

Recognition of pollen-derived phosphatidyl-ethanolamine by human CD1d-restricted $\gamma\delta$ T cells

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Background: Evidences from mice and human beings indicate that $\gamma\delta$ T cells could be relevant in recognition of stress-induced self and/or yet unidentified inhaled foreign antigens. Their specificity differs from classic MHC-restricted $\alpha\beta$ T cells and involves the immunoglobulin-like structure of the $\gamma\delta$ T-cell receptor with the recognition of small organic molecules, alkylamines, and self lipid compounds presented by CD1⁺ dendritic cells.

Objective: Because CD1 receptors are mainly devoted to lipid antigen presentation, we sought to determine whether exogenous pollen membrane lipids may act as allergens for CD1-restricted $\gamma\delta$ T cells.

Methods: Peripheral blood and nasal mucosa-associated $\gamma\delta$ T cells were cloned from normal controls and cypress-sensitive subjects and tested for their antigen specificity and CD1-restriction with phospholipids extracted from tree pollen grains, as well with other natural or synthetic compounds. Phospholipid reactivity of cloned $\gamma\delta$ T cells was measured by mean of proliferative response and cytokine release as well as by testing their helper activity on IgE production *in vitro* and *in vivo*.

Results: Cloned $\gamma\delta$ T lymphocytes from subjects with allergy, but not normal controls, were found to recognize pollen-derived phosphatidyl-ethanolamine (PE) in a CD1d-restricted fashion. Only 16:0/18:2 and 18:2/18:2 PE were stimulatory, whereas no response was recorded for disaturated PE, phosphatidylcholine, neutral lipids, or protein extract.

Proliferating clones secreted both T_H1-type and T_H2-type cytokines and drove IgE production *in vitro* and *in vivo*.

Conclusion: CD1d-restricted $\gamma\delta$ T cells specific for phospholipids can represent a key mucosal regulatory subset for the control of early host reactivity against tree pollens.

Clinical implications: By knowing how lipid allergen constituents interact with mucosal immune system, we can expand our possibilities in diagnostic and therapeutic interventions. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: Cypress pollen, olive pollen, phospholipids, phosphatidyl-ethanolamine, rhinitis, nasal mucosa, $\gamma\delta$ T lymphocytes, T-cell clones, CD1-restriction, cytokines, IL-4, skin prick tests, specific IgE

Studies in mice suggest that $\gamma\delta$ T cells have several functions. They not only mediate host defence in preventing lethal herpes virus 1 encephalitis,¹ or protecting against malaria,² tuberculosis,³ and listeriosis,⁴ but also have a fundamental role in immunoregulation.⁵

Several lines of evidence also implicate $\gamma\delta$ T cells as important in human immunity.⁶ Polyclonal V γ 2V δ 2 T cells expand *in vivo*, and typically increase to 8% to 60% of peripheral blood T cells during a variety of infectious diseases such as tuberculosis,⁷ salmonellosis,⁸ brucellosis,⁹ and toxoplasmosis.¹⁰ A subset of CD4⁺V δ 1⁺ $\gamma\delta$ T cells has been described in the respiratory tract of subjects with allergy as well.¹¹

Given the emerging role of $\gamma\delta$ T cells in host defence and immunoregulation, it is surprising that few natural antigens recognized by $\gamma\delta$ T cells have been identified. Such antigen specificity is remarkably different from MHC-restricted recognition of bound peptides by $\alpha\beta$ T-cell receptors (TCRs), and involves the immunoglobulin-like structure of the $\gamma\delta$ TCR¹² with the recognition of unprocessed peptides,¹³ small organic phosphate molecules,¹⁴ or alkylamines derived from microbes and edible plants.¹⁵

The epithelial-associated $\gamma\delta$ T cells can recognize stress-induced self-antigens, which would enable them to monitor multiple insults to the epithelium.¹⁶ This could explain the compartmentalized distribution of cells carrying identical TCR within mucosal surfaces.¹⁷ However,

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Abbreviations used

α -GalCer:	α -Galactosyl-ceramide
APC:	Antigen-presenting cell
MS:	Mass spectrometry
NC:	Normal control
NM:	Nasal mucosa
PB:	Peripheral blood-derived
PC:	Phosphatidyl-choline
PE:	Phosphatidyl-ethanolamine
PG:	Phosphatidyl-glycerol
TCR:	T-cell receptor

data from human beings and mice seem to indicate the relevance of mucosa-associated $\gamma\delta$ T cells in allergen recognition and airway inflammation, perhaps mediated by interaction of foreign pollen antigens with CD1⁺ dendritic cells.¹⁸⁻²⁰ Because it is now clearly established that CD1 receptors are almost exclusively addressed to the recognition of lipid antigens by T cells,²⁰ we sought to determine whether exogenous lipids extracted by the membrane of Mediterranean tree pollens (*Cupressus arizonica* and *Olea europea*) may act as antigens for $\gamma\delta$ T cells in subjects with allergy.

METHODS

Subjects and cell sources

After obtaining approval of the local Ethics Committee and informed consent of the participating subjects, PBMCs from 4 cypress-sensitive and 4 age-matched and sex-matched healthy controls were collected during the cypress pollinating season (from January to March in central Italy). Specimens of the inferior turbinate were surgically removed from the allergic subjects and from 2 of the 4 controls, which had chronic vasomotor rhinitis and nasal polyposis and did not display any sensitization to common allergens. None of the subjects was receiving anti-inflammatory therapy for their problems.

All participants received skin prick test for common allergens, including cypress, grass, weed, tree pollen mixture, house dust mite, cat, dog, and moulds. A positive response was defined as a wheal ≥ 3 mm in diameter. Total serum and specific IgE were measured by using the Pharmacia UniCap system (Pharmacia Diagnostics, Uppsala, Sweden). Normal subjects did not show a positive response to SPT, and serum specific IgE were absent.

PBMCs were cultured in RPMI 1640 medium (Gibco-BRL, Life-Technologies, Gaithersburg, Md) supplemented with 2 mmol/L L-glutamine (Gibco), 20 μ mol/L 2-mercaptoethanol, 100 U/mL penicillin, 50 μ g/mL streptomycin (complete medium), and 10% FCS in 24-well flat-bottomed culture plates (Costar, Cambridge, Mass) in a final volume of 3 mL.

Natural and synthetic lipids

Lipids were extracted from cypress and olive pollens following the procedure of Folch et al.²¹ The methanol/chloroform extract was evaporated nearly to dryness under N₂ and chromatographed on silica gel G thin layer chromatography plates by using chloroform/methanol/water (65:25:4 by volume) as solvent. Pure phospholipid standards were used to identify phospholipid classes, which were detected by staining with dichloro-fluorescein. Finally, phospholipids

were quantified by phosphorus assay after digestion with 70% perchloric acid.²²

Synthetic phospholipids were obtained commercially and used without further purification. 1-Palmitoyl-2-linoleoyl-sn-glycero-3-phospho-rac-(1-glycerol) sodium salt (PG; 16:0/18:2), dipalmitoyl-sn-glycero-3-phosphoethanolamine (PE; 16:0/16:0), 1-hexadecanoyl-2-[(*cis,cis*)-9,12-octadecadienoyl]-sn-glycero-3-phosphoethanolamine (PE 18:2/16:0), dilinoleoyl-sn-glycero-3-phosphoethanolamine (PE 18:2/18:2), and dipalmitoyl-sn-glycero-3-phosphocholine (PC; 16:0/16:0) were purchased from Avanti Polar Lipids (Alabaster, Ala). Naturally occurring PEs from egg yolk, liver, or brain tissue were obtained from Avanti Polar Lipids. All phospholipids suspensions were prepared in absolute ethanol.

Electrospray ionization mass spectroscopy of pollen PE

Electrospray ionization mass spectrometric (MS) analysis was performed on a triple-quadrupole tandem mass spectrometer (Quattro Ultima, Micromass, Manchester, United Kingdom) equipped with an electrospray ionisation interface. Total phospholipid extracts were dissolved in 25 μ L methanol:chloroform:water:NH₄OH (7:2:0.8:0.2, vol:vol) for single stage and tandem MS analysis of PE, and were analysed by nanospray electrospray ionization-MS. Dry heated nitrogen was used as both the cone and desolvation gas (70 and 450 L/h respectively), and dry argon was used as the collision gas (3.5 $\times 10^{-3}$ millibar). All data were recorded at mass resolution, as a signal average of 10 scans/collection, with a scan time of 12 seconds.

PE species was preferentially detected by using negative ionization. Identities of PE species were determined by neutral loss scans of phospho-ethanolamine (m/z 141⁺). The predominant molecular species present for each ion peak was determined by analysis of fatty acyl fragments generated by collision gas-induced fragmentation under negative ionization. The major precursor for PE molecular ion [M-H]⁻ was fragmented.

Generation of $\gamma\delta$ T-cell clones

Mononuclear cell suspensions were exposed to purified cypress-derived total lipid extract (1-5 μ g/mL) in bulk cultures (12-well culture plates; Costar, Cambridge, Mass), without adding cytokines or growth factors for almost two weeks. Nasal mucosa (NM) specimens were homogenized on a cell strainer and recovered cells suspended in complete medium supplemented with recombinant human IL-2 (50-100 U/mL; EuroClone, Weatherby, United Kingdom) without adding antigens.

Proliferating T-cell lines were tested *in vitro* for antigen specificity by incubating 2×10^4 T-cell blasts in triplicate 200- μ L cultures in the presence of 10^5 irradiated autologous feeder cells plus purified phospholipids (0.1-1 μ g/mL) in 96-well round-bottomed plates (Costar) for 72 hours at 37°C, in a humidified atmosphere of 5% CO₂. After pulsing for 16 hours with 0.5 μ Ci ³H-thymidine (Amersham, Amersham, United Kingdom), radionuclide uptake was measured by scintillation counting. Responses were considered positive when the stimulation index (ratio between the mean cpm obtained in triplicate cultures with autologous irradiated PBMCs plus antigen and the mean cpm obtained in triplicate cultures containing autologous irradiated PBMCs alone) was >10 .

Phospholipid-specific T-cell blasts were depleted of $\alpha\beta^+$ T cells by negative magnetic selection using an antihuman $\alpha\beta$ mAb and a magnetic beads-coated mouse antihuman IgG as secondary antibody (all from Serotec, Marseille, France). After 2 cycles on the Miltenyi Vario Macs (Miltenyi Biotec srl, Bologna, Italy) equipped with a LD column, eluted TCR $\alpha\beta^+$ blasts were seeded at 0.5, 1, and 2 cells per well in 96-well round-bottomed plates (Nunc, Denmark) with 10^5 irradiated (5000 rads) autologous T-depleted

PBMCs as feeder cells, 1% (vol/vol) phytohemagglutinin, and recombinant IL-2 (20 U/mL) in complete medium supplemented with 10% heat-inactivated human AB serum. Growing microcultures were then expanded at weekly intervals with 10^5 irradiated feeder cells and rIL-2.

Immunophenotype and intracellular cytokines of emerged clones were detected by flow cytometry using the method of Jung.²³ Samples were stained with Cy-chrome-conjugated anti-CD4 (PharMingen, San Diego, Calif), fluorescein isothiocyanate-conjugated anti-CD161 (NKR-P1A Ag, PharMingen), anti-CD8 (PharMingen), anti- $\alpha\beta$ (Serotec, Marseille, France), and anti- $\gamma\delta$, V γ 9, V δ 1, and V δ 2 (Serotec) mAbs. Thereafter, cells were fixed by incubation for 20 minutes with 2% formaldehyde (Sigma, St Louis, Mo) and permeabilized by incubation for 10 minutes in PBS supplemented with 2% FCS, 2 mmol/L Na $_2$ S $_2$ O $_3$, and 0.5% saponin (Sigma). Permeabilized cells were incubated with phycoerythrin-labeled anti-recombinant human IL-4 and fluorescein isothiocyanate anti-rhIFN- γ mAbs (Becton Dickinson, San Jose, Calif). Two-color immunofluorescent staining was used to enumerate cytokine-positive cells in lymphocyte cell subpopulations (FACScan flow cytometer, Becton Dickinson). The Lysis II program (Becton Dickinson) was used to analyze the results.

CD1d-restriction of phospholipid recognition

TCR $\gamma\delta$ T-cell clones were stimulated with phospholipids in the presence or absence of mAbs directed toward single CD1 epitopes (ie, anti-CD1a, b, c, and d) at a working concentration of 20 μ g/mL. Control experiments were performed with mouse IgG, anti-MHC class I and II mAbs. Human CD1 transfectants were prepared from cervical carcinoma cell line HeLa with the expression vector pSR α -Neo containing cDNA inserts encoding CD1a, CD1b, CD1c, or CD1d according to previously described methods.²⁴ A total of 10^5 irradiated (50 Gy) CD1 transfectants were seeded with 2×10^4 cloned T cells in a 96 round-bottom plate (Costar) and exposed to PE (0.1 to 20 μ g/mL) and cultured for 96 hours in 5% CO $_2$ atmosphere, pulsed with 1 μ Ci of ^{3} H-Thymidine, and harvested 16 hours later for β scintillation counting by using a Beckman scintillation counter (Beckman Coulter, Fullerton, Calif). Control samples were represented by irradiated HeLa cells plus phospholipids. Results are expressed as absolute cpm ($\pm$ SD) count of triplicate culture wells.

To avoid interferences with membrane-bound phospholipids, in some experiments, CD1d-transfected HeLa cells were incubated for 1 hour on ice with 2 mL 0.9% NaCl containing 75 mmol/L 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide-HCl (Pierce Chemical Co, Rockford, Ill), followed by extensive washing in RPMI 1640. Specific responses were evaluated by ^{3} H-thymidine incorporation (as above) after 5 days culture.

Functional activity of CD1d-restricted $\gamma\delta$ T-cell clones

PE-specific CD1d-restricted $\gamma\delta$ T cell clones (2×10^4 cells/well) were cultured with irradiated HeLa transfectants (10^5 cells/well) and synthetic PE compounds for 48 hours. IL-2 and IL-4 production was measured by using commercial reagents (R&D Systems, Minneapolis, Minn) in the recovered supernatants, and the results were expressed as pg/mL.

For *in vitro* IgE production, $\gamma\delta$ T-cell clones were cocultured with autologous T-depleted PBMCs for 10 days, and IgE were measured in the supernatants of stimulated samples by capture ELISA. The mAb for IgE capture (clone HP 6029) was purchased from Zymed (San Francisco, Calif). Immunoplates (Nunc, Wiesbaden, Germany) were coated overnight at 4°C with the capture antibody diluted in 0.1 mol/L bicarbonate buffer. Blocking was performed by adding 2% BSA/TRIS-buffered saline for 1 hour, followed by several washings. Supernatants and internal standards were then added to the wells

and incubated in duplicate for 2 hours. After several washes, the biotinylated antihuman IgE mAb (Vector Laboratories, Inc, Burlington, Calif) was added. Plates were incubated for another hour with alkaline phosphatase-conjugated streptavidin. After the final reaction with phosphatase substrate (Sigma), plates were read in a microplate ELISA reader at 405 nm, and the amount of IgE was calculated according to the standard curve. The variations of readings in duplicate cultures never exceeded 15%. Values are expressed in ng/mL.

In vivo helper activity on IgE production

For *in vivo* skin testing, 11 cypress-sensitive and 16 normal controls were further enrolled in the study. Purified pollen-derived and synthetic phospholipids were dissolved in absolute ethanol and used for skin prick tests at the concentration of 10 μ g/mL. One hundred microliters of phospholipid solution was applied over the skin of forearms of subjects with allergy and control subjects. After puncturing with a sterile lancet, the mean of wheal and flare maximal diameters (mm $\pm$ SD) was measured after 15 minutes.

For detection of PE-specific IgE, sera from all 15 subjects and those from 20 healthy controls were placed on microplates previously coated with pollen-derived PE and control neutral lipids. For coating procedure, lipids were dissolved in *n*-hexane to a concentration of 50 μ g/mL. One hundred microliters of this solution were added to wells of 96-well microtiter plates. After evaporation of the solvent in air, the plates were blocked for 2 hours with 10% FBS diluted in PBS containing 2 mmol/L Ca $^{2+}$ (binding buffer). Tested sera were diluted in the binding buffer in the presence of 10% serum at an initial concentration of 6.7 nmol/L. Serial 2-fold dilutions were prepared in the plates (100 μ L per well), which were thereafter incubated for 24 hours at room temperature. The plates were washed, and binding of specific IgE was detected by adding a goat antihuman IgE (diluted 1:200) antibody conjugated to alkaline phosphatase (Southern Biotechnology, Birmingham, Ala). The reaction was developed by using chromogenic substrate 4-nitrophenyl phosphate disodium salt solution, followed by reading plates at 405 nm using a microplate reader (Bio-Tek Instruments Inc, Winooski, Vt).

Statistical analysis

Statistical comparisons in T-cell proliferation and other functional assays were made by using 1-way ANOVA. Differences in the mean percentages and SDs were calculated by the Mann-Whitney *U* test. *P* values less than .05 were considered significant.

RESULTS

Circulating and mucosal-associated $\gamma\delta$ T cells recognize pollen-derived phospholipids

Only 1 out of 5 V δ 2 $^{+}$ $\gamma\delta$ T-cell clones obtained from peripheral blood-derived (PB) of normal subjects was found to be responsive, whereas the majority of phospholipid-specific $\gamma\delta$ T cell clones, most of which expressed CD4, were derived from the PB (3 out of the 6 obtained) or NM (6 out of the 12 obtained) of subjects with allergy (Table I).

The 6 $\gamma\delta$ T-cell clones obtained from NM of subjects with allergy were V δ 1 $^{+}$ CD4 $^{+}$, and half of them coexpressed the CD161 molecule (see this article's Fig E1, A, in the Online Repository at www.jacionline.org). This has been described in subjects with allergy with rhinitis²⁵ and/or asthma.¹¹ Among purified extracts derived from both olive and cypress pollens, PE was the main phospholipid to which T-cell clones were found to be responsive

TABLE I. Phenotypal and functional characteristics of PE-specific $\gamma\delta$ T-cell clones

Clone	Phenotype	V δ -TCR	CD161 ⁺	IL-4	IFN- γ
PB-Q5	CD4	V δ 1	No	++	++
PB-S7	CD4	V δ 2	Yes	+	+++
PB-W11	CD4-CD8-	V δ 2	No	+++	+
NM-A3	CD4	V δ 1	Yes	+++	+
NM-1B	CD4	V δ 1	Yes	++	++
NM-H6	CD4	V δ 1	Yes	+++	+
NM-12C	CD4	V δ 1	No	+++	+
NM-L21	CD4-CD8-	V δ 1	No	+++	++
NM-S3	CD4	V δ 1	No	+++	++
NC-PB-3C	CD4-CD8-	V δ 2	No	++	+++

in vitro (see this article's Fig E1, B, in the Online Repository at www.jacionline.org). The recognition of such phospholipid was CD1d-restricted. Indeed, blocking experiments with mAbs against CD1 epitopes showed that PE reactivity was largely dependent on CD1d molecule on antigen-presenting cells (APCs; see this article's Fig E1, C, in the Online Repository at www.jacionline.org).

Mass spectrometry analysis of cypress and olive PE composition exhibited a restricted distribution dominated by species containing the fatty acid linoleate (C18:2_{n-6}), so the major species was di-linoleoyl (18:2/18:2), followed by palmitoyl-linoleyl (16:0/18:2) PE. There were no plasmenyl or plasmanyl-PE species, nor disaturated or mono-unsaturated species in PE from pollens (data not shown).

Fine antigen specificity of $\gamma\delta$ T-cell clones

The pattern of *in vitro* response to natural and synthetic phospholipids is represented in Fig 1. Relevant dose-dependent IL-2 production by PB-Q5 (Fig 1, A) and PB-S7 (Fig 1, B) was observed mainly to synthetic 16:0/18:2 and 18:2/18:2 PE, whereas synthetic 16:0/16:0 or 18:0/18:0 PE or control 16:0/16:0 PC did not significantly increase IL-2 secretion above basal values. The same clones proliferated mostly against cypress-derived and, to a lesser extent, olive-derived PE. The TcRV δ 2⁺ normal control (NC)-PB-3C clone was responsive *in vitro* to synthetic 16:0/18:2 PE, but weakly to 18:2/18:2 PE or pollen-derived PE (Fig 1, C). However, this clone proliferated mostly to egg-derived PE, a finding not observed with $\gamma\delta$ T-cell clones from subjects with allergy (Fig 1, A and B).

The antigen specificity of mucosal-derived $\gamma\delta$ T-cell clones from subjects with allergy was quite similar to that observed in those PB-derived. Again, unsaturated 16:0/18:2 PE and 18:2/18:2 PE were the main compounds to which such clones showed significant *in vitro* IL-2 production (data not shown). Disaturated PE species and other natural extracts, such as brain and egg PE, as well as 16:0/16:0 PC were not stimulatory (data not shown).

The response of all tested clones toward pollen-derived or synthetic phospholipids was substantially maintained by CD1d⁺ HeLa cells that had been exposed to fixation. In particular, none of the selected clones acquired

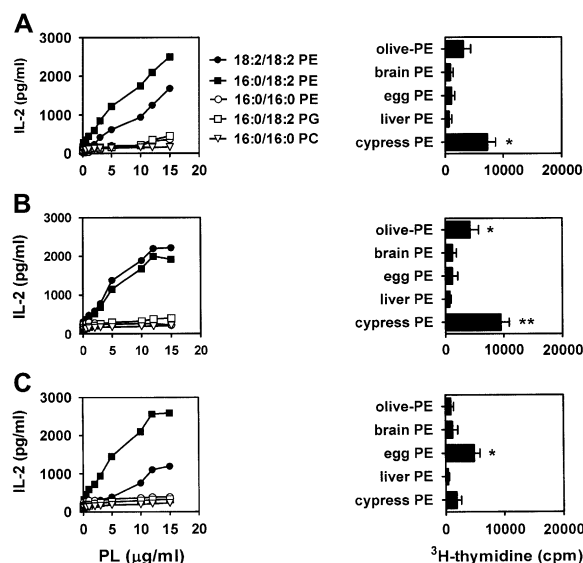


FIG 1. Fine antigen specificity of $\gamma\delta$ T-cell clones. Natural and synthetic phospholipid (PL) compounds were used to stimulate IL-2 production (left) and proliferation (right) of $\gamma\delta$ T-cell clones. **A**, PB-Q5 TCRV δ 1⁺CD4⁺ clone. **B**, PB-S7 TCRV δ 2⁺CD4⁺ clone. **C**, NC-PB-3C TCRV δ 2⁺CD4⁺CD8⁻ clone. **P* < .05; ***P* < .001).

autoreactivity against HeLa CD1d⁺, as demonstrated by the absence of ³H-thymidine incorporation (mean: 585 ± 250 cpm) without adding exogenous phospholipids.

PE-specific $\gamma\delta$ T cells display helper activity on IgE production

There is conflicting evidence from animal studies that CD1-restricted (TCR invariant natural killer [iNK-T] or classic natural killer [NK-T]) T cells could be necessary for the development of many features of experimental allergy, including high specific IgE production.^{26,27} Because $\gamma\delta$ ⁺ T-cell clones derived from PB and nasal mucosa of subjects with allergy were able to recognize pollen-derived PE, we tested their ability to help autologous B cells synthesize *in vitro* IgE on pollen PE stimulation. All studied clones are depicted in Fig 2. Those derived from nasal mucosa of subjects with allergy were able to produce IL-4, as well as to induce IgE secretion by autologous non-T cells, on PE stimulation. The capacity to help IgE production was present in both $\gamma\delta$ ⁺CD4⁺CD161⁻ and CD4⁺CD161⁺ clones (Fig 2, NM-1B and NM-12C, respectively), whereas the $\gamma\delta$ T-cell clone from normal subjects (Fig 2, NC-PB-3C) neither proliferated against pollen-derived PE nor produced IgE *in vitro*, even if it was able to secrete IL-4 in response to synthetic 16:0/18:2 PE or brain and egg PE.

To investigate in deep the clinical relevance of T_H2 helper activity of $\gamma\delta$ T cells, we assessed cutaneous skin test reactivity to purified pollen-derived PE and synthetic compounds (10 μ g/mL), as well as the presence of circulating PE-specific IgE in both patients and controls. For such *in vivo* testing, 15 cypress-sensitive and 20 control subjects were investigated. Negative controls,

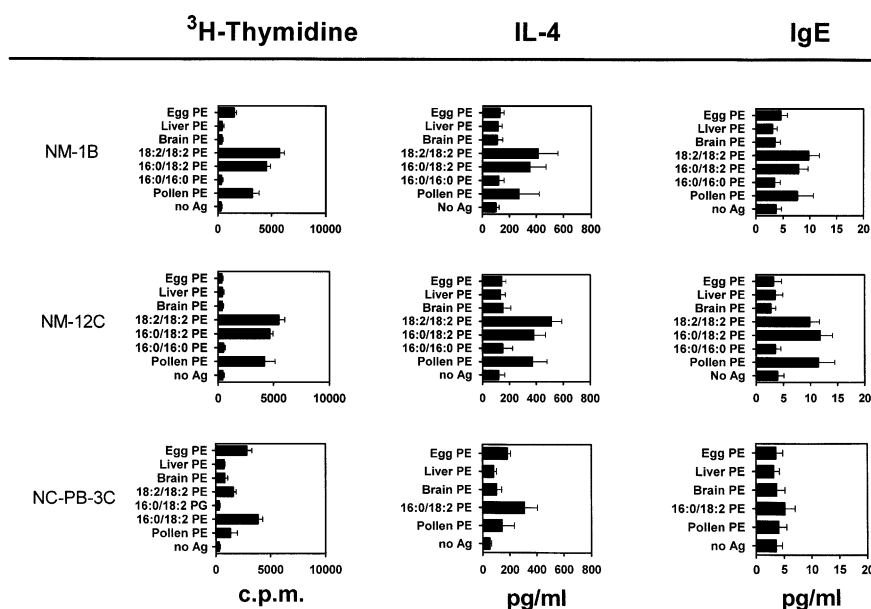


FIG 2. *In vitro* helper activity of NM $\gamma\delta$ T-cell clones. NM-derived T-cell clones from subjects with allergy proliferate, secrete IL-4, and induce IgE production by autologous B lymphocytes when pulsed with pollen-derived or synthetic unsaturated PE ($P < .001$). The NC clone, despite showing discrete IL-4 production with pollen and egg PE ($P < .05$) as well as with 16:0/18:2 PE ($P < .001$), does not display significant helper effect on IgE production *in vitro*. Ag, antigen.

which were represented by vehicle ethanol solution and pollen-derived neutral lipids, determined an erythematous reaction never exceeding 2 mm maximal diameter in forearms of healthy controls. Subjects with allergy displayed a clear wheal-and-flare reaction when tested with cypress-derived PE, and a mild reaction with olive-derived PE (see this article's Fig E2, A, in the Online Repository at www.jacionline.org). Moreover, they did not react to egg-derived, brain-derived, or liver-derived extracts (1.4 ± 0.6 mm). No positive reactions to pollen-derived PE were recorded in normal subjects. Circulating specific IgE to cypress-derived and, to a lesser extent, to olive-derived PE were present in the sera from subjects with allergy, as detected by an ELISA technique, whereas they were absent in normal controls (see this article's Fig E2, B, in the Online Repository at www.jacionline.org). Such reactivity could be inhibited *in vitro* by absorption of sera with serial dilution of pollen-derived PE (50% inhibition with 1 $\mu\text{g/mL}$ PE), but not with pollen-derived neutral lipids (data not shown).

DISCUSSION

Sphingolipid and phospholipid compounds can apparently bind CD1 and function as antigens for CD1-restricted T cells. By using mouse hybridomas, Gumperz et al²⁸ have drawn the conclusion that phospholipids, particularly phosphatidyl-inositol and PE, may represent a major class of self-antigens recognized by CD1d-restricted $\alpha\beta^+$ T cells. Some of the T cells that recognize these antigens

may also respond to α -galactosyl-ceramide (α -GalCer).²⁰ However, recent evidence suggests that phospholipids, and particularly PE, from membrane of tumor cell lines or those extracted from soy, brain, liver, and egg can stimulate the proliferation and regulatory functions of double-negative $V\alpha_{24}V\beta_{11}\text{TCR}^+\text{iNKT}$ cells.²⁹ Pollen grains are known to contain many unsaturated fatty acids necessary to allow pollen germination, and these could potentially contribute to the immunogenicity of CD1-presented lipid antigens.³⁰ Indeed, we have previously demonstrated that phospholipids from cypress pollen membrane play a definite role in interacting with dendritic cells expressing surface CD1a and CD1d.³¹ These molecules are critical in pollen grains capture and in the subsequent presentation of phospholipids to $\alpha\beta^+$ T cells.

Our knowledge about lipid antigen recognition by $\gamma\delta$ T-cell clones is limited to the reactivity against self lipids by $V\delta 1^+$ T cells that is restricted by CD1c.³² These T cells are usually double-negative, do not express the CD161 receptor (natural killer receptor-protein 1A [NKR-P1A]), and produce almost exclusively IFN- γ . The candidate self lipid antigens they recognize are mannosyl-1-phosphodolichols compounds, which are structurally related to the mycobacterial hexosyl-1-phosphoisoprenoids presented by CD1c to $\alpha\beta$ T cells.³³

The CD1d-restricted PE recognition by $\gamma\delta$ T-cell clones is a surprising finding in human beings, because to date, such restriction was confined to regulatory NK-T cells, with a particular TCR repertoire ($V\alpha_{24}\text{-J}\alpha_{18}\text{-V}\beta_{11}$) specific for synthetic α -GalCer.³⁴ In mice, an extensive characterization of T-cell hybridomas derived from NK-

T-cell subpopulations revealed the existence of a significant proportion of autoreactive NK-T cells (with a V α 14 invariant TCR³⁵) similar to that found in human beings, but also the presence of non-V α 14 T cells (as much as 55% of NK1.1⁺ CD4 T-cell hybridomas) that failed to exhibit CD1d autoreactivity and did not respond to the surrogate antigen α -GalCer.³⁶ Similar T cells have been recently found to recognize polar phospholipids derived from a tumor cell-line, but also naturally occurring PE species derived from plants, brain, liver, and egg.²⁹ Moreover, phospholipid-specific T-cell clones with classic $\alpha\beta$ TCR coexpressing CD161 are present in the blood of subjects with allergy.³¹

The described nasal $\gamma\delta$ T-cell reactivity against pollen phospholipids emphasizes a new mechanism of mucosal immune reactivity that seems to be exclusive of subjects with allergy. Binding to CD1d of antigenic PE is favored by the presence of unsaturated acyl chains in the *cis* configuration.²⁹ Nonbilayer phospholipid arrangements (hexagonally packed cylinders of the hexagonal tubular phase) occur in about 40% of membrane lipids from pollens, particularly phosphatidyl-serine, cardiolipin, PE, and phosphatidyl-choline.³⁰ Electron microscopy studies demonstrate that these nonbilayer structures appear as protuberances on the surface of the membrane.³⁷ In this manner, acyl side chains of phospholipids are exposed and may thus enter the CD1 pocket, making the contact between pollen grain membrane and dendritic cell stable. Instead, of some CD1-restricted $\alpha\beta$ ⁺ T cells, the described $\gamma\delta$ T-cell clones do not display reactivity against CD1d transfected HeLa cells. Therefore, they presumably do not recognize autologous phospholipids. Most importantly, because of the similarities in phospholipid composition of tree pollens tested, olive-derived PEs have been able to induce some functional activation in $\gamma\delta$ T-cell clones derived from cypress-sensitive subjects. Indeed, enhanced *in vitro* proliferation, weak T_H2-like helper activity on IgE production and nonsignificant skin test reactivity has been demonstrated. This is in line with the lack of polymorphism of CD1 receptors that might be the adaptive consequence of binding lipid antigens with conserved hydrophobic moieties.²⁰

The increasing number of reports demonstrating $\gamma\delta$ T-cell function in various infectious models along with a growing list of antigen recognition by their TCRs, is consistent with a role in the early immune response to foreign nonprotein antigens.³⁸⁻⁴¹ Our current data, together with previously reported expansion of IL-4-producing $\gamma\delta$ T cells in the airways of subjects with asthma^{11,41} and the presence at the mucosal sites of APCs that express CD1 molecules,^{18,31} point out 2 new concepts. First, lipids from pollen grains may be recognized as antigens by the human immune system. Second, because the rapid mucosal capture of pollens by APCs can be mediated by CD1 molecules, lipid-specific CD1d-restricted $\gamma\delta$ T cells may function as first-line mucosa-associated lymphocytes, which act as key regulators of cytokine, chemokine and inflammatory environment governing the onset and persistence of the allergic immune response.

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