



Therapeutic targets for olive pollen allergy defined by gene markers modulated by Ole e 1-derived peptides



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ABSTRACT

Two regions of Ole e 1, the major olive-pollen allergen, have been characterized as T-cell epitopes, one as immunodominant region (aa91–130) and the other, as mainly recognized by non-allergic subjects (aa10–31). This report tries to characterize the specific relevance of these epitopes in the allergic response to olive pollen by analyzing the secreted cytokines and the gene expression profiles induced after specific stimulation of peripheral blood mononuclear cells (PBMCs). PBMCs from olive pollen-allergic and non-allergic control subjects were stimulated with olive-pollen extract and Ole e 1 dodecapeptides containing relevant T-cell epitopes. Levels of cytokines were measured in cellular supernatants and gene expression was determined by microarrays, on the RNAs extracted from PBMCs. One hundred eighty-nine differential genes (fold change >2 or <−2, $P < 0.05$) were validated by qRT-PCR in a large population.

It was not possible to define a pattern of response according to the overall cytokine results but interesting differences were observed, mainly in the regulatory cytokines. Principal component (PCA) gene-expression analysis defined clusters that correlated with the experimental conditions in the group of allergic subjects. Gene expression and functional analyses revealed differential genes and pathways among the experimental conditions. A set of 51 genes (many essential to T-cell tolerance and homeostasis) correlated with the response to aa10–31 of Ole e 1. In conclusion, two peptides derived from Ole e 1 could regulate the immune response in allergic patients, by gene-expression modification of several regulation-related genes. These results open new research ways to the regulation of allergy by *Oleaceae* family members.

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1. Introduction

Allergen-specific CD4⁺ helper T-cell (Th) generation is the initial event leading to the development of allergic disease. Type I allergy

Abbreviations: PBMCs, peripheral blood mononuclear cells; qRT-PCR, quantitative real-time polymerase chain reaction; PCA, principal component analysis; Tregs, regulatory T-cells; EAACI, European Academy of Allergology and Clinical Immunology; GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

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is caused by an inappropriate Th2 response to environmental allergens (El Biaze et al., 2003; Schleimer et al., 1989; Walker et al., 1991) leading to antigen-specific IgE production as well as recruitment and activation of proinflammatory cells (e.g., eosinophils and mast cells) in mucosal target organs (Hakansson et al., 1997, 1998; Till et al., 1997). In contrast, under similar exposure conditions, tolerance to allergens is maintained in non-allergic individuals. A deficit in immune tolerance is considered to cause allergy in pre-disposed individuals. Peripheral T-cell tolerance is characterized by functional inactivation due to specific T-cell subtypes with an immunosuppressive function, generically named regulatory T-cells (Tregs) (Akdis, 2008). IL-10 and TGF- β , secreted by these Tregs, may play an important role (Robinson, 2009; Akdis and Akdis, 2009; Palomares et al., 2010).

Olive tree pollen is one of the most important causes of pollinosis in the Mediterranean countries and some parts of Australia and North America. Olive pollen induces mainly nasal and conjunctival symptoms, although it may cause asthma exacerbation in areas with high antigenic load. The major olive allergen, Ole e 1, recognized by around 80% of allergic subjects, is a single polypeptide chain of 145 aa (Villalba et al., 1993; Lauzurica et al., 1988) that shows a high degree of sequence homology and IgE crossreactivity to the main allergens in other *Oleaceae* family pollens such as lilac, privet and, in particular, to ash, a relevant allergen source in areas such as Central Europe and North America. These facts make possible their use as a marker allergen to detect genuine sensitization to this family and in particular to ash (Martín-Orozco et al., 1994; Niederberger et al., 2002; Castro et al., 2007; Lombardero et al., 2002).

Ole e 1 has at least 4 B-cell epitopes (Martín-Orozco et al., 1994) and 2 regions, aa91 to 102 and aa109 to 130 that are defined as immunodominant T-cell epitopes (Cárdaba et al., 1998). Several works have analyzed the IgG and IgE B-cell epitopes of Ole e 1 for developing a molecule-based therapeutic strategy (González et al., 2006; Marazuela et al., 2008; Twaroch et al., 2011).

On the other hand, it was published how continuous exposure to olive pollen lowered the likelihood of patients to be sensitized to olive-pollen allergens (Geller-Bernstein et al., 2002; Florido et al., 1999). Also, a pilot study showed how stimulation with Ole e 1 peptides induced a different cytokine profile in peripheral blood mononuclear cells (PBMCs) from olive pollen-allergic patients compared to non-allergic subjects, according to IL-10 (Cárdaba et al., 2007), being the peptides that included aa10–31 Ole e 1 region, mainly recognized by non-allergic subjects, and postulated as possible immunomodulator peptides. More recently, we have described the decrease of TGF- β sera levels and Foxp3 mRNA expression in olive pollen-allergic subjects, compared with healthy controls and subjects with specific treatment, facts that indicated the lack of regulatory mechanisms in olive pollen-allergic subjects during the pollen season (Aguerrí et al., 2012). Further, we have defined a gene-expression pattern that makes it possible to discriminate different clinical conditions related with olive-pollen response in subjects with high exposure (Aguerrí et al., 2013).

This report explores how Ole e 1-derived peptides affect gene expression and examines the capacity of these peptides to modulate the levels of Th1/Th2 and Treg cytokines by studying the stimulation of PBMCs of responder and non-responder subjects to this allergen. This study could be important to understand the behavior of Ole e 1 peptides and their capacity to modulate genes and/or pathways that play essential roles in this disease, as well as to establish biomarkers, that might be useful as new therapeutic tools to modulate olive-pollen allergy and allergy to other members of the *Oleaceae* family.

2. Material and methods

2.1. Subjects

The study population comprised 15 untreated olive pollen-allergic patients and 14 non-allergic controls selected from a previous immunological study (Aguerrí et al., 2012). The subjects were unrelated and recruited and diagnosed at the Allergy Service of hospitals in Andalusia (Spain), a region in southern Spain with particularly high pollen counts during the pollen season and high prevalence of asthma. Subjects were selected in 2 olive pollen exposure conditions: April to June, with very high pollen counts, and outside the pollen season, October to December, with low pollen counts.

Non-allergic control was composed by subjects with no history of respiratory allergies.

Olive pollen-allergic patients fulfilled the following criteria: seasonal rhinitis and/or asthma from April to June, a positive skin prick test result for *O. europaea* pollen extract (ALK Abelló, Madrid, Spain), and no previous immunotherapy.

All subjects underwent skin prick testing for olive pollen allergy with whole *O. europaea* extract (ALK Abelló) and a battery of common allergens (Aguerrí et al., 2012), following the EAACI recommendations (EAACI, 1989).

The exclusion criteria were: current smoking, age outside the correct range, less than 10 years' residence in the study area, and corticosteroid or anti-histaminic treatment.

Informed consent was obtained from each subject. Ethical approval for the study was obtained from the Ethical and Research Committee of the participating hospitals. Biological samples were obtained from Biobanco-FJD, IIS-Fundación Jiménez Díaz Madrid.

Total IgE and *O. europaea* specific IgE antibodies determinations were previously described (Aguerrí et al., 2012).

2.2. Olive tree pollen extract and Ole e 1 peptide purification

Olive tree pollen was obtained from Allergom AB, Sweden. The pollen (5% w/v) was extracted with 50 mM ammonium bicarbonate, pH 8.0, containing 1 mM phenyl-methyl-sulfonyl-fluoride, followed by centrifugation at 12 000 $\times$ g for 20 min at 4 °C. The lyophilized supernatant was stored at –20 °C.

Ole e 1 dodecapeptides were synthesized (purity >90%) according to the Ole e 1 amino-acid sequence (Villalba et al., 1993). The peptides used were: p2 (aa11–22: FHIQQQVYCDTC) and p3 (aa22–33: CRAGFITELSEF) selected for being peptides mainly recognized by non-allergic subjects and inducers of IL-10 (Cárdaba et al., 1998, 2007) and p10 (aa91–102: NEIPTEGWAKPS), p12 (aa109–120: TVNGTTRTVNPL), and p13 (aa119–130: PLGFFKKEALPK) described as T-cell immunodominant regions (Cárdaba et al., 1998).

2.3. PBMC culture

PBMCs were isolated from heparin-containing peripheral blood samples taken during and outside pollen season, by gradient centrifugation on Lymphoprep (Comercial Rafer, Zaragoza, Spain) following the manufacturer's instructions. PBMCs (10^6 cells) were cultured in RPMI 1640 supplemented with 5% FBS inactivated, 1% L-glutamine, 1% peni-streptomycin and 1 mM sodium pyruvate (Flow Laboratories, Irvine, UK) in the absence or presence of olive pollen stimuli: olive pollen extract (*Olea europaea*, 25 μ g/ml), peptides 2 + 3 (p2 + 3, 5 μ g/ml), peptides 10 + 12 + 13 (p10 + 12 + 13, 5 μ g/ml) and PHA (2.5 μ g/ml) as positive control, during 24 h 37 °C, 5% CO₂ in humidified air. Concentration and combination of stimuli were based on previous assays (Cárdaba et al., 1998).

After stimulation, the cells were isolated by centrifugation (10 min, 720 g). All the supernatants were used to measure soluble cytokine levels. Total RNA of 6 patients from each group and pollen-exposure moment was extracted using Trizol method (Invitrogen, Carlsbad, CA). A total of 96 RNA samples were studied (6 patients $\times$ 2 groups $\times$ 2 exposure moments $\times$ 4 experimental conditions).

2.4. Soluble cytokine levels measurement

Levels of soluble IL-2, IFN- γ (Th1 cytokine), IL-4 and IL-5 (Th2 cytokines), IL-10 (Treg cytokine) were analyzed in the cell supernatants by flow cytometry, using the BD Cytometric Bead Array (CBA), human Th1/Th2 Cytokine Kit (Becton Dickinson, BD, San Diego, CA) and the BD CBA Human Soluble Protein Flex Set System (BD) for IL-13 (Th2 cytokine). Flow cytometric analyses were

performed using a FACSCalibur flow cytometer (BD). Data were acquired and analyzed by Becton Dickinson (BD) Cytometric Bead Array (CBA) Software.

TGF- β (Treg cytokine) supernatants levels were determined by ELISA (BD).

2.5. Statistical analysis

Data were analyzed using the Fisher, χ^2 test, 2-factor ANOVA test (for statistical comparison of quantitative parameters) and multiple regression analysis to examine quantitative traits (total IgE, specific IgE to *O. europaea* extract, and cytokines levels). Multiple comparisons were analyzed with the ANOVA test with the post hoc contrast “Bonferroni”.

2.6. Microarray preparation and analyses

Microarray experiments were performed according to the MIAME guidelines.

Ninety-six total RNA samples (using a pooling strategy) were studied. Equimolar amounts of RNA (1:1 from each condition) from 2 independent samples were pooled prior labeling and hybridization strategy. Three independent pools were created for each experimental condition (non-stimulated cells, stimulated with *Olea* pollen extract, stimulated with peptides 2 + 3 and stimulated with peptides 10 + 12 + 13), for each group (non-allergic control and allergic patients) and for each pollen-exposure moment (during and outside). Therefore, the total number of RNAs analyzed by microarrays was forty eight.

Microarray targets were prepared from 200 ng of total RNA using the Affymetrix kit (Santa Clara, CA, USA) for reverse transcription and a double round of in vitro transcription. HuGe U133 plus 2.0 GeneChip were hybridized with 10 μ g of cDNA and processed by manufacturer's protocol. This process was performed in Scientific Park of Complutense, Madrid, Spain.

2.7. Image and data analysis

Preliminary microarray analyses included assessment of data quality by standard quality checks and principal component assays (PCA). First-line check, background corrections, and quantile normalization of the data were performed by Robust Multiarray Averaging (RMA) method. Data analysis was performed using Partek Genomics Suite software.

2.8. Microarray analysis

All primary data discussed in this work have been deposited in NCBI's Gene Expression Omnibus and are accessible through GSE54522 accession numbers.

The analyses were carried out using the Babelomics web suite (Medina et al., 2010).

2.9. Differential expression

Differential gene expression assessment of all comparisons was carried out using limma moderated *t*-statistics. The *P*-values were corrected for multiple testing using the correction of Benjamini Hochberg (Benjamini and Hochberg, 1995).

2.10. Functional profiling

Gene set analysis was carried out by the logistic regression models as previously described (Sartor et al., 2009). Functional blocks from two data-bases, GO Biological Process and KEGG Pathways

Table 1
Demographic and clinical characteristics of study population.

Study population		n	Mean age (years)	Sex (%) female/male	IN	OUT		Clinical diagnosis
Group						Total IgE (IU/ml)	Specific IgE (kAU/l)	
Non-allergic control	14		37 $\pm$ 10	46/54*	61.76 $\pm$ 36.08*	67.88 $\pm$ 61.01*	0.34 $\pm$ 0.00*	Non-allergic
Untreated Olive pollen-allergic patients	15		33 $\pm$ 9	86/14*	144.26 $\pm$ 127.66*	185.87 $\pm$ 174.29*	44.79 $\pm$ 37.29*	82% Asthma and rhinitis 18% Rhinitis

IU: International Units; AU: Arbitrary Units.

* *P* < 0.05 comparing both groups.

were used. For each comparison, the number of functional statistical significant blocks (adjusted P value <0.05), with differential genes enrichment, expressed by positive or negative LOR (log odds ratio) was obtained.

2.11. Validation of differential gene-expression by qRT-PCR

One hundred eighty-nine genes were selected from differential gene expression results in samples obtained outside pollen season studied, according to fold-change value (>2 or <-2), P value <0.05 , and based on their representativeness of differential pathways and biological processes. These genes were validated in a cohort of 10 allergic subjects selected according to the same criteria as the initial population, using qRT-PCR.

Forty RNAs samples (10 patients $\times$ 4 experimental conditions) were studied, using a pooling strategy from 2 independent samples. A total of 20 pools by duplicated, were analyzed by qRT-PCR. We used 384-well micro fluidic cards (Applied Biosystem, Foster City, CA, USA) with the 189 genes and 3 reference genes (18S, GAPDH, β 2-Microglobuline). Two-hundred nanograms of total individual RNA were sent to the Scientific Park of Cantoblanco, Madrid, Spain, to be analyzed by qRT-PCR using the TaqMan Gene Expression System (Applied Biosystems). Specific mRNA expression from each gene was normalized to 18S rRNA gene, and calculated according to the cycle threshold (C_t) method. Statistical analyses were performed with StatMiner program.

Functional analyses of 51 genes selected were performed using the DAVID Bioinformatics Resources 6.7 program (<http://david.abcc.ncifcrf.gov/>) and the analysis of protein interactions was carried out with the STRING 9.0 (known and predicted protein–protein interactions) free program (<http://string-db.org/>).

3. Results

3.1. Subjects

The demographic and clinical parameters of the population studied are summarized in Table 1.

The mean levels of *Olea europaea*-specific IgE antibodies and total IgE underwent a statistically significant increase in the allergic group ($P < 0.05$); in these subjects, higher levels were obtained outside pollen season than during pollen season, as has been described previously (Aguerre et al., 2012).

3.2. Levels of cytokines in supernatant of stimulated PBMCs

No clear pattern of secreted cytokines made it possible to distinguish between both groups. Mean levels of Th1 and Th2 cytokines were overall low (data not shown) and specifically, IL-2 and IFN- γ mean levels were higher in healthy control group in the most of experimental conditions, and undetectable for IL-13, except for allergic patient samples outside pollen season stimulated with *Olea europaea* extract and with p10 + 12 + 13 Ole e 1 peptides.

Figs. 1 and 2 summarize the mean data of regulatory cytokines (IL-10 and TGF- β). The results showed higher IL-10 levels in allergic patients except for patient samples stimulated with p2 + 3 and p10 + 12 + 13 outside pollen season (Fig. 1B). Interestingly, during pollen season (Fig. 1A), patient samples stimulated with p2 + 3 showed the highest IL-10 levels. In contrast, the mean TGF- β levels (Fig. 2) were higher in control than in allergic samples, except for patient samples stimulated with p2 + 3 and p10 + 12 + 13 outside pollen season, the same stimuli that showed the lowest levels of IL-10.

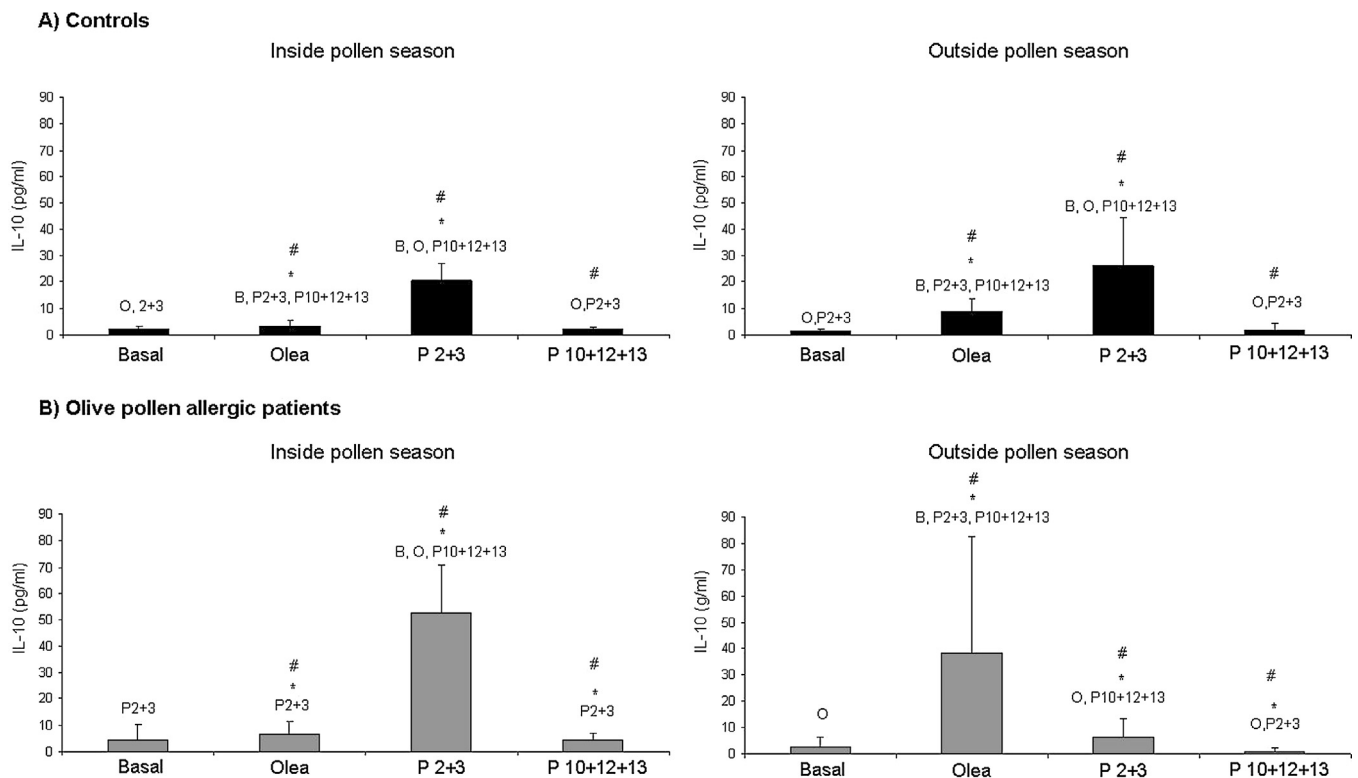


Fig. 1. Levels of IL-10 in supernatant of PBMCs unstimulated and stimulated. # Statistically significant comparisons ($P < 0.05$) between control and patient group in the same stimuli and pollen season moment. * Statistically significant comparisons ($P < 0.05$) in the same subject groups, between the two season moments. B (basal), O (olea), P2 + 3 (peptides 2 + 3), P10 + 12 + 13 (peptides 10 + 12 + 13); statistically significant comparisons ($P < 0.05$) in the same subject group and pollen season moment, among different stimuli.

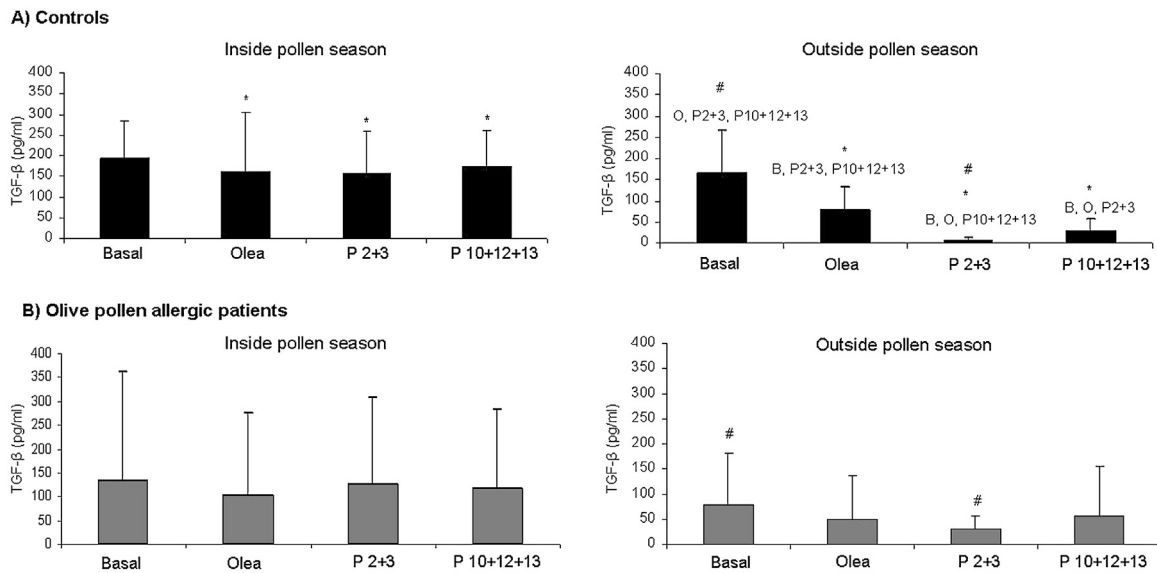


Fig. 2. Levels of TGF- β in supernatant of PBMCs unstimulated and stimulated. # Statistically significant comparisons ($P < 0.05$) between control and patient group in the same stimuli and pollen season moment. * Statistically significant comparisons ($P < 0.05$) in the same subject groups, between the two season moments. B (basal), O (olea), P2 + 3 (peptides 2 + 3), P10 + 12 + 13 (peptides 10 + 12 + 13); statistically significant comparisons ($P < 0.05$) in the same subject group and pollen season moment, among different stimuli.

3.3. Gene expression clustering according to experimental conditions

Whole-genome gene-expression analysis was performed on 24 pools of RNA samples from each group. Specifically, these 24 samples were 3 pools of two independent RNA samples $\times$ 4 experimental conditions (basal, olea extract, peptides 2 + 3, peptides 10 + 12 + 13) $\times$ 2 exposure-pollen moment, for each group of study, control and allergic subjects.

After data normalization, a principal component analysis (PCA) was performed. This unsupervised analysis did not show a clear

pattern in the control group; however, allergic-subject samples grouped in clusters correlated with experimental conditions (Fig. 3). This fact could indicate that the stimuli modulate different genes in allergic and non-allergic subjects.

In order to define specific gene differences, two kinds of analyses were performed: First, differential gene-expression comparisons and functional analyses, by KEGG and gene-ontology, of the four experimental conditions between control group and allergic subjects group in the two pollen-exposure moments. The number of statistically significant genes among the different conditions studied is described in the Supplementary Results (basal data were

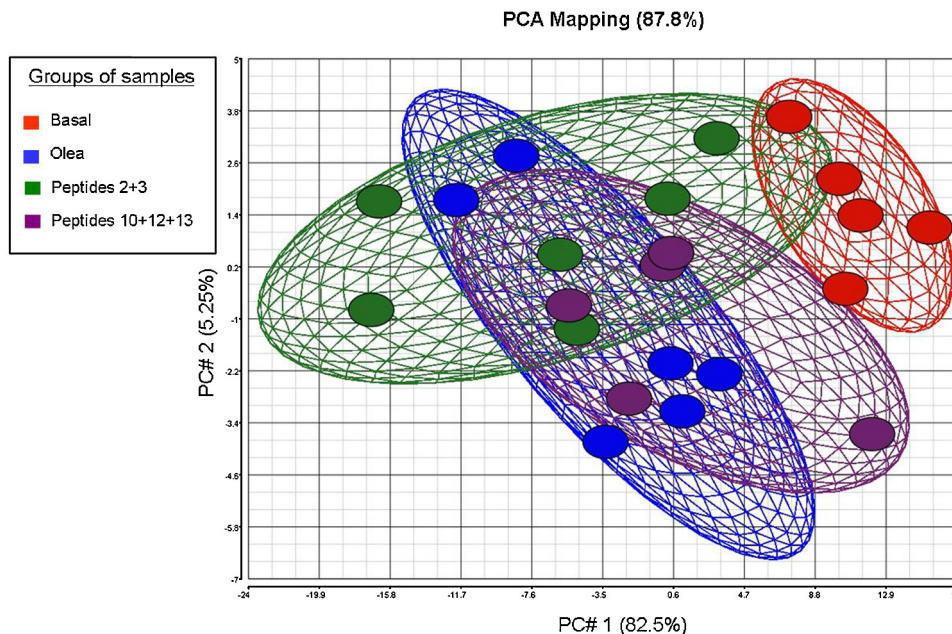


Fig. 3. Principal component analysis (PCA) to detect global genetic variations among olive pollen-allergic samples from 4 experimental conditions. The gene-expression analysis by unsupervised PCA showed how it was possible to draw a clear division and separate the data points from basal samples and stimulated with extract of *Olea europaea*, peptides 2 + 3 of Ole e 1, and peptides 10 + 12 + 13 of Ole e 1 ($P < 0.001$).

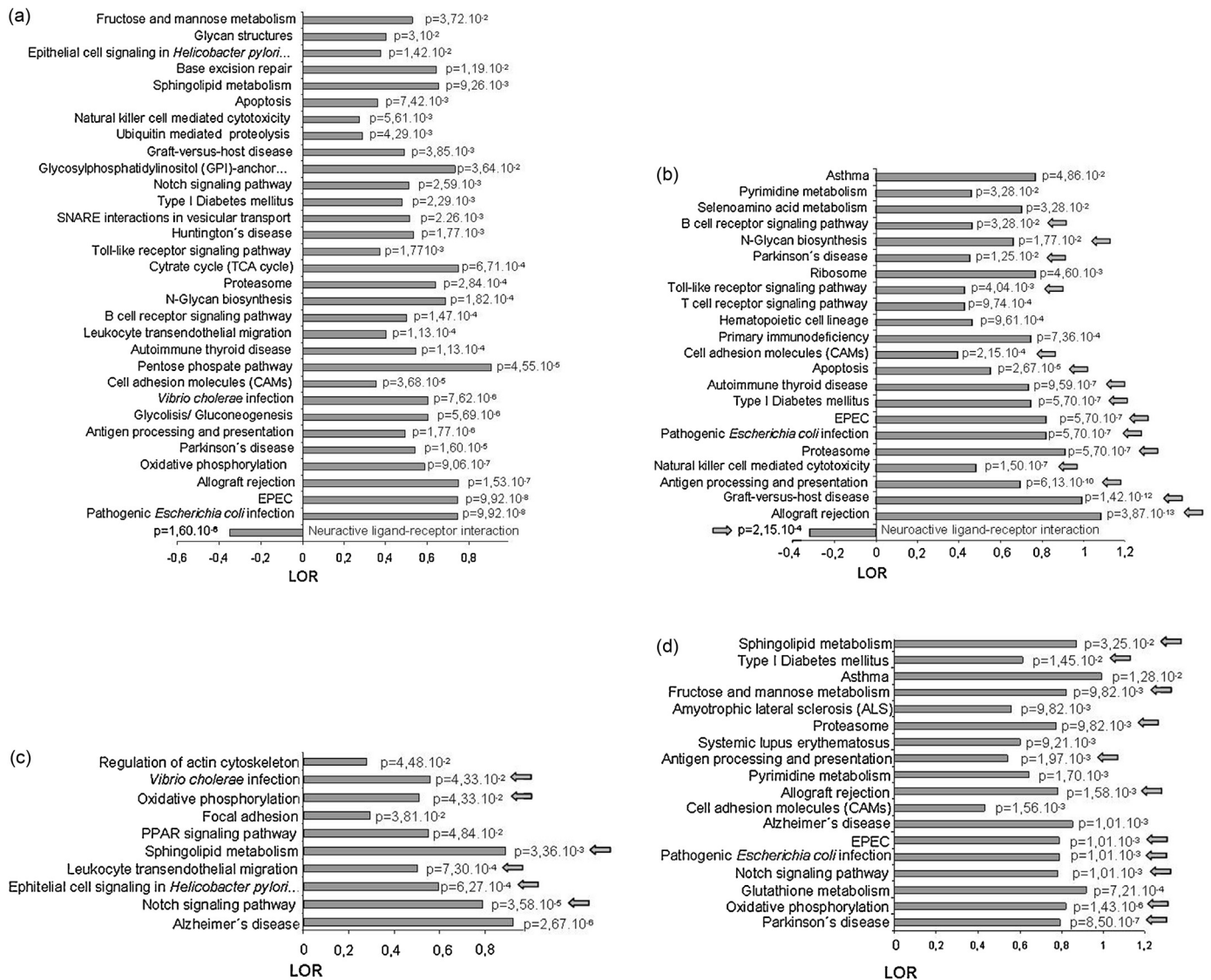


Fig. 4. Differential pathways ($p_{\text{adjusted}} < 0.05$) between samples from olive pollen-allergic patients and non-allergic controls, obtained outside pollen season. (A) Differential pathways between basal samples ($n = 32$) (Agueri et al., 2013). (B) Differential pathways between *Olea europaea* total extract stimulation samples ($n = 23$). (C) Differential pathways between samples stimulated with peptides 2 + 3 ($n = 10$). (D) Differential pathways between samples stimulated with peptides 10 + 12 + 13 ($n = 18$). LOR: odds ratio logarithm. Arrows indicate differential pathways shared with basal samples.

published previously) (Agueri et al., 2013). As our main focus was to analyze the influence of the stimuli, the analyses were mainly centered on samples stimulated outside pollen season, as this condition was likely to be more representative of what could happen biologically. Fig. 4 summarizes the statistically significant pathways between control and allergic groups, highlighting those that are shared among basal sample and each stimulus, outside pollen season.

Second, specific comparisons among stimuli and basal samples, into the allergic subjects group (outside pollen season). The results of differential gene-expression analyses between samples with or without stimulation are detailed in the Supplementary Results. Functional analyses revealed fewer differences between stimulation with peptides and basal samples than between *Olea europaea*-extract stimulation and basal samples.

Including both kinds of analyses, 189 transcripts were selected for validation for being statistically significant in at least one comparison, to have a fold change higher or lower than 2 and to be

representative of the major number of pathways and biological process possible.

3.4. Validation of differential gene expression by qRT-PCR

As part of the analysis of these 189 transcripts in 10 allergic patients using an unsupervised hierarchical model, the samples were classified according to the expression of these genes. It was possible to distinguish between samples stimulated with peptides 2 + 3 and the other experimental conditions (Fig. 5).

For a better understanding of these results, the samples were reanalyzed according to the expression of 51 genes (Supplementary Table 1) that showed the highest statistical differences comparing the peptides 2 + 3 stimulus to the other experimental conditions. This analysis defined the response to peptides 2 + 3 in 100% of the cases. The functional analysis of these genes revealed 5 specific clusters that included genes that are essential in the maintenance of the peripheral T-cell tolerance and in the

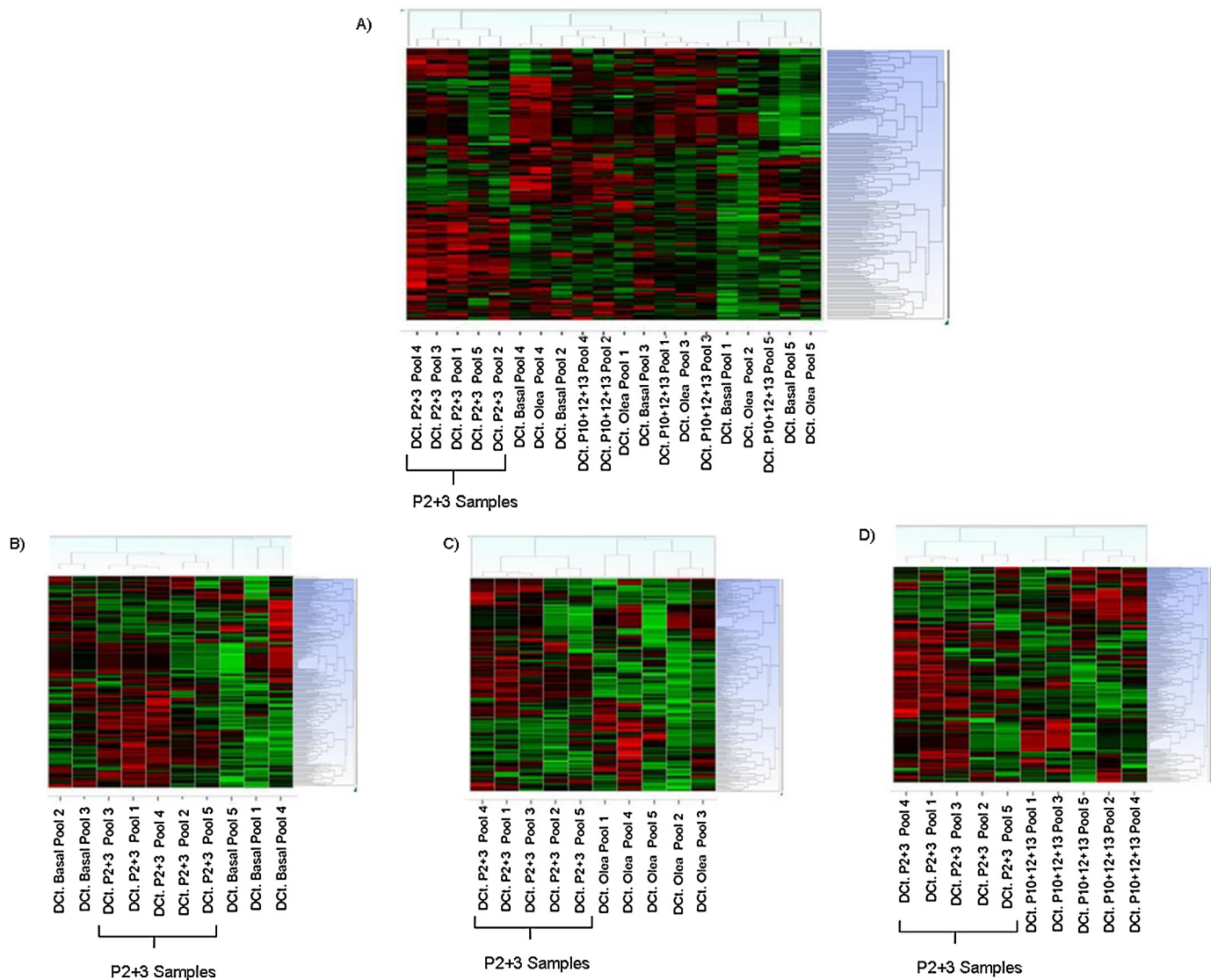


Fig. 5. Unsupervised hierarchical model grouping the samples stimulated with peptides 2 + 3 in allergic subjects, according to gene expression of the 189 most differential genes. (A) Classification of all the samples of the study. (B) Classification of peptides 2 + 3 peptides samples and basal samples. (C) Classification of peptides 2 + 3 samples and *Olea europaea* total extract stimulation samples. (D) Classification of peptides 2 + 3 samples and peptides 10 + 12 + 13.

Table 2
Functional annotation clustering of the 51 genes specifically regulated by peptides 2 + 3.

1. Immune-response E.S.: 6.05	Positive regulation of immune system process Chemotaxis Cytokine activity Response to bacterium Regulation of cell proliferation Others CD36, DAB2, FCN1, APOE, LRP1, STAB1	CD86, EBI3, C1QC, IL1B, IL2RA, IL6, TLR4 CCL20, CCL22, CXCL1, FPR3, IL1B, IL6 EBI3, CCL20, CCL22, CXCL1, INHBA, IL1A, IL1B, IL24, IL6 CCL20, IL1B, IL6, PTGS2, STAB1, TLR4 CD86, EBI3, CXCL1, GPNMB, APOE, IL1A, IL1B, IL2RA, IL6, PTGS2 FCN1, PI4K2A, TREM1, TREM2
2. Endocytosis E.S.: 2.68		
3. Epidermal growth factor E.S.: 1.76	ADAM28, ITGB8, LRP1, PTGS2, STAB1	
4. Lysosome E.S.: 1.61	FUCA1, GAA, LGMN, PSAP	
5. Wound healing E.S.: 1.57	CD36, IL1B, IL6, SERPINB2, TFPI2	
6. Miscellaneous	CAMK1, CD84, FAAH2, DNMT1, GDE1, ME1, MIR155HG, MYOF, RNASE1, SEMA6B, SPINK1, ST14, TGFB1, UBASH3B, WNT5A, ZNF467	

E.S.: enrichment score.

allergic patients after stimulation with Ole e 1 peptides (Cárdaba et al., 2007) and of a decrease in TGF- β sera levels in patients with olive-pollen allergy patients during pollen season (Aguerre et al., 2012), suggest the implication of regulatory mechanisms in the olive-pollen and Ole e 1 response, and more important, pointing to how these mechanisms of regulation could be different according to the stimuli and the environmental moment of pollen exposure. Although these data have to be corroborated in a bigger population and by stricter curves of cytokines secretion, but they point to the idea that the moment of pollen exposure could be very important for defining different immunotherapy strategies. This idea is in agreement with 2 recent reports that describe the importance of keeping in mind the presence of allergens in the environment for immunotherapy decisions (Wambre et al., 2010) and for interpreting the results of individual clinical trials (Durham et al., 2014).

The other main objective was to use massive-genomic tools to search for profiles of gene-expression or deregulated biological pathways having the ability to differentiate samples from allergic and non-allergic subjects stimulated with olive pollen-related stimuli. First, the fact that only the samples from allergic subjects were clustered by PCA according to stimulation conditions (unsupervised method) (Fig. 3) showed the different global behavior against specific stimuli between healthy and allergic subjects. Also, this result highlights the likelihood of finding genomic differences among the response to specific stimuli in allergic subjects.

Gene expression and functional analyses revealed many pathways and biological processes that are differentially expressed in the different experimental conditions studied. Fig. 4 summarizes the statistically significant pathways among controls and allergic groups and the 4 kinds of samples analyzed outside pollen season. Curiously, the comparison of basal samples between control and allergic groups showed a very high number of statistically increased pathways in allergic subjects, related and unrelated with immune response, as was previously described (Aguerre et al., 2013), but interestingly, the comparisons between groups of *Olea europaea* extract and stimulated samples with Ole e 1 T-cell peptides showed that the stimuli, in concordance with cytokine results and global gene-expression analysis, seem to modulate some specific pathways. Indeed, peptides 2 + 3 showed the lowest number of differential pathways compared with controls, and this was the only stimulus that did not induce the asthma pathway. In addition, comparisons of the 4 experimental conditions (basal and stimuli), in the allergic subjects group revealed that there were fewer differential pathways and biological process deregulated comparing peptides stimulation and basal, than between *Olea europaea* extract stimulation and basal, and there were several differences among stimuli.

The validation of the 189 most significant differential transcripts confirmed that these genes were differentially expressed among the experimental conditions, but specifically, their gene expression defined the response of the samples stimulated with peptides 2 + 3 (Fig. 5). Subsequent analysis of the most differential genes according to peptides 2 + 3 stimulation provided a profile of 51 genes (Supplementary Table 1). Heat maps of these genes (data not shown) were able to differentiate 100% of the samples stimulated with p2 + 3 from the other conditions. To our knowledge, this is the first report which describes a molecular profile that defines specific gene-expression modulation by allergen T-cell epitopes.

The functional analysis of these 51 genes revealed 5 main functional annotations clustering (Table 2) and one miscellaneous cluster. The main clustering was immune response and included 15 genes that codify proteins implicated in immune-allergic responses, and essential genes in the maintenance of the peripheral T-cell tolerance and inflammatory response. The analysis of these data with programs for protein interactions (Fig. 6) showed a group of proteins around a central core, including proteins related with immune response.

Overall, peptides 2 + 3 derived from the major Ole e 1 antigen appear to regulate the immune response in patients allergic to olive pollen, modifying the gene expression of many genes highly related to regulation such as DNMT1 (an enzyme associated with DNA methylation), LGMN (a gene implicated in human Treg cell function, which is induced upon T-cell receptor stimulation), DAB2 (a FOXP3 target gene required for Treg), EBI3 (subunit of IL-35 related to regulation of immune response), CD84 (a leukocyte receptor that may modulate Fc ϵ R-mediated signaling and plays a protective role against allergic responses), MIR155 and other attractive genes such as FPR3 (an innate immunity receptor that could be implicated in nasal tissue remodeling), TREM1, ALOX5, PTGS2 and several adhesion molecules ligands (CCL22, CCL20, etc.), cytokines and chemokines, and 2 genes, C1Q and STAB1, described as candidate biomarkers of early efficacy of allergen immunotherapy as the hallmark of a regulatory innate immune response predictive of clinical tolerance (Zimmer et al., 2012). These findings are consistent with our cytokine results and the description of an amino-acid region in Ole e 1, which includes these immunomodulatory peptides, as non-allergenic (Twaroch et al., 2011).

Specific immunotherapy is currently the only curative therapy, and is able to modify the course of the disease. However, the use of extracts potentially creates a risk among other causes, due to the possibility to sensitize against minority components. In olive pollen, this fact may be particularly relevant if we consider the greater clinical severity associated with minor pollen allergens (Quiralte et al., 2005).

Our study provides very interesting information on how the stimulus directly influences gene expression and, in particular, how the peptides 2 + 3 (pointed out as theoretically immunoregulatory epitopes) have a completely different behavior in gene expression of olive pollen-allergic subjects compared to *Olea europaea* extract and Ole e 1 immunodominant peptides, modulating a specific genetic profile, in which many of these genes are related to immunoregulation.

Regulatory T-cell epitopes (Tregitopes) were defined few years ago (De Groot et al., 2008) as peptides with special characteristics, emphasizing among others, to have a pattern of “promiscuous binding to Major Histocompatibility Complex” and to induce a regulatory T-cell profile, with IL-10 secretion among other soluble mediators (Elyaman et al., 2011). Their use in therapies for autoimmune diseases is starting to be considered (Elyaman et al., 2011). According to our global results, these Ole e 1 peptides act as “like-Tregitopes” with a high immunoregulator potential that should be more extensively studied.

In conclusion, we have demonstrated how, according to the stimuli used and the environmental moment of pollen exposure, different mechanisms of regulation could be implicated in olive-pollen response. Also, we have defined a profile of 51 genes specifically modulated by aa10–31 Ole e 1 which could be very useful as new therapeutic targets, opening new ways of research on the regulation of this disease.

Author contribution statement

D. Calzada, M. Aguerre, and B. Cárda have worked in all project steps: Design of the study, experimental work, results discussion, and manuscript drafting.

S. Baos collaborated in part of experimental work.

D. Montaner, M. Mata, and J. Dopazo have performed the statistical analysis.

J. Quiralte and F. Florido performed the patient selection and collaborated in the design of study.

C. Lahoz collaborated in the design of the study, results discussion, and manuscript elaboration.

Conflict of interest statement

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.molimm.2014.12.002>.

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SUPPLEMENTARY INFORMATION of:

Therapeutic targets for Olive pollen allergy defined by gene-markers modulated by Ole e 1-derived peptides

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SUPPLEMENTARY RESULTS

The highest number of statistical significant differential genes ($P < 0.05$ and fold change higher or lower than 2 or -2) was obtained when basal samples of allergic group was compared with basal samples of controls, being of 2045 upregulated genes and 1025 downregulated genes. In relation with stimuli, the number of modulated genes was lower: 994 upregulated vs 739 downregulated comparing samples stimulated with *Olea europaea* extract from allergic subjects with stimulated control samples, 866 upregulated vs 524 downregulated comparing samples of allergic subjects stimulated with peptides 2+3 and the same stimulus in controls and, 436 upregulated vs 582 downregulated genes comparing samples of allergic subjects stimulated with peptides 10+12+13 with controls.

The second type of statistical analysis was performed only among allergic group samples. The differential gene-expression analyses between samples with or without stimulation showed a total of 2118 differentially expressed ($P < 0.05$) between *Olea europaea* extract (930 upregulated and 1188 downregulated genes) and basal samples, 1626 between peptides 2+3 stimulated (763 upregulated and 863 downregulated) and basal samples and 1477 genes between peptides 10+12+13 (600 upregulated and 877 downregulated) and basal conditions. Functional analysis revealed that there was lower differential pathways and biological process between peptides stimulation and basal, than *Olea europaea* extract stimulation and basal.

Table S1. Description of the main genes modulated by peptides 2+3 of Ole e 1 allergen.

LocusLink Gene Symbol	LocusLink Gene Name	Location	Function
ABHD2	Abhydrolase domain containing 2	15q26.1	Non- described.
ADAM28	ADAM metallopeptidase domain 28	8p21.2	This gene encodes a member of the ADAM (disintegrin and metalloprotease domain) family. Implicated in biological processes involving cell-cell and cell-matrix interactions.
APOE	Apolipoprotein E	19q13.2	Essential for the normal catabolism of triglyceride-rich lipoprotein constituents.
CAMK1	Calcium/calmodulin-dependent protein kinase I,	3p25.3	Catalytic activity.
C1QC	Complement component 1, q subcomponent, C chain	1p36.11	This gene encodes a major constituent of the human complement subcomponent C1q.
CCL20	Chemokine (C-C motif) ligand 20	2q36.3	Chemotactic factor that attracts lymphocytes and, slightly, neutrophils, but not monocytes. Inhibits proliferation of myeloid progenitors. May be involved in formation and function of the mucosal lymphoid tissues by attracting lymphocytes and dendritic cells towards epithelial cells.
CCL22	Chemokine (C-C motif) ligand 22	16q13	May play a role in the trafficking of activated/effector T-lymphocytes to inflammatory sites and other aspects of activated T-lymphocyte physiology. Chemotactic for monocytes, dendritic cells and natural killer cells.
CD36	CD36 molecule (thrombospondin receptor)	7q11	Important functions as a cell adhesion molecule.
CD84	CD84 molecule	1q24	This gene encodes a membrane glycoprotein that is a member of the signaling lymphocyte activation molecule (SLAM) family.
CD86	CD86 molecule	3q21	The protein is expressed by antigen-presenting cells, and it is the ligand for CD28 antigen and cytotoxic T-lymphocyte-associated protein 4, at the cell surface of T cells. Binding with CD28 is a costimulatory signal for activation of the T-cell.
CXCL1	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	9q34	This protein plays a role in inflammation and as chemoattractant for neutrophils
DAB2	Disabled homolog 2, mitogen-responsive phosphoprotein (Drosophila)	5p13	It is involved in regulation of cytoskeleton-based functions by regulating cell positioning, inducing macrophage adhesion, or regulating protein trafficking by interacting with different proteins implicated in endocytosis.
DNMT1	DNA (cytosine-5-)-methyltransferase 1	19p13.2	The gene encoded by a enzyme implicated in the establishment and regulation of tissue-specific patterns of methylated cytosine residues.
EBI3	Epstein-Barr virus induced 3	19p13.3	EBI3 associates with p28 to form IL-27 (anti-inflammatory) and with p35 to form IL35 (important in immune regulation).
FAAH2	fatty acid amide hydrolase 2	Xp11.21	The encoded enzyme is able to catalyze the hydrolysis of a broad range of bioactive lipids.
FCN1	ficolin (collagen/fibrinogen domain containing) 1	9q34	It is predominantly expressed in the peripheral blood leukocytes, and has been postulated as a plasma protein with elastin-binding activity.

FPR3	formyl peptide receptor 3	19q13.3-q13.4	Formyl peptide receptors are involved in antibacterial host defense and inflammation. Activation of FPRs mediates induction of neutrophil chemotaxis, production of reactive oxygen species (ROS) to clear damaged cells and stimulation of degranulation of neutrophils
FUCA1	Fucosidase, alpha-L- 1, tissue	1p34	The protein encoded by this gene is a lysosomal enzyme involved in the degradation of fucose-containing glycoproteins and glycolipids
GAA	Glucosidase, alpha; acid	17q25.2-q25.3	This gene encodes by acid alpha-glucosidase essential for the degradation of glycogen to glucose in lysosomes.
GDE1	Glycerophosphodiester phosphodiesterase 1	16p12-p11.2	It has glycerophosphoinositol phosphodiesterase activity. It can be modulated by G-protein signaling pathways.
GPMB	Glycoprotein (transmembrane) nmb	7p15	
IL1A	Interleukin 1, alpha	2q14	The protein encoded by these genes is pleiotropic and an important mediator of the inflammatory response. Involved in several cellular activities, including cell proliferation, differentiation, and apoptosis.
IL1B	Interleukin 1, beta	2q14	
IL2RA	Interleukin 2 receptor, alpha	10p15-p14	This gene encodes a part of IL2R.
IL6	Interleukin 6	7p21	This gene encodes a cytokine implicated in inflammation and the maturation of B cells.
IL24	Interleukin 24	1q32	Immunoregulation.
INHBA	Inhibin, beta A	7p15-p13	Inhibins/activins are involved in regulating diverse functions such as hypothalamic and pituitary hormone secretion, gonadal hormone secretion, germ cell development and maturation, erythroid differentiation, insulin secretion, nerve cell survival, embryonic axial development or bone growth, depending on their subunit composition.
ITGB8	Integrin, beta 8	7p21.1	It mediates cell-cell and cell-extracellular matrix interactions and this complex plays a role in human airway epithelial proliferation.
LGMN	Legumain	14q32.1	This cysteine protease is involved in the processing of bacterial peptides and endogenous proteins for MHC class II presentation in the lysosomal/endosomal systems.
LRP1	Low density lipoprotein receptor-related protein 1	12q13-q14	The protein encoded by this gene is an endocytic receptor involved in several cellular processes, including intracellular signaling, lipid homeostasis, and clearance of apoptotic cells.
MEPG	Macrophage expressed gene 1	11q12.1	Expressed in macrophages and peripheral blood monocytes.
ME 1	Malic enzyme 1, NADP(+)-dependent, cytosolic	6q12	This gene encodes a cytosolic NADP-dependent enzyme that generates NADPH for fatty acid biosynthesis. Their activity, the reversible oxidative decarboxylation of malate, links the glycolytic and citric acid cycles.
MIR155HG	MIR155 host gene (non-protein coding)	21q21.3	miRNA155, are endogenous noncoding RNAs of about 22 nucleotides that regulate mRNAs by targeting them for cleavage or translational repression.
MYOF	Myoferlin	10q24	The protein encoded by this gene is a type II membrane protein (plasma and nuclear membranes), member of the ferlin family. The protein contains C2 domains that play a role in calcium-mediated membrane fusion events, suggesting that it may be involved in membrane regeneration and repair.
PI4K2A	Phosphatidylinositol 4-kinase type 2 alpha	10q24	Phosphatidylinositolpolyposphates (PtdInsPs) are centrally involved in many biologic processes, ranging from cell growth and organization of the actin cytoskeleton to endo- and exocytosis.

PSAP	Prosaposin	10q21-q22	This gene encodes a highly conserved glycoprotein which is a precursor for 4 cleavage products: saposins A, B, C, and D.
PTGS2	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	1q25.2-q25.3	Prostaglandin-endoperoxide synthase (PTGS), also known as cyclooxygenase, is the key enzyme in prostaglandin biosynthesis, and acts both as a dioxygenase and as a peroxidase. May have a role as a major mediator of inflammation and/or for prostanoid signaling in activity-dependent plasticity, induced by cytokines and mitogens
RNASE1	Ribonuclease, RNase A family, 1 (pancreatic)	14q11.2	This gene encodes a member of the pancreatic-type of secretory ribonucleases, a subset of the ribonuclease.
SEMA6B	Sema domain, transmembrane domain (TM), and cytoplasmic domain, (semaphorin) 6B	19p13.3	Located in axonal terminals and might have a role in immunoregulation.
SERPINB2	Serpin peptidase inhibitor, clade B (ovalbumin), member 2	18q21.3	Inhibits urokinase-type plasminogen activator.
SPINK1	Serine peptidase inhibitor	5q32	Mutations in this gene are associated with hereditary pancreatitis.
STAB1	Stabilin 1	3p21.1	Lymphocyte homing.
ST14	Suppression of tumorigenicity 14 (colon carcinoma)	11q24-q25	Integral membrane serin protease.
TFPI2	Tissue factor pathway inhibitor 2	7q22	Proteinase inhibitor. May play a role in the regulation of plasmin-mediated matrix remodelling.
TGFBI	Transforming growth factor, beta-induced, 68kDa	5q31	
TLR4	Toll-like receptor 4	9q33.1	Pathogen recognition and activation of innate immunity.
TREM1	Triggering receptor expressed on myeloid cells 1	6p21.1	This receptor amplifies neutrophil and monocyte-mediated inflammatory responses.
TREM2	Triggering receptor expressed on myeloid cells 2	6p21.1	The encoded protein is involved in inflammation by triggering the production of constitutive inflammatory cytokines.
UBASH3B	Ubiquitin associated and SH3 domain containing B	11q24.1	The encoded protein was found to inhibit endocytosis of epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor.
WNT5A	Wingless-type MMTV integration site family, member 5A	3p21-p14	Ligand for members of the frizzled family of seven transmembrane receptors. Can activate or inhibit canonical Wnt signaling, depending on receptor context. In the presence of FZD4, activates beta-catenin signaling. In the presence of ROR2, inhibits the canonical Wnt pathway by promoting beta-catenin degradation through a GSK3-independent pathway which involves down-regulation of beta-catenin-induced reporter gene expression.
ZNF467	Zinc finger protein 467	7q36.1	Involved in transcriptional regulation.