Human CD1-restricted T cell recognition of lipids from pollens

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Plant pollens are an important source of environmental antigens that stimulate allergic responses. In addition to acting as vehicles for foreign protein antigens, they contain lipids that incorporate saturated and unsaturated fatty acids, which are necessary in the reproduction of higher plants. The CD1 family of nonpolymorphic major histocompatibility complex–related molecules is highly conserved in mammals, and has been shown to present microbial and self lipids to T cells. Here, we provide evidence that pollen lipids may be recognized as antigens by human T cells through a CD1-dependent pathway. Among phospholipids extracted from cypress grains, phosphatidyl-choline and phosphatidyl-ethanolamine were able to stimulate the proliferation of T cells from cypress-sensitive subjects. Recognition of phospholipids involved multiple cell types, mostly $CD4^+$ T cell receptor for antigen (TCR)$\alpha\beta^+$, some $CD4^-CD8^-$ TCR$\gamma\delta^+$, but rarely Vα24*i*$^+$ natural killer–T cells, and required $CD1a^+$ and $CD1d^+$ antigen presenting cell. The responding T cells secreted both interleukin (IL)-4 and interferon-γ, in some cases IL-10 and transforming growth factor-β, and could provide help for immunoglobulin E (IgE) production. Responses to pollen phospholipids were maximally evident in blood samples obtained from allergic subjects during pollinating season, uniformly absent in *Mycobacterium tuberculosis*–exposed health care workers, but occasionally seen in nonallergic subjects. Finally, allergic, but not normal subjects, displayed circulating specific IgE and cutaneous weal and flare reactions to phospholipids.

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Abbreviations used: BAL, bronchoalveolar lavage; BCG, Bacillus Calmette-Guérin; ESI, electrospray ionization; MS, mass spectrometry; PC, phosphatidylcholine; PL, phospholipids.

The mechanisms of recognition of pollen grains as allergens by the immune system have been debated for a considerable time, and because most of the proteins described as sensitizing agents are contained inside the intine membrane and/or in the cytoplasmic region, it is difficult to explain their rapid delivery in vivo to the host immune system (1, 2). Traditional models suggest that nonspecific receptors such as C-type lectins and integrins bind surface glycoproteins, favoring phagocytosis by alveolar macrophages of the whole pollen grain and/or of starch granules (<5 μm) derived from grasses (3). This in turn permits direct interaction of TCRs with antigen-derived peptides presented by MHC-encoded antigen-presenting molecules, consistent with the current central paradigm for antigen presentation to T cells (4).

However, peptides are not the sole physiological target of T cell responses, as T cells can recognize a variety of lipid antigens by means of CD1 proteins, a family of nonpolymorphic antigen presenting molecules (5) that have evolved to present lipids and glycolipids to the mammalian immune system (6, 7). Of the many potential immune functions for CD1, a compelling case can be made for a role in host defense, especially against intracellular pathogens (8). In addition, CD1-restricted T cells are involved in regulation of autoimmune responses (9, 10) and in immunosurveillance against tumors (11). Human CD1 genes on chromosome 1 encode a diversified family of proteins that are subdivided into group I (including CD1a, -b, and -c) and group II (CD1d; reference 12). The CD1 proteins are expressed on professional APCs (13), and can

present bacterial or synthetic lipids and glycolipids to various T cell subpopulations (14, 15), which in turn mediate effector functions including cytolysis and IFN-γ secretion that are known to be protective for the host (16).

Currently, it is not known if natural lipids from nonbacterial and/or nonmammalian species may be recognized as antigens in a CD1-restricted fashion by the human immune system, apart from the glycolipid α-galactosyl ceramide (17). In mice, Vα14*i* NKT cells can recognize ceramide-based glycolipids as well as synthetic PL antigens in a CD1d-restricted manner (18), and have recently been demonstrated as fundamental for the development of allergen-induced airway hyperreactivity (19). This suggested to us the possibility that lipids contained within pollen grains, a source of environmental antigens that are frequently associated with airway hyperreactivity in humans, might be capable of activating CD1-restricted T cell responses. Pollen grains are known to contain many unsaturated fatty acids that are necessary to allow pollen germination, and these could potentially contribute to the immunogenicity of CD1-presented lipid antigen (20–22).

Our results suggest that phospholipids (PLs) at the pollen surface may be of functional relevance for the capture of the pollen grain by APCs and its recognition by the immune system of sensitive subjects. T cell clones specific for pollen PLs possessed functional properties similar to those of regulatory T cells, secreted both Th1 and Th2 type cytokines and displayed helper activity for IgE production. Thus, CD1-restricted PL-specific T cells could have a central role in regulating the immune response in allergic individuals.

RESULTS

A possible role for CD1a and CD1d molecules in capture of cypress pollen

Both upper (23) and lower airways (24) are known to contain large numbers of DCs and macrophages, which are cell types that have the potential to express CD1 proteins. Previous studies have reported the frequent expression of CD1a on airway derived DCs from allergic subjects (25). We extended these observations by examining the presence of CD1d on DCs or other types of mononuclear cells in the airways, and sought to determine if the expression of CD1 proteins could be relevant to the capture of pollens in vivo and in vitro. Immunophenotyping of bronchoalveolar lavage (BAL)-derived mononuclear cells revealed the presence of $CD11c^+CD86^+$ DCs in asthmatic subjects, as compared with healthy controls (unpublished data), thus confirming the existence in the airways of atopic individuals of professional APCs (23–25). As shown in Fig. 1 a, a variety of

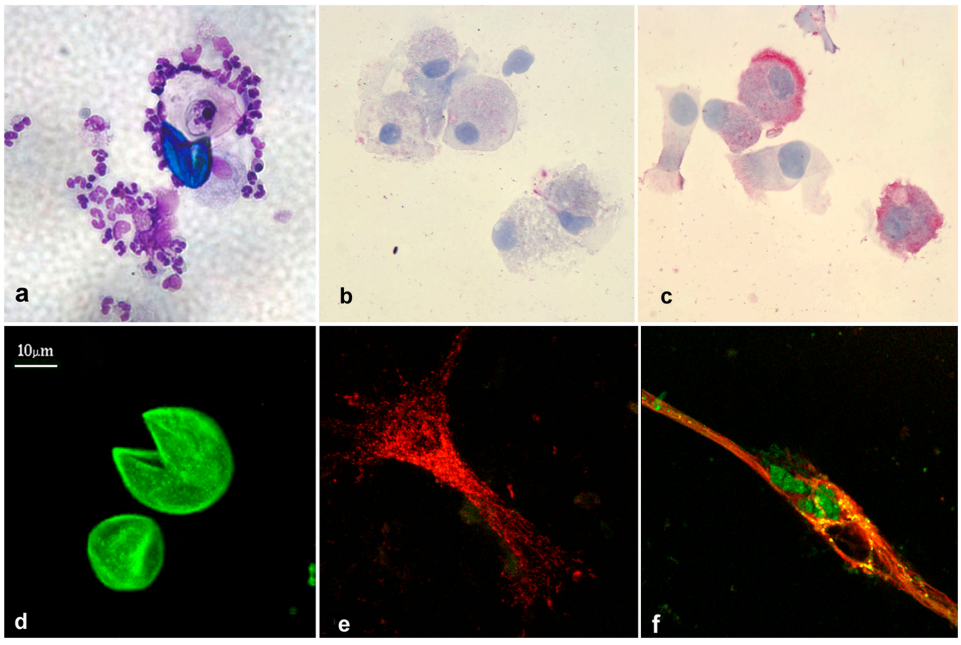

Figure 1. Interactions between cypress pollen and $CD1d^+$ DCs. Pollen capture in vivo. (a) The pollen grain constituted by a dark blue exine envelope with extruded cytoplasmic material as appear in a BAL, sampled during pollinating season (May-Grünwald-Giemsa; 400×). The cellular infiltrate contains pulmonary DCs, macrophages, and lymphocytes. (b) A BAL cytospin sample, representative of those obtained from controls ($n = 10$), as it appeared after labeling with anti-human CD1d mAb (clone NOR3.2, working dilution 1:10) and staining with alkaline phosphatase/antialkaline phosphatase technique. Cells with morphology consistent with macrophages and DCs did not show surface or intracellular staining. (c) $CD1d^+$ APC in BAL cytospin from an allergic patient representative of all samples obtained from all allergic subjects ($n = 15$). (d) Pollen grain stained with fluorescein diacetate (0.1 mM); (e) DC stained with lipophylic dye 4-[4-(dihexadecylamino)styryl]-*N*-methylquinolinium iodine (DiQ; 1 mM); (f) a DiQ-labeled (red) DC after coculture with fluorescein diacetate–labeled (green) pollen grain for 15 min. Labeled fragments of the pollen grain have colocalized with DiQ-labeled membranes, as evidenced by the merged orange color.

mononuclear cell types present in BAL samples of asthmatic subjects were able to interact with inhaled pollen grains. In addition, staining with a CD1d-specific mAb revealed that many strongly $CD1d^+$ cells with morphology consistent with macrophages or DCs were present in the BAL suspensions from asthmatic subjects but not in BAL from normal controls (Fig. 1, b and c). Using confocal fluorescence microscopy, we then followed pollen grain capture by in vitro activated $CD1^+$ DCs. Intact pollen grains were first labeled using fluorescein diacetate (Fig. 1 d), and then combined with monocyte-derived DCs previously stained with DiQ {4-[4-(dihexadecylamino)styryl]-*N*-methylquinolinium iodide} (Fig. 1 e) to highlight both the surface and internal membranes of the cells. The subsequent ingestion of the fluorescent grain was evident starting at 15 min after incubation, with a progressive concentration over time within cytoplasmic vesicles and cell membranes (Fig. 1 f).

Adopting an in vitro culture system, we found that monocyte-derived DCs that expressed detectable surface levels of multiple CD1 proteins (Fig. 2 a) could efficiently bind pollen grains in vitro. This interaction could be blocked by preincubation of DCs with antibodies directed against the cell surface–expressed conformation of CD1d and, to a lesser extent, CD1a proteins. In contrast, a nonbinding control Ig or mAbs specific for MHC class I, CD1b, or CD1c molecules had no effect on pollen binding by the cells (Fig. 2 b). Because polar lipids, and particularly PLs, may bind the hydrophobic pocket of CD1a and CD1d molecules (16, 26), we tested the ability of lipids extracted from cypress pollen to saturate CD1 receptors on DCs and thereby block pollen grain binding. Only polar PL extract was able to inhibit pollen grain adhesion to DCs, as compared with the neutral lipid or protein pollen extract (Fig. 2 c). However, because DCs use multiple redundant surface receptors to bind foreign substances, many of which functionally recognize hydrophobic portions of both protein and lipid molecules (27), we also tested the ability of CD1 expression in a nonphagocytic cell type to mediate pollen grain binding to the cell surface. HeLa cells, a transformed human cell line of epithelial origin, that were stably transfected for expression of CD1d could bind cypress pollen grains, whereas mock-transfected HeLa cells that did not express CD1 showed minimal binding (Fig. 2 d). Such binding could be inhibited by pretreatment of the CD1d-transfected HeLa cells with mAbs against CD1d, but was unaffected by pretreatment with mAbs against other CD1 molecules or nonbinding control Ig. In addition, pretreatment of $CD1d^+$ HeLa cells with cypress PLs could partially (69 ± 7%) inhibit this interaction. Collectively, these data indicated that DCs bound pollen grains by specific interaction of CD1 molecules with surface PLs.

Analysis of PL components of cypress pollen

To identify specific ligands that might mediate this interaction, we performed a direct chemical analysis of isolated cy-

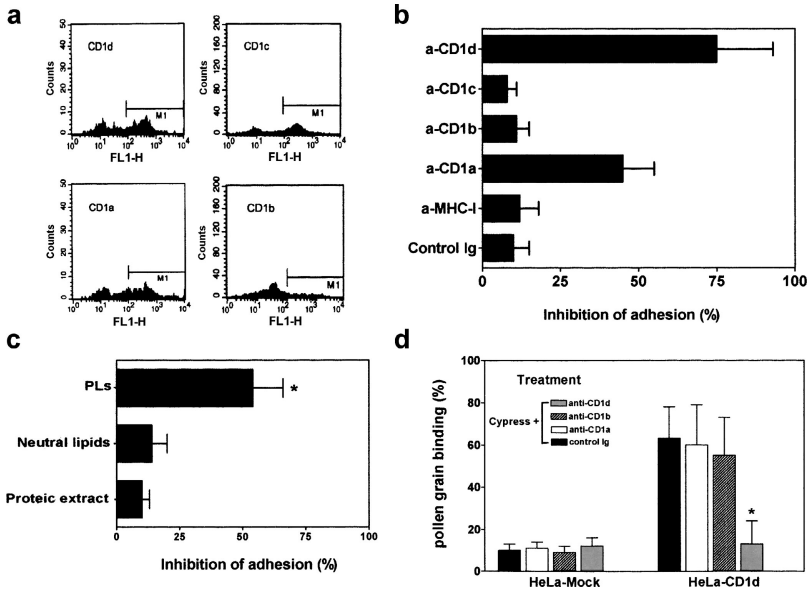

Figure 2. CD1-dependence of pollen capture in vitro. (a) Expression of CD1 receptors on monocyte-derived DCs. (b) Pre-treatment of DCs with anti-human CD1d and anti-CD1a mAbs (20 μg/ml) was able to inhibit adherence of pollen grains to DCs (*, $P < 0.005$). Results are expressed as mean percentages (±SD) of inhibition with respect to samples incubated with control Ig. (c) Polar PLs, as opposed to neutral lipids or protein extract from cypress pollen, were able to saturate CD1 receptors on DCs at a concentration of 10 μg/ml, thus preventing further grain capture (*, $P < 0.005$). (d) HeLa CD1d transfectants bound cypress pollen grains and such binding could be specifically inhibited by preincubation with anti-CD1d mAb (*, $P < 0.005$). For each of the 10 different preparations, 500 cells were counted by each of three independent observers, who were blinded to the identity of the samples.

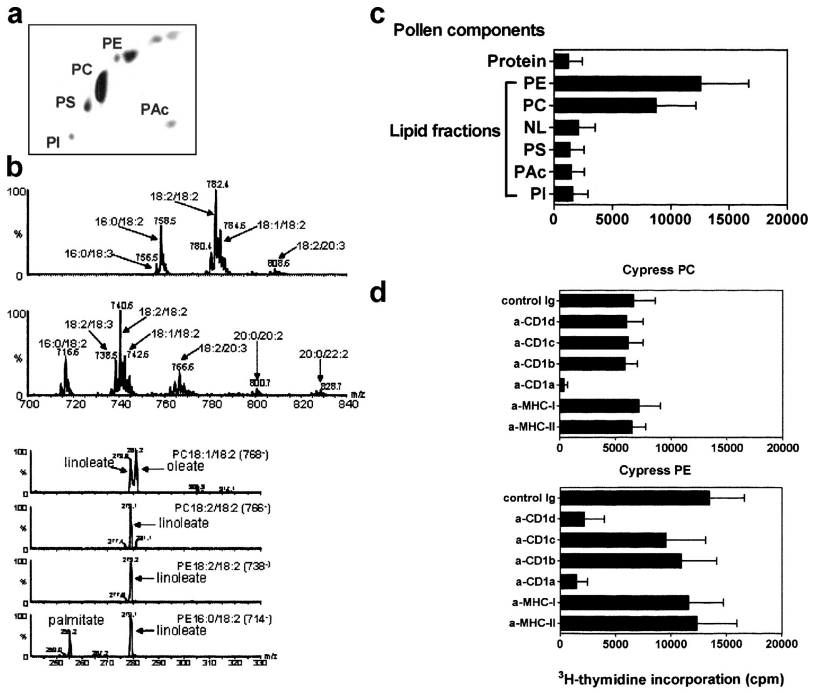

Figure 3. Biochemical characterization of PLs extracted from cypress pollen. (a) Bidimensional TLC of PLs. The main species represented were phosphatidyl-choline (PC), phosphatidyl-ethanolamine (PE), phosphatidyl-serine (PS), phosphatidyl-inositol (PI), and free phosphatidic acid (PAc). (b) Electrospray ionization MS analysis of purified PC and PE from cypress pollen. (c) In vitro [^{3}H]thymidine incorporation by T cell lines to pollen lipids or control protein (previously defatted) cypress pollen extract. (d) CD1 restriction of PC and PE recognition. Mean values ± SD of single PC- and PE-specific T cell lines representative of all tested in triplicate.

press pollen lipids. Two-dimensional TLC of the lipid composition of the membranes from *Cupressus arizonica* and *Cupressus sempervirens* pollens showed a variety of PLs (Fig. 3 a) that were similar to those previously described for *Typha latifolia* or *Brassica napus* pollens (28, 29). Phosphatidyl-choline (PC) represented greater than 50% of total PL, followed by phosphatidyl-ethanolamine (PE; 15%) and then smaller amounts of the other PL classes.

Molecular species compositions of TLC-purified pollen PC and PE were determined by tandem mass spectrometry (MS)/MS. Precursor scans for molecules giving rise after collision-induced fragmentation to a protonated phosphocholine head group with m/z 184^+ allowed identification and structural determination of the PC molecules (Fig. 3 b, top panel). Similarly, molecules characterized by the neutral loss of phosphoethanolamine (m/z 141^+; not depicted) were analyzed by collision-induced dissociation MS to resolve the precise structure of diacyl PE species (Fig. 3 b, middle panel). Inspection of the electrospray ionization $(ESI)^-$ scan confirmed the absence of either plasmenyl or plasmanyl PE species. The molecular species components of the major ions present in the PC and PE spectra were identified by product ion scans of the $[M-15]^-$ ion for PC and the $[M-H]^-$ ion for PE (Fig. 3 b, bottom four panels). Both PC and PE compositions exhibited a restricted distribution dominated by species containing the fatty acid linoleate ($C18:2_{n-6}$), such that the major species in each case was dilinoleoyl (18:2/18:2). There were no disaturated or monounsaturated species in either PC or PE from pollen.

CD1-restricted T cell responses against pollen-derived PLs

T cell lines from cypress-sensitive subjects displayed significant in vitro proliferative responses mainly to cypress PE and, to a lesser extent, PC (Fig. 3 c). These responses appeared to be specific, because neither PE nor PC stimulated proliferation of a panel of other human T cell lines derived from in vitro activation with unrelated protein substances, such as tetanus toxoid (unpublished data) or a protein extract from previously defatted cypress pollen. Similarly, in vitro [^{3}H]thymidine incorporation in T cell lines was not stimulated by incubation with neutral lipids extracted from cypress pollen or with other pollen PLs, such as phosphatidyl-serine, phosphatidyl-inositol, or phosphatidic acid (Fig. 3 c).

The type of antigen-presenting molecules involved in presentation of PC and PE to specific T cell lines was investigated with the use of blocking mAbs. Pretreatment of autologous DCs with anti-CD1a and CD1d mAbs inhibited proliferation of most PL-specific T cell lines (Fig. 3 d). Anti-CD1b, anti-CD1c, or anti-MHC mAbs did not modify the responses to cypress PLs. It is noteworthy that the effects of antibodies specific for CD1a and CD1d on the proliferative activity of PL-specific T cell lines correlated

Table I. Characteristics of phospholipid-specific T cell clones from cypress-sensitive subjects

Clone	Phenotype	CD1 restriction	Cypress PC	16:0/16:0 PC	16:0/18:2 PC	18:1/16:0 PC	18:1/18:1 PC	18:2/18:2 PC	Brain PC	Liver PC	Egg PC
1D3	TCRαβ$^+$CD4$^+$	CD1A	+	+++	0	0	0	0	++	0	+
1G5	TCRαβ$^+$CD4$^+$	CD1A	+++	0	+	0	0	+++	0	0	0
3F9	TCRVαβ24$^+$CD4$^+$	CD1A	+	+++	0	0	0	0	++	0	++
3G1	TCRαβ$^+$CD4$^+$	CD1D	++	0	0	0	0	+++	0	0	0
5A2	TCRαβ$^+$CD4$^+$	CD1A	++	0	+	0	+	++++	0	+	0
5B7	TCRαβ$^+$CD4$^+$	CD1A	+++	0	0	0	+	++	0	+	0
5B9	TCRαβ$^+$CD4$^+$	CD1A	+	0	0	0	0	+++	0	0	0
6D1	TCRαβ$^+$CD4$^+$	CD1D	++	0	+	0	0	+++	0	0	0
6D7	TCRαβ$^+$CD4$^+$	CD1A	++	0	0	0	0	++	0	0	0

		CD1	Cypress PE	16:0/16:0 PE	16:0/18:2 PE	18:1/18:1 PE	18:2/18:2 PE	16:0/18:2 PG	Brain PE	Liver PE	Egg PE
B1.5	TCRαβ$^+$CD4$^+$	CD1D	+++	0	+	0	++	0	0	0	0
C2	TCRαβ$^+$CD4$^+$	CD1D	+++	0	++	+	++	0	0	0	0
D4	TCRαβ$^+$CD4$^+$	CD1D	++	0	0	0	0	+++	0	0	0
E1.1	TCRαβ$^+$CD4$^+$	CD1D	+++	0	0	0	++	0	0	0	0
F1	TCRαβ$^+$CD4$^+$	CD1D	+++	0	++	0	++	0	0	0	0
F3	TCRαβ$^+$CD4$^+$	CD1D	+++	0	0	0	0	+++	0	0	0
L5	TCRαβ$^+$CD4$^+$	CD1D	+++	0	++	+	0	0	0	0	0
S7	TCRVα1$^+$CD4$^+$	CD1D	+++	0	+++	0	+	0	0	0	++
N3	TCRVα24$^+$CD161$^+$CD4$^-$CD8$^-$	CD1D	++	0	++	0	++++	0	0	+	+
P3	TCRαβ$^+$CD4$^+$	CD1D	++	0	++	0	+++	0	0	0	0
P13	TCRαβ$^+$CD4$^+$	CD1D	++	0	0	0	0	+++	0	0	0
EA3	TCRαβ$^+$CD4$^+$	CD1D	+++	0	+++	0	++	0	0	0	+
EA6	TCRVδ1$^+$CD161$^+$	CD1D	+	0	++++	0	++	0	0	+	0
FA3	TCRαβ$^+$CD4$^+$CD161$^+$	CD1D	+++	0	+	0	+++	0	0	+	0
FA8	TCRαβ$^+$CD4$^+$	CD1D	++	0	++	0	+++	0	0	0	0
GA10	TCRαβ$^+$CD4$^+$	CD1D	+	0	++	0	++	0	0	0	0
DD6	TCRαβ$^+$CD4$^+$CD161$^+$	CD1D	++	0	0	0	0	++	0	0	0
DE10	TCRαβ$^+$CD4$^+$	CD1D	++	0	++	+	+	0	0	0	0
Q5	TCRVδ1$^+$CD4$^+$CD161$^+$	CD1A	+	+++	0	0	0	0	++	0	++

Antigen reactivity was measured by IL-4 production in 48 h culture supernatants: between 10 and 50 pg/ml (0), between 60 and 150 pg/ml (+), between 160 and 250 pg/ml (++), and over 260 pg/ml (+++/++++).

strongly with the effects of these antibodies on the binding of pollen grains by DCs (Fig. 2)

Isolation of PL-specific T cell clones

The CD1-restricted T cell lines were then cloned to identify their fine antigenic specificity. Cloning efficiency was 9.5% for PC- and 18% for PE-specific T cell lines. In contrast, using the same protocol of in vitro stimulation with cypress-derived PLs, cloning efficiency in healthy normal subjects was 0% for PC and 2% for PE. The majority of PL-specific T cell clones produced both IL-4 and IFN-γ, and thus were identified as Th0 cells (unpublished data). Most T cell clones derived from PC-specific T cell lines were CD4$^+$, expressed TCRαβ, and were mainly restricted by CD1a (Table I, top section). Their fine antigen specificity was investigated using synthetic PC species selected from the compositional analyses shown in Fig. 3 b. The highest in vitro proliferation and IL-4 production were observed to the predominant PC18:2/18:2 species, a response comparable to the reactivity against a natural pollen-derived PC mixture (Table I, top section). Two CD4$^+$ clones (1D3 and 3F9), with weak cypress PC reactivity, responded in vitro to synthetic dipalmitoyl PC (PC 16:0/16:0), a species not present in naturally occurring cypress-derived PC. It is interesting to note that these T cell clones displayed significant IL-4 production also after stimulation with brain- and egg-derived PC (Table I, top section). The 3F9 T cell clone was CD1a-restricted and expressed Vα24 and Vβ11 TCR gene segments based on specific mAb staining. Only two of eight PC 18:2/18:2-specific T cell clones were CD1d restricted. An overall weaker reactivity

Table II. Characteristics of PE-specific T cell clones from normal subjects

Clone	Phenotype	CD1 restriction	Cypress PE	16:0/16:0 PE	16:0/18:2 PE	18:1/18:1 PE	18:2/18:2 PE	16:0/18:2 PG	Brain PE	Liver PE	Egg PE
NC-A2	TCRαβ$^+$CD4$^+$CD161$^+$	CD1D	+	0	+	0	0	++	++	0	+++
NC-S22	TCRαβ$^+$CD4$^+$	CD1D	++	0	++	0	++	0	+	+	++
NC-D4	TCRVα24$^+$CD161$^+$ CD4$^-$CD8$^-$	CD1D	+	0	0	0	0	++	0	++	0
NC-F.1	TCRαβ$^+$CD4$^+$	CD1D	+	0	0	0	+	0	++	++	++
NC-FP5	TCRαβ$^+$CD4$^+$	CD1D	+	0	+	0	++	0	+	+	+++

Antigen reactivity was measured by IL-4 production in 48 h culture supernatants: between 10 and 50 pg/ml (0), between 60 and 150 pg/ml (+), between 160 and 250 pg/ml (++), and over 260 pg/ml.

was found against either PC 16:0/18:2 or PC 18:1/18:1, whereas no proliferative response was recorded with PC 18:1/16:0 as the stimulating agent (Table I).

The majority of T cell clones derived from allergic subjects displayed reactivity toward cypress PE mixture (Table I, bottom section). Only one clone (Q5) was purely CD1a restricted. It reacted with PE 16:0/16:0 and to a lesser extent with egg-derived PE, and expressed a γδ TCR that used the Vδ1 gene product. Functionally, it secreted significant amounts of IL-4 when exposed in vitro to PE 16:0/16:0 or to egg-derived PE. Group II CD1d-restricted T cell clones in this panel included one example that expressed the Vα24/Vβ11 *i*NKT cell-associated TCR (N3) and also one γδ T cell clone (EA6) that coexpressed the NK marker CD161. The *i*NKT cell clone N3 responded vigorously to cypress-derived PE, as well as to synthetic PE 18:2/18:2, and to a lesser extent toward liver-derived PE. Three CD4$^+$ clones reacted exclusively with a phosphatidylglycerol 16:0/18:2, whereas the majority reacted with either PE 16:0/18:2 or PE 18:2/18:2 or both of these PLs. In contrast, there were very few clones with reactivity toward PE 18:1/18:1.

To examine the requirement for PL internalization by APCs for CD1 presentation, we assessed the ability of HeLa CD1d$^+$ transfectants to stimulate responses when subjected to mild chemical fixation before loading with stimulating PLs. The fixation of the APC surface prevents internalization of CD1 or exogenous lipids to intracellular compartments, which is required for some but not all previously studied examples of lipid antigen presentation (30). Because aldehyde fixatives caused strong augmentation of autoreactivity of NKT cell clones to CD1d$^+$ APC, we instead used the chemical cross-linker ECDI as a fixative (14). As illustrated in the Fig. 4, the response of clones toward cypress-derived or synthetic PLs was substantially maintained by CD1d$^+$ HeLa cells that had been subjected to fixation. In particular, none of the selected clones (two CD4$^+$ TCRαβ$^+$ and one Vδ1$^+$ CD4$^+$) acquired autoreactivity against HeLa CD1d$^+$, as demonstrated by the absence of [^{3}H]thymidine incorporation (mean: 585 ± 250 cpm) without adding exogenous PLs. In addition, antigen specificity of tested clones was maintained in terms of IL-4 production and proliferation.

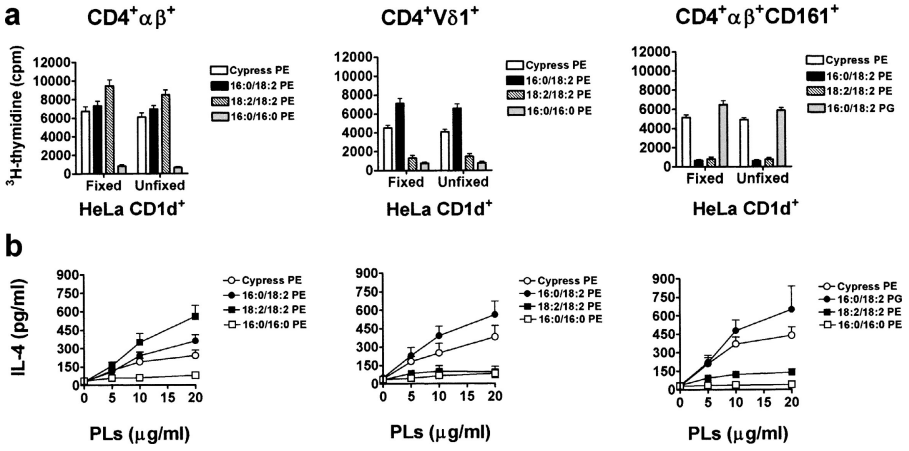

Figure 4. Specificity of antigen recognition by PL-specific CD4$^+$ αβ$^+$ or γδ$^+$ T cell clones. (a) Effect of fixation on PL presentation by CD1d-transfected HeLa cells. (b) IL-4 production by T cell clones after in vitro pulsing with cypress-derived or synthetic PLs. Note: for CD4$^+$αβ$^+$ clones, data are referred to C2, F1, and P3, for CD4$^+$Vδ1$^+$ clones data are referred to S7 and EA6, whereas for CD4$^+$αβ$^+$CD161$^+$ data are referred to DD6 clone. All illustrated in Table I.

Functional characteristics of CD1-restricted T cell clones with unconventional TCR

In some cases we have observed that T cell clones specific for cypress-derived PLs did coexpress NK markers, or displayed a particular TCR (Table I). In addition, they often respond to PL mixtures of different origin with even greater [^{3}H]thymidine incorporation as for cypress PLs. Because regulatory functions have been described among CD1-restricted T cells, mainly on the basis of their cytokine profile induced by encounter of environmental antigens (31), we searched for the possibility that some of our clones could secrete, together with IL-4 and IFN-γ, also IL-10 or TGF-β1 in response to antigen stimulation. Among clones tested, we selected the CD1a-restricted 16:0/16:0 PE-specific T cell clone Q5 coexpressing CD161, CD4, and TCR Vδ1 receptor, the double-negative CD161$^+$ Vα24$^+$ Vβ11$^+$ CD1d-restricted T cell clone (N3), which reacted with 18:2/18:2 PE and the CD4$^+$ TCRVα24$^+$ clone, CD1a-restricted, specific for 16:0/16:0 PC (3F9). Functionally, all these clones were able to produce both IL-4 and IFN-γ after in vitro stimulation with cypress-derived PL mixtures or corresponding synthetic lipid species (Fig. 5). The Q5 T cell clone responded to egg-derived PE mixture with multiple cytokine secretion, particularly TGF-β1 ($P < 0.001$). N3 T cell clone responded vigorously to cypress-derived PE, while displaying a weaker proliferative response to liver-derived PE, with increase in IL-10 production ($P < 0.05$ vs. unstimulated cultures). Finally, the 3F9 clone showed discrete IL-10 and TGF-β1 production after brain and egg PC mixtures stimulation ($P < 0.001$ vs. unstimulated cultures). Such cytokine secretion pattern was not influenced by the presence of CD1a$^+$ or CD1d$^+$ HeLa transfectants, because the cytokine production in the absence of added lipids was always negligible. Proliferative activity, as measured by [^{3}H]thymidine uptake, and cytokine secretion patterns were concordant mainly for N3 T cell clone, which is specific for 18:2/18:2 PE. The two other clones proliferated mainly in response to disaturated (16:0) PC or PE, which are absent on cypress pollen PL extracts.

PL-specific T cell clones from nonallergic subjects

Although likely to be present at a significantly lower frequency, CD1-restricted and PL-specific T cell clones could also be isolated from normal nonallergic subjects. The phenotypic and functional characteristics of five such PE-specific T cell clones isolated from normal subjects are listed in Table II. All but one were classical TCR αβ$^+$ CD4$^+$ cells and one (NC-D4) TCR Vα24$^+$ CD161$^+$ CD4 and CD8 negative. Their responses to in vitro stimulation with cypress-derived PE were in all cases CD1d-restricted and characterized by significant IL-4 production (ranging from 125 to 210 pg/ml). Of interest was their vigorous IL-4 production after stimulation with egg-, brain-, or liver-derived PLs. This finding was quite different from the pattern we observed in allergic subjects, in which there was a clear bias toward responses to plant-derived PLs, as compared with animal-derived ones (Table I).

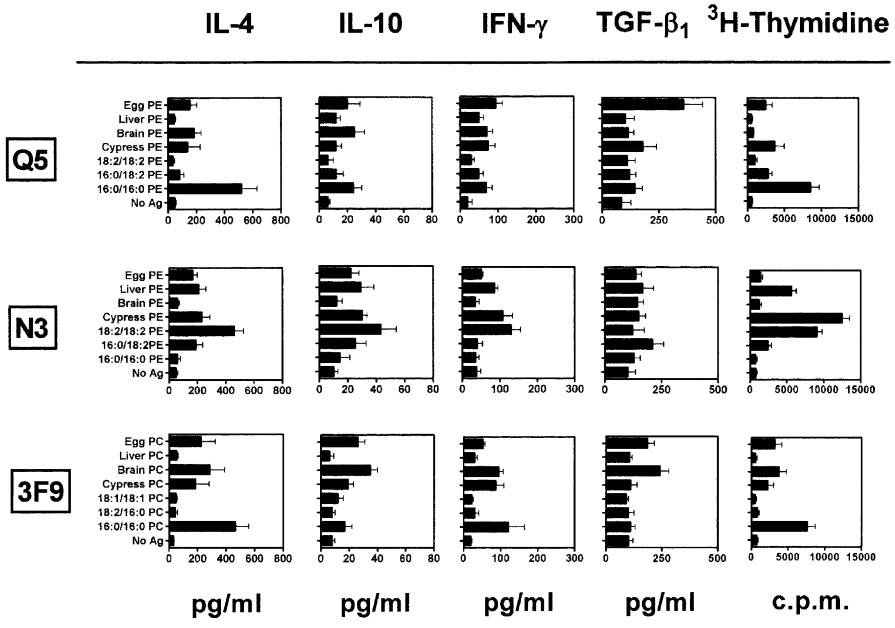

Figure 5. Functional characteristics of selected PL-specific T cell clones. For cytokine production by T cell clones and proliferation assays, mitomycin C treated HeLa cells transfected with CD1a (for clones Q5 and 3F9) or CD1d (for clone N3) were used as APCs. Stimulating antigens consisted of 10 μg/ml of cypress, brain, liver, and egg PL mixtures (PC or PE), as well as of synthetic PL species. Secreted IL-4, IL-10, TGF-β1, and IFN-γ (mean ± SD of pg/ml in the supernatants of triplicate cultures) were measured after 48 h of culture by ELISA. [^{3}H]thymidine incorporation (mean cpm ± SD of triplicate cultures) after 5 d of culture was measured by scintillation counting. See Table I for CD1 restriction.

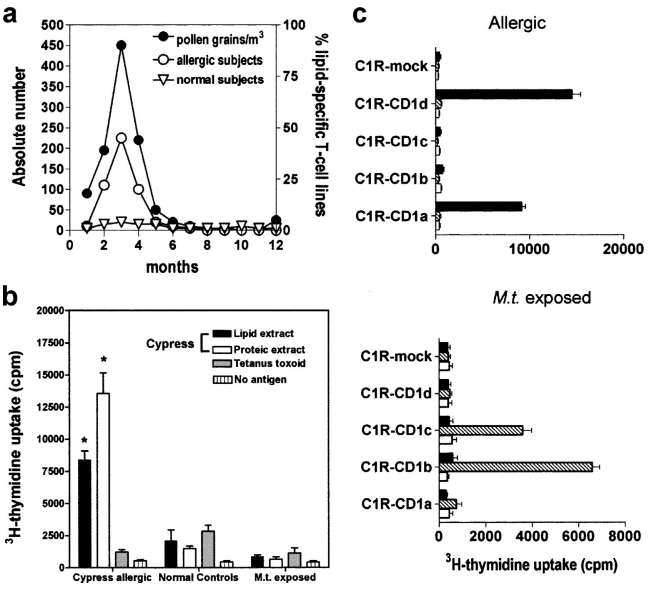

Figure 6. Response to cypress pollen PLs in allergic subjects is specific and shows seasonal variability. (a) Correlation over a 1-yr period between atmospheric levels of cypress pollen (absolute counts, referred to the right y axis) in the region inhabited by the study subjects and the frequency of PL-specific T cell lines (percentage of responding T cell lines calculated over the total number obtained, referred to the left y axis). (b) Mean values (±SD) of proliferative responses of PBMC to stimulation with cypress antigens. A clear response ($P < 0.005$) was seen in allergic, as compared with normal controls or *M. tuberculosis* exposed subjects. (c) CD1a- and CD1d-restricted proliferative response in T cell lines of allergic subjects (top) toward pollen-derived PLs (10 μg/ml; filled square), mycolic acid stimulation (10 μg/ml; shaded square), or medium alone (open square). The bottom panel shows the responses of T cell lines derived from *M. tuberculosis*–exposed subjects. One representative experiment of all performed in 14 patients and 10 controls is depicted.

Correlation of PL-specific responses with atopic history and pollen exposure

Because cypress pollination in Italy is typically present from January to March (32), we undertook a survey of the frequency of PL-specific T cell lines over a 1-yr period. In allergic patients, the average number and proportion of PL-specific T cell lines recovered after in vitro culture paralleled the presence of cypress pollen grains in the atmosphere, following the characteristic seasonal distribution (Fig. 6 a). There were very few inducible PL-specific T cell lines in the peripheral blood of normal controls or in the same allergic subjects sampled during nonpollinating periods. The relevance of this phenomenon was also assessed in freshly isolated PBMC by exposing cells to cypress PLs and protein extracts, or to an irrelevant antigen, such as tetanus toxoid (Fig. 6 b). Cypress-sensitive subjects displayed in vitro proliferative responses to either lipid or protein cypress extracts, as compared with *Mycobacterium*-exposed health care workers. In normal controls we found in rare cases a slight [^{3}H]thymidine incorporation above the basal values, in line with results obtained with the few PE-specific T cell clones we were able to isolate from PBMC. Such results suggest that PL reactivity of normal subjects, even if present at very low levels, did not correlate with the seasonal cypress pollen count variations, as observed in allergic patients.

We also examined the reactivity of PL-specific T cell lines derived from cypress-sensitive subjects to purified mycolic acids from *Mycobacterium tuberculosis*, which has previously been identified as a CD1-presented lipid antigen in humans (10, 11). Because IL-12 and IFN-γ are powerful suppressors of Th2 activity, lack of frequent exposure to infections that stimulate production of these cytokines has been speculated to increase the risk of developing atopy (33). For instance, Bacillus Calmette-Guérin (BCG) infection of mice either 4 or 12 wk before allergen airway challenge results in significant reduction of eosinophils within the lungs with respect to uninfected controls (34). In humans, neonatal BCG vaccination or, alternatively, evidence of an immunologic memory of this vaccination is associated with a reduced prevalence of allergic sensitization, asthma, eczema, and hay fever during childhood (35).

Unlike previously published findings with human T cell lines derived from in vitro stimulation with mycobacterial lipid products (36, 37), we did not find in allergic subjects any significant reactivity to mycolic acid under our experimental conditions (Fig. 6 c). In contrast, health care workers with documented exposure to and latent infection with *M. tuberculosis* responded in a CD1b- and CD1c-restricted manner to mycolic acids, but did not respond to cypress-derived PLs in vitro.

Role of PL-specific T cells in IgE production and in vivo hypersensitivity to pollen PLs

There is conflicting evidence from animal studies on the requirement of classical CD1-restricted *i*NKT cells for the development of various features of experimental allergy, including high specific IgE production (19, 38–40) but this function has not been assessed to our knowledge for other subsets of CD1-restricted T cells. We tested the ability of PE-specific TCRαβ$^+$ CD4$^+$ cell clones, from both patients and controls, to help autologous B cells synthesize IgE in vitro upon cypress PE stimulation. Clones derived from allergic patients, but not from controls, were able to induce IgE secretion when cocultured with autologous B cells and stimulatory cypress PE or synthetic extracts, with a less pronounced effect for those coexpressing CD161 (Fig. 7 a). However, such a helper effect on in vitro IgE production couldn't always reflect a similar behavior on clinical allergic diseases. To evaluate the potential in vivo significance of PL recognition in cypress pollen allergy, we assessed cutaneous skin prick test reactivity to purified cypress-derived PLs and synthetic compounds (10 μg/ml) in allergic and healthy subjects. Negative control reagents, which were represented by vehicle ethanol solution and synthetic 16:0/16:0 PC or PE, caused a mild erythematous

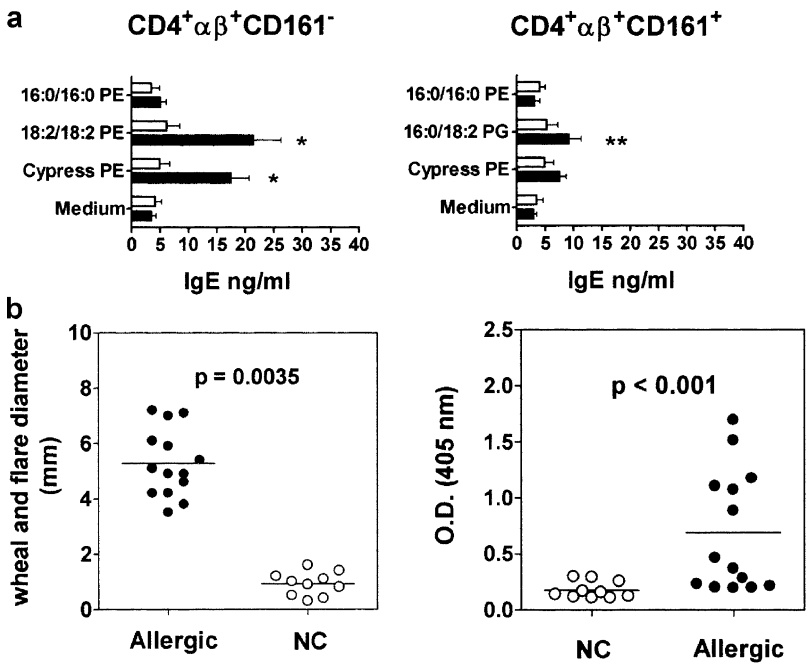

Figure 7. IgE reactivity toward pollen PLs. (a) Two representative experiments of in vitro IgE production by autologous B cells cocultured with $CD4^+$ $TCR\alpha\beta^+$ cell clones, expressing or not the CD161 molecule, and stimulated with cypress PE or synthetic PLs. Filled bars refer to clones from allergic subjects (clones B1.5 and FA8 $\alpha\beta^+CD161^-$ and clone DD6 $\alpha\beta^+CD161^+$, as reported in Table I), open bars to those of normal controls (clone NC-FP5 $\alpha\beta^+CD161^-$ and clone NC-A2 $\alpha\beta^+CD161^+$, as reported in Table II). *, $P < 0.001$ with respect to unstimulated, 16:0/16:0 PE-stimulated or normal control clones. **, $P < 0.05$ with respect to unstimulated cultures. (b) In the left panel is the average of mean wheal and flare reactions to cypress-derived PLs in study population. The right panel represents the circulating PE-specific IgE (mean OD at 405 nm) as detected by ELISA assay. NC, normal controls.

reaction never exceeding 2 mm maximal diameter in forearms of healthy controls. Allergic subjects displayed a mean 5.27 ± 1.23 mm diameter of reaction when tested with cypress-derived PLs, or with synthetic 18:2/18:2 PE, whereas there was a 1.4 ± 1.2 mm wheal and flare reaction with negative control reagents. Moreover, they did not respond to egg-, brain-, or liver-derived PL extracts (1.1 ± 0.6 mm). No positive reactions to pollen-derived or synthetic PLs were recorded in normal subjects or in *Mycobacterium*-exposed heath care workers (wheal and flare diameters 0.92 ± 0.43 mm; Fig. 7 b, left).

The presence of wheal and flare reactions against the same PLs that were recognized by CD1-restricted T cells suggested that these T cells might be responsible for the synthesis of PL-specific IgE antibodies. In fact, in 6 out of 14 allergic subjects there was a clear binding of circulating IgE to 18:2/18:2 PE-coated plastic wells, as revealed by the rise in O.D. by more than 2 SD above that of controls (0.177 ± 0.076). The average of resulting values was significantly higher in patients than in normal controls (Fig. 7 b, right).

DISCUSSION

Our studies have provided novel information concerning recognition of lipid antigens by human T lymphocytes, and, at the same time, they may shed new light on the mechanisms governing the allergic immune response. The first important finding is that PLs from pollen membranes play a crucial role in the early phases of the allergic immune response because they are involved in the capture of pollen by $CD1^+$ DCs, which is likely to be required for their subsequent recruitment of pollen-specific T cells. Nonbilayer PL arrangements (hexagonally packed cylinders of the hexagonal tubular phase) occur in ~40% of membrane lipids from pollens, particularly phosphatidylserine, cardiolipin, PE, and PC (20). Electron microscopy studies demonstrate that these nonbilayer structures appear as protuberances on the surface of the membrane (41). In this manner, acyl side chains of PLs are exposed and may thus enter the CD1 pocket, making the contact between pollen grain and DC stable. Although we have not studied the mechanism allowing pollen internalization by CD1 molecules in detail, this is the first example to our knowledge of antigen-presenting molecules that behave as receptors capable of binding to, and perhaps favoring the uptake of the presented antigens. This feature is likely the consequence of the unique capacity of CD1 molecules to bind and exchange lipids also on the cell surface (9, 12, 42) and of the particular structural features of PLs on the surface of pollen grain.

Obviously, CD1 molecules are unlikely to be the only receptors by which APC can interact with pollens. APCs

posses a wide array of molecules (including scavenger receptors, mannose receptors, chemokine, and adhesion molecule receptors, etc.) for binding to diverse ligands (3, 43). Pollen grains in general, or starch granules from grasses in particular, possess also glycosylated constituents (mannose residues, amylose, and amylopectine) in their external exine membrane, which may interact with some of these receptors. (44) That tree pollens in particular can use a CD1-dependent interaction with mucosal DCs to gain access to the deeper mucosal surfaces of allergic subjects is an intriguing possibility, but will require further study for confirmation. However, the recent demonstration that pollen-associated lipid mediators, such as phytoprostanes, can modulate in vitro some DC functions by inhibiting IL-12 p70 production and potentiating their Th2-polarizing capacity toward naive T cells seems to be in line with our observations and point out the relevance of lipid pollen fractions (either secreted or membrane associated) in influencing the adaptive DC-dependent immune response (45).

Our results suggest that PC 18:2/18:2, a predominant component of cypress PC, could be one of the specific targets in pollen grain capture and recognition by $CD1a^+$ DCs, as well as a stimulating antigen for some responding T cell clones from allergic subjects. There is increasing interest in studying lipid antigens recognized in the context of CD1a, particularly because the three-dimensional structure of this molecule has recently been determined by x-ray crystallography (46). In this regard, few CD1a-restricted clones have been described so far. One of these was autoreactive and derived from a patient with systemic lupus erythematosus, whereas a second $CD8^+$ T cell clone was specific for mycobacterial antigen, which has recently been identified as a lipopeptide (47). CD1a-restricted T cell clones that respond to the common mammalian sphingoglycolipid sulfatide have also been isolated from normal human subjects (10). More recently a series of double-negative TCR $\alpha\beta^+$ T cell clones have been described that recognize *Mycobacterium leprae*–derived nonpeptide antigens in the context of $CD1a^+$ Langerhans dendritic cells (48). Before our study, no CD1a-restricted PL-specific T cell clones had been reported, nor had membrane PL been demonstrated to be involved in CD1a-mediated pollen grain capture. Langerhans-derived DCs in the epidermis adopted the C-type lectin langerin, a pattern recognition receptor of the innate immune system, to capture sugar-containing nonpeptide molecules (42). The CD1-mediated PL binding and uptake described in the current study could represent an adaptive response of the immune system that facilitate this type of restriction in allergic subjects, because $CD1a^+$ or $CD1d^+$ DCs are uniformly absent in the airways of normal subjects (25).

Another interesting antigen specificity was noted toward PC 16:0/16:0, a species not present as a component of PL mixtures extracted from cypress pollen. This antigen specificity might represent a novel example of epitope spreading during the immune response (49), in which the initial reactivity against pollen PLs may recruit T cells with higher avidity for different PL antigens. The physiological roles of these T cells after encountering an environmental antigen at mucosal surfaces could be to protect the host from potentially dangerous dietary or inhaled products, as well as to restrain any tendency toward autoreactivity or damaging hypersensitivity, as demonstrated by the cytokine secretion pattern (similar to that of regulatory T cells) they acquire when pulsed with stimulating PLs (50). Alternatively, because PC 16:0/16:0 is the most abundant PL present in the pulmonary surfactant (51), it may become a cross-reacting autoantigen in allergic patients with important clinical consequences. Indeed, the PL composition of human respiratory surfactant is frequently altered during allergic respiratory diseases (52, 53). It is interesting to speculate that cross-reactivity between similar surfactant PL compounds containing palmitate acyl chains and allergen-triggered reactivity of mucosal T cells against PLs in asthmatic airways may perhaps explain the worsening of airway inflammation observed in asthmatic patients treated with natural porcine surfactant and then challenged in vivo with allergen (54).

There are few descriptions of CD1d-mediated recognition of PLs by T cells. It was previously noted that biotinylated PE 16:0/16:0 can bind human CD1d molecules on APC, although this did not lead to stimulation of CD1d-restricted NKT cell hybridomas specific for the glycolipid α-galactosyl-ceramide (55). One murine Vα14*i* NKT cell hybridoma reactive to CD1d transfected tumor cells was found to respond strongly to purified PE, PG, and PI, identified as potential self-antigens presented by mCD1d1 to regulatory NKT cells (26). More recently, the same NKT cell hybridoma was found to recognize also natural PEs of other cellular origins, including *Escherichia coli*, mammalian tissues, and soybean (18). Such recognition correlated with the degree of unsaturation of the PEs, with plant PE being the most potent activator. Our data confirm that plant-derived, unsaturated PE are the main PL antigens recognized by a variety of T cell clones derived from allergic subjects, which consist mainly of TCR $\alpha\beta^+$ $CD4^+$ clones. Therefore, the nature of the acyl chains in the lipid antigens contributes enormously to immunogenicity. This appears a general phenomenon, not limited to one TCR. Apart from the theoretical cross-reactivity with some constituents of human surfactant, such T cell clones display a limited reactivity toward PL extracted from mammalian tissues, such as liver- and brain tissue–derived PLs. Thus, autoreactivity, although not completely excluded, does not seems to be their major functional property.

The clones isolated from allergic subjects showed a tendency to produce both IFN-γ and IL-4, thus suggesting that they participate in both inflammatory and allergic responses. Regarding the latter, the observed in vitro IL-4 response to cypress-derived PLs by PBMC from allergic subjects, and the functional T helper activity for IgE production, seems to indicate that most PL-specific $\alpha\beta$ $CD4^+$ and $\gamma\delta^+$ T cell clones could be viewed as predominantly of the Th2-type.

Such a conclusion can be drawn not only on the basis of functional in vitro testing, but also in consideration of the presented evidence of cutaneous immediate reactivity toward cypress PLs and the demonstration that circulating IgE antibodies specific for these antigens do exist in allergic subjects. Such findings are in keeping with previous reports (56, 57) and, more generally, with helper activity by CD1-restricted T cells (58). The contribution of PL-specific T cells to allergic reactions in the lung of pollen-sensitive patients is not demonstrable with in vitro experiments, and therefore we can only speculate about their role in the pathogenesis of this disease. Lipid-reactive T cells, such as *i*NKT cells expressing the invariant TCR Vα14 have been associated with the establishment of allergic reactions in mouse experimental models of allergy (19, 38, 59). Whereas the ligands responsible for triggering iNKT cells in these models are not currently known, our studies have identified T cells with known antigen specificities that might have important functions. In the case that plant PL–specific T cells are activated in the lung, they could locally exert their effector functions. This is in keeping with the presence in the respiratory tract of asthmatic subjects of $CD1d^+$ and $CD1a^+$ DCs coexpressing the CD86 molecule (60, 61), and of $CD1d^+$ DCs in the lamina propria of the duodenal mucosa of cow's milk-sensitive subjects (62).

In conclusion, the CD1-restricted T cell clones described in the current study represent, to our knowledge, the first description of human T cell populations able to recognize naturally occurring foreign PLs associated with a strong environmental allergen and with a clinical evidence of allergic disease. CD1 molecules play a fundamental role in the presentation of microbial antigens, and a regulatory role for CD1d-restricted NKT cells has been strengthened by a number of recent studies (63, 64, 65). Now a new piece can be added to the puzzle, linking such T cell subpopulations to the control of host reactivity against autologous PLs and to environmental allergens, such as the lipids present in tree pollen membranes.

MATERIALS AND METHODS

Phospholipids. Unless stated otherwise, all PLs were obtained commercially and used without further purification. Dipalmitoyl-*sn*-glycero-3-phosphocholine (PC 16:0/16:0), 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (PC 16:0/18:2), 1-oleoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine (PC 18:1/16:0), 1,2-di[(cis)-9-octadecenoyl]-*sn*-glycero-3-phosphocholine (PC 18:1/18:1), dilinoleoyl-*sn*-glycero-3-phosphocholine (PC 18:2/18:2), 1-palmitoyl-2-linoleoyl-sn-glycero-3-phoshpho-rac-(1-glycerol) sodium salt (PG 16:0/18:2), and dilinoleoyl-*sn*-glycero-3-phosphoethanolamine (PE 18:2/18:2) were purchased from Avanti Polar Lipids, Inc. Dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (PE 16:0/16:0), 1,2-Di[(cis)-9-octadecenoyl]-*sn*-glycero-3-phosphoethanolamine (PE 18:1/18:1), and 1-hexadecanoyl-2-[(cis,cis)-9,12-octadecadienoyl]-*sn*-glycero-3-phosphoethanolamine (PE 16:0/18:2) were purchased from Sigma-Aldrich. Naturally occurring PC and PE from egg yolk, liver, or brain tissue were obtained from Avanti Polar Lipids, Inc. All PLs suspensions were prepared in absolute ethanol.

Preparation of cypress pollen lipids. Lipids were extracted from pollen after the procedure of Folch et al. (66). The methanol/chloroform extract was evaporated nearly to dryness under N_2 and chromatographed on silica gel G TLC plates, using chloroform/methanol/water (65: 25: 4 by vol), as the solvent. Phospholipid classes were separated by two-dimensional TLC (6.5 × 6.5 cm; PE SIL G 250 μm; Whatman) with (a) chloroform/methanol/1.6 M ammonia (70:30:5, by volume) and (b) chloroform/methanol/acetone/acetic acid/water (75:15:30:15:7.5 by volume). Spots were revealed by staining with dichlorofluorescein, scraped off the plate, and eluted with chloroform/methanol/acetic acid/water (50:39:1:10 by volume). Samples were freed from dichlorofluorescein by washing three times with 3 ml of 4 N ammonia and three times with methanol/water (45:10 by vol). Trace amount of silica gel were removed by centrifugation. Finally, PLs were quantified by phosphorus assay after digestion with 70% perchloric acid (67).

Electrospray ionization mass spectroscopy of cypress pollen PLs. ESI mass spectrometric analysis was performed on a triple-quadruple tandem mass spectrometer (Quattro *Ultima*; Micromass) equipped with an ESI interface. Total PL extracts were dissolved in 25 μl of methanol/chloroform/water/NH_4OH (7:2:0.8:0.2, vol/vol) for single stage and tandem MS analysis of PC and PE and were analyzed by nanospray ESI–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 (3.5×10^{-3} mbar). All data was recorded at mass resolution, as a signal average of 10 scans/collection, with a scan time of 12 s.

PC species were detected by positive ionization, whereas PE was preferentially detected using negative ionization. After fragmentation with argon gas, PC molecules produced a fragment with m/z 184^+, corresponding to the protonated phosphocholine headgroup, and precursor scans of the m/z184 moiety provided diagnostic determination of PC. Identities of PE species were determined by neutral loss scans of phosphoethanolamine (m/z 141^+). The predominant molecular species present for each ion peak resolved was determined by analysis of fatty acyl fragments generated by collision gas-induced fragmentation under negative ionization. The major precursor ion for PC in negative ionization represented loss of one methyl group $[M\text{-}15]^-$, whereas for PE the molecular ion $[M\text{-}H]^-$ was fragmented.

Study subjects and cell sources. 14 cypress-sensitive subjects (eight males and six females, age range 19–45 yr) were enrolled in the study on the basis of the concordance between their clinical history (January to March rhinoconjunctivitis and/or asthma) and the results of cutaneous skin prick tests and serum specific IgE levels, as detected with commercial reagents. We avoided enrollment of sensitive subjects who clinically experienced symptoms also in other months of the year, indicating a broader allergen sensitization (including dust mites, *Parietaria officinalis*, or *Graminaceae*). This selection is in line with the epidemiological characteristics of cypress-sensitive adult population in the Mediterranean area (32), in which 84% of cases with the above-mentioned characteristics are represented by monosensitive subjects with scarce evidence of atopic inheritance in their families and in which symptoms related to cypress allergy often develop in adult age. Controls were represented by two distinct groups of age-and sex-matched subjects: 10 health care workers with documented exposure to and latent infection with *M. tuberculosis* and 10 healthy subjects without clinically evident allergic diseases.

BAL samples from five cypress-sensitive, nine adjunctive dust mite-sensitive asthmatic subjects, and from 10 normal volunteers were used as sources of pulmonary macrophages/dendritic cells for morphological and immunocytochemical studies. Informed consent to all invasive procedures was obtained from participants and the study was approved by the local Ethical Committee.

Peripheral blood from the cypress-sensitive subjects, health care workers, and 10 normal controls were repeatedly collected during 1 yr. Processed samples were centrifuged over a Ficoll-Hypaque density gradient and mononuclear cells resuspended in RPMI 1640 supplemented with 10% FCS (GIBCO BRL). Circulating adherent monocytes were differentiated in vitro into DCs by culturing with rhGM–CSF (25 ng/ml; EuroClone) and recombinant human interleukin 4 (rhIL-4; 200 U/ml; EuroClone) for 5 d and used as $CD1^+$ APC.

Evaluation of pollen grain–dendritic cell interactions. Immunophenotyping was performed on BAL mononuclear cell suspensions, as previously described (39), by means of the following mAbs: purified anti–human CD11c (clone BU15; Serotec), CD80 and CD86 (clones BB1 and BU63; Ancell), followed by fluorescein-conjugated goat anti–mouse Ab of appropriate isotype controls (Southern Biotechnology Associates, Inc.). For morphological observation, cytospin preparations of BAL mononuclear cell suspensions (0.5×10^6/ml) were stained with May-Grünwald-Giemsa, whereas for immunocytochemistry preparations CD1d molecules were detected by labeling with anti-CD1d mAb (clone NOR3.2; Serotec) at working dilution of 0.2 μg/ml, and further developed by a three-stage immuno–alkaline phosphatase/antialkaline phosphatase technique, as previously described (39).

Monocyte-derived DC (10^6/ml) were incubated on polylysine-treated coverslips with cypress pollen (5×10^4 grains/ml) for 30 min at 37°C. Adherence of grains to the cells was scored at 30 min. For each preparation, 500 cells were counted by each of three independent observers, who were blinded to the identity of the samples, and the percentages ± SD of cells with bound grains were calculated. Phase-contrast images were obtained with an Olympus AX70 microscope through a SPOT-RT cooled color digital camera (Diagnostic Instruments) with the SPOT-RT 3.1 software. For receptor-mediated inhibition of pollen grain adhesion, the DC suspensions were preincubated with the following purified mAbs (20 μg/ml): anti–human CD1a (clone NA1/34), CD1b (clone M-T101), CD1c (clone L161), and CD1d (clone NOR3.2; all from Serotec), anti-MHC class I and II (clones G46-2.6 and G46-6; BD Biosciences). For receptor blockade, cypress-derived PLs were administered to dendritic cells at various concentrations, ranging from 0.1 to 10 μg/ml. Subsequent pollen adhesion was evaluated described before. In some adhesion experiments the HeLa cell line, mock, or CD1d transfected (14), was adopted with the same experimental procedure described above for DC. The time-course of pollen grain ingestion was evaluated on dendritic cells incubated at 37°C with 4-[4-(dihexadecylamino)styryl]-*N*-methylquinolinium iodine (DiQ; Molecular Probes) at the labeling concentration of 1 μM for 45 min. Images were obtained using a confocal apparatus (MRC-1024; Bio-Rad Laboratories, Inc.) equipped with an krypton-argon laser on Olympus IMT-2 inverted microscope using a 60×, 1.4 NA objective. 0.1 mM fluorescein diacetate (Molecular Probes) in PBS was adopted for labeling pollen grain suspensions.

Generation of PL-specific T cell lines and clones. $0.5–1 \times 10^6$ nonadherent PBMC per ml from each sample (the 14 cypress-sensitive and the 10 healthy subjects) were cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine (GIBCO BRL), 20 μM 2-mercaptoethanol, 100 U/ml penicillin, 50 μg/ml streptomycin (complete medium), and 10% FCS with autologous dendritic cells in 24-well flat-bottomed culture plates (Costar Corporation) in a final volume of 3 ml. A total of six wells per subject were run at each collection time point during one year (i.e., every month from December to March, and every two months thereafter). Cells were exposed to TLC-purified cypress-derived PC and PE (10 μg/ml) without adding cytokines or growth factors and restimulated every 2 wk with $CD1^+$ autologous DC plus cypress PL extracts. PBMC of control *M. tuberculosis*–exposed health care workers were cultured as above with 10 μg/ml mycolic acid (Sigma-Aldrich) dissolved in absolute ethanol. Proliferating lines were further expanded with recombinant human interleukin 2 (rhIL-2; 50–100 U/ml; EuroClone) and then tested in vitro for antigen specificity, as previously described in detail (15).

For limiting-dilution cloning of PL-specific T cell lines, T cell blasts were seeded at 0.5, 1, and 2 cells per well in 96-well round-bottomed plates (Nunclone) with 10^5 irradiated (5,000 rads) autologous T cell–depleted PBMC as feeder cells, 1:2,000 (final dilution) PHA-P (Difco) and rIL-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. To expand established clones, T cell blasts were restimulated every 3 wk with 0.1% PHA-P and irradiated autologous feeder cells.

Immunophenotype of emerging clones was detected by flow cytometry with the following mAbs: Cy-chrome–conjugated anti-CD4 (clone RPA-T4; BD Biosciences), phycoerythrin-conjugated anti-Vβ11 (clone C21; Serotec), fluorescein (FITC)-conjugated anti-CD161 (NKR-P1A Ag, clone DX12; BD Biosciences), anti-CD8 (clone LT8; Serotec), anti-αβ (clone BMA031; Serotec), anti Vα24 (clone C15; Serotec), anti-pan TCR γδ (clone 5A6.E9; T Cell Diagnostics) or -Vγ9 (clone 7A5; T Cell Diagnostics), -Vδ1, and -Vδ2 (clones TS-1 and 15D; all from T Cell Diagnostics). In some experiments three-color immunofluorescence data were analyzed on gated Cy-chrome-$CD4^+$ cells. Forward and side light scatter was used to optimize gating of lymphocytes, and fluorescence analysis was performed using Lysis II software (Becton Dickinson)

Functional characterization of PL-specific T cells. For evaluation of CD1 restriction, T cell lines and clones were stimulated in the presence or absence of mAbs directed toward single CD1 epitopes (i.e., anti-CD1a, b, c, and d; Serotec) at working concentration of 20 μg/ml. Control experiments were performed with mouse IgG, anti-MHC class I and II mAbs (as mentioned in the Evaluation of pollen grain–dendritic cell interactions section). Stable CD1 transfectants of cervical carcinoma cell line HeLa or C1R lymphoblastoid B cell line expressing CD1a, CD1b, CD1c, or CD1d and CD1-negative mock transfectants, have been previously described (68). Mitomycin C–treated (1 h at 37°C with 150 μg/ml; Sigma-Aldrich) or irradiated (50 Gy) CD1 transfectants (10^5 per well) were seeded with cloned T cells (2×10^4 per well) in a 96–round-bottom plate (Costar) in 0.2 ml medium alone or containing either PC or PE (from 1 to 20 μg/ml). After culturing for 96 h in 5% CO_2 atmosphere, wells were pulsed with 1 μCi of [^{3}H]thymidine and harvested 16 h later for β scintillation counting using a scintillation counter (Beckman Coulter). Results were expressed as mean cpm (± SD) of triplicate culture wells.

For experiments examining PL presentation by fixed APCs, mock- or CD1d-transfected HeLa cells were incubated for 1 h on ice with 2 ml 0.9% NaCl containing 75 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide-HCl (ECDI; Pierce Chemical Co.) followed by extensive washing in RPMI 1640.

Specific response and CD1 restrictions were evaluated by [^{3}H]thymidine incorporation, as above, after 5 d of culture, and/or by measurement of IL-4 (Immunotech) and IFN-γ (R & D Systems) production using commercial kits with supernatants recovered after 48 h.

For measurement of helper activity for in vitro IgE production, T cell clones (10^5 cells per well) were cocultured with autologous T cell depleted PBMC (10^5 per well) as a source of B cells for 10 d in a final volume of 0.2 ml, and IgE levels were measured in the supernatants of stimulated samples by capture ELISA. The mouse IgM for human IgE capture (clone HP 6061) was purchased from Southern Biotechnology Associates, Inc. Immunoplates (Nunc) were coated overnight at 4°C with the capture antibody diluted in 0.1 M bicarbonate buffer. Blocking was performed by adding 2% bovine serum albumin/Tris-buffered saline for 1 h, followed by several washings. Supernatants and internal standards were then added to the wells and incubated in duplicate for 2 h. After several washes, the biotinylated anti–human IgE detection mAb (clone HP6029; Southern Biotechnology Associates, Inc.) was added. Plates were incubated for another hour with alkaline phosphatase–conjugated streptavidin. After the final reaction with phosphatase substrate (Sigma-Aldrich), 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 nanograms/milliliter.

In vivo evaluation of IgE reactivity against pollen PLs. Purified cypress-derived and synthetic PLs were dissolved in absolute ethanol and used for skin prick tests at the working concentration of 10 μg/ml. 100 μl of PL solution was applied over the skin of forearms of allergic and control subjects, and, after puncturing with a sterile lancet, the mean wheal and flare reaction maximal diameters (mm ± SD) were measured after 15 min.

Circulating specific IgE to PLs were measured by an ELISA assay. In brief, half the wells of microtiter ELISA plates (Polysorp; Nunc) were

coated with each of the following anionic PLs at 50 μg/ml: synthetic dilinoleoyl-*sn*-glycero-3-phosphoethanolamine (PE 18:2/18:2, from Avanti Polar Lipids, Inc.) and cypress-derived PE in ethanol, the other half with ethanol alone, and air dried overnight at 4°C. After blocking with 1% serum albumin (Sigma-Aldrich) in Tris for 1 h at 37°C and three washes with PBS, serum diluted 1:10 in 1% Tris/albumin was added in duplicate to both PL-coated and -uncoated halves of the plates. After incubation for 3 h at 37°C and three washes with PBS, alkaline phosphatase-conjugated goat anti–human IgE (Southern Biotechnology Associates, Inc.) was added at 1:1,000 dilution, for 1 h at 37°C. Color was developed by adding dinitrophenyl phosphate (1 mg/ml [Sigma-Aldrich] solution containing 1 M $MgCl_2$ in bicarbonate buffer, pH 9.6, after three washes with PBS and two with bicarbonate buffer. The plates were incubated at 37°C and read after 1 h at 405 nm (with reference 490 nm). Background values were obtained from wells containing no antigen, and their absorbances subtracted from all sample readings. The results were expressed as a mean OD. Values >2 SDs above the mean of healthy controls were deemed to be positive.

Statistical analysis. Study populations were stratified according to age and sex. Statistical comparison in dendritic cell–pollen adhesion experiments and in T cell proliferation assays were calculated by the Mann-Whitney U test. We used Kruskall-Wallis one-way analysis of variance (with a significance level of 0.05) for statistical evaluation of the differences in cytokine production, IgE secretion by different T cell clones and circulating specific IgE assay. The Statistical Package for the Social Sciences (SPSS, Inc.), version 11.0, was used for all statistical computations.

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