

# *Olea europaea* pollen lipids activate invariant natural killer T cells by upregulating CD1d expression on dendritic cells

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**Background:** Invariant natural killer T (iNKT) cells recognize lipids presented by CD1d and have been implicated in the pathogenesis of allergic asthma. Recognition of plant pollen lipids by iNKT cells and their role in allergic responses are poorly defined.

**Objective:** Our goal was to investigate whether iNKT cells can be activated by monocyte-derived dendritic cells (DCs) exposed to lipid antigens from *Olea europaea*.

**Methods:** DCs generated *in vitro* were exposed to *O. europaea* pollen grains or lipids isolated from them. Expression of lipid-presenting molecules (CD1), as well as maturation markers (HLA-DR, HLA-I, CD86, and CD80 molecules), on DCs was analyzed. iNKT cell activation after coculture with DCs was evaluated based on expansion, cytokine production, and cytotoxicity tests.

**Results:** DCs upregulated CD1d and CD86 expression and downregulated CD1a expression after exposure to a whole extract of olive pollen lipids. CD1d and CD1a were regulated at the transcriptional level in a peroxisome proliferator-activated receptor  $\gamma$  activation-dependent manner. Polar lipids, diacylglycerols, free fatty acids, and triacylglycerols isolated from pollen grains upregulate CD1d. The increase in CD1d expression on the DC cell surface induced by polar lipids was not regulated at the RNA level. iNKT cells efficiently recognize DCs treated with the different lipids isolated from olive pollen grains.

**Conclusions:** Lipids from *O. europaea* pollen upregulate CD1d and CD86 molecules on DCs, which are then able to activate iNKT cells through a CD1d-dependent pathway. (J Allergy Clin Immunol 2012;■■■:■■■-■■■.)

**Key words:** Pollen lipids, *Olea europaea*, dendritic cells, invariant natural killer T cells, CD1d, peroxisome proliferator-activated receptor  $\gamma$

## Abbreviations used

DC: Dendritic cell  
FFA: Free fatty acid  
iDC: Immature dendritic cell  
iNKT: Invariant natural killer T  
PPAR $\gamma$ : Peroxisome proliferator-activated receptor  $\gamma$

Natural killer T cells are CD1d-restricted T lymphocytes that recognize lipid antigens presented by CD1d molecules. Invariant natural killer T (iNKT) cells are characterized by the use of a restricted T-cell receptor repertoire: V $\alpha$ 24/J $\alpha$ 18, pairing with preference with the V $\beta$ 11 chain.<sup>1</sup> iNKT cells are cytotoxic and able to secrete both T<sub>H</sub>1 and T<sub>H</sub>2 cytokines. The most potent activator of iNKT cells is  $\alpha$ -galactosylceramide.<sup>2</sup> In addition, a number of exogenous and endogenous glycolipid ligands have been described to be presented by CD1d to iNKT cells.<sup>3,4</sup> iNKT cells are considered regulatory T cells, which can be important in infections,<sup>5</sup> antitumor responses, autoimmunity, and inflammation, including allergic responses.<sup>6</sup>

Studies performed in experimental allergic asthma models suggest a critical function of iNKT cells in the development of allergen-induced airway hyperreactivity independent from conventional CD4<sup>+</sup> T cells.<sup>7-9</sup> In contrast, it has been described that iNKT cells are dispensable for T cell-dependent allergic airway inflammation.<sup>10</sup> In human subjects some investigators have reported high levels of iNKT cells in the lungs of asthmatic patients.<sup>11-17</sup> By contrast, other groups did not observe significant differences in iNKT cell levels between healthy control subjects and asthmatic patients.<sup>18-22</sup>

Responses of CD1a- and CD1d-restricted T cells have been described against phospholipids extracted from cypress pollen in allergic patients.<sup>23</sup> Activation of  $\gamma\delta$  T lymphocytes from allergic patients against pollen-derived phosphatidylethanolamine in a CD1d-restricted manner has been observed.<sup>24</sup> In addition, isoprostanes (lipid plant hormone precursors), which are present in pollens, have been described to inhibit IL-12 human dendritic cell (DC) production and induce T<sub>H</sub>2 polarization in naive T cells.<sup>25</sup>

DCs might play an important role in presenting lipid antigens to T cells and natural killer T cells<sup>26</sup>; however, not much is known about the effect of lipids on DC activation or maturation and how they are recognized by human natural killer T cells in allergic responses.

In the present work we analyzed the phenotypic changes observed in DCs when exposed to pollen lipids derived from *Olea europaea* (olive) and also their recognition by human iNKT cells.

## METHODS

### Olive pollen lipid preparation

Lipids from olive pollen grains (ALK-Abelló, Hørsholm, Denmark) were extracted after diethyl ether treatment, collecting the supernatant after

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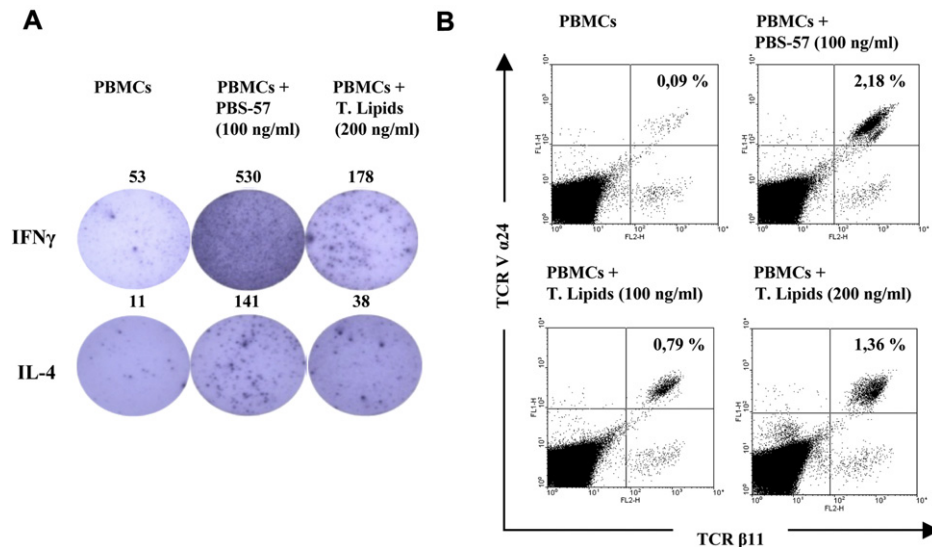


**FIG 1.** iNKT cell activation by olive pollen lipids. **A**, Numbers represent spot-forming cells detected by using the ELISpot. For IFN- $\gamma$ , the mean was  $30 \pm 16$  in control DCs versus  $151 \pm 31$  in olive pollen lipid-treated DCs. For IL-4, the mean was  $8 \pm 2$  in control DCs versus  $32 \pm 5$  in lipid-treated DCs. **B**, iNKT cell expansion as measured by percentages of double-positive cells:  $0.16 \pm 0.13$  in control DCs versus  $1.6 \pm 1.7$  in olive pollen lipid-treated (200 ng/mL) PBMCs. One representative experiment of 7 is shown. *TCR*, T-cell receptor; *T. Lipids*, total lipids.

incubation and centrifugation of pollen grains. Extracts were evaporated nearly to dryness in an  $N_2$  atmosphere. Subsequently, lipids were dissolved in chloroform/methanol (2:1 by volume) and chromatographed on Silica Gel G (Merck Millipore, Darmstadt, Germany), thin-layer chromatographic plates by using chloroform/methanol/water (70:30:1 by volume) as a solvent. Bands were revealed by staining with iodine, scraped off the plate, and eluted by washing with chloroform and methanol. The relative mobility of each band was used to identify crude fractions that were polar lipids, diacylglycerols, free fatty acids (FFAs), and triacylglycerols. Total and polar lipid levels were quantified by using the phosphorus assay.<sup>27</sup>

### Aqueous pollen extracts

Aqueous pollen extracts were generated by incubation of pollen grains in RPMI-1640 (30 mg/mL) for 30 minutes at 37°C, followed by centrifugation (for 20 minutes at 3345g) and sterile filtration.<sup>25</sup>

### Lipid-free pollen extracts

Lipid-free pollen extracts were generated by means of incubation of pollen grains with diethyl ether (0.5% wt/vol) by using stirring on ice for 3 hours. Subsequently, samples were dried in a hood. The defatted olive pollen was homogenized in 50 mmol/L ammonium bicarbonate and 1 mmol/L phenylmethylsulfonyl fluoride, and the proteins were quantified by means of bicinchoninic acid assay.

### Human iNKT cell expansion and isolation

iNKT cells were expanded from total PBMCs by treatment with 100 U/mL rhIL-2 in the presence of PBS-57 (100 ng/mL)<sup>28</sup> for 2 weeks. iNKT cells (V $\alpha$ 24<sup>+</sup>V $\beta$ 11<sup>+</sup> cells) were positively selected by using magnetic separation (Miltenyi Biotec, Bergisch Gladbach, Germany).

### Cytotoxicity assay

Cytotoxicity was measured with the nonradioactive Cytotoxicity Detection Kit LDH (Roche, Mannheim, Germany), according to the manufacturer's instructions. All cytotoxicity assays were performed in triplicate. iNKT cells were used as effector cells, and untreated or treated immature dendritic cells

(iDCs) were used as target cells. A PBS-57–positive control was added to iDCs at 100 ng/mL.

For information on real-time quantitative RT-PCR, measurement of cytokines by means of ELISA and ELISpot, antibodies, flow cytometric analysis, immunofluorescence, DC generation, olive pollen lipid treatment, and statistical analysis, see the [Methods](http://www.jacionline.org) section in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org).

## RESULTS

### Whole lipid extract from olive pollen is able to activate and stimulate iNKT proliferation from PBMCs

To determine whether lipids from olive pollen were able to stimulate human T cells, we first isolated PBMCs from healthy blood donors that were subsequently treated *in vitro* with the whole lipid extract from olive grain pollen, and the production of IFN- $\gamma$  and IL-4 was evaluated by using ELISpot. Our results demonstrated that both IFN- $\gamma$  and IL-4 levels were increased in pollen lipid-treated PBMCs (Fig 1, A). To investigate whether iNKT cells were activated by the whole lipid fraction, we assessed the expansion of T-cell receptor V $\alpha$ 24/V $\beta$ 11<sup>+</sup> T cells in the PBMC cultures. As shown in Fig 1, B, we observed a significant expansion of likely iNKT cells in response to pollen lipids.

### Olive pollen grains activate DCs *in vitro*

DCs are probably the main antigen-presenting cells for iNKTs, and thus we decided to analyze the interaction between intact olive pollen grains and DCs. We observed iDC adhesion to pollen grains in just 15 minutes after coincubation, with an increase in the number of interacting cells over time (Fig 2, A). In this *in vitro* system we analyzed the surface expression of CD1 molecules and DC maturation markers. After 48 hours

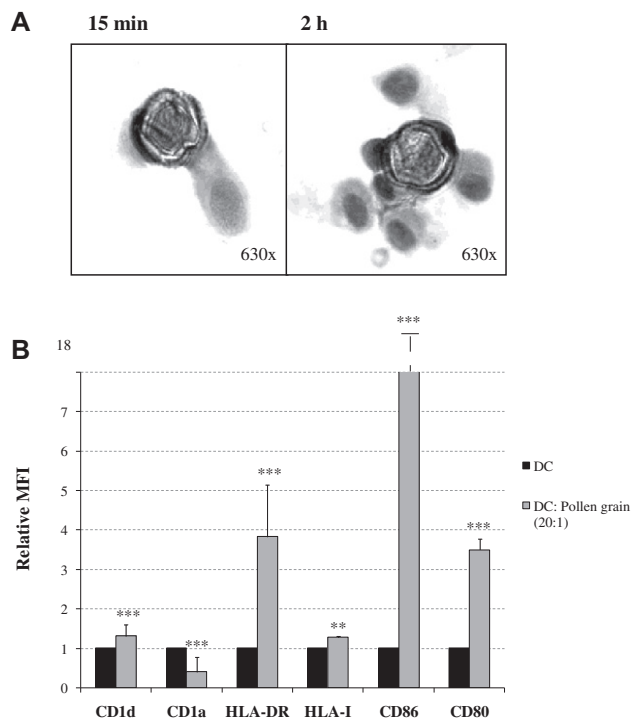


**FIG 2.** Interactions between olive pollen and DCs. **A**, Pollen capture *in vitro* at 15 minutes and 2 hours. **B**, Mean fluorescence intensity (MFI)  $\pm$  SD normalized to control cells (CD1d,  $n = 12$ ; CD1a,  $n = 7$ ; CD86 and HLA-DR,  $n = 6$ ; and HLA-I and CD80,  $n = 3$ ). \*\* $P < .03$  and \*\*\* $P < .01$ .

in culture, we found that DCs displayed a statistically significant increase in CD1d, HLA-DR, HLA class I, CD80, and CD86 expression and a statistically significant decrease in CD1a expression in the presence of olive pollen grains (Fig 2, B).

### iDCs treated with olive pollen lipids are able to activate iNKT cells

Lipid-treated or untreated iDCs were cocultured with previously isolated iNKT cells and cytotoxic killing assays were carried out to assess whether iDCs could activate iNKT cells after treatment with olive pollen lipids. We found that iNKT cells efficiently lysed iDCs treated with olive pollen lipids, as shown in Fig 3, A, whereas the lysis detected with iDCs treated with pollen aqueous extract was similar to that seen in control specimens. In addition, we observed an increase in the production of IFN- $\gamma$  and IL-4 in response to pollen lipid-treated iDCs (Fig 3, B), which was comparable with that observed when cells were exposed to PBS-57.

### Olive pollen lipids increase surface CD86 and CD1d expression on iDCs

We decided to study the phenotypic and functional effects of pollen lipids on monocyte-derived iDCs. After exposure to pollen lipids, DCs became flat and enlarged, suggesting their activation (see Fig E1 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). We found that cell-surface levels of CD86 were increased in the lipid-treated iDCs when compared with those seen in nontreated or aqueous extract-treated iDCs. This

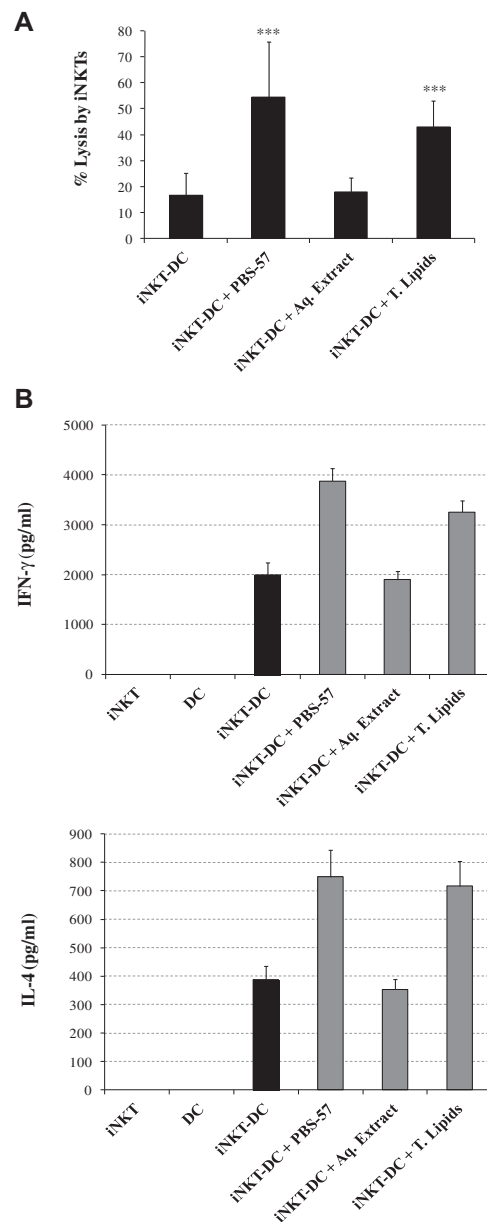


**FIG 3.** Isolated iNKT cells are activated by olive pollen lipids. **A**, DC lysis by iNKT cells. Mean percentage of lysis  $\pm$  SD is represented ( $n = 10$ ). **B**, IFN- $\gamma$  and IL-4 detection by iNKT cells in response to DCs exposed to lipids. Total lipids (T. Lipids) and PBS-57 were added at 100 ng/mL. Results represent triplicate samples  $\pm$  SD from a representative experiment ( $n = 3$ ). \*\*\* $P < .01$ . Aq. Extract, Aqueous extract.

suggests the induction of a semimaturational state on these DCs because the rest of the maturation markers (CD80, HLA-DR, and HLA class I) were not modified, as observed in Fig 4, A. Interestingly, CD1d expression was dramatically increased in the presence of pollen lipids; in contrast, CD1a expression underwent a significant decrease compared with that seen in untreated cells, resembling what happened when the iDCs were exposed to olive pollen grains. When DCs were exposed to a lipid-free extract of pollen, we observed DC maturation, displaying an increase in HLA-DR, CD86, and CD80 expression without inducing changes in the expression of CD1 molecules, as shown in Fig 4, A. This is in contrast to what we observed in the pollen grains

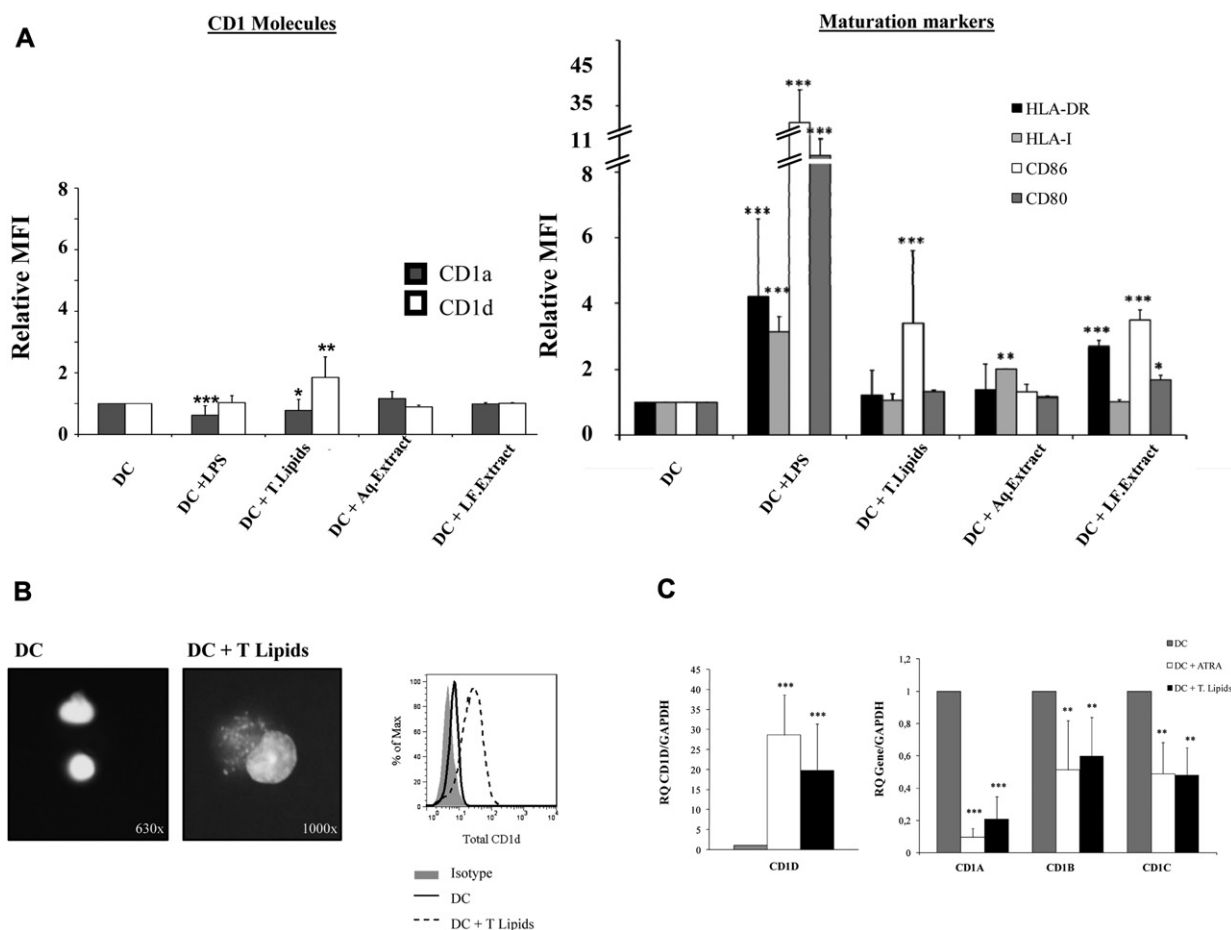


**FIG 4.** Olive pollen lipid-treated DCs. **A**, Relative mean fluorescence intensity (MFI)  $\pm$  SD in independent experiments (CD1d,  $n = 24$ ; CD1a, CD86, and HLA-DR,  $n = 17$ ; CD80 and HLA-I,  $n = 6$ ). **B**, Total CD1d protein levels measured by means of fluorescence microscopy showing control cells (DCs; left) and DCs exposed to lipids (right) and flow cytometry (far right). **C**, Relative quantification (RQ; mean  $\pm$  SD) of CD1D ( $n = 8$ ), CD1A ( $n = 6$ ), CD1B, and CD1C ( $n = 4$ ) gene expression. Aq. Extract, Aqueous extract; LF. Extract, lipid-free extract; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; T. Lipids, total lipids. \* $P < .05$ , \*\* $P < .03$ , and \*\*\* $P < .01$ .

in which there was an upregulation in both CD1d and HLA-DR expression.

### Olive pollen lipids increase total protein and mRNA levels of CD1d in iDCs

To investigate the mechanisms causing the increased surface expression of CD1d by olive pollen lipids, we first examined whether the higher CD1d surface expression was due to an increase in total (intracellular and extracellular) protein levels. Thus CD1d intracellular staining was assessed by means of flow cytometry and fluorescence microscopy. As shown in Fig 4, B, total CD1d levels were increased in pollen lipid-treated iDCs compared with those seen in untreated cells. We also carried out real-time PCR to evaluate the levels of CD1D, CD1A, CD1B, CD1C, and HLA-DR mRNA. All-trans retinoic acid was used as a positive control because it upregulates CD1d gene expression. Olive pollen lipid-treated cells expressed significantly higher mRNA levels for CD1D than control cells, whereas they displayed decreased CD1A, CD1B, and CD1C levels (Fig 4, C). The expression of HLA-DR mRNA was lower in the

DCs treated with lipids and especially in the DCs matured with LPS (see Fig E2 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)).

### CD1d upregulation in olive pollen lipid-treated iDCs and their capacity to stimulate iNKT cells are linked with peroxisome proliferator-activated receptor $\gamma$ activation

Stimulation of the lipid-activated transcription factor peroxisome proliferator-activated receptor (PPAR $\gamma$ ) turns on retinoic acid synthesis by inducing enzymes that increase intracellular all-trans retinoic acid from retinol, inducing the transcription of CD1d, whereas CD1a levels are decreased<sup>29</sup>; furthermore, PPAR $\gamma$  is activated by fatty acyl compounds.<sup>30</sup> Therefore we decided to investigate whether lipid components of olive pollen, which is rich in unsaturated fatty acids, were associated with the upregulation of CD1d through PPAR $\gamma$  activation. To this end, we used GW9662, a selective irreversible PPAR $\gamma$  antagonist. Rosiglitazone, a PPAR $\gamma$  agonist, was used as a positive control. As we previously observed, pollen lipid-treated iDCs expressed

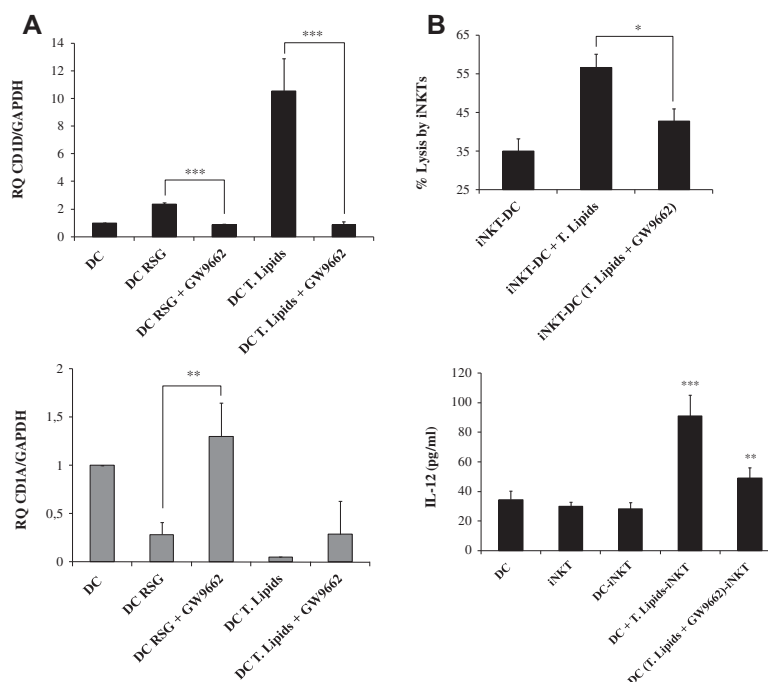


**FIG 5.** The PPAR $\gamma$  antagonist GW9662 reverses CD1d upregulation and natural killer T cell activation induced by pollen lipids in DCs. **A**, Relative quantification (RQ) of CD1D and CD1A transcript levels in pollen lipid-treated DCs in the presence or absence of GW9662 (mean  $\pm$  SDs,  $n = 4$ ). **B**, Activation of iNKT cells: cytotoxicity and induction of IL-12 production cells by DCs in the presence or absence of GW9662 (mean  $\pm$  SDs,  $n = 3$ ). GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; RSG, rosiglitazone; T. Lipids, total lipids. \* $P < .05$ , \*\* $P < .03$ , and \*\*\* $P < .01$ .

increased levels of CD1d on the cell surface, whereas CD1a expression was decreased in comparison with that seen in control cells, and the presence of GW9662 partially restored CD1d and CD1a surface expression in pollen lipid-treated iDCs (Fig 5, A). Surface protein levels correlated with transcriptional changes in CD1A and CD1D levels (see Fig E3 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)).

### Analysis of olive pollen lipids

We performed separation of the whole lipid components by using thin layer chromatography on silica plates in which we identified the most important classes of lipids in olive pollen<sup>31</sup>: polar lipids, diacylglycerols, FFAs, and triacylglycerols (see Fig E4 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)).

iDCs were cultured for 48 hours in the presence of each type of lipid, and cell-surface expression of CD1a, CD1d, HLA-DR, and CD86 was tested by using flow cytometry. Each lipid fraction (phospholipids, diacylglycerols, FFAs, and triacylglycerols) was able to upregulate CD1d protein expression on the cell surfaces of DCs (Fig 6, A). CD86 showed a significant increase in polar lipid-treated DCs, but no changes were found in CD1a and HLA-DR expression. Interestingly, we also observed a significant increase in the transcriptional expression of CD1D in DCs cultured with FFAs, whereas polar lipid-treated iDCs did not show a significant augmentation of CD1D mRNA levels compared with levels seen in control cells (Fig 6, B), indicating that the increased expression of CD1d by polar lipids derived from olive pollen is not regulated at the RNA level, unlike FFAs present in the whole lipid extract. Furthermore, the CD1d surface expression in polar lipid-treated DCs was not dependent on PPAR $\gamma$  activation, as experiments

with its antagonist GW9662 indicate, because treatment with GW9662 did not reverse the CD1d upregulation produced by polar lipids on the DC surface in contrast with the inhibition observed when whole lipid extract or the FFA fraction derived from olive pollen were used (Fig 6, C). In addition, we found that DCs could activate iNKT cells after treatment with each lipid fraction, as shown by using cytotoxic killing assays in which iNKT cells significantly lysed polar lipid- and FFA-treated DCs in comparison with control cells (Fig 6, D).

In summary, our results indicate that the different lipids of olive pollen can affect the phenotype of DCs, inducing CD1d expression, which results in iNKT activation.

### DISCUSSION

Little is known about the possible recognition of lipids present in pollen grains and its capacity to stimulate iNKT cells. Our data showed an activation of iNKT cells exposed to olive pollen lipids *in vitro*. DCs are probably the main antigen-presenting cells for iNKT cells *in vivo*, and previous studies have implicated them in the bronchial mucosa during the allergic response.<sup>23,32-34</sup> In our *in vitro* culture system monocyte-derived DCs were able to surround and enclose olive pollen grains, with their plasmatic membranes subsequently displaying a dramatic increase in maturation markers, such as CD86, CD80, and HLA-DR, probably as a consequence of DC activation through receptors recognizing molecular structures on the pollen grains other than lipids. After the encounter with pollen grains, the DCs display an increase in CD1d expression together with a CD1a downregulation when compared with that seen in immature DCs. Although no digestion of the pollen grains was

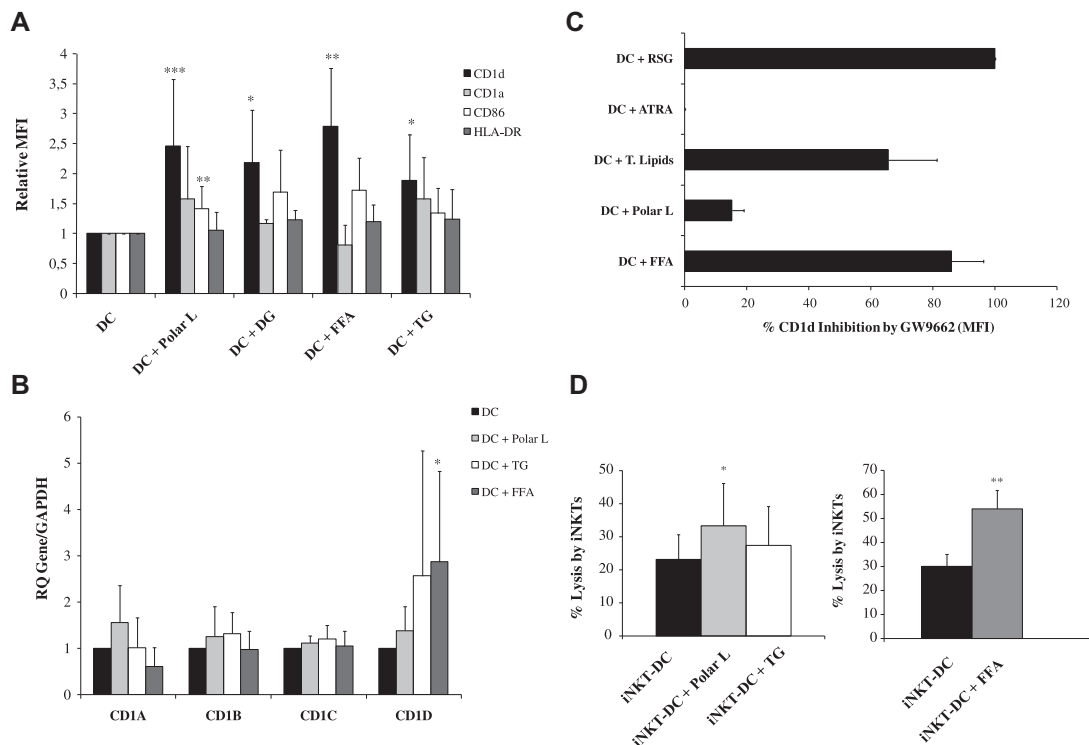


**FIG 6.** Pollen lipid species-treated DCs. **A**, Relative mean fluorescence intensity (MFI)  $\pm$  SD (CD1d and CD1a,  $n = 10$ ; CD86 and HLA-DR,  $n = 5$ ) on treated or untreated DCs determined by means of fluorescence-activated cell sorting. **B**, Relative quantification (RQ) of CD1 mRNAs by using real-time PCR (means  $\pm$  SDs of CD1d,  $n = 7$ ; CD1a,  $n = 3$ ). **C**, Percentage of CD1d inhibition (MFI)  $\pm$  SD by GW9662 in DCs with or without total lipids, FFAs, or polar lipids. Results are normalized to rosiglitazone-treated cells. **D**, The mean percentage of lysis  $\pm$  SD is represented for polar lipid- and triacylglycerol (TG)-treated DCs ( $n = 5$ ) and for FFA-treated DCs ( $n = 3$ ). ATRA, All-trans retinoic acid; Polar L, polar lipids; RSG, rosiglitazone; T. Lipids, total lipids. \* $P < .05$ , \*\* $P < .03$ , and \*\*\* $P < .01$ .

observed, it is possible that lipids from the exine or from the pollen coat that fills the exine cavities might interact with DCs or that small fragments from the pollen grains could be released and endocytosed by the DCs. It is worth noting that exposure of DCs to lipid extracts produces a much more pronounced increase in CD1d expression that, in this case, was not accompanied with upregulation of HLA-DR or CD80 and only a subtle increase in CD86 expression, and thus the expression of CD1d and the maturation markers are probably not functionally linked. Supporting this, the lipid-free extract of the pollen was able to induce a full maturation of DCs without inducing expression of CD1d. We also observed this behavior in DCs exposed to delipidized extracts from other plant allergens (results not shown). It is conceivable that DCs could uptake lipids from the pollen grains that would then modify the transcriptional regulation of the CD1 genes. Indeed, this was the case when we treated DCs with whole lipid extracts from olive pollen in which we found an upregulation of CD1d mRNA, whereas the CD1a mRNA was dramatically downregulated and levels of HLA-DR mRNA were slightly downregulated. Our work provides evidence that PPAR $\gamma$  is activated in response to lipid molecules present in olive pollen grains and that this activation is responsible for the CD1d expression profile in treated DCs.

Previous studies have shown direct interactions of fatty acids and eicosanoids with PPAR $\gamma$  and PPAR $\alpha$ .<sup>30</sup> It is known that olive

pollen grains contain increased percentages of these polyunsaturated fatty acids mainly in phospholipids, glycolipids, and triacylglycerol acyl chains.<sup>31</sup> In our study FFAs from olive pollen lipids increased CD1d surface expression at the transcriptional level. Nevertheless, polar lipids (including phospholipids and glycolipids) were able to increase CD1d surface expression with no augmentation in the levels of CD1d mRNA, suggesting that a posttranscriptional regulatory mechanism is involved. Increased surface expression of CD1d, which is not regulated at the transcriptional level, has been previously shown in DCs and macrophages infected with the intracellular pathogen *Listeria monocytogenes*.<sup>35</sup> Our data strongly suggest an activation of iNKT cells by pollen lipids. We cannot exclude that some lipids from the pollen could be presented directly to iNKT cells; however, the major mechanism of iNKT cell activation is probably indirect, most likely from PPAR $\gamma$  activation by the fatty acids present in the lipid fractions. This activation of iNKT cells *in vitro* could indicate a possible expansion and cytokine secretion in the respiratory airways.<sup>17</sup> Other groups had demonstrated that substances present in the environment (pollution, dust, bacteria, and virus) were able to activate iNKT cells and worsen inflammatory allergic responses.<sup>36,37</sup> It is possible that lipids presented in the allergens could act as “adjuvants” to generate allergic responses in susceptible subjects. The role of pollen lipids is not restricted exclusively to the activation of iNKT cells because they have demonstrated several effects on DCs, as well as other

types of T cells.<sup>23-25</sup> Allergy is a complex response, and it is necessary to take into account the role of conventional and nonconventional T cells. Our results indicate that iNKT cells are efficiently activated by DCs exposed to lipids present in the grains of *O europaea*, supporting a role of these cells in the pathogenesis of allergy against olive pollen.

We thank Dr Paul B. Savage for the PBS-57 and Centro de Transfusiones de la Comunidad de Madrid for the buffy coats.

**Clinical implications: DCs treated with lipids isolated from *O europaea* pollen grains upregulated CD1d expression on the cell surface and were able to activate iNKT cells. This suggests that iNKT cells could be important in allergic responses.**

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## METHODS

### Measurement of cytokines by using ELISA and ELISpot

Untreated or treated iDCs were cocultured in 96-well flat-bottom plates with iNKT cells (ratio 1:1) for 48 hours, and supernatants were analyzed for IL-4 and IFN- $\gamma$  by using the sandwich ELISA assay (R&D Systems, Minneapolis, Minn), according to the manufacturer's instructions. All ELISA reactions were done in triplicate.

Pollen lipid- or PBS-57 (100 ng/mL)-treated PBMCs were restimulated for 24 hours in 96-well human IFN- $\gamma$  or IL-4 ELISpot plates (Millipore, Temecula, Calif) after 2 weeks in culture. ELISpot plate development was performed according to the manufacturer's recommendations.

### Real-time quantitative RT-PCR

Total RNA was isolated with TRIZOL reagent (Invitrogen, San Diego, Calif), and reverse transcription was performed with the First Strand cDNA Synthesis Kit for RT-PCR (AMV; Roche). Quantitative PCR was performed with real-time PCR TaqMan assays (ABI PRISM 7700; Applied Biosystems, Foster City, Calif). The comparative  $2^{-\Delta\Delta C_t}$  method was used to quantify transcripts.

### DC generation and olive pollen lipid treatment

Monocytes were obtained from buffy coats by means of Ficoll gradient centrifugation and magnetic cell separation with anti-CD14-conjugated microbeads (Miltenyi Biotec). To obtain iDCs, monocytes were resuspended into 24-well culture dishes at a density of  $1 \times 10^6$  cells/mL and cultured in RPMI-1640 (Invitrogen), 10% FCS (Harlan Indianapolis, Ind) supplemented with 1000 U/mL rhIL-4, and 1000 U/mL rhGM-CSF (ImmunoTools, Friesoythe, Germany) for 5 days.

iDCs were treated for 48 hours with whole lipid extract from olive pollen (200 ng/mL). The thin layer chromatography fractions were as follows: polar lipids (100 ng/mL), diglycerides, FFAs, and triglycerides or vehicle control (dimethyl sulfoxide). iDCs were also treated with 100 ng/mL LPS (Sigma-Aldrich,

St Louis, Mo), polar lipids 100 nmol/L all-trans retinoic acid (Sigma-Aldrich), 2.5  $\mu$ mol/L rosiglitazone (Alexis Biochemicals, San Diego, Calif), or 5  $\mu$ mol/L PPAR $\gamma$  antagonist GW9662 (Alexis Biochemicals).

### Antibodies, flow cytometric analysis, and immunofluorescence

Primary conjugated antibodies used in this study were as follows: anti-human CD1d-phycoerythrin (PE; clone CD1d42; BD PharMingen, San Jose, Calif); anti-CD1a (clone OKT6; from American Type Culture Collection); anti-CD1b (clone 4A76) and anti-CD1c (clone L161; Immunotech); anti-CD86-fluorescein isothiocyanate (clone 2331 FUN-1), anti-CD80-PE (clone I307.4), and anti-HLA-DR-PE (clone TU361 BD PharMingen); and anti-HLA class I (A, B, and C; clone W6/32; American Type Culture Collection). For surface staining, cells were washed and incubated with specific antibodies for 30 minutes at 4°C. For intracellular staining, cells were fixed with 2% paraformaldehyde and permeabilized in PBS containing 0.1% saponin and 1% BSA for 15 minutes at room temperature and assessed for fluorescence intensity by using a FACSCalibur cytometer (Becton Dickinson, San Jose, Calif).

For the immunofluorescence experiments, treated or untreated iDCs were allowed to adhere to the glass slides at 37°C. Cells were fixed in 3% paraformaldehyde for 15 minutes and permeabilized in PBS containing 0.3% saponin before staining with anti-CD1d. Cover slips were mounted in Vectashield with 4'-6-diamidino-2-phenylindole dihydrochloride and photographed by using fluorescence microscopy (Zeiss Axio Imager A1; Zeiss, Oberkochen, Germany).

### Statistical analysis

Mean values were compared by using the unpaired Student *t* test. All statistical analyses were performed with the Statgraphics program (Statpoint Technologies, Warrenton, Va). Statistically significant differences were represented as follows: \**P* < .05, \*\**P* < .03, and \*\*\**P* < .01.

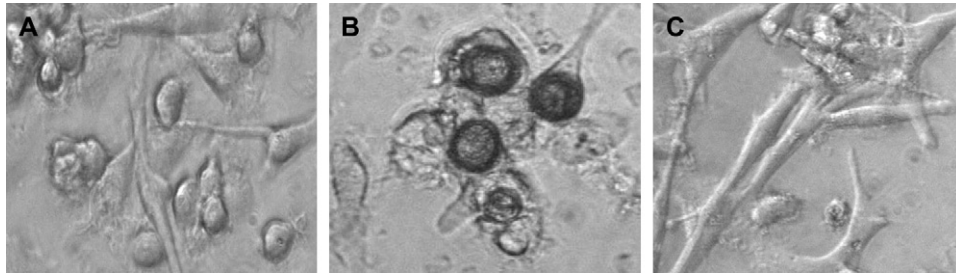


**FIG E1.** Monocyte derived–DCs photographed with light inverted microscopy (original magnification  $\times 40$ ). **A**, Control DCs. **B**, Olive pollen grain–stimulated DCs after 48 hours in culture. Several cells enclose pollen grains with their cytoplasm. **C**, DCs treated with whole lipid extract from pollen acquire flat and enlarged morphology.

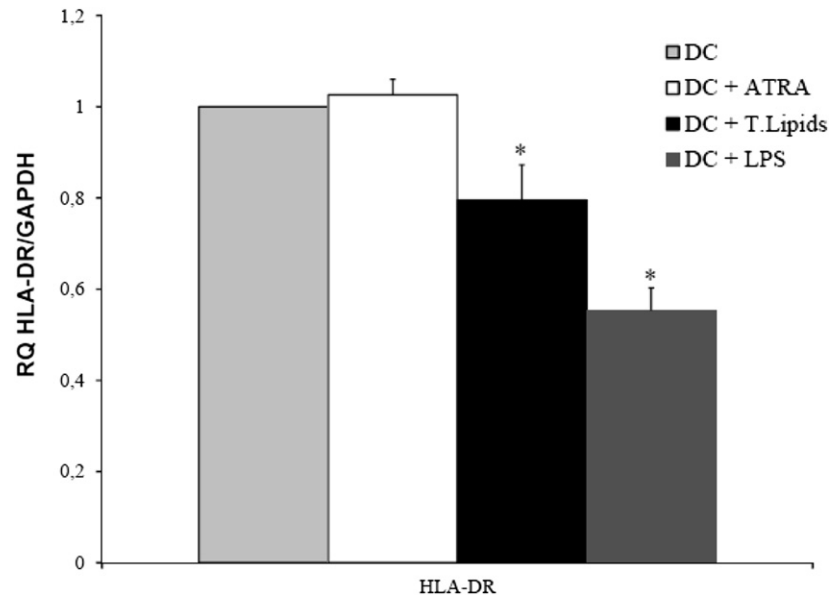


**FIG E2.** HLA-DR mRNA quantification by means of quantitative PCR. Relative quantification (*RQ*) of RNA transcript levels in DCs treated with all-trans retinoic acid (*ATRA*) and total lipids (*T. Lipids*) from pollen and LPS referred to control DCs (mean  $\pm$  SDs,  $n = 3$ ). \* $P < .05$ .

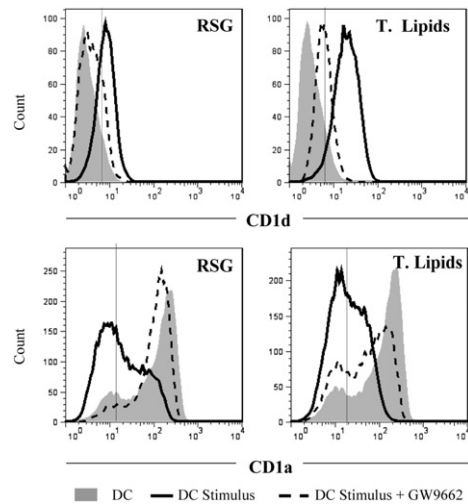


**FIG E3.** The PPAR $\gamma$  antagonist GW9662 reverses CD1d upregulation by pollen lipids in DCs. CD1a and CD1d expression was measured by using fluorescence-activated cell sorting in lipid-treated or untreated DCs in the presence or absence of GW9662. One representative experiment of 4 is shown. *RSG*, Rosiglitazone; *T. Lipids*, total lipids.

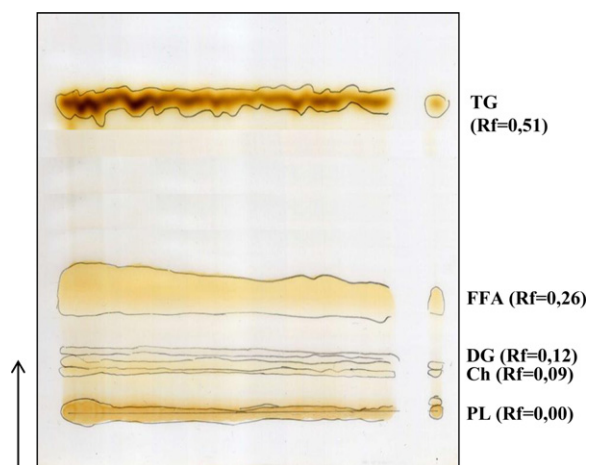


**FIG E4.** Chemical analysis of olive pollen lipids by means of thin layer chromatography. The main species represented are polar lipids, diacylglycerols (*DG*), FFAs, and triacylglycerols (*TG*), attending to its grade of polarity in the solvent. *Rf* represents the relative mobility of each lipid. *Ch*, Cholesterol; *PL*, phospholipids.