Human Invariant Natural Killer T Cells Respond to Antigen-Presenting Cells Exposed to Lipids from *Olea europaea* Pollen

Beatriz Abos Gracia^a Juan López Relano^a Ana Revilla^b Lourdes Castro^c
Mayte Villalba^c Beatriz Martín Adrados^a Jose Ramon Regueiro^a
Edgar Fernández-Malavé^a Eduardo Martínez Naves^a
Manuel Gómez del Moral^b

Departments of ^aImmunology and ^bCell Biology, Faculty of Medicine, and 12 de Octubre Health Research Institute (imas12), and ^cDepartment of Biochemistry and Molecular Biology, Faculty of Chemistry, Complutense University, Madrid, Spain

Keywords

CD1d · Invariant natural killer T cells · *Olea europaea* · Pollen lipids

Abstract

Background: Allergic sensitization might be influenced by the lipids present in allergens, which can be recognized by natural killer T (NKT) cells on antigen-presenting cells (APCs). The aim of this study was to analyze the effect of olive pollen lipids in human APCs, including monocytes as well as monocyte-derived macrophages (Mφ) and dendritic cells (DCs). **Methods:** Lipids were extracted from olive (*Olea europaea*) pollen grains. Invariant (i)NKT cells, monocytes, Mφ, and DCs were obtained from buffy coats of healthy blood donors, and their cell phenotype was determined by flow cytometry. iNKT cytotoxicity was measured using a lactate dehydrogenase assay. Gene expression of *CD1A* and *CD1D* was performed by RT-PCR, and the production of IL-6, IL-10, IL-12, and TNF-α cytokines by monocytes, Mφ, and DCs was measured by ELISA. **Results:** Our results showed that monocytes and monocyte-derived Mφ treated with olive pollen lipids

strongly activate iNKT cells. We observed several phenotypic modifications in the APCs upon exposure to pollen-derived lipids. Both Mφ and monocytes treated with olive pollen lipids showed an increase in *CD1D* gene expression, whereas upregulation of cell surface CD1d protein occurred only in Mφ. Furthermore, DCs differentiated in the presence of human serum enhance their surface CD1d expression when exposed to olive pollen lipids. Finally, olive pollen lipids were able to stimulate the production of IL-6 but downregulated the production of lipopolysaccharide-induced IL-10 by Mφ. **Conclusions:** Olive pollen lipids alter the phenotype of monocytes, Mφ, and DCs, resulting in the activation of NKT cells, which have the potential to influence allergic immune responses.

© 2017 S. Karger AG, Basel

Introduction

Allergic responses are mainly characterized by the activation of T helper type 2 (Th2) lymphocytes and the subsequent production of IgE. Most allergens recognized

for both Th2 cells and IgE immunoglobulins are proteins, but it is not known what causes a protein to become an allergen. The majority of allergens contain, in addition to proteins, other substances such as carbohydrates and lipids, the role of which in the immune response leading to allergy is largely unknown. A group of specialized T lymphocytes recognize lipids or glycolipids instead of proteins. These T cells are restricted by CD1, a family of MHC-like molecules. CD1 molecules can be divided in 2 groups according to sequence homology and tissue distribution [1]. Human CD1a, CD1b, and CD1c molecules are members of group I and group II is formed by CD1d. CD1a–c molecules are expressed in professional antigen-presenting cells (APCs) and are involved in the defense against microbial infections, like mycobacteria [2]. CD1d is also expressed on professional APCs including dendritic cells (DCs), macrophages (M ϕ), monocytes, and some B cells. CD1d molecules present lipidic antigens to a specialized type of T lymphocyte called NKT cells [3, 4]. NKT cells have the ability to secrete a wide range of cytokines including INF- γ , IL-4, IL-13, and IL-17, and they have been described to have regulatory properties in different immune responses [5]. They are able to respond against lipidic antigens from pathogens, autoantigens, or allergens [6].

We have recently reported that lipids from *Olea europaea* pollen are able to change the phenotype and function of monocyte-derived DCs differentiated in the presence of fetal calf serum (FCS). After exposure to the lipids, DCs upregulate CD1d and CD86 molecules on their cell surface and activate iNKT cells through a CD1d-dependent pathway, suggesting that lipids can influence the immune response against pollen antigens [7].

We analyze here the effect of olive pollen lipids in other types of CD1d-expressing cells, i.e., monocytes and M ϕ . In addition, we study the action of lipids from olive pollen in the phenotype and function of monocyte-derived DCs differentiated in the presence of human serum, because these cells display a profile of CD1 surface expression which is different from that induced in cells differentiated in the presence of FCS. This is due to the fact that human serum components (such as lysophosphatidic acid and cardiolipin) are able to inhibit the expression of CD1a–c while upregulating CD1d expression on immature (i)DCs [8].

Our results show that different types of CD1d-expressing APCs cells exposed to lipids from *O. europaea* display a unique phenotype and are able to activate iNKT cells.

Materials and Methods

Preparation of Olive Pollen Lipids

Lipids from olive pollen grains (ALK-Abelló, Hørsholm, Denmark) were extracted after diethyl ether incubation and centrifugation. The supernatant was collected and evaporated nearly to dryness in an N₂ atmosphere. Next, the lipids were dissolved on chloroform/methanol (2:1 by volume) and chromatographed on silica gel G thin-layer chromatographic plates by using chloroform/methanol/water (70:30:1 by volume) as a solvent. Bands were stained 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: polar lipids, diacylglycerols, free fatty acids and triacylglycerols. Total and polar lipids were quantified by a phosphorous assay [7].

Aqueous pollen extracts were generated by incubation of pollen grains in RPMI-1640 (30 mg/mL) for 30 min at 37°C followed by centrifugation (20 min at 3,345 g) and sterile filtration.

M ϕ DC Generation and Cell Stimulation

First, monocytes were obtained from buffy coats by Ficoll gradient centrifugation and magnetic cell separation using anti-CD14-conjugated microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). In order to obtain iDCs and M ϕ , monocytes were resuspended into 24-well culture dishes at a density of 1×10^6 cells/mL, and cultured in RPMI-1640 (Invitrogen) and 10% FCS (Harlan) or human serum (Invitrogen) for the experiments with DCs, and with 1% of antibiotic. Cells were supplemented with 1,000 U/mL recombinant human (rh)IL-4 and 1,000 U/mL rhGM-CSF (ImmunoTools) for 5 days to obtain DCs and with 1,000 U/mL rhGM-CSF to get M ϕ .

Monocytes, M ϕ , and iDCs were treated with whole-lipid extract from olive pollen (200 ng/mL) or vehicle control (DMSO) during 48 h. Cells were treated with 100 ng/mL of lipopolysaccharide (LPS; Sigma-Aldrich), 100 nM of all-trans retinoic acid (ATRA) (Sigma-Aldrich), or 100 ng/mL of PBS-57, an analog of the α -galactosylceramide, when indicated.

Flow Cytometry

The primary conjugated Abs used in this study were: anti-human CD1d-PE, clone CD1d42 (BD Pharmingen); anti-CD1a, clone OKT6 (from American Type Culture Collection, ATCC); anti-CD1b, clone 4A76 (Immunotech) and anti-CD1c, clone L161 (Immunotech); anti-CD86-FITC, clone 2331 FUN-1; anti-CD80-PE, clone I307.4; and anti-HLA-DR-PE, clone TU36 (BD Pharmingen). For surface staining, cells were washed and incubated with specific antibodies for 30 min at 4°C, protected from light. Cells were washed with PBS and assessed for fluorescence intensity using a FACSCalibur cytometer (Becton Dickinson).

Real-Time Quantitative RT-PCR

Total RNA was isolated using TRIZOL reagent (Invitrogen), and reverse transcription was performed with the first-strand cDNA synthesis kit for RT-PCR (AMV) (Roche). Quantitative PCR was performed using real-time PCR (ABI PRISM 7700; Applied Biosystems): 30 cycles at 95°C for 10 s and at 60°C for 60 s using UPL assays (Roche). Primers for human *CD1D*, *CD1A*, and *GAPDH* were as follows: *CD1D* (L-GAAGTGAAGAAGCAAGTGAAGC; R-GGGTAGAATCCTAGAACATGG), *CD1A* (L-CCC-ATGAATTGCAGTTTGAA; R-AGCTTCCTGAGACCTTTCCA),

Fig. 1. Isolated iNKT cells are activated by olive pollen lipid-treated monocytes (Mo) and macrophages Mφ. **a** Mean of the percentage of lysis $\pm$ SD of Mo cultured in the presence or absence of PBS-57, pollen aqueous extract (Aq. Extract), and total-pollen lip-

ids (T. Lipids) by iNKT cells ($n = 4$) after 48 h. **b** Percentage of lysis of Mφ stimulated or not stimulated with the indicated stimulus by iNKT cells (mean $\pm$ SD of the triplicates of a representative experiment). ** $p < 0.03$; *** $p < 0.01$.

and *GAPDH* (L-GCTCTCTGCTCCTCCTGTTC; R-ACGACC-AAATCCGTTGACTC). All PCR reactions were done in duplicate, with 1 control reaction containing no RT enzyme and *GAPDH* as the housekeeping gene. The comparative $2^{-\Delta\Delta C_t}$ method was used to quantify transcripts.

Human iNKT Cell Expansion and Isolation

iNKT cells were expanded from total peripheral blood mononuclear cells by treatment with 100 U/mL rhIL-2 in the presence of PBS-57 (100 ng/mL), for 2 weeks. iNKT cells were then positively selected by magnetic separation (Miltenyi Biotec) with the specific anti-Vα24⁺ and anti-Vβ11⁺ antibodies (Beckman Coulter).

Cytotoxicity Assay

Cytotoxicity was measured using the nonradioactive lactate dehydrogenase cytotoxicity detection kit (Roche, Mannheim, Germany), according to the manufacturer's instructions. All cytotoxicity assays were performed in triplicate. The percentage of specific lysis was calculated as follows: $100 \times (\text{experimental value} - \text{low control}) / (\text{high control} - \text{low control})$. iNKT cells were used as effector cells and untreated or treated monocytes and Mφ as target cells. PBS-57-positive control was added to the cells at a concentration of 100 ng/mL.

Cytokine Quantification

Levels of IL-6, IL-10, IL-12, and TNF-α produced by human monocytes, Mφ, and DCs (differentiated with human serum) were determined, before and 48 h after stimulation, by using a commercially available kit (Invitrogen, Thermo Fisher Scientific), according to the manufacturer's recommendations. Cells were stimulated with vehicle (DMSO); olive pollen lipids (200 ng/mL); olive pollen lipids (200 ng/mL) plus LPS (100 ng/mL); and LPS alone (100

ng/mL). In all assays, diluent negative controls, standards, and samples were tested in duplicate. Mean OD₄₅₀ readings obtained from diluent negative controls were subtracted from all readings (samples and controls) before making standard curves that were used to calculate sample concentrations. The ELISA reader ELx800 (BioTek) was used for OD measurement.

Statistical Analysis

Mean values were compared using the unpaired Student *t* test. All statistical analyses were performed with Statgraphics software. Statistically significant differences were represented as: * $p < 0.05$, ** $p < 0.03$, and *** $p < 0.01$.

Results

Monocytes and Monocyte-Derived Mφ Treated with Olive Pollen Lipids Are Able to Activate iNKT Cells

To assess whether monocytes and Mφ could activate iNKT cells after treatment with olive pollen lipids, we performed "in vitro" cytotoxic killing assays. We found that lipid-treated monocytes as well as monocyte-derived Mφ were efficiently lysed by iNKT cells (Fig. 1). In contrast, no significant lysis was detected when cells were cultured in the presence of the aqueous extract of the pollen. In addition, the cytotoxic effect observed with lipid-treated cells was comparable to that observed when monocytes or Mφ were exposed to PBS-57, a CD1d-binding glycolipid that efficiently activates iNKT cells.

Fig. 2. Olive pollen lipid-treated macrophages (Mφ). **a, b** Surface expression of CD1a, CD1b, CD1c, and CD1d on monocyte-derived Mφ cultured in the presence or absence of LPS, ATRA, or total-pollen lipids (T. Lipids) during 48 h. **a** Relative mean fluorescence intensity (MFI) $\pm$ SDs of 3–5 independent experiments (CD1d and CD1a, $n = 5$; CD1b and CD1c, $n = 3$). **b** Representative histograms. **c** CD1d surface expression on Mφ treated or not treated with polar

lipids (Polar L.) or triacylglycerols (TGs). Max, maximum. Data are shown as representative histograms from 3 independent experiments. **d, e** HLA-DR and CD86 surface expression on control, LPS-, ATRA-, or T. Lipid-stimulated Mφ. **d** Relative MFI $\pm$ SD of 4 independent experiments. **e** Representative histograms. ** $p < 0.03$; *** $p < 0.01$.

Olive Pollen Lipids Induce Phenotypic Changes on Monocytes and Monocyte-Derived Mφ

We next decided to investigate if APCs show phenotypic changes after being exposed to pollen lipids. For this purpose, we used flow cytometry to analyze the expression of CD1, HLA-DR, and CD86 molecules on the

cell surface of monocytes, and monocyte-derived Mφ differentiated under standard culture conditions (with FCS).

We found that cell-surface levels of CD1d, and, to a lesser extent, CD1c were increased on the cell surface of the Mφ after 48 h of exposure to pollen lipids. The levels

of CD1d were comparable to those observed after treating the cells with ATRA (Fig. 2a, b), an agent known to enhance the CD1d cell surface expression on DCs [7, 9]. When the total-lipid extract was fractionated, we found an increase of CD1d expression on cells treated with both polar and apolar lipid fractions (Fig. 2c). Thus, the recognition of lipid-treated M ϕ by iNKT cells in the “in vitro” cytotoxicity tests correlates with the levels of CD1d expression on the surface of target cells.

No changes were found in CD86 or HLA-DR expression after the exposure of M ϕ to total-lipid extract (Fig. 2d, e).

We next examined the phenotype of monocytes exposed to pollen lipids, which showed no significant changes on CD1d cell surface expression (Fig. 3a, b). In contrast, we found that group I CD1 molecules (CD1a–c) significantly decreased their expression on monocytes in the presence of the pollen lipids (Fig. 3a, b). Furthermore, when the monocytes were exposed to different fractions of the pollen lipids, we observed a subtle but statistically significant increase of CD1d that was induced by the polar lipid fraction (Fig. 3c, d). Olive pollen lipid treatment did not change the expression of CD86 or HLA-DR on monocytes (Fig. 3e, f).

Thus, monocytes treated with total-pollen lipids did not increase their CD1d expression on the cell surface, but they were efficiently recognized by iNKT cells. In order to evaluate the transcriptional level of CD1d when cells were treated with olive pollen lipids, we carried out real-time PCR experiments in which ATRA exposure was used as a control. We observed a significant increase in the mRNA levels for *CD1D* in both, lipid-treated monocytes and M ϕ in comparison with control cells (Fig. 3g).

The results showed a correlation between protein levels and transcriptional changes in CD1d for lipid-treated M ϕ (also observed in iDCs differentiated in the presence of FCS [7]. In contrast, no parallelism was observed in the case of monocytes, which display an increase in CD1d at the transcriptional level, but not in the surface protein expression after culture with the olive pollen lipids.

DCs Differentiated in the Presence of Human Serum Enhance Their CD1d Expression When Exposed to Olive-Pollen Lipids

We next decided to characterize the phenotypic effects of olive pollen lipids on a different kind of CD1d-expressing cell, i.e., monocyte-derived DCs differentiated in the presence of human serum (HS-DCs).

We assessed the phenotype of pollen lipid-treated or untreated HS-DCs by flow cytometry. Our results showed

a significant increase in CD1d and CD1a cell surface expression in pollen lipid-treated HS-DCs when compared to controls (Fig. 4a, b). The increase of CD1a cell surface expression in lipid-treated HS-DCs contrasts with what we previously observed in standard serum conditions [7], i.e., exposure to pollen lipids downregulated CD1a on the cell surface. No changes were observed on CD1b, CD1c, and class II (DR) (Fig. 4c, d). We also found a subtle increase in the cell surface levels of CD86 in pollen lipid-treated HS-DCs when compared with those seen in controls (the same result was previously obtained in FCS-DCs) (Fig. 4c, d).

Next, we analyzed the effect of different lipidic fractions isolated in the phenotype of iDCs which were cultured for 48 h in the presence of polar or apolar (triacylglycerol) lipids. Both types of lipid fractions were able to induce upregulation of CD1d and CD1a on the cell surface of iDCs in a dose-dependent manner (Fig. 5a, b). The levels of *CD1D* and *CD1A* mRNAs were assessed by real-time PCR experiments in which we used rosiglitazone, a PPAR γ agonist, as a positive control because it upregulates the *CD1D* mRNA expression. As a further control, we treated the cells with GW9662, a selective irreversible PPAR γ antagonist. No changes were observed in the *CD1D* mRNA levels of lipid-exposed iDCs when compared to controls. Moreover, pollen lipid-treated HS-DCs expressed higher levels of *CD1A* mRNA (although this was not statistically significant) than the control cells. The increase of *CD1A* levels does not appear to be PPAR γ -dependent, since treatment with rosiglitazone decreased *CD1A* mRNA levels and these were further increased when the cells were treated with lipids plus GW9662. This situation is completely different from what happens in the case of DCs differentiated in the presence of FCS [7].

Olive Pollen Lipids Modulate the Cytokine Profile Secretion of Human M ϕ

Finally, we analyzed the profile secretion of IL-6, IL-10, IL-12, and TNF- α by monocytes, M ϕ , and DCs (differentiated in the presence of human serum) upon olive pollen lipid stimulation (Fig. 6). Our results showed no significant changes in the secretion of IL-6, IL-10, IL-12, or TNF α in monocytes or DCs treated with or without olive pollen lipids. In contrast, we observed that olive pollen lipids were able to induce the secretion of moderate amounts of IL-6 by M ϕ . Furthermore, the amount of IL-10 secreted by LPS-treated M ϕ was reduced in the presence of olive pollen lipids.

Fig. 3. Olive pollen lipid-treated monocytes (Mo). **a, b** Surface expression of CD1a, CD1b, CD1c, and CD1d on purified Mo cultured in the presence or absence of LPS, ATRA, or total-pollen lipids (T. Lipids) after 48 h. **a** Relative mean fluorescence intensity (MFI) $\pm$ SD from 3 independent experiments. **b** Representative histograms. **c, d** Surface expression of CD1d on purified Mo treated with or without polar lipids (Polar L.), diacylglycerols (DGs), free fatty acids (FFAs), and triacylglycerols (TGs) during 48 h. **c** The relative MFI $\pm$ SD of 3 independent experiments. **d** Repre-

sentative histograms of CD1d cell surface expression on Mo treated with or without Polar L. or TGs. **e, f** HLA-DR and CD86 surface expression on control, ATRA-, LPS-, or T. Lipid-stimulated Mo. **e** Relative MFI $\pm$ SD of 3 independent experiments. **f** Representative histograms. **g** Effect of ATRA and T. Lipids on the transcription of *CD1D* on Mo-derived macrophages or purified Mo. Data from 3 independent experiments are shown as mean gene expression levels relative to the expression level of an endogenous control (*GAPDH*) $\pm$ SD. * $p < 0.05$; ** $p < 0.03$; *** $p < 0.01$.

Fig. 4. Olive pollen lipid-treated DCs cultured in medium with human serum. **a, b** Surface expression of CD1a, CD1b, CD1c, and CD1d on monocyte-derived DCs cultured in human serum medium in the presence or absence of ATRA, pollen aqueous extract (Aq. Extract), or total-pollen lipids (T. Lipids) during 48 h. **a** Relative mean fluorescence intensity (MFI) $\pm$ SD from 3–8 indepen-

dent experiments (CD1d, $n = 8$; CD1a, $n = 7$; CD1b and CD1c, $n = 3$). **b** Representative histograms. **c, d** HLA-DR and CD86 surface expression on control, ATRA-, Aq. Extract-, or T. Lipid-stimulated DCs cultured in medium with human serum. **c** Relative MFI $\pm$ SD of 3 independent experiments. **d** Representative histograms. * $p < 0.05$; *** $p < 0.01$.

Discussion

The mechanisms leading to allergic sensitization are not completely understood, and they are not only influenced by the allergen itself but also by other molecules, such as lipids, that are directly bound as ligands by the

allergen or are present in the allergy source [10]. Lipids from the pollen coat are codelivered with the allergens and can modulate the immune responses of predisposed subjects by interacting with the innate immune system and iNKT cells, and they can potentially induce NKT cell-dependent allergy disease and asthma [7, 11, 12].

Fig. 5. DCs treated in the presence of pollen lipidic species in medium with human serum. **a** CD1d and CD1a surface expression on DCs treated in the presence or absence of polar lipids (Polar L.) or triacylglycerols (TGs) at different concentrations in human serum medium during 48 h. Data shown are representative histograms from 3 independent experiments. Max, maximum. **b** Relative mean fluorescence intensity (MFI) $\pm$ SD of CD1d and CD1a on

treated (Polar L. or TGs, at 100 ng/mL) or untreated DCs in human serum medium from 3 independent experiments. **c** Transcriptional expression of *CD1D* and *CD1A* on DCs treated in the presence or absence of rosiglitazone (RSG) or total-pollen lipids (T. Lipids) and with or without the PPAR- γ inhibitor GW9662, in medium with human serum or FCS ($n = 3$). ** $p < 0.03$; *** $p < 0.01$.

The results presented here showed that human iNKTs can efficiently recognize monocytes and M ϕ which have been exposed to pollen-lipid extracts. This is in agreement with previous studies in both animal models and human subjects suggesting that iNKT cells might signifi-

cantly affect the course of asthma or respiratory allergy [13–16].

Our previous results [7] showed that lipids from the olive pollen coat can modulate the immune response by upregulating CD1d expression on monocyte-derived

Fig. 6. Production of IL-6, IL-10, IL-12, and TNF-α cytokines expressed in pg/mL by monocytes, Mφ, and DCs before (T0) and after 48 h stimulation with DMSO as negative control (DMSO), olive pollen lipids (OL), OL and lipopolysaccharide (OL + LPS),

and LPS. The average cytokine concentration of 4 independent experiments (pg/mL) ± SD is shown. * $p < 0.05$; ** $p < 0.03$; *** $p < 0.01$.

DCs differentiated in the presence of FCS. Here, we extended the type of cells that can be recognized by NKT cells, including Mφ and DCs differentiated in the presence of human serum. In both cell types, treatment with pollen lipids led to an increase of CD1d on the cell surface, which could be linked to the iNKT cell response. The presence of normal human serum during the differentiation of iDCs from monocytes inhibits the expression of CD1a, CD1b, and CD1c, but not CD1d [17–19], when compared to DCs differentiated in the presence of FCS. It is interesting that olive pollen lipids increase both CD1a and CD1d expression on iDCs differentiated in the pres-

ence of human serum, a more physiological situation than FCS. It has been described that, in vivo, numbers of CD1d+ DCs or Mφ [20] or CD1a+ Langerhans cells or DCs are increased and express high levels of CD1a protein in airway epithelial cells and mucosa in seasonal allergy rhinitis sufferers [21] as well as in allergic bronchial asthmatics [22–25].

Lysophosphatidic acid and cardiolipin, present in human serum, are the lipids that inhibit the transcription of CD1a, CD1b, and CD1c (group I) while upregulating CD1d in a PPARγ-dependent manner [26]. Our data show that *CD1A* mRNA is upregulated when iDCs differenti-

ated in the presence of human serum are treated with total lipids, and that this phenomenon is not dependent on PPAR γ . Furthermore, the upregulation of CD1d protein on the surface of these cells is not due to an increase in *CD1D* transcription, indicating that posttranscriptional regulatory mechanisms are involved in the CD1d upregulation at the protein level. Increased surface expression of CD1d, which is not regulated at the transcriptional level, has been previously shown in DCs and M ϕ infected with the intracellular pathogen *Listeria monocytogenes* [27] as well as in FCS-derived DCs treated with polar lipids [7].

It has been described that activation of iNKT cells might occur in response to the upregulation of CD1d on the surface of APCs. Cells can increase their CD1d levels after exposure to proinflammatory cytokines, pathogens, ATRA, or lipidic substances from allergens [20, 27, 28].

The correlation between protein levels and transcriptional changes in CD1d for lipid-treated M ϕ and DCs [7] contrasts with our observations in monocytes, in which increased CD1d at the transcriptional level did not correlate with the enhanced expression of the protein on the cell surface after culturing the cells with the olive pollen lipids. This suggests a differential CD1d expression control mechanism in stimulated monocytes compared to DCs and M ϕ . iNKT cells can be activated by the CD1d-mediated presentation of antigenic lipids with no need of CD1d upregulation [29, 30], thus our observations in monocytes may suggest that the lipids from the olive pollen can be presented directly by CD1d molecules to iNKT cells. In addition, we observed that olive pollen lipids can change the cytokine profile secretion of M ϕ , a fact that can also influence the response of NKT cells or other types of cells involved in allergic responses. The increase

of IL-6 production, together with the downregulation of IL-10, that we observed could well be linked to a proinflammatory response against the protein components of the olive pollen in the presence of pollen lipids.

The data showed in this article indicate a strong possibility that CD1-dependent presentation of exogenous lipids, together with changes in the cytokine profiles of APCs induced by those lipids, may be an important and not well explored aspect of allergic diseases. For example, an increased frequency of bee and wasp venom responsive to CD1a-restricted T cells in allergic individuals was recently described [31]. Furthermore, endogenous lipids from Brazil nut can act as adjuvants for the immune response against Ber e 1 allergen and iNKT cells play a crucial role in this response [12].

In summary, our results show that olive pollen-lipid molecular species have the potential to stimulate APCs, resulting in the activation of NKT cells, thus reinforcing the idea that these cells might actively participate in the development of allergic responses.

Acknowledgements

This work was supported by grants from the Spanish Instituto de Salud Carlos III (ref. PI08-00125 and PI13-00218 to E.M.N.) and the Universidad Complutense de Madrid (ref. 920631 to M.G.M. and E.M.N.) B.A.G. was the recipient of a Predoctoral Fellowship from the Universidad Complutense de Madrid.

Disclosure Statement

All authors declare that they have no conflicts of interest regarding this study.

References

- Calabi F, Jarvis JM, Martin L, Milstein C: Two classes of CD1 genes. *Eur J Immunol* 1989 19: 285–292.
- Adams EJ: Lipid presentation by human CD1 molecules and the diverse T cell populations that respond to them. *Curr Opin Immunol* 2014;26:1–6.
- Salio M, Silk JD, Jones EY, Cerundolo V: Biology of CD1- and MR1-restricted T cells. *Annu Rev Immunol* 2014;32:323–366.
- McEwen-Smith RM, Salio M, Cerundolo V: CD1d-dependent endogenous and exogenous lipid antigen presentation. *Curr Opin Immunol* 2015;34:116–125.
- Brennan P, Brigl M, Brenner MB: Invariant natural killer T cells: an innate activation scheme linked to diverse effector functions. *Nat Rev Immunol* 2013;13:101–117.
- Berzins SP, Ritchie DS: Natural killer T cells: drivers or passengers in preventing human disease? *Nat Rev Immunol* 2014;14:640–646.
- Abós-Gracia B, del Moral MG, López-Relaño J, Viana-Huete V, Castro L, Villalba M, et al: *Olea europaea* pollen lipids activate invariant natural killer T cells by upregulating CD1d expression on dendritic cells. *J Allergy Clin Immunol* 2013;131:1393–1399.
- Leslie DS, Dascher CC, Cembrola K, Townes MA, Hava DL, Hugendubler LC, et al: Serum lipids regulate dendritic cell CD1 expression and function. *Immunology* 2008;125:289–301.
- Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Motoki K, et al: CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. *Science* 1997;278:1626–1629.
- Bublin M, Eiwegger T, Breiteneder H: Do lipids influence the allergic sensitization process? *J Allergy Clin Immunol* 2014;134:521–529.
- Agea E, Russano A, Bistoni O, Mannucci R, Nicoletti I, Corazzi L, et al: Human CD1-restricted T cell recognition of lipids from pollens. *J Exp Med* 2005;202:295–308.

- 12 Mirotti L, Florsheim E, Rundqvist L, Larsson G, Spinozzi F, Leite-de-Moraes M, et al: Lipids are required for the development of Brazil nut allergy: the role of mouse and human iNKT cells. *Allergy* 2013;68:74–83.
- 13 Lisbonne M, Diem S, de Castro Keller A, Lefort J, Araujo LM, Hachem P, et al: Cutting edge: invariant V alpha 14 NKT cells are required for allergen-induced airway inflammation and hyperreactivity in an experimental asthma model. *J Immunol* 2003;171:1637–1641.
- 14 Yamamoto H, Okamoto Y, Horiguchi S, Kunii N, Yonekura S, Nakayama T: Detection of natural killer T cells in the sinus mucosa from asthmatics with chronic sinusitis. *Allergy* 2007;62:1451–1455.
- 15 Koh YI, Shim JU, Wi J, Kwon YE: The role of natural killer T cells in the pathogenesis of acute exacerbation of human asthma. *Int Arch Allergy Immunol* 2012;158:131–141.
- 16 Bashir ME, Lui JH, Palniveelu R, Naclerio RM, Preuss D: Pollen lipidomics: lipid profiling exposes a notable diversity in 22 allergenic pollen and potential biomarkers of the allergic immune response. *PLoS One* 2013;8:e57566.
- 17 Panther E, Idzko M, Corinti S, Ferrari D, Herouy Y, Mockenhaupt M, et al: The influence of lysophosphatidic acid on the functions of human dendritic cells. *J Immunol* 2002;169:4129–4135.
- 18 Martino A, Volpe E, Baldini PM: The influence of lysophosphatidic acid on the immunophenotypic differentiation of human monocytes into dendritic cells. *Haematologica* 2006;91:1273–1274.
- 19 Leslie DS, Dascher CC, Cembrola K, Townes MA, Hava DL, Hugendubler LC, et al: Serum lipids regulate dendritic cell CD1 expression and function. *Immunology* 2008;125:289–301.
- 20 Spinozzi F, Porcelli SA: Recognition of lipids from pollens by CD1-restricted T cells. *Immunol Allergy Clin North Am* 2007;27:79–92.
- 21 Till SJ, Jacobson MR, O'Brien F, Durham SR, KleinJan A, Fokkens WJ, et al: Recruitment of CD1a+ Langerhans cells to the nasal mucosa in seasonal allergic rhinitis and effects of topical corticosteroid therapy. *Allergy* 2001;56:126–131.
- 22 Möller GM, Overbeek SE, Van Helden-Meeuwse CG, Van Haarst JM, Prens EP, Mulder PG, et al: Increased numbers of dendritic cells in the bronchial mucosa of atopic asthmatic patients: downregulation by inhaled corticosteroids. *Clin Exp Allergy* 1996;26:517–524.
- 23 Agea E, Forenza N, Piattoni S, Russano A, Monaco A, Flenghi L, et al: Expression of B7 co-stimulatory molecules and CD1a antigen by alveolar macrophages in allergic bronchial asthma. *Clin Exp Allergy* 1998;28:1359–1367.
- 24 Bertorelli G, Bocchino V, Zhou X, Zanini A, Bernini MV, Damia R, et al: Dendritic cell number is related to IL-4 expression in the airways of atopic asthmatic subjects. *Allergy* 2000;55:449–454.
- 25 Tanaka A, Minoguchi K, Samson KT, Oda N, Yokoe T, Tazaki T, et al: Inhibitory effects of suplantat tosilate on the differentiation and function of monocyte-derived dendritic cells from patients with asthma. *Clin Exp Allergy* 2007;37:1083–1089.
- 26 McIntyre TM, Pontsler AV, Silva AR, St Hilaire A, Xu Y, Hinshaw JC, et al: Identification of an intracellular receptor for lysophosphatidic acid (LPA): LPA is a transcellular PPAR-gamma agonist. *Proc Natl Acad Sci USA* 2003;100:131–136.
- 27 Raghuraman G, Geng Y, Wang CR: IFN-beta-mediated up-regulation of CD1d in bacteria-infected APCs. *J Immunol* 2006;177:7841–7848.
- 28 Zajonc DM, Girardi E: Recognition of microbial glycolipids by natural killer T cells. *Front Immunol* 2015;6:400.
- 29 Spada FM, Borriello F, Sugita M, Watts GF, Koezuka Y, Porcelli SA: Low expression level but potent antigen presenting function of CD1d on monocyte lineage cells. *Eur J Immunol* 2000;30:3468–3477.
- 30 Salio M, Speak AO, Shepherd D, Polzella P, Illarionov PA, Veerapen N, et al: Modulation of human natural killer T cell ligands on TLR-mediated antigen-presenting cell activation. *Proc Natl Acad Sci USA* 2007;104:20490–20495.
- 31 Subramaniam S, Aslam A, Misbah SA, Salio M, Cerundolo V, Moody DB, Ogg G: Elevated and cross-responsive CD1a-reactive T cells in bee and wasp venom allergic individuals. *Eur J Immunol* 2016;46:242–252.