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A CD1d-Dependent Antagonist Inhibits the Activation of Invariant NKT Cells and Prevents Development of Allergen-Induced Airway Hyperreactivity

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The prevalence of asthma continues to increase in westernized countries, and optimal treatment remains a significant therapeutic challenge. Recently, CD1d-restricted invariant NKT (iNKT) cells were found to play a critical role in the induction of airway hyperreactivity (AHR) in animal models and are associated with asthma in humans. To test whether iNKT cell-targeted therapy could be used to treat allergen-induced airway disease, mice were sensitized with OVA and treated with di-palmitoyl-phosphatidylethanolamine polyethylene glycol (DPPE-PEG), a CD1d-binding lipid antagonist. A single dose of DPPE-PEG prevented the development of AHR and pulmonary infiltration of lymphocytes upon OVA challenge, but had no effect on the development of OVA-specific Th2 responses. In addition, DPPE-PEG completely prevented the development of AHR after administration of α -galactosylceramide (α -GalCer) intranasally. Furthermore, we demonstrate that DPPE-PEG acts as antagonist to α -GalCer and competes with α -GalCer for binding to CD1d. Finally, we show that DPPE-PEG completely inhibits the α -GalCer-induced phosphorylation of ERK tyrosine kinase in iNKT cells, suggesting that DPPE-PEG specifically blocks TCR signaling and thus activation of iNKT cells. Because iNKT cells play a critical role in the development of AHR, the inhibition of iNKT activation by DPPE-PEG suggests a novel approach to treat iNKT cell-mediated diseases such as asthma. *The Journal of Immunology*, 2010, 184: 000–000.

Asthma is a major public health problem that has increased markedly in prevalence in the past two decades (1). Asthma is caused by Th2-driven inflammatory responses, which enhance airway and peripheral blood eosinophilia, induce airway hyperreactivity (AHR), and elevate serum IgE (2). Conventional class II MHC restricted CD4⁺ Th2 cells orchestrate the inflammation in asthma (3) by secreting key cytokines like IL-4 and IL-13 (4, 5). Conventional CD4⁺ T cells recognize exogenous Ags and initiate an

allergic inflammation in the lungs. In mouse models of asthma, elimination of CD4⁺ cells abrogates the development of AHR (2). Although Th2-driven immune responses are vitally important during the development of asthma (6), a Th2 response alone is not sufficient to induce this disease (6, 7). Th2-biased allergen sensitization can occur independently of asthma, perhaps explaining the fact that only one third of individuals with allergic rhinitis develop asthma (8).

The CD4 surface molecule is expressed by conventional CD4⁺ T cells and CD1d-restricted NKT cells with an invariant TCR (invariant NKT [iNKT] cells), which may also play a crucial role in the development of allergen-induced AHR. Moreover, in several mouse models of asthma, AHR failed to develop in the absence of iNKT cells producing IL-4 and IL-13, although Th2 responses developed normally (9–11). Recent studies in humans with asthma also suggest that a significant number of CD3⁺ CD4⁺ cells in the lungs of patients with persistent asthma are iNKT cells (9, 12–14).

iNKT cells constitute a lymphocyte subpopulation that is abundant in the thymus, spleen, liver, and bone marrow and is also present in lungs (9, 10, 15). iNKT cells express surface markers that are characteristic of both NK cells and conventional T cells. When activated, iNKT cells rapidly produce large quantities of various cytokines, including IL-4 and IFN- γ , which influence subsequent adaptive immune responses and the polarization of conventional $\alpha\beta$ -TCR⁺ T cells (16). Besides allergic diseases, iNKT cells have been shown to regulate the development of a wide array of autoimmune, antimicrobial, antitumor, and antitransplant immune responses that depend on their capacity to produce large quantities of cytokines (17–19).

The largest and best studied population of NKT cells recognizes glycolipid Ags presented by the nonpolymorphic MHC class I-like protein CD1d and expresses an invariant V α 14-J α 18 TCR in mice, or an invariant V α 24-J α 18 TCR in humans. Several studies suggest that phosphatidylethanolamine and liposomes can bind to

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Abbreviations used in this paper: AHR, airway hyperreactivity; BAL, bronchoalveolar lavage; BODIPY, boron-dipyrromethene; Cdyn, dynamic compliance; DPPE, di-palmitoyl-phosphatidylethanolamine; EOS, eosinophils; iNKT, invariant NKT; α -GalCer, α -galactosylceramide; LYM, lymphocyte; MO, monocyte; NEU, neutrophils; PEG, polyethyleneglycol; PenH, enhanced pause; p-ERK, phosphorylated ERK; RL, lung resistance; WT, wild type.

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CD1d and prevent iNKT cell activation (20–22). There are many types of liposomes that can be differentiated by their lipid composition, surface charge, steric interactions, and the number of lamellae. In medicine, liposomes are used to encapsulate a variety of drugs and provide a slow release repository, thereby providing a prolonged therapeutic effect. Although conventional liposomes can encapsulate a variety of drugs, they are recognized *in vivo* by the cells of the reticuloendothelial system and are cleared rapidly from the circulation (23, 24). Unlike conventional liposomes, sterically stabilized liposomes such as di-palmitoyl-phosphatidyl-ethanolamine (DPPE) covalently attached to polyethyleneglycol (PEG) are biologically hyporeactive because of specific coating strategies of their surface (20–22). Many PEG derivatives have already been approved for clinical and pharmaceutical use.

To examine the role of iNKT cells in the development of allergic diseases and asthma in greater detail, and to determine whether iNKT cells might serve as a target for asthma-specific therapy, the functional capacities of the CD1d-binding antagonist DPPE-PEG was evaluated. Our results indicate that treatment of sensitized mice with DPPE-PEG-inhibited cytokine production such as IL-4 and IFN- γ by iNKT cells and thus blocked the development of AHR in a murine model using OVA. In contrast, DPPE-PEG had no significant effect on Ag-specific Th2 cells. Furthermore, DPPE-PEG completely inhibited AHR induced by the iNKT cell ligand α -galactosylceramide (α -GalCer) and acts as antagonist to CD1d/ α -GalCer binding, thus blocking the ERK phosphorylation pathway in iNKT cells. The ability to prevent iNKT activation and AHR with a CD1d-dependent antagonist lends further support to the hypothesis that iNKT cells are required for the development of AHR. These findings suggest that an iNKT cell-specific antagonist such as DPPE-PEG may be used in a novel therapeutic approach for allergic inflammatory responses and other disorders characterized by inappropriate iNKT cell activation.

Materials and Methods

Mice and Ags

Female BALB/cByj mice (6–8-wk-old) were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were maintained in a pathogen-free mouse colony at the Keck School of Medicine, University of Southern California, and Massachusetts General Hospital under protocols approved by the Institutional Animal Care and Use Committee.

Reagents

Anti-CD3 mAb was purchased from BD Pharmingen (San Diego, CA). α -GalCer was purchased from Axxora (San Diego, CA). DPPE-PEG-350 (1,2-Bis(diphenylphosphino)ethane [DPPE] coupled to polyethylene glycol monomethylether [PEG] with an average m.w. of 350 g/mol) was purchased from Avanti Polar Lipids (Alabaster, AL) and dissolved in DMSO at the concentration of 1 mg/ml (final concentration of DMSO, 0.01–0.05%). In Biacore experiments, DPPE-PEG was dissolved in 95% ethanol (final concentration of ethanol <0.1%).

To induce AHR, mice were immunized *i.p.* with 50 μ g LPS-free OVA (Worthington Biochemical, Lakewood, NJ) in 2 mg alum in a volume of 0.5 ml. Ten days later, mice were lightly anesthetized with methoxoflurane and challenged with OVA (50 μ g) intranasally on 3 consecutive days. Boron-dipyrromethene (BODIPY)- α -GalCer was prepared by slight modifications of the procedure as described before (25).

Flow cytometric analysis

Cells were preincubated with anti-Fc receptor mAb 2.4G2 and normal rat serum and were washed before staining. iNKT cells were identified using various Ab combinations that included PE conjugated CD1d:PBS-57 (α -GalCer analog) loaded tetramer (National Institutes of Health, National Institute of Allergy and Infectious Diseases, MHC tetramer core facility, Atlanta GA), TCR β -PECy5 or allophycocyanin (clone H57-597; eBioscience, San Diego, CA) and CD4 allophycocyanin or allophycocyanin-Alexa750 (clone RM4-5; eBioscience). Cells were acquired on the FACS

Canto 8 color flow cytometer (BD Biosciences, San Jose, CA), and 10,000 events within the iNKT cell gate were collected. The data were analyzed with FlowJo 6.2 software (Tree Star, Ashland, OR).

For BODIPY- α -GalCer staining, the cells were incubated with 10 ng/ml BODIPY- α -GalCer for 45 min at 4°C. The cells were washed three times with cold PBS + 2% FCS and were analyzed on the FACS Canto II 8 color flow cytometer (BD Biosciences).

Restimulation of lymph node cells *in vitro*

Cells isolated from lymph nodes of OVA-primed mice were restimulated *in vitro* (5×10^5 cells per well in a 96-well plate) with various concentrations of OVA. Supernatants were collected after 4 d and assayed for IL-4 and IFN- γ by ELISA as previously described (10).

In vitro assays with iNKT cells

Splenocytes cells (5×10^6 per ml) pooled from BALB/cJ mice ($n = 4$) were cultured in anti-CD3 mAb (2 μ g/ml)-precoated 24-well plates in complete RPMI 1640 $\pm$ increasing concentrations of DPPE-PEG350 or cultured in 24-well plates in complete RPMI 1640 $\pm$ α -GalCer (100 ng/ml) $\pm$ increasing concentrations of DPPE-PEG350. After 48 h, supernatants were gathered and examined for IL-4 and IFN- γ by ELISA as described earlier (26). iNKT cell lines were prepared as described before (27) with some modifications. iNKT cells were negatively selected from splenocytes, using a mixture of biotinylated mAbs against CD19, CD62-L, and CD11c, followed by incubation with antibiotin microbeads. The samples were subsequently enriched using magnetic cell sorting and flow cytometry.

Surface plasmon resonance

MCD1d was expressed and purified as previously described (28). The ability of DPPE-PEG to inhibit binding of α -GalCer to CD1d was determined by staining with the CD1d/ α -GalCer complex-specific Ab L317 (29) using a Biacore 3000 (Biacore, Piscataway, NJ). Histidine-tagged mCD1d was incubated with DPPE-PEG (5 μ g/ml) or vehicle overnight before the addition of a dose range of α -GalCer. The relative number of CD1d/ α -GalCer complexes was detected by immobilizing the histidine-tagged CD1d/lipid-mixture onto a Ni-coated nitrilotriacetic acid sensor chip. A saturating dose of L317 (10 μ g/ml) was injected for 1 min at a flow rate of 25 μ l/min. The extent of binding was analyzed 5 s after the end of the injection. Data are shown as concentration of α -GalCer versus L317 binding (Biacore Response Units) in triplicate injections $\pm$ SD.

Measurement of DPPE-PEG affinity to human and mouse CD1d

DPPE-PEG or α -GalCer was used as competitors against biotinylated 18:1 PE lipid for binding to hCD1d molecule (Fig. 5C, *left panel*) and to mCD1d molecule (Fig. 5C, *right panel*). In all assays, Maxisorp ELISA (Nunc, Naperville, IL) plates were coated with anti-mouse IgG1 Ab (Pierce, Rockford, IL) for overnight and then incubated with hCD1d:mIgG or mCD1d:mIgG dimer (BD Biosciences) for 2 h. Serial-diluted glycolipids (DPPE-PEG or α -GalCer) were then added onto the plate in the presence of 1 μ g/ml biotinylated 18:1 PE lipid. After 24 h of incubation at 37°C, the amount of bound biotinylated 18:1 PE lipid was determined by adding streptavidin-HRP and TMB substrate. Inhibition curves were fitted with sigmoidal dose-response formula using Prism 4.0 software (Prism Software, Irvine, CA). Results represent an average of three independent assays and are expressed as mean $\pm$ SD. Statistical analysis was done in each curve fitting, and $p < 0.001$ was considered significantly different.

To test the specificity of the binding of α -GalCer to CD1d, dendritic cells were positively isolated from splenocytes of BALB/c or CD1d knockout mice after incubation with CD11c Microbeads (Miltenyi, Bergisch Gladbach, Germany) and sorting using an AutoMACS Pro separator (Miltenyi) according to the manufacturer's instructions. Subsequently, cells were stained with 10 ng/ml BODIPY- α -GalCer and analyzed by flow cytometry.

Cytokine ELISAs

Cytokine secretion following *in vitro* challenge with OVA and control peptides were determined by ELISA as described earlier (10). The following mAbs R4-6A2 and XMG1.2-biotin for IFN- γ and 11B11 and BVD6-24G2-biotin for IL-4 were used for capture and detection.

Induction of AHR and measurement of airway responsiveness

AHR responses were assessed by methacholine-induced airflow obstruction in conscious mice placed in a whole-body plethysmograph (Buxco Electronics, Troy, NY) as described previously (10). In some experiments, AHR was assessed by invasive measurement of airway resistance, in which

anesthetized and tracheostomized mice were mechanically ventilated using a modified version of a method described earlier (30). Aerosolized methacholine was administered for 20 breaths in increasing concentrations (1.25, 2.5, 5, and 10 mg/ml of methacholine). We continuously computed lung resistance (R_L) and dynamic compliance (C_{dyn}) by fitting flow, volume, and pressure to an equation of motion. To induce α -GalCer-dependent AHR, α -GalCer was administered intranasally (1 μ g α -GalCer in 50 μ l PBS) to mice anesthetized with ketamine and xylazine as described before (31).

ERK1/2 phosphorylation

L2 cells (CD1d expressing APCs, a kind gift of Dr. Mark Exley, Harvard Medical School, Boston, MA) were resuspended at a concentration of 1 million cells per ml and pulsed with α -GalCer or DPPE-PEG (100 ng/ml each) for 2 h at 37°C as described before with some modification (32). The cells were subsequently washed, readjusted at the concentration of 10×10^6 per ml, and added to iNKT cell lines at the concentration of 5×10^6 per ml. The mixture was incubated at 37°C for various periods of time, fixed, and stained for intracellular phosphorylated ERK (p-ERK1/2) (BD Pharmingen) expression by flow cytometry as described before (32).

Collection and analysis of bronchoalveolar lavage fluid

Mice were lethally anesthetized with phenobarbital 450 mg/kg i.p. The trachea was cannulated and the lungs were lavaged three times with 0.3 ml of PBS, and the collected fluid was pooled. Cell counts and analysis of the bronchoalveolar lavage (BAL) was performed as described before (10).

Results

DPPE-PEG inhibits α -GalCer-dependent activation of iNKT cells in vitro

To determine whether activation of iNKT cells by α -GalCer could specifically be prevented by DPPE-PEG, splenocytes were incubated with α -GalCer (100 ng/ml) or plate-bound anti-CD3. The addition of increasing concentrations of DPPE-PEG inhibited secretion of IL-4 and, to a lesser extent, IFN- γ by α -GalCer-stimulated iNKT cells. Our titrating experiment revealed that 25 μ g/ml DPPE-PEG will inhibit cytokine release from iNKT cells significantly; however, to achieve 100% blocking we need to use 100 μ g/ml of DPPE-PEG in culture (data not shown). Treatment with DPPE-PEG preferentially inhibited the release of IFN- γ , but increased the release of IL-4 when T cells were activated by anti-CD3 (Fig. 1A). Importantly, α -GalCer-stimulated cytokine secretion by iNKT cells was significantly inhibited at concentrations of DPPE-PEG that had no or minimal effect on cytokine production by T cells activated by anti-CD3. We observed similar results when the iNKT cell hybridoma line DN32.D3 cells were stimulated by anti-CD3 and treated with DPPE-PEG (data not shown).

We also tested the influence of DPPE-PEG on the activation of DN32 cells by α -GalCer. DN32 cells were loaded with 200 μ g/ml DPPE-PEG and stimulated with titrating doses of α -GalCer. Treatment with DPPE-PEG inhibited IL-2 release from DN32 cells, particularly at suboptimal concentrations of α -GalCer (Fig. 1B).

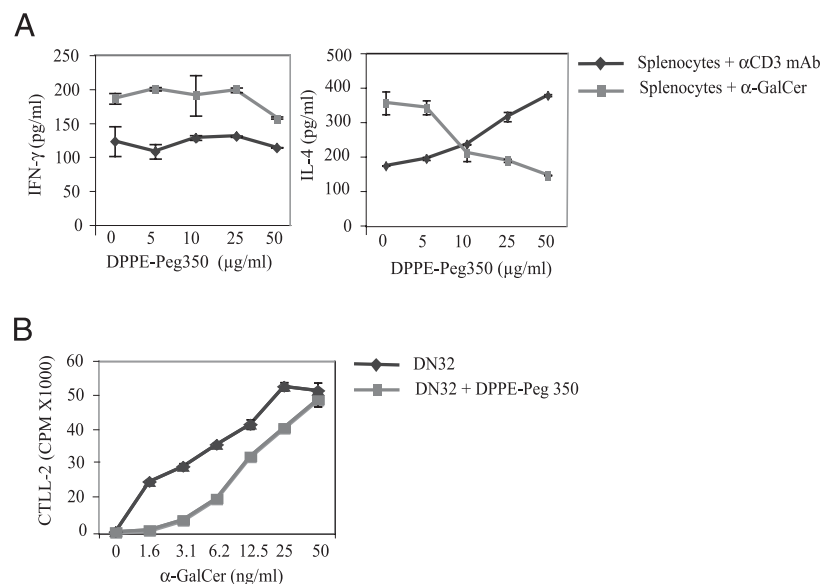
DPPE-PEG treatment inhibits the development of allergic AHR

To determine whether DPPE-PEG may be used to treat Th2 cell-mediated immune responses, we tested DPPE-PEG in a murine model of airway inflammation using OVA as an Ag. In this model, an asthma-like phenotype with AHR, eosinophilic airway inflammation, and mucus hypersecretion is induced by systemic sensitization with OVA followed by intrapulmonary challenge with OVA (Fig. 2A), and we have previously demonstrated that iNKT cells are required for the induction of allergic diseases in this model (10). In this study, we show that a single injection of DPPE-PEG (250 μ g i.v.) after systemic sensitization with OVA completely prevented the development of AHR on intranasal challenge with OVA in BALB/c mice (Fig. 2B). AHR was measured by enhanced pause (PenH) in a whole-body plethysmograph (Fig. 2B) and was confirmed by direct measurement of R_L and C_{dyn} in anesthetized, tracheostomized, intubated, and mechanically ventilated BALB/c mice (Fig. 2C). In addition, treatment with DPPE-PEG effectively inhibited the development of eosinophilic airway inflammation in OVA-immunized BALB/c mice, thus significantly reducing the number of eosinophils, lymphocytes, and macrophages in the BAL fluid as compared with the OVA-immunized control group (Fig. 2D). Moreover, histologic examination of H&E- and PAS-stained lung sections isolated from OVA-immunized mice revealed no significant differences between saline (negative) control and DPPE-PEG-treated mice (Fig. 2E). In contrast, OVA-sensitized and challenged mice that did not receive DPPE-PEG developed strong cellular infiltrates and showed narrowing and mucous plugging of the airways. From those results, we conclude that systemic administration of DPPE-PEG is able to prevent allergic airway disease induced by OVA.

DPPE-PEG does not inhibit OVA specific Th2 responses

The pathology of the described murine model is known to depend on CD4⁺ OVA-specific Th2 cells. Hence, lymphocytes from sensitized animals secrete large amounts of IL-4, IL-5, and IL-13 in response to restimulation with Ag in vitro (10, 33). Because treatment with DPPE-PEG inhibited the development of AHR, we

FIGURE 1. DPPE-PEG inhibits α -GalCer-dependent activation of iNKT cells in vitro. **A**, Splenocytes cells (5×10^6 per ml) pooled from BALB/c mice ($n = 4$) were cultured in anti-CD3 mAb-precoated (2 μ g/ml) 24-well plates in complete RPMI 1640 $\pm$ increasing concentrations of DPPE-PEG, or cultured in 24-well plates in complete RPMI $\pm$ α -GalCer (100 ng/ml) $\pm$ increasing concentrations of DPPE-PEG. After 48 h, supernatants were gathered and examined for IL-4 and IFN- γ by ELISA. ELISA data are representative of three separate experiments each and are shown as mean $\pm$ SD for triplicate samples. **B**, DN32 NKT hybridoma cells were incubated with 200 μ g/ml of DPPE-PEG for 4 h and cultured with increasing concentrations of α -GalCer for 48 h. Culture supernatants were collected and IL-2 expression analyzed with IL-2 dependent cell line CTLL-2. Data are means $\pm$ SD of triplicate cultures.



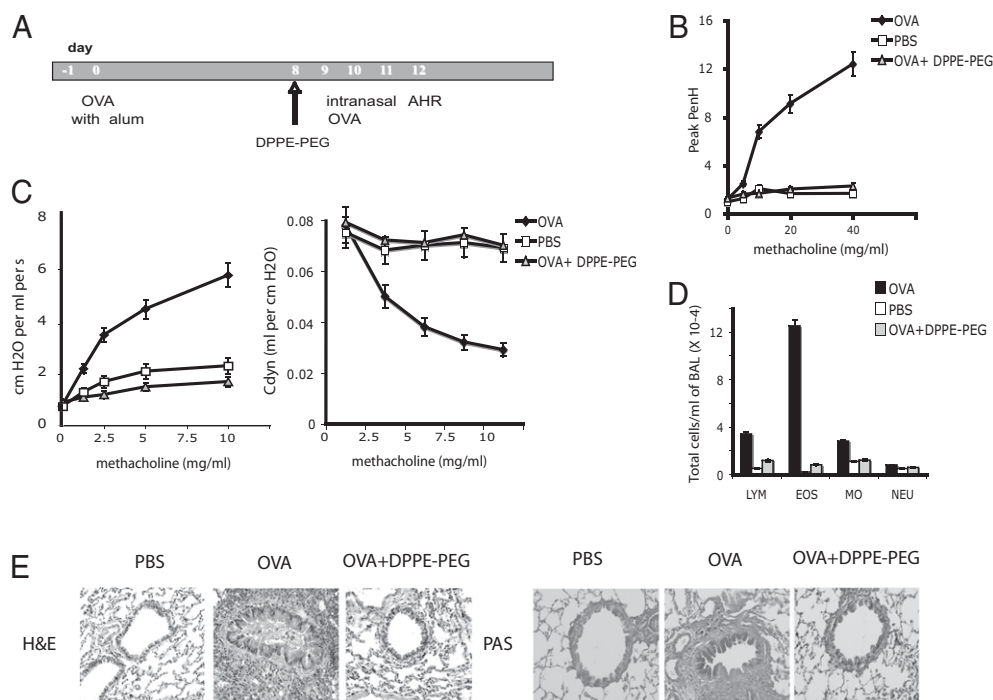


FIGURE 2. AHR is inhibited by the administration of DPPE-PEG. **A**, A cohort of five BALB/c mice were immunized with OVA i.p. on day 0, followed by intranasal OVA challenges on days 9, 10, and 11. DPPE-PEG (250 μ g) was injected i.v. on day 8, and AHR was measured on day 12. **B**, DPPE-PEG prevents AHR and eosinophilic airway inflammation. Methacholine-induced AHR was measured. Administration of DPPE-PEG completely inhibited AHR in OVA-immunized mice (measured by PenH). Data are the mean $\pm$ SEM PenH and are representative of three experiments. **C**, Invasive measurement of airway resistance was performed in BALB/c mice that received DPPE-PEG compared with the PBS-treated group. AHR was assessed by changes in airway resistance (R_L , cm of H₂O/ml/s) in response to methacholine in anesthetized, tracheostomized, intubated, and mechanically ventilated mice. Data represent the mean $\pm$ SEM of four mice per group. **D**, The increased cell number in the BAL fluid of OVA-immunized mice was almost completely abrogated by DPPE-PEG. BAL fluid from the mice was analyzed 3 h after airway measurements, shown as the number of cells per ml of BAL fluid. EOS, eosinophils; LYM, lymphocyte; MO, monocyte; NEU, neutrophils. **E**, DPPE-PEG inhibits airway inflammation. Left panel (H&E and PAS staining): lung tissue from an untreated control mouse showing normal airway and surrounding parenchyma. The airway mucosa is characterized by low cuboidal cells with minimal intracytoplasmic mucus and absence of peribronchiolar inflammatory infiltrates. Middle panel (H&E and PAS staining): numerous inflammatory cells surrounding the airways and streaks of mucus in the lumen characterize lung tissue from an OVA-treated mouse. The bronchiolar epithelium has hyperplastic columnar epithelial cells with abundant intracytoplasmic accumulations of mucus, as well as eosinophils and mononuclear cells in the peribronchial space. Right panel (H&E and PAS staining): lung parenchyma of an OVA-sensitized mouse that has received DPPE-PEG, showing minimal mucus production and negligible cellular infiltration. Bronchiolar mucosae consist of low cuboidal epithelium with an absence of peribronchiolar inflammatory infiltrates (original magnification $\times 400$).

asked whether administration of a CD1d-dependent antagonist prior to airway challenge was able to modify systemic OVA-specific Th2 responses. Bronchial lymph node cells were isolated from OVA-sensitized mice treated with either DPPE-PEG or PBS and restimulated in vitro with 100 μ g/ml OVA 1 d after the induction of AHR. DPPE-PEG had no significant effect on the levels of IL-4, IL-5, or IL-13 secreted by T cells (Fig. 3). No OVA-specific cytokine secretion was observed when the animals had not been sensitized with OVA on day 0 (data not shown). Consequently, the iNKT-dependent development of AHR, but not OVA-specific MHC class II-dependent Th2 responses, can be inhibited by DPPE-PEG.

DPPE-PEG treatment inhibits the development of α -GalCer-induced AHR

We previously demonstrated that mice challenged intranasally with α -GalCer develop severe AHR within 24 h, which appears to be caused by the activation of local iNKT cells in the lungs (31, 34). To determine whether DPPE-PEG could be used in this model of iNKT cell-dependent AHR, a single dose of DPPE-PEG (100 μ g) with or without α -GalCer was administered intranasally to a cohort of naive BALB/c mice. AHR was measured 24 h later by PenH in a whole-body plethysmograph (Fig. 4A) and was confirmed by

direct measurement of R_L and C_{dyn} in anesthetized, tracheostomized, intubated, and mechanically ventilated BALB/c mice (Fig. 4B). A single dose of DPPE-PEG completely prevented the development of AHR in the group that received DPPE-PEG and α -GalCer when compared with α -GalCer alone. As a control, administration of DPPE-PEG alone showed no effect on the induction of AHR or pulmonary inflammation (data not shown). Moreover, histologic examination of H&E and PAS-stained lung sections revealed no significant differences between vehicle (negative) control and DPPE-PEG treated mice (Fig. 4C). In contrast, α -GalCer treated mice that did not receive DPPE-PEG developed severe cellular infiltrates, and significant accumulations of mucus were found on PAS staining. We conclude from these results that DPPE-PEG prevented iNKT cell-dependent α -GalCer-induced AHR.

DPPE-PEG acts as CD1d antagonist

Although several reports suggest that liposomes may bind to CD1d (20–22), no data have demonstrated direct binding of liposome-derived lipids to CD1d. To test this, increasing doses of DPPE-PEG were used to competitively block the binding of α -GalCer to CD1d. In a first set of experiments, the ability of DPPE-PEG to block the binding of α -GalCer conjugated to the fluorochrome

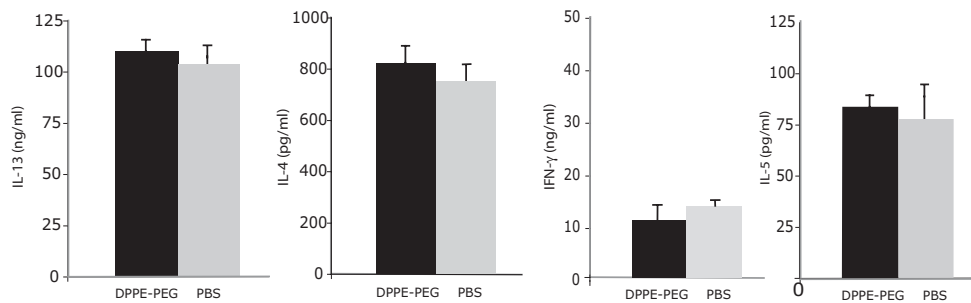


FIGURE 3. DPPE-PEG does not inhibit the development of OVA-specific Th2 responses. Bronchial lymph nodes were removed from DPPE-PEG treated or PBS treated mice and restimulated with 100 μ g OVA *in vitro*. After 72 h, supernatants were collected and cytokine production was analyzed by ELISA. ELISA data are representative of three separate experiments ($n = 4$) and are shown as mean $\pm$ SD for triplicate samples.

BODIPY was assessed. In the absence of DPPE-PEG, DN32 iNKT cell hybridomas expressing CD1d displayed intense staining with α -GalCer BODIPY (Fig. 5A). On increasing doses of DPPE-PEG, the fluorescence of DN32 cells loaded with α -GalCer BODIPY significantly decreased, suggesting that DPPE-PEG was in competition with α -GalCer BODIPY in binding to CD1d. We performed additional control experiments to address the specificity of α -GalCer BODIPY staining. We stained dendritic cells isolated from the spleen of wild type (WT) versus CD1d knockout mice and stained them with α -GalCer BODIPY. Our results suggest that α -GalCer BODIPY stain specifically the CD1d⁺ cells from WT

mice and fail to stain dendritic cells from CD1d knockout mice, suggesting that our staining is CD1d-dependent and specific (Fig. 5B). Furthermore, we also monitored the CD1d expression on DN32 cells in experiment 5A, and we did not observe any alternations in the level of CD1d in the presence of increasing dose of DPPE-PEG (data not shown).

To directly test the ability of DPPE-PEG to compete with α -GalCer for binding with CD1d, we had two approaches. First, a competitive ELISA was performed between biotinylated 18:1 PE lipid (a pollen-derived, phosphatidyl-ethanolamine known as a CD1d ligand) (35) at a fixed concentration and increasing

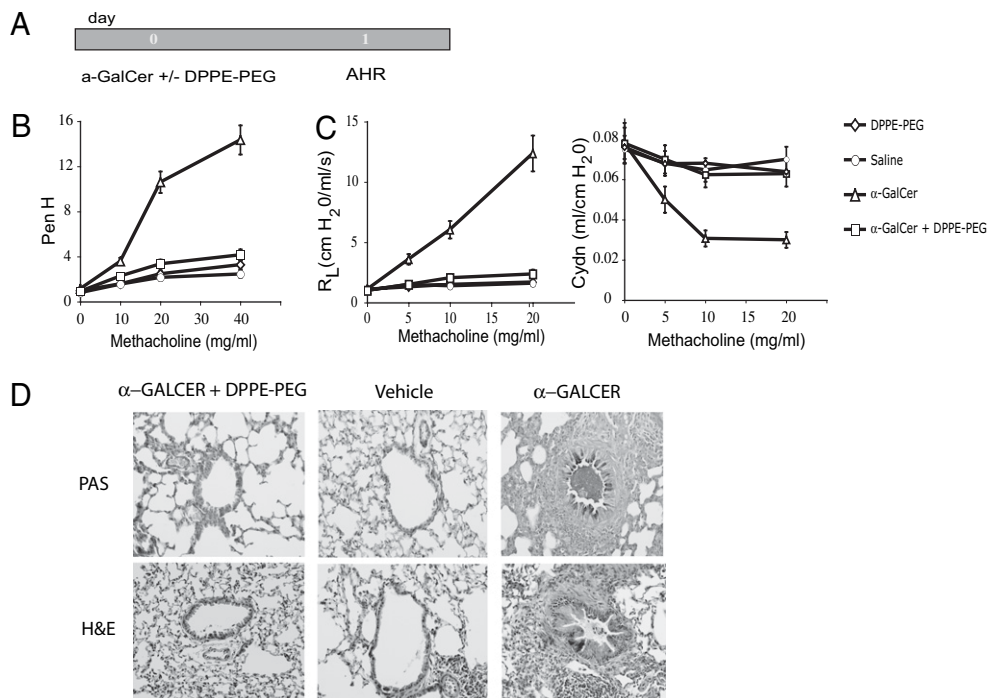


FIGURE 4. DPPE-PEG inhibits the induction of α -GalCer induced AHR. **A**, A cohort of five BALB/c mice were intranasally challenged with 1 μ g α -GalCer with or without 100 μ g DPPE-PEG. After 24 h, increasing concentrations of methacholine were used to assess AHR. **B**, DPPE-PEG prevents AHR. Methacholine-induced AHR was measured by PenH on a cohort of BALB/c mice that received DPPE-PEG, compared with the PBS-treated group. Data are the mean $\pm$ SEM PenH and are representative of three experiments. **C**, Invasive measurement of airway resistance was performed in BALB/c mice that received DPPE-PEG, compared with the PBS-treated group. AHR was assessed by changes in airway resistance (R_L , cm of H₂O/ml/s) and Cdyn (ml/cm of H₂O) in response to methacholine in anesthetized, tracheostomized, intubated, and mechanically ventilated mice. Data represent the mean $\pm$ SEM of five mice per group. **D**, DPPE-PEG inhibits airway inflammation. Left panel (H&E and PAS staining): lung parenchyma of a mouse treated with α -GalCer that has received DPPE-PEG, showing minimal mucus production and minor cellular infiltration. Bronchial mucosa consists of low cuboidal epithelium with an absence of peribronchiolar inflammatory infiltrates (original magnification $\times 400$). Middle panel (H&E and PAS staining): lung tissue from vehicle control mouse showing normal airway and surrounding parenchyma. The airway mucosa is characterized by low intracytoplasmic mucus and absence of peribronchiolar inflammatory infiltrates. Right panel (H&E and PAS staining): lung tissue from an α -GalCer-treated mouse shows vast amounts of inflammatory cells surrounding the airways and strong mucus production in the lumen. The bronchiolar epithelium has hyperplastic columnar epithelial cells with abundant intracytoplasmic accumulations of mucus, as well as eosinophils and mononuclear cells in the peribronchial space.

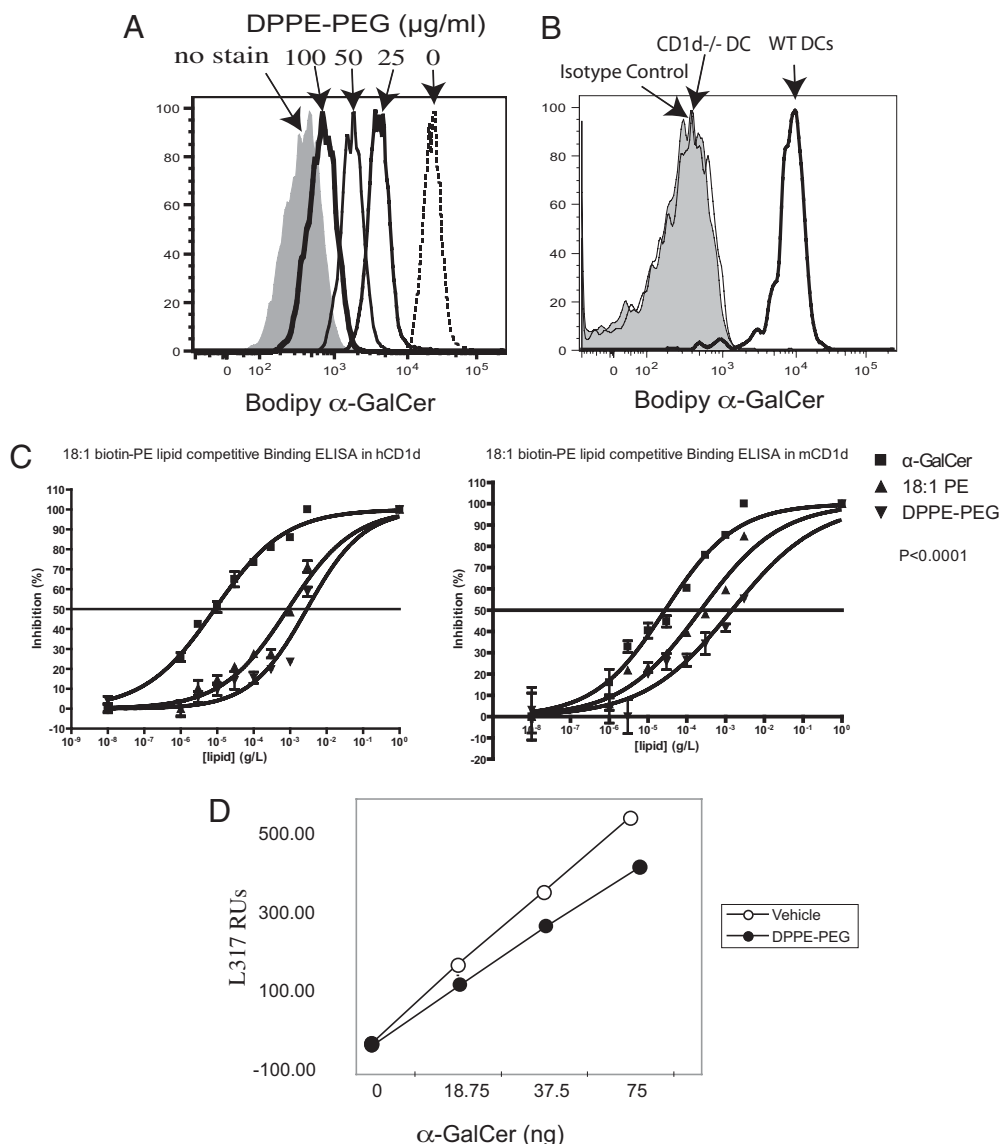


FIGURE 5. DPPE-PEG acts as CD1d antagonist. **A**, DN32 cells (1×10^6) were incubated at 37°C with titrating concentration of DPPE-PEG (100, 50, 25, and 0 μg/ml) for 4 h. Subsequently, the cells were washed and stained with 10 ng/ml of BODIPY-α-GalCer as described in *Materials and Methods*. The histogram data are representative of three separate experiments. **B**, CD11c⁺ cells were positively selected by magnetic cell sorting from WT (thick line) or CD1d^{-/-} (thin line) splenocytes and stained with BODIPY-α-GalCer as described in *Materials and Methods*. The results were compared with BODIPY isotype control (shaded histogram). The histogram data are representative of three separate experiments. **C**, Human CD1d:mIgG or mouse CD1d:mIgG dimers were incubated with plate-bound anti-mouse IgG1 Ab for 2 h. Next, biotinylated 18:1 PE lipid at 1 μg/ml was used as a competitor against serially diluted DPPE-PEG or α-GalCer for the binding to hCD1d or mCD1d molecules. The amount of biotinylated 18:1 PE lipid bound was determined by adding streptavidin-HRP and TMB substrate. Inhibition curves were fitted with a sigmoidal dose-response formula. Results are representative of three independent assays and expressed as mean $\pm$ SD. Statistical analysis was done in each curve fitting, and $p < 0.001$ was considered significantly different. **D**, Histidine-tagged mCD1d was incubated with DPPE-PEG (5 μg/ml dissolved in ethyl alcohol) or vehicle (ethyl alcohol) overnight before the addition of a dose range of α-GalCer. The following day, the relative number of CD1d/α-GalCer complexes was detected by immobilizing the histidine-tagged CD1d/lipid-mixture onto a Ni-coated nitrilotriacetic acid sensor chip (Biacore). A saturating dose of L317 (10 μg/ml) was injected for 1 min at a flow rate of 25 μl/min. The extent of binding was analyzed 5 s after the end of the injection. Data are shown as concentration of α-GalCer versus L317 binding (Biacore Response Units) in triplicate injections $\pm$ SD. These data are representative of two independent experiments. For all of the concentrations of α-GalCer, p values were calculated using a one-way ANOVA Bonferroni multiple comparison test ($p < 0.001$).

concentration of α-GalCer or DPPE-PEG for the binding to human or mouse CD1d molecules. The quantity of biotinylated 18:1 PE lipid bound to CD1d was measured by addition of streptavidin-HRP and chromogenic substrate. The final results were reported as the half maximal inhibitory concentration or IC₅₀. As shown in Fig. 5C (left panel), α-GalCer appears to be a strong CD1d agonist to human CD1d, because low concentrations of α-GalCer were needed to inhibit binding of 18:1 PE lipid. DPPE-PEG is also capable of inhibiting binding of 18:1 PE lipid to CD1d at higher

concentrations (Table I). Similar results were seen in competitive ELISAs using murine CD1d (right panel). To confirm these results, we performed a Biacore surface plasmon resonance assay. Mouse CD1d-α-GalCer complexes were immobilized on Biacore chips. Subsequently, binding of an mAb (L317) specific for CD1d-α-GalCer complexes (29) was used to determine the ability of DPPE-PEG to inhibit binding of α-GalCer to CD1d, hence the ability of L317 to detect CD1d-α-GalCer complexes. Using the Biacore assay, we found that DPPE-PEG significantly inhibited

Table I. IC50 in 18:1 PE lipid competitive binding ELISA

	Log IC50 in hCD1d (g/l)	Log IC50 in mCD1d (g/l)
α-GalCer	−5.07 ± 0.10	−4.56 ± 0.12
18:1 PE lipid	−3.06 ± 0.14	−3.64 ± 0.14
DPPE-PEG	−2.54 ± 0.21	−2.84 ± 0.21

IC50 in the 18:1 PE lipid competitive binding ELISA was calculated by Prism 4.0 software from the inhibition curves. Results represent the average of three independent assays and are expressed as mean ± SD. In each assay, IC50 was compared by one-way ANOVA, and $p < 0.001$ was considered significantly different.

the formation of CD1d–α-GalCer complexes (Fig. 5D). To conclude, DPPE-PEG binds to CD1d and competitively inhibits binding of 18:1 PE lipid to CD1d. However, binding capacity of DPPE-PEG to CD1d appears to be weaker than α-GalCer, which is the definition of TCR antagonist.

We next tested whether DPPE-PEG as an antagonist can block the TCR signaling on iNKT cells. We analyzed whether DPPE-PEG had any influence on the phosphorylation of ERKs in iNKT cells on activation with α-GalCer. iNKT cell lines were prepared as described in *Materials and Methods* and incubated with either DPPE-PEG alone or combination of DPPE-PEG and α-GalCer. As expected, iNKT cells incubated with α-GalCer–induced ERK phosphorylation as early as 1 min, and ERK phosphorylation was still detectable after 60 min, although at much lower levels (Fig. 6, upper panel). The addition of DPPE-PEG (100 μg/ml) resulted in a complete blockade of the α-GalCer–dependent ERK phosphorylation at several time points (Fig. 6, lower panel). DPPE-PEG at lower levels of 25 μg/ml and 12.5 μg/ml was able to only partially inhibit the ERK phosphorylation by α-GalCer. These results suggest that DPPE-PEG can directly block α-GalCer–induced phosphorylation of ERK, thereby inhibiting TCR signaling and activation of iNKT cells.

Discussion

Whereas conventional CD4⁺ T cells play a major role in asthma by recognizing exogenous Ags and by initiating allergic inflammatory responses in the lungs, we and others have shown that iNKT cells are also important effector cells in asthma. Thus, iNKT cell-deficient mice failed to develop AHR and have substantially reduced eosinophilia after sensitization and challenge with allergen, whereas Th2 responses developed normally (10, 11, 36). The requirement for iNKT cells is specific, because adoptive transfer of iNKT cells from WT mice reestablished the development of AHR in iNKT cell deficient Jα18^{−/−} mice. In confirmation of

those studies, we and others recently found that IL-17RB⁺ iNKT cells play an important role in IL-25 mediated AHR in mice (37, 38). Furthermore, direct activation of pulmonary iNKT cells by intranasal α-GalCer was sufficient to induce AHR and airway inflammation in the complete absence of class II MHC-restricted T cells in mice (31). In addition, studies examining sputum, sinus mucosa, and BAL of patients with asthma, as well as analysis of BAL fluids from patients with COPD, revealed a significant increase in the number of iNKT cells (13, 39–41). Thus, it seems clear that iNKT cells are present in the lungs of patients with lung diseases such as asthma, albeit with varying percentages and frequencies. The studies regarding iNKT cells in mice and nonhuman primates strongly suggest that this cell type might play an important role in allergic diseases and asthma (10, 11, 31, 38, 39, 42–46).

In the current study, the ability of DPPE-PEG to act as a pharmacologic antagonist of iNKT cell function was evaluated. We observed that sensitized BALB/c mice treated with DPPE-PEG i.v. showed a significant decrease in AHR, as measured by PenH, airway resistance, and Cdyn, as well as a reduction in airway inflammation as determined by histologic staining of lung sections. To our knowledge, this report is the first to target iNKT cells to treat allergic diseases, and our data indicate for the first time that iNKT cell inhibition by a specific antagonist can be used to effectively treat allergic diseases such as asthma.

To answer the question whether DPPE-PEG binds directly to CD1d and may thus act as a receptor agonist, we established several approaches, including competition assays using cellular and molecular techniques. We observed that binding of α-GalCer, conjugated to the fluorochrome BODIPY, was inhibited by increasing doses of DPPE-PEG (Fig. 5). This resulted in decreased fluorescence by DN32 cells loaded with α-GalCer BODIPY, suggesting a competition between α-GalCer and DPPE-PEG that involves both intracellular processing and surface binding to CD1d. In addition, we assessed the inhibitory effect of DPPE-PEG on the formation of CD1d–α-GalCer complexes on Biacore chips (Fig. 5D). We found that DPPE-PEG inhibits the formation of CD1d–α-GalCer complexes in a dose-dependent manner. To confirm our results, we performed competition ELISAs to human and mouse CD1d. In these assays, DPPE-PEG and α-GalCer both competed with 18:1 lipid conjugated to PE, and the results were reported as inhibition curves or IC50 inhibitory concentration. To summarize, we show that DPPE-PEG binds to CD1d, albeit in a much higher concentration than a strong receptor agonist such as α-GalCer. This feature is characteristic of agonistic peptides or glycolipids, because they usually bind to TCR molecules with much lower affinity and can reduce the activity of TCR-associated phosphorylation

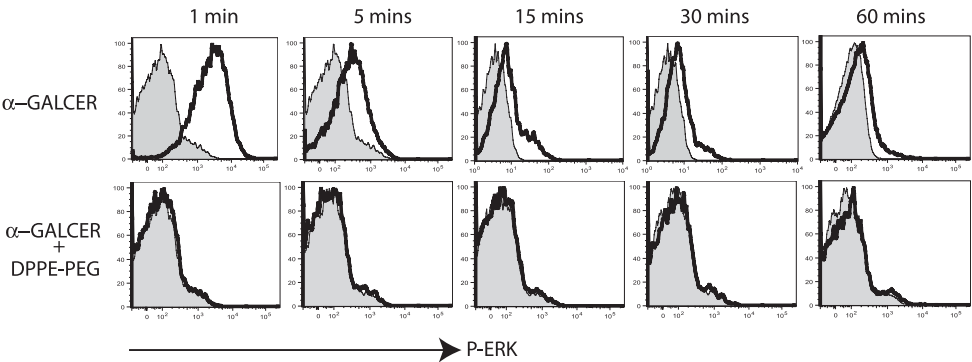


FIGURE 6. DPPE-PEG blocks ERK phosphorylation pathway (FACS histogram for p-ERK1/2 expression in intracellularly stained iNKT lines activated as described in *Materials and Methods*). The FACS profile shows p-ERK1/2 activation of iNKT cells by APCs pulsed with α-GalCer alone (upper panel) or α-GalCer plus DPPE-PEG (lower panel) at basal rates (time point 0, shaded histograms) and at various time points after incubation (thick lines).

such as ERK or induce an altered phosphorylation of the CD3 chain (47–49). Two approaches have been developed in the past to block the function of specific receptors: receptor antagonism or specific inhibition. In general, receptor antagonists bind to the specific receptor, thereby competitively blocking the binding of the receptor agonist. In contrast, an inhibitor usually blocks the pathway of a receptor and usually does not bind to the receptor itself (50). Because DPPE-PEG binds to CD1d and competes with α -GalCer in receptor binding, we conclude that DPPE-PEG acts as a specific CD1d receptor antagonist.

In addition to the alteration of TCR signaling, it has been proposed that antagonists, owing to their faster dissociation rates, can block TCR serial engagement by the agonist peptide (51, 52), resulting in the inhibition of T cell activation. To evaluate the inhibitory effect of DPPE-PEG on iNKT cells at the level of TCR signaling, the ability of DPPE-PEG to block α -GalCer-dependent p-ERK phosphorylation was determined. Whereas α -GalCer strongly induced p-ERK phosphorylation in iNKT cells, the addition of DPPE-PEG completely blocked the p-ERK phosphorylation in response to α -GalCer (Fig. 6). Consequently, we suggest that DPPE-PEG specifically inhibits activation of iNKT cells by α -GalCer. In fact, a single dose of DPPE-PEG significantly inhibited AHR induced by the intranasal administration of α -GalCer in WT mice, demonstrating an efficient inhibition of iNKT cell-induced pulmonary inflammation by the administration of DPPE-PEG (Fig. 4).

Although iNKT cells play an important role in the development of AHR, this process also requires the presence of allergen-specific CD4⁺ Th2 cells that respond to allergen. Th2 cells also produce IL-4, IL-5, and IL-13 and interact with mast cells, basophils, and eosinophils to mediate airway inflammation. However, many allergens, such as house dust mite, pollens, and rye grass, consist of several components, including proteins and glycolipids (53). Several studies have shown that priming with a purified protein alone does not induce airway inflammation and might indeed lead to the development of tolerance (33, 54). Interestingly, co-administration of a purified protein with glycolipids such as α -GalCer lead to the development of severe asthma by activating both NKT cells and T cells (34). We believe that glycolipids may act as adjuvants during the induction phase of allergic diseases, and asthma and IL-4 and IL-13 production by NKT cells potentiate the development and action of Th2 cells in the lungs (10).

Fig. 1 shows that increasing doses of DPPE-PEG exaggerated the release of IL-4 in cultures in which splenocytes were incubated with anti-CD3, whereas levels of IFN- γ decreased. We believe that the decrease in IFN- γ by blocking iNKT cells in the cultures created a culture environment for the T cells to produce higher levels of IL-4 in vitro. In contrast, secretion of IL-4, IL-5, IL-13 or IFN- γ upon restimulation with anti-CD3 or with OVA in vitro was not affected after treatment of mice with DPPE-PEG, indicating that DPPE-PEG has no effect on T cell responses mediated by MHC presentation pathways (Fig. 3).

These results confirm that DPPE-PEG acts as a direct antagonist against the binding of α -GalCer to CD1d, and thus activation of iNKT cells, and that the inhibition of iNKT cells by DPPE-PEG may be an effective strategy to treat allergic diseases such as asthma. Although DPPE-PEG inhibited binding of α -GalCer to CD1d using both live cells and immobilized CD1d complexes, it is important to note that the inhibitory effect of DPPE-PEG was significantly greater using live iNKT cells than using immobilized CD1d- α -GalCer complexes. This difference in DPPE-PEG antagonism implies that DPPE-PEG acts more efficiently when intracellular processing of CD1d is available and intact. This might indicate that DPPE-PEG binds to CD1d intracellularly and thereby

inhibits loading of CD1d ligands such as α -GalCer. Given that DPPE-PEG did not have any effect on T cell responses in vitro and in vivo, these data confirm that DPPE-PEG acts selectively on CD1d and not on MHC pathways.

Although it is known that glycolipids derived from *Sphingomonas* spp. or cypress tree pollen can activate iNKT cells, it is reasonable to believe that these and other glycolipids may enter the lungs, activate local iNKT cells, and trigger asthmatic responses directly, without the influence of additional Ags (31, 53).

Given the evidence that iNKT cells are both necessary and sufficient for the induction of AHR in animal models, such as mice and nonhuman primates, iNKT cell antagonists might provide a novel strategy to treat allergic diseases. In this study, we show for the first time that DPPE-PEG selectively antagonizes iNKT cells, thereby inhibiting allergic inflammation of the airways in an experimental model. iNKT cell-based therapies using DPPE-PEG may provide a novel and effective regimen in treating patients with unwanted iNKT cell activation such as allergic disease and asthma.

Disclosures

S.A.P. is a paid consultant for Vaccinex, Inc. of Rochester, NY and is an inventor on several pending patent applications related to the use of glycosylceramides for treatment of diseases.

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