 T cells (CD19$^-$ *i*Vα14$^-$ CD8α$^-$ TCR-β$^+$ CD4$^+$) from BALB/c or Jα18$^{-/-}$ mice challenged with OVA/HDE. (F) Cytokine production of lung CD44$^{high}$ conventional CD4 T cells (CD19$^-$ *i*Vα14$^-$ CD8α$^-$ TCR-β$^+$ CD4$^+$) ex vivo after the indicated treatments. Lymphocytes were kept 2 h in the presence of degranulation inhibitors to accumulate intracellular cytokines without further stimulation. The graph summarizes data from two or three independent experiments, with three to four mice per group. All cytokine values obtained from the BALB/c + OVA/HDE group were significantly different ($P < 0.05$) from the values obtained from the Jα18$^{-/-}$ + OVA/HDE group. Error bars indicate mean ± SEM.

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cell antigen. Preliminary results of biochemical analyses suggest that the Vα14*i* NKT cell–specific antigenic activities of at least some HDEs are not glycosphingolipid (unpublished data). However, a more thorough analysis of many different HDEs will be required to determine whether this conclusion can be generalized.

As several bacteria contain *i*NKT cell antigens, we consider it most likely that the immunostimulatory material for *i*NKT cells detected in HDEs was of bacterial origin. HDEs are known to contain a variety of microbial products, and in a previous publication, we reported that HDEs have MyD88-dependent adjuvant activity (Ng et al., 2006). The results presented in this study demonstrate a predominant role for Vα14*i* NKT cells in mediating the stimulatory effect of HDEs. These findings are not contradictory, as the activation of *i*NKT cells to putatively weak self-antigens requires a synergistic contribution of MyD88 signals (Mattner et al., 2005), leading to cytokine secretion by APCs (Brigl et al., 2003; Paget et al., 2007). Therefore, we hypothesize that APC-derived cytokines could also be required for an *i*NKT cell response to microbial antigens that are weak and/or at low concentration. However, the presence of Vα14*i* NKT cell antigen detected in the CD1d-coated plate assay and the ability of DCs deficient for TLR signaling to stimulate *i*NKT cells in vivo suggest that the *i*NKT cell–dependent adjuvant activity of HDEs required antigen recognition.

Regardless of the nature of the antigen, the variation in the ability of different extracts to stimulate *i*NKT cells suggests diversity in the composition of HDEs from different homes (Figs. 1 and 2). Furthermore, although most HDEs activated *i*NKT cells in all the in vitro assays, some exhibited mouse versus human species specificity or a requirement for a particular Vβ gene, suggesting that the *i*NKT cell antigens contained within these HDEs could have differing TCR affinities or that they preferentially activate subsets of these cells. We speculate that the concentration and/or potency of *i*NKT cell antigens found in different environments could contribute to the striking differences in the frequency of *i*NKT cells observed in the peripheral blood of normal individuals (Kronenberg, 2005).

A surprising result from these experiments was the degree to which the adjuvant activity of our HDE standard was dependent on the presence of Vα14*i* NKT cells (Fig. 4). In analyzing the response to OVA alone versus OVA/HDE in $J\alpha18^{-/-}$ mice, an *i*NKT cell–independent adjuvant effect was observed; however, this effect was relatively minor. In contrast, the prominent role of Vα14*i* NKT cells in our i.n. sensitization experiments is consistent with other studies showing an important effect of *i*NKT cells on immune responses in the lung, not only in models of asthma and chronic obstructive pulmonary disease (Lisbonne et al., 2003; Bilenki et al., 2004; Kim et al., 2008; Kim et al., 2009; Pichavant et al., 2008) but also in the response to infectious agents (Nieuwenhuis et al., 2002; Kawakami et al., 2003). In the context of i.n. sensitization with OVA/HDE, production by Vα14*i* NKT cells of all cytokines tested was increased (Fig. 4), including Th2 cytokines and IL-17A, which have been implicated in asthma causation in other models (Bilenki et al., 2004; Meyer et al., 2006; Pichavant et al., 2008; Matangkasombut et al., 2009).

Importantly, our data revealed a synergy in the cytokine responses of Vα14*i* NKT cells and conventional CD4 T cells in the lung after OVA/HDE vaccination (Fig. 5). It has been reported previously that concurrent activation of *i*NKT cells with glycolipid antigen can augment the adaptive immune responses of both T and B cells (Cerundolo et al., 2009). The novel finding we report here is the enhancing effect of an ongoing adaptive immune response on the simultaneous activation of Vα14*i* NKT cells. Three weekly i.n. exposures to HDE standard alone induced less cytokine production by Vα14*i* NKT cells in vivo than i.n. vaccination with OVA/HDE (Fig. 5). As OVA does not contain *i*NKT cell antigens, we hypothesize that the adaptive anti-OVA response functioned to augment the response of Vα14*i* NKT cells, although the mechanism mediating enhanced Vα14*i* NKT cell cytokines remains to be determined. This positive feedback of adaptive immune responses on innate-like *i*NKT cells may have been missed previously because relatively early responses were analyzed, whereas in this study a more prolonged immunization protocol was used. Innate cell-derived cytokines IL-12 and IFN-α/β have been reported to augment CD1d-dependent *i*NKT cell activation (Brigl et al., 2003; Mattner et al., 2005; Paget et al., 2007), which was especially crucial for stimulation with presumably weak antigens. However, in our experiments, activation of the innate immune system by TLR-Ls present in the HDE standard apparently had a far weaker synergistic effect on antigen-specific Vα14*i* NKT cell activation than the ongoing adaptive immune response elicited by OVA.

In this context, it might be surprising that the response of conventional $CD4^+$ T cells was comparably low when mice were i.n. immunized with HDE standard alone or OVA alone. However, although the 100-μg OVA vaccination dose provided ample T cell antigens, it provided hardly stimulating ligands for the innate immune system. In contrast, the 20-μl HDE dose used for these experiments should have provided larger amounts of danger signals, but based on our reasoning, only limited amounts of any one specific T cell antigen. HDEs are known to contain several traditional allergens, including house dust mite, molds, and animal dander. However, based on reported concentrations of common indoor allergens found in house dust (Cho et al., 2006), we estimate the delivery of 0.16 μg of each relevant allergen per dose of HDE standard, or ~1/625 of the OVA dose used for these experiments.

In summary, our novel findings include, first, the discovery that antigens for mouse and human *i*NKT cells are widely distributed in living indoor environments. Second, we demonstrate that the adjuvant activity of HDEs is largely dependent on Vα14*i* NKT cells. Third, in the context of repeated antigen exposures in the lung, not only does Vα14*i* NKT cell activation contribute to the response by conventional $CD4^+$ T cells, but the conventional $CD4^+$ T lymphocyte response augments *i*NKT cell activation as well. Based on these findings, we suggest that the airway milieu induced by repeated

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stimulation with *i*NKT and conventional CD4 T cell antigens could act as a feed-forward loop that might contribute to the genesis of asthma and other allergic diseases, selectively in those environments more enriched for these antigens.

## MATERIALS AND METHODS

**Mice.** All mice were housed under specified pathogen–free conditions at the animal facilities of the La Jolla Institute for Allergy and Immunology and the University of California, San Diego in accordance with the Institutional Animal Care Committee guidelines. All experiments were performed in an Association for Assessment and Accreditation of Laboratory Animal Care International–accredited facility with prior approval of the La Jolla Institute for Allergy and Immunology Animal Care Committee (Institutional Animal Care and Use Committee) in accordance with the Public Health Service Policy. C57BL/6 and BALB/c mice were purchased from the Jackson Laboratory. MyD88 (Adachi et al., 1998)- and Trif (*Lps2*; Hoebe et al., 2003; Yamamoto et al., 2003)-deficient animals have been described previously and were used as double-deficient animals. B6.129-*Tcra-J*$^{tm1Tgi}$ (*Jα18*$^{-/-}$) mice (Cui et al., 1997) and CD1d-deficient mice (*CD1d*$^{-/-}$; Mendiratta et al., 1997) on the C57BL/6 background were the gift of M. Taniguchi (RIKEN Institute, Yokohama, Japan) and L. Van Kaer (Vanderbilt University, Nashville, TN), respectively, and were crossed onto the BALB/c background.

**Reagents and mAbs.** α-GalCer ([2*S*,3*S*,4*R*]-1-O-[a-D-galactopyranosyl]-*N*-hexacosanoyl-2-amino-1,3,4-octadecanetriol) was obtained from the Kirin pharmaceutical research corporation. Lipopeptide Pam-3-Cys (P-3-C; EMC Microcollections) and *Escherichia coli* 026-B6 LPS (Sigma-Aldrich) were purchased from commercial vendors. Whole body extracts from the dust mites *D. farinae* and *D. pteronyssinus* were obtained from Greer Laboratories. mAbs against the following mouse antigens were used in this study: CD1d (1B1), CD3-ε (145.2C11; 17A2), CD4 (GK1.5; RM4-5), CD8α (53-6.7; 5H10), CD11b (M1/70), CD11c (HL3), CD19 (1D3; 6D5), CD24 (M1/69), CD25 (PC61.5), CD44 (IM7), CD45.1 (A20), CD45.2 (104), CD45R (B220; RA3-6B2), CD69 (H1.2F3), TCR-β (H57-597), Thy1.2 (30-H12; 53–2.1), NK1.1 (PK136), Ly6CG (Gr1), TNF (MP6-XT22), IL-4 (11B11), and IFN-γ (XMG1.2). Antibodies were purchased from BD, BioLegend, eBioscience, or Invitrogen. The mAbs against human Vα24 (C15) and Vβ11 (C21) were obtained from Beckman Coulter. Antibodies were biotinylated or conjugated to Pacific blue, Alexa Fluor 405, Pacific orange, V500, FITC, Alexa Fluor 488, PE, PE–Texas red, PerCP-Cy5.5, PE-Cy7, APC, Alexa Fluor 647, Alexa Fluor 700, APC-Cy7, APC–Alexa Fluor 750, or APC-eFluor780. Anti–mouse CD16/32 antibody used for Fc receptor blocking was isolated in our laboratory. Purification of mouse CD1d, preparation of PE- and APC-conjugated α-GalCer–loaded CD1d tetramers, and staining of cells were performed as described previously (Matsuda et al., 2000).

**Preparation of HDEs.** Methods used for the collection and processing of house dust samples have been described in detail previously (Ng et al., 2006). In brief, with approval from our institution's human subjects committee, dust samples were obtained by vacuuming a single carpeted bedroom within suburban homes. Collected house dust was run through a course sieve to remove large particulate matter and suspended in sterile PBS at 100 mg/ml. House dust suspensions were then placed on a rotor at room temperature for 18 h, filtered through glass wool, and finally through 0.22-μm Steriflip filters to obtain sterile HDEs. For select experiments, we prepared an HDE standard by combining individual HDEs found to have high levels of *i*NKT cell antigen based on a mouse *i*NKT cell hybridoma assay that is described in the next section.

**Cell lines and CD1d-coated plate assay.** The CD1d-reactive Vα14*i* NKT cell hybridomas DN3A4-1.2, DN3A4-1.4, and 2H4, as well as the CD1d-reactive non-*i*NKT cell hybridoma 24 have been described previously (Brossay et al., 1998; Burdin et al., 2000). The OVA (OVA$^{257–264}$)-specific hybridoma B3Z (Sanderson and Shastri, 1994) was a gift of S. Schoenberger (La Jolla Institute for Allergy and Immunology, La Jolla, CA). Stimulation of *i*NKT cell hybridomas on CD1d-coated plates was performed as described previously (Sidobre et al., 2002). Aliquots of individual HDEs found to have high levels of *i*NKT cell ligands were combined to prepare an HDE standard that was used for the in vivo experiments. Human PBMCs were obtained from a healthy donor in accordance with the La Jolla Institute for Allergy and Immunology Normal Blood Donor Umbrella Program (#VD-057-0909). The human NKT lines #59 and #2225 were generated from normal human donor PBMCs and maintained as described previously (Rogers et al., 2004). In brief, *i*NKT cells were stimulated every 9–12 d with α-GalCer–pulsed, irradiated (3,000 R) PBMCs and 30 ng/ml recombinant human IL-2 (BioLegend). TCR expression was >95% Vα24$^{+}$ Vβ11$^{+}$. To detect *i*NKT cell antigens, human PBMCs ($4 \times 10^6$ cells in 0.4 ml) were pulsed for 4–20 h at 37°C in a $CO_2$ incubator with 1 ng/ml α-GalCer or 1:10 dilutions of the indicated HDEs in AIM V medium (Invitrogen). Cells were then washed twice, and $2 \times 10^5$ pulsed PBMCs were co-cultured in duplicate with $4 \times 10^4$ *i*NKT cells in 200 μl in 96-well flat-bottom plates. After 72 h, human GM-CSF in supernatants was determined by ELISA using coating and detection antibodies (clones BVD2-23B6 and BVD2-21C11) from BioLegend. Recombinant cytokines were purchased from BD. In brief, Nunc plates (Thermo Fisher Scientific) were coated overnight with 1–2 μg/ml of coating antibody in carbonate buffer. Wells were blocked with PBS + 1% BSA before the addition of test supernatants. Bound cytokine was detected with biotinylated anticytokine antibody followed by streptavidin–horseradish peroxidase (SouthernBiotech). Wells were developed with TMB chromogen (Dako) and stopped with 2 N sulfuric acid before reading at 450 nm.

**DC adoptive transfer experiments.** Mouse BM-DCs were prepared by culturing BM progenitor cells from mice deficient for both MyD88 and Trif (BM-DC$^{DKO}$) with mouse GM-CSF (PeproTech) for 6–8 d. BM-DC$^{DKO}$ were incubated in vitro with 750 ng/ml LPS, 50 ng/ml α-GalCer, or 50 μl/ml HDE standard for 4–5 h and washed, and $5 \times 10^5$ pulsed BM-DC$^{DKO}$ were injected i.v. into naive recipient mice. *i*NKT and NK cell responses were analyzed 12–16 h later by flow cytometry. For CD1d blockade in vivo, 200 μg α-CD1d antibodies (1B1) were injected i.v. together with the indicated BM-DCs.

**Flow cytometry.** For staining of cell surface molecules, cells were suspended in staining buffer (PBS, 1% BSA, and 0.01% $NaN_3$) and stained with fluorochrome-conjugated mAb (0.1–1 μg/$10^6$ cells) for 15 min in a total volume of 50 μl. FcγR-blocking antibody α-CD16/32 (2.4G2) and unconjugated rat IgG (Jackson ImmunoResearch Laboratories, Inc.) were added to prevent nonspecific binding. If biotin-conjugated mAb was used, cell-bound mAb was detected with streptavidin conjugates (1:200) in a second incubation step. Staining of T cells with α-GalCer–loaded CD1d tetramers was performed as described previously (Matsuda et al., 2000). In brief, cells were stained with the tetramer together with other surface mAbs in staining buffer at 4°C for 30 min. For analysis of intracellular cytokines, cells were fixed and permeabilized using the Cytofix/Cytoperm reagents (BD) for 10 min at 37°C. Cells were washed twice and incubated for 30 min with fluorochrome-conjugated mAb and unconjugated rat IgG in Perm/Wash solution (BD), which was followed by an additional 5-min incubation in Perm/Wash solution without mAb. For in vitro experiments intended for intracellular staining, GolgiPlug and GolgiStop (BD) were added for the last 4 h of incubation. For ex vivo experiments intended for intracellular staining of IL-17A, lymphocytes were cultured for 2 h in the presence of GolgiPlug and GolgiStop. Cells were analyzed with FACSCalibur or LSR II (BD), and data were processed with CellQuest Pro (BD) or FlowJo (Tree Star) software. Graphs derived from digital data are displayed on a biexponential scale.

**Airway allergen sensitization and challenge model.** Naive mice ($n$ = 4–6 per group) received a series of three weekly i.n. 100-μg OVA vaccinations with or without 20 μl HDE standard. 3 wk after completing sensitization, all mice received two i.n. 10-μg OVA challenges, delivered 6 d apart, and airway and immunological responses were assessed the next day (experimental outline in Fig. 4 A). Mice were lightly anesthetized (isoflurane; Abbott Laboratories)

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before the i.n. or pharyngeal-laryngeal delivery, and all reagents were delivered in a total volume of 30 µl PBS. 18 h after the final OVA challenge, mice were sacrificed, lungs were lavaged with 800 µl PBS, and BALF was collected. Total BALF cell counts were determined with a hemocytometer. In addition, BALF cytospin slides were prepared, fixed in acetone, and Wright-Giemsa stained. A blinded observer determined the percentage of eosinophils, neutrophils, and mononuclear cells on each slide by counting a minimum of 150 cells in random high-power fields with a light microscope (Carl Zeiss). Lung tissue was flash frozen, cryosectioned, acetone-fixed onto poly-L-lysine–coated slides, and stained with hematoxylin-eosin, peroxidase/3,3′-diaminobenzidine, and periodic acid Schiff by standardized techniques. To quantitate peribronchial inflammation, eosinophil infiltration, and airway mucous production, a scoring system (0–5) was devised in which a blinded observer scored four to eight airways per mouse for each of these parameters. Mean inflammation scores were determined by averaging the total cellular infiltration, eosinophil infiltration, and airway mucous production scores for each mouse group as described previously (Ng et al., 2006; Lam et al., 2008).

Sera were obtained from vaccinated and airway allergen–challenged mice at sacrifice to measure antigen-specific IgG1, IgG2a, and IgE levels by ELISA as described previously (Ng et al., 2006; Lam et al., 2008). Samples were compared with high-titer anti-OVA IgG1, IgG2a, and IgE standards, with end point titrations of $1.2 \times 10^7$ U/ml for IgG1, $1.5 \times 10^5$ U/ml for IgG2a, and 512 U/ml for IgE. To remove IgG and improve the sensitivity of the OVA-specific IgE ELISA, serum samples were preincubated with protein G–Sepharose beads (Pharmacia). OVA-specific BLN cytokine responses were assessed as described previously (Ng et al., 2006; Lam et al., 2008). In brief, BLNs harvested from each group of experimental mice were pooled, and single-cell suspensions were prepared by enzymatic digestion with 300 U/ml Collagenase VIII (Sigma-Aldrich) and 1.5 µg/ml DNase-I (Sigma-Aldrich). BLN cells were cultured in triplicate at $10^6$ cells/ml in media with or without 50 µg/ml OVA for 72 h before harvesting supernatants. Cytokine levels in culture supernatants were determined by ELISA using BD reagents according to the manufacturer's recommendations. BLN cytokine responses were calculated by subtracting background cytokine production (generally ≤0.05 ng/ml) from responses of BLN cells cultured with OVA.

In some experiments, lymphocyte responses in the lung and BALF were analyzed 16 h after a single challenge or after the end of the sensitization phase (three weekly challenges). Lung tissue was first cut into small sections and enzymatically digested with Collagenase VIII and DNase-I, as described for BLNs in the previous paragraph.

**Statistical analysis.** Results are expressed as mean ± SEM. Comparisons were drawn using a two-tailed Student's *t* test (Excel; Microsoft) or analysis of variance test (Prism; GraphPad Software). Each experiment was repeated at least twice. Graphs were generated with Prism software.

**Online supplemental material.** Fig. S1 shows the trans-activation of NK cells, as measured by up-regulation of CD69, after the injection of BM-DC$^{DKO}$ as described in Fig. 1. Fig. S2 presents representative major basic protein– and periodic acid Schiff–stained lung sections of mice described in Fig. 4. Fig. S3 summarizes the percentage of CD4$^+$ and CD4$^-$ *i*NKT cells in the lung of experimental mice and their contribution to cytokine responses described in Fig. 5. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20102229/DC1.

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## SUPPLEMENTAL MATERIAL

**Wingender et al., http://www.jem.org/cgi/content/full/jem.20102229/DC1**

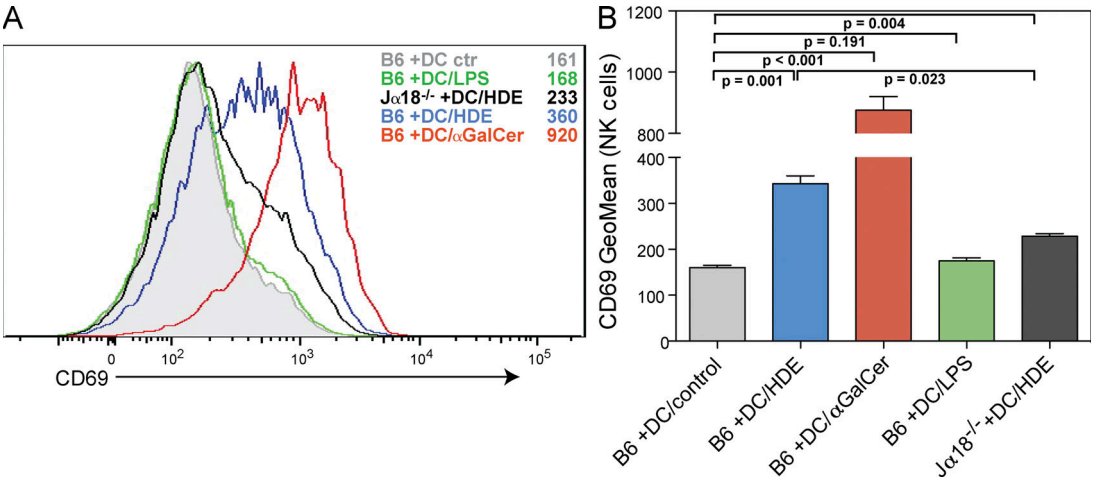


**Figure S1. Trans-activation of NK cells is largely Vα14*i* NKT cell dependent.** DCs were generated with GM-CSF from BM of MyD88 and Trif double-deficient animals (BM-DC$^{DKO}$; DC in the graph). BM-DC$^{DKO}$ were washed and incubated with the indicated substances (50 µl/ml HDE, 150 ng/ml LPS, and 50 ng/ml α-GalCer) for 5 h, and 5 × $10^5$ BM-DC$^{DKO}$ were injected i.v. into C57BL/6 mice (B6) or Jα18-deficient mice (Jα18$^{-/-}$). 15 h later, splenic NK cells were analyzed. (A and B) Representative expression of CD69 by splenic NK cells from single mice (A) and a summary of data from at least three mice per group from a representative experiment (B). The numbers in the histogram denote the geometric mean values of CD69 on NK cells. Closed, DC/control (ctr) into C57BL/6 mice (first number); green, DC/LPS into C57BL/6 mice (second number); black, DC/HDE into Jα18$^{-/-}$ mice (third number); blue, DC/HDE into C57BL/6 mice (fourth number); red, DC/α-GalCer into C57BL/6 mice (fifth number). Representative data from one of four independent experiments, with three to four mice per group, are shown.

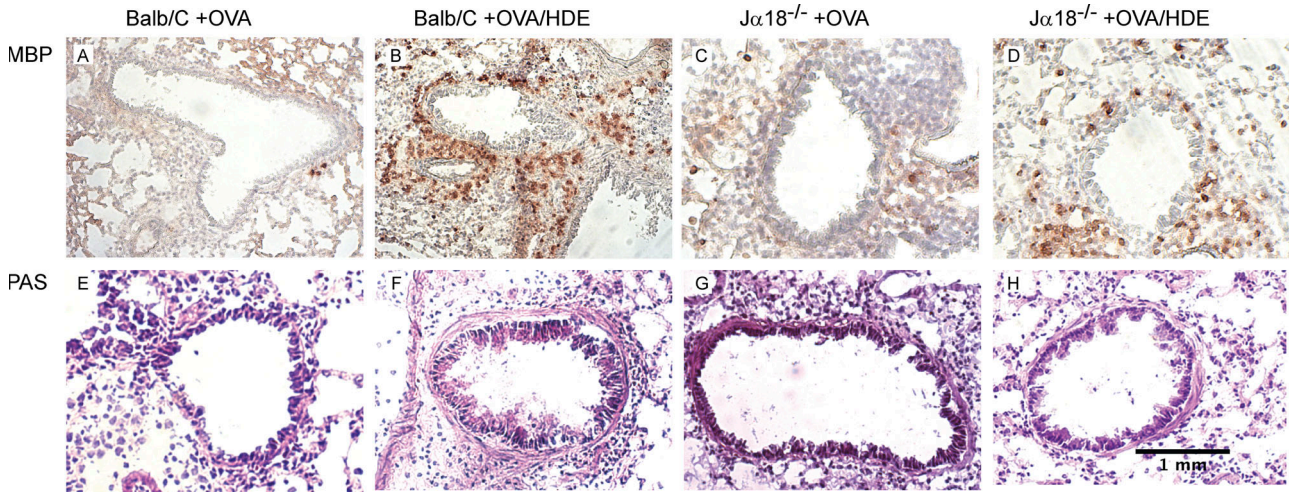


**Figure S2. Vα14*i* NKT cells contribute to the adjuvant effect of HDE during allergen-induced airway inflammation.** BALB/c mice and Jα18$^{-/-}$ mice on the BALB/c background were sensitized three times, once per week (days 0, 7, and 14) with chicken OVA alone (100 µg/mouse) or together with HDE standard. On days 35 and 41, the mice were challenged with OVA alone (10 µg/mouse) and analyzed 24 h after the last challenge. Tissue from the indicated animals was fixed, and histology was prepared. (A–D) Staining of eosinophils by major basic protein (MBP) mAb demonstrated a large influx of eosinophils in wild-type mice sensitized with OVA and HDE (B). This influx was less pronounced in Jα18$^{-/-}$ animals (D). (E–H) Periodic acid Schiff (PAS) staining of the airway epithelium demonstrated an increased frequency of large, pink, mucin-secreting cells in the airways of OVA/HDE-sensitized wild-type (F) compared with Jα18$^{-/-}$ (H) mice. Representative data of two independent experiments, with three to four mice per group, are shown.

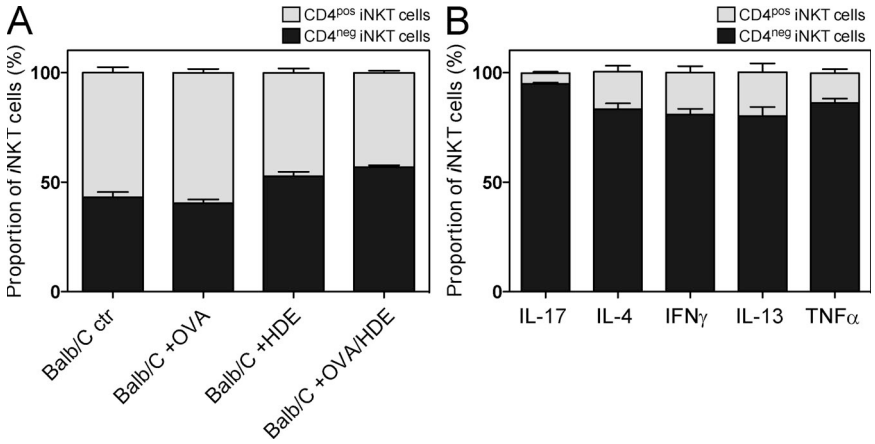


**Figure S3. The role of CD4⁻ Vα14*i* NKT cells during allergen-induced airway inflammation.** BALB/c mice were vaccinated three times, once per week (days 0, 7, and 14) with chicken OVA alone (100 µg/mouse), HDE alone, or with OVA together with HDE and analyzed on day 15. Representative data of three independent experiments, with two to five mice per group, are shown. (A) Relative proportion of CD4⁺ and CD4⁻ *i*NKT cells in the lung after the indicated treatments. $p_{(OVA\ vs\ ctr)} = 0.395$; $p_{(HDE\ vs\ ctr)} = 0.007$; $p_{(OVA/HDE\ vs\ ctr)} = 0.001$. (B) Production of the indicated cytokines by lung CD4⁺ and CD4⁻ *i*NKT cells from mice challenged with OVA/HDE. Background values have been subtracted. The CD4⁻ fraction of lung *i*NKT cells was responsible for at least 60% of respective cytokines produced by *i*NKT cells for all cytokines in all experiments. Error bars indicate mean ± SEM.