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ORIGINAL ARTICLE

Basic and Translational Allergy Immunology

Unique molecular signatures typify skin inflammation induced by chemical allergens and irritants

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Abstract

Background: Skin exposure to chemicals may induce an inflammatory disease known as contact dermatitis (CD). Distinguishing the allergic and irritant forms of CD often proves challenging in the clinic.

Methods: To characterize the molecular signatures of chemical-induced skin inflammation, we conducted a comprehensive transcriptomic analysis on the skin lesions of 47 patients with positive patch tests to reference contact allergens and nonallergenic irritants.

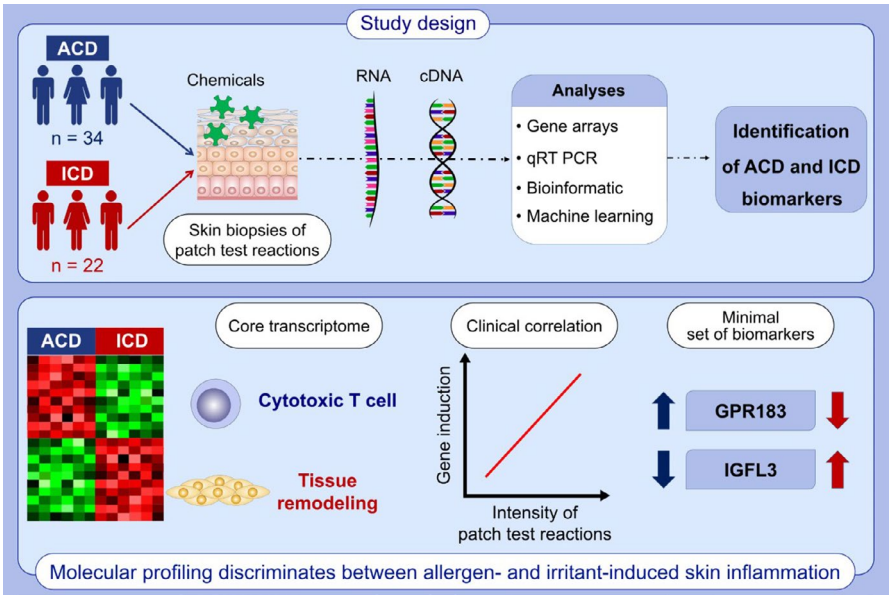
Results: A clear segregation was observed between allergen- and irritant-induced gene profiles. Distinct modules pertaining to the epidermal compartment, metabolism, and proliferation were induced by both contact allergens and irritants; whereas only contact allergens prompted strong activation of adaptive immunity, notably of cytotoxic T-cell responses. Our results also confirmed that: (a) unique pathways characterize allergen- and irritant-induced dermatitis; (b) the intensity of the clinical reaction correlates with the magnitude of immune activation. Finally, using a machine-learning approach, we identified and validated several minimal combinations of biomarkers to distinguish contact allergy from irritation.

Conclusion: These results highlight the value of molecular profiling of chemical-induced skin inflammation for improving the diagnosis of allergic versus irritant contact dermatitis.

KEYWORDS
allergic contact dermatitis, chemical allergens, chemical irritants, biomarker, transcriptomic profiling

Abbreviations: ACD, Allergic contact dermatitis; CD, Contact dermatitis; CTL, cytotoxic CD8+ T cell; DEG, Differentially expressed gene; Fc, Fold change; FDR, False discovery rate; GNLY, Granulysin; GZMB, Granzyme B; ICD, Irritant contact dermatitis; IFNG, Interferon gamma; MI, Methylisothiazolinone; PCA, Principal component analysis; PT, Patch test; SLS, Sodium lauryl sulfate; Teff, Effector T cells; Tmem, Memory T cells.

Marine-Alexia Lefevre, Audrey Nosbaum, Jean-François Nicolas and Marc Vocanson contributed equally to this work.



GRAPHICAL ABSTRACT

The core transcriptome of allergen- and irritant-induced reactions consists of cytotoxic T-cell-and tissue remodeling-related transcripts, respectively. The magnitude of gene activation correlates with the intensity of the clinical reactions. Machine-learning approach identifies several minimal combinations of biomarkers to distinguish allergic versus irritant contact dermatitis.

1 | INTRODUCTION

Contact dermatitis (CD), or contact eczema, is a skin disease affecting approximately 15% to 20% of the general population in industrialized countries.[1,2] CD is the most frequent occupational disease in many European countries.[3] The direct (treatment and compensation) and indirect (sick leave and lack of productivity) costs associated with the disease have resulted in the economic burden of CD being estimated at over 5 billion Euros per year in Europe.[4] CD typically presents as acute inflammation with itching, redness, edema, vesicles, and oozing, as well as thickening and crusting in its chronic form. CD is induced by repeated skin contacts with external agents. A wide range of substances has been implicated in the disease, notably chemicals with irritant and/or allergenic properties, which are responsible for the irritant (ICD,[5]) and allergic (ACD,[6]) types of CD respectively. Available data indicate that ICD accounts for approximately 70% to 80% of all cases of occupational CD in industrialized countries.[7]

The mechanisms by which chemicals cause ICD are poorly understood, and vary from disorganization of cell membrane lipid bilayers to damage of barrier proteins.[8] ICD may be caused by acute exposure to corrosive substances such as acids and bases, which trigger intense cell necrosis and major skin barrier disruption, or by chronic exposure to mild irritants such as detergents.[9] In both cases, irritation is most likely triggered by injured cells releasing reactive oxygen species (ROS) and ATP in response to stress, and generating damage-associated molecular patterns (DAMPs) involving high-mobility group box 1 (HMGB1), heat-shock proteins, and interleukin (IL)-1a. These alterations are then detected by the innate receptors of surrounding healthy cells,[10] resulting in the release of a myriad of inflammatory mediators (cytokines, chemokines, arachidonic acid derivatives, proteases, etc.) within minutes to hours after contact.[11,12] The reaction is further amplified by infiltration from the blood of inflammatory monocytes, neutrophils, etc.[13]

ACD is the prototype of a type IV delayed-type hypersensitivity reaction, which develops in individuals who have previously been sensitized to chemical allergens (also referred to as haptens). The most common clinically relevant allergens associated with occupational or nonoccupational exposure include protein-reactive compounds, such as metals, fragrances, preservatives, or rubbers.[14] ACD is mediated by the recruitment and reactivation of allergen-specific effector/memory T cells (Teff/mem) – key cells involved in the immune response to allergens and skin inflammation.[15] Akin to irritants, certain contact allergens have toxic/proinflammatory properties and activate danger recognition platforms (Toll-like and NOD-like receptors) through the release of stress and DAMP signals to induce T-cell sensitization and ACD development.[16]

Although ICD and ACD involve different pathogenic molecular mechanisms,[17,18] their symptoms and presentation are often similar, making it difficult to distinguish between the two CD forms without performing allergology tests, such as patch tests.[19] Although the robustness of patch tests has long been established, this method can sometimes give inconclusive results, leading to problems in disease management. This is particularly common for patients with highly sensitive skin: these patients mount positive responses both to reference allergens and to noninflammatory doses of irritant controls, leading to questions about the specificity of allergen-induced reactions. A recent 20-year retrospective analysis revealed that 30%–40% of weak positive patch test reactions were not reproduced on retesting.[20,21] This suggests a lack of

persistence of skin allergy in some patients, or that some of the skin test results were false positives, occurring due to the irritant properties of chemicals applied for several days under occlusion.[22]

There is now great interest in developing new diagnostic approaches that could help clinicians make a reliable diagnosis of CD. A recent study concluded that an immunohistochemical phenotyping approach, based on a minimal set of markers (CD3, CD4, CD8, etc.), was not reliable enough to discriminate between the cellular events shaping the ACD and the ICD reactions.[23] Alternatively, several investigators have explored the molecular signatures induced by clinically relevant chemical allergens in human skin.[24-28] They identified pathways in CD that were distinct from those occurring in dermatoses such as psoriasis and atopic dermatitis,[24,29] and Fortino *et al*. recently proposed that gene profiling could be of major value for discriminating between ICD and ACD.[28]

In this study, we then conducted a comprehensive analysis to compare the reactions induced by a large set of allergens (n = 6) and irritants (n = 3), as well as different degrees of clinical reactions, in order to identify ACD and ICD biomarkers robust enough to allow for the variability of clinical contexts.

Our results identified the core signatures of allergen- and irritant-induced skin inflammation, opening the way for the development of new approaches to improve ACD diagnosis.

2 | METHODS

A brief description of the Methods is provided below. Additional details about study design, gene arrays, qRT-PCR experiments, and bioinformatic and biostatic analyses are provided in the Supplemental Methods section.

Patients suspected for skin allergy were enrolled in the study after being referred to the hospital for routine diagnosis of contact dermatitis. They were patch tested either with the contact allergens: nickel, methylisothiazolinone [MI], and linalool hydroperoxide, or the drug allergens: amoxicillin, piperacillin/tazobactam, and acetazolamide, and/or the irritants: SLS, nonanoic acid, and cantharidin (Table 1). At 72 h, the patch test sites were evaluated for reactivity, and reactions were clinically graded as negative (–), doubtful (+/?), or positive (1+, 2+ or 3+).[19] Biopsies (3 × 3 mm) were collected from positive and negative patch-tested skin, as well as from control areas including nonlesional skin or vehicle (petrolatum or isopropanol) patch-tested skin. Total RNA was then extracted from the biopsies and used for Affymetrix PrimeView Human Gene Expression array and qRT-PCR analysis.

The study was approved by the local ethics committee (N°19-145 CHU Lyon), and written informed consent was obtained from each participant.

3 | RESULTS

Forty-seven patients suspected for ACD (34 females and 13 males; aged 29–88 years, median 61 years) were enrolled in this study and received patch tests (PT) to different chemicals. Overall, thirty-six patients developed positive reactions to contact allergens (nickel, n = 10; MI, n = 6; linalool hydroperoxide, n = 10) and/or chemical irritants (SLS, n = 7; nonanoic acid, n = 4; cantharidin, n = 7). These patients were biopsied and their samples were subjected to extensive genomic and molecular profiling. In parallel, we biopsied 11 patients with suspected drug allergies, who developed positive patch tests to drug allergens (amoxicillin, n = 6; acetazolamide, n = 1; piperacillin/tazobactam, n = 1) and/or irritants (SLS; n = 4). These patients were included in the study because patch test reactions to drugs are thought to exhibit clinical, histological, and pathophysiologic mechanisms similar to those of reactions to contact allergens.

Patient clinical features and patch test scores are detailed in Table 1. All patch test reactions were positive (graded as 1+, 2+, 3+), except for two patients with doubtful (+/?) reactions to linalool hydroperoxide and MI, and five patients with negative reactions to SLS.

3.1 | Molecular profiling discriminates between contact allergy and irritation

We first executed a hypothesis-free principal component analysis (PCA) of data from the full set of genes measured on the arrays. This PCA demonstrated that all allergen and irritant samples were segregated from their respective controls, with the exception of the negative SLS patch test reaction samples (Figure 1). This PCA also showed that the expression profiles of the majority of allergen samples (including those of the drug allergens) diverged from those of the irritant samples. All allergen and irritant samples were on the same side of PC1, suggesting that gene expression was partially shared. However, PC1/PC2 analysis revealed clear differences in expression (Figure 1). Interestingly, the expression profiles of nickel, linalool hydroperoxide, and amoxicillin samples differed from each other. No differences were observed when nickel samples were compared with MI samples. Similar differential profiles were observed with the irritant molecules, notably between cantharidin and SLS (Figure 1).

Although differences in the magnitude of the respective patch test reactions between sample specimens (Table 1) may have an impact on the observed PCA variations, these results confirm that the molecular signatures of positive patch test reactions to allergens and irritants are distinct, and that molecule-dependent profiles exist within each group.[24-28]

3.2 | Main signaling pathways differentiating allergen- and irritant-induced inflammatory responses

We then searched to define the genes and signaling pathways that differed between the allergen and irritant samples. We first evaluated the number of differentially expressed genes (DEGs) between samples from each patient in each group and their respective controls. The five SLS samples that did not exhibit positive clinical responses

TABLE 1 Patient features and patch test results

Patient		Patch test			
Patient number	Gender/age	Indication	Class	Compound	Result
1	M/78	Eczema	Irritant	Cantharidin	2+
			Vehicle	Petrolatum	–
2	F/57	Eczema	Irritant	Cantharidin	3+
			Vehicle	Petrolatum	–
3	F/66	Eczema	Irritant	Cantharidin	3+
			Vehicle	Petrolatum	–
4	F/43	Eczema	Irritant	Cantharidin	1+
			Vehicle	Petrolatum	–
5	M/67	Eczema	Irritant	Cantharidin	2+
			Vehicle	Petrolatum	–
6	F/52	Eczema	Allergen	Linalool hydroperoxide	+/?
			Vehicle	Petrolatum	–
7	F/50	Eczema	Allergen	Nickel	2+
			Vehicle	Petrolatum	–
8	M/66	Eczema	Allergen	Methylisothiazolinone	2+
			Nonlesional skin	-	–
9	F/50	Eczema	Irritant	Cantharidin	3+
			Allergen	Nickel	1+
			Vehicle	Petrolatum	–
10	F/61	Eczema	Irritant	Cantharidin	3+
			Vehicle	Petrolatum	–
11	F/40	Eczema	Allergen	Methylisothiazolinone	3+
			Allergen	Nickel	3+
			Vehicle	Petrolatum	–
12	F/68	Eczema	Allergen	Linalool hydroperoxide	2+
			Vehicle	Petrolatum	–
13	F/41	Eczema	Allergen	Nickel	3+
			Vehicle	Petrolatum	–
14	F/41	Eczema	Allergen	Nickel	2+
			Vehicle	Petrolatum	–
15	F/60	Eczema	Allergen	Methylisothiazolinone	3+
			Vehicle	Petrolatum	–
16	F/62	Eczema	Allergen	Nickel	1+
			Vehicle	Petrolatum	–
17	F/53	Eczema	Allergen	Nickel	2+
			Vehicle	Petrolatum	–
18	F/34	Eczema	Allergen	Methylisothiazolinone	2+
			Vehicle	Petrolatum	–
19	M/42	Eczema	Allergen	Linalool hydroperoxide	2+
			Vehicle	Petrolatum	–
20	F/66	Eczema	Allergen	Linalool hydroperoxide	1+
			Nonlesional skin	-	–
21	F/65	Eczema	Allergen	Linalool hydroperoxide	3+
			Vehicle	Petrolatum	–

(Continues)

TABLE 1 (Continued)

Patient		Patch test			
Patient number	Gender/age	Indication	Class	Compound	Result
22	F/67	Eczema	Allergen	Methylisothiazolinone	2+
			Vehicle	Petrolatum	–
23	F/57	Eczema	Allergen	Linalool hydroperoxide	2+
			Vehicle	Petrolatum	–
24	F/76	Eczema	Allergen	Methylisothiazolinone	+/?
			Vehicle	Petrolatum	–
25	F/35	Eczema	Irritant	SLS	3+
			Vehicle	Petrolatum	–
26	F/50	Eczema	Allergen	Nickel	1+
			Vehicle	Petrolatum	–
27	F/48	Eczema	Irritant	Nonanoic acid	1+
			Vehicle	Isopropanol	–
28	F/29	Eczema	Allergen	Linalool hydroperoxide	3+
			Irritant	Nonanoic acid	1+
			Nonlesional skin	-	–
29	M/44	Eczema	Irritant	Nonanoic acid	1+
			Vehicle	Isopropanol	–
30	M/62	Eczema	Irritant	Nonanoic acid	1+
			Vehicle	Isopropanol	–
31	F/29	Eczema	Allergen	Linalool hydroperoxide	1+
			Irritant	SLS	–
			Vehicle	Petrolatum	–
32	F/57	Eczema	Allergen	Nickel	1+
			Irritant	SLS	–
			Vehicle	Petrolatum	–
33	F/44	Eczema	Allergen	Nickel	1+
			Irritant	SLS	–
			Vehicle	Petrolatum	–
34	F/71	Eczema	Allergen	Linalool hydroperoxide	1+
			Irritant	SLS	–
			Vehicle	Petrolatum	–
35	M/54	Eczema	Allergen	Linalool hydroperoxide	2+
			Irritant	SLS	–
			Vehicle	Petrolatum	–
36	M/86	Eczema	Irritant	SLS	1+
			Vehicle	Petrolatum	–
37	M/88	cADRs	Allergen	Amoxicillin	1+
			Irritant	SLS	1+
			Nonlesional skin	-	–
38	M/75	cADRs	Irritant	SLS	1+
			Nonlesional skin	-	–
39	F/78	cADRs	Allergen	Acetazolamide	3+
			Vehicle	Petrolatum	–

(Continues)

TABLE 1 (Continued)

Patient		Patch test			
Patient number	Gender/age	Indication	Class	Compound	Result
40	M/43	cADRs	Allergen	Amoxicillin	2+
			Vehicle	Petrolatum	–
41	F/66	cADRs	Allergen	Amoxicillin	1+
			Vehicle	Petrolatum	–
42	F/68	cADRs	Irritant	SLS	1+
			Vehicle	Petrolatum	–
43	F/73	cADRs	Allergen	Amoxicillin	2+
			Nonlesional skin	-	–
44	M/64	cADRs	Allergen	Amoxicillin	1+
			Vehicle	Petrolatum	–
45	F/68	cADRs	Allergen	Piperacillin/Tazobactam	2+
			Nonlesional skin	-	–
46	M/73	cADRs	Allergen	Amoxicillin	1+
			Nonlesional skin	-	–
47	F/68	cADRs	Irritant	SLS	1+
			Vehicle	Petrolatum	–

Note: Patch test reactions were scored at 72 h in accordance with International Contact Dermatitis Research Group (ICDRG) criteria and European Society of Contact Dermatitis (ESCD) guidance.

–, negative reaction; +/?, doubtful reaction (erythema only, no infiltration); 1+, mild positive reaction (redness, induration, and possibly papules); 2+, strong positive reaction (erythema, induration, papules, and vesicles); 3+, extreme positive reaction (intense erythema, induration, and coalescing vesicles); F, Female; M, Male; cADRs, cutaneous Adverse Drug Reactions.

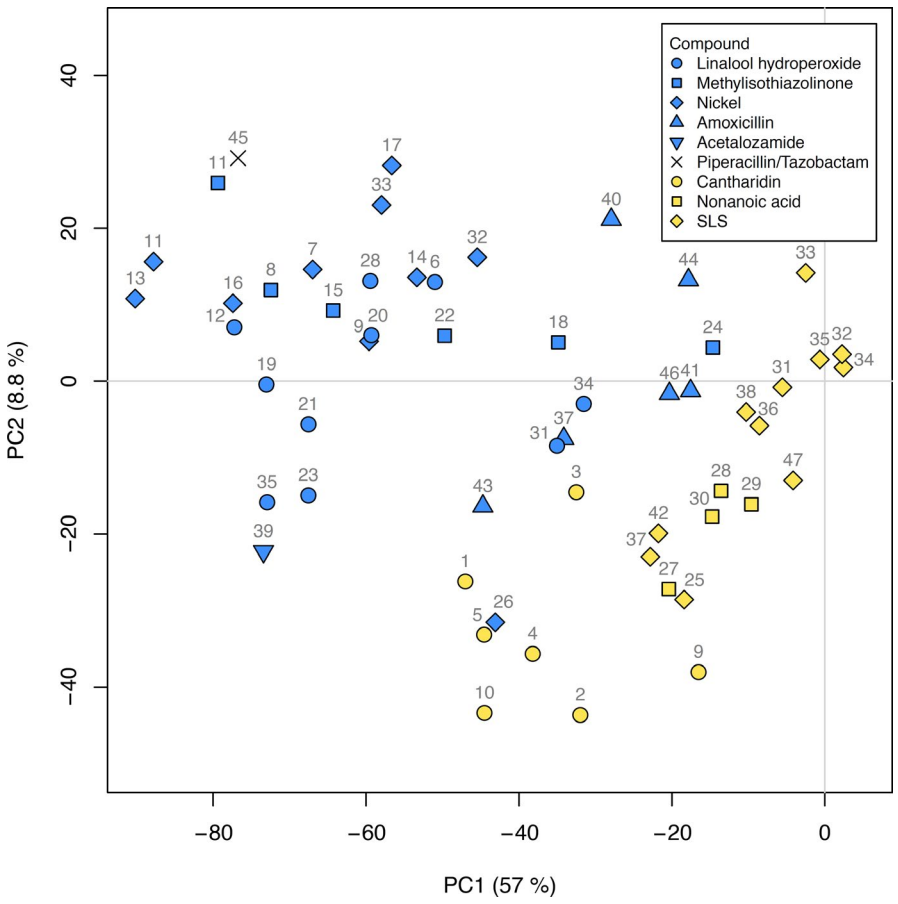


FIGURE 1 Intra-individual comparison of gene expression in allergen and irritant samples. Principal component analysis performed on the full set of genes measured on the arrays. To compensate for donor-induced variability, the gene expression level of the control sample from each donor was subtracted from that of the corresponding treated sample from the same donor. Results depict the sample location (symbols) in the first two principal components (PCs), with the controls a coordinate 0. Together, the first two components represented approximately 65% of the variance. Patient numbers are reported above each sample

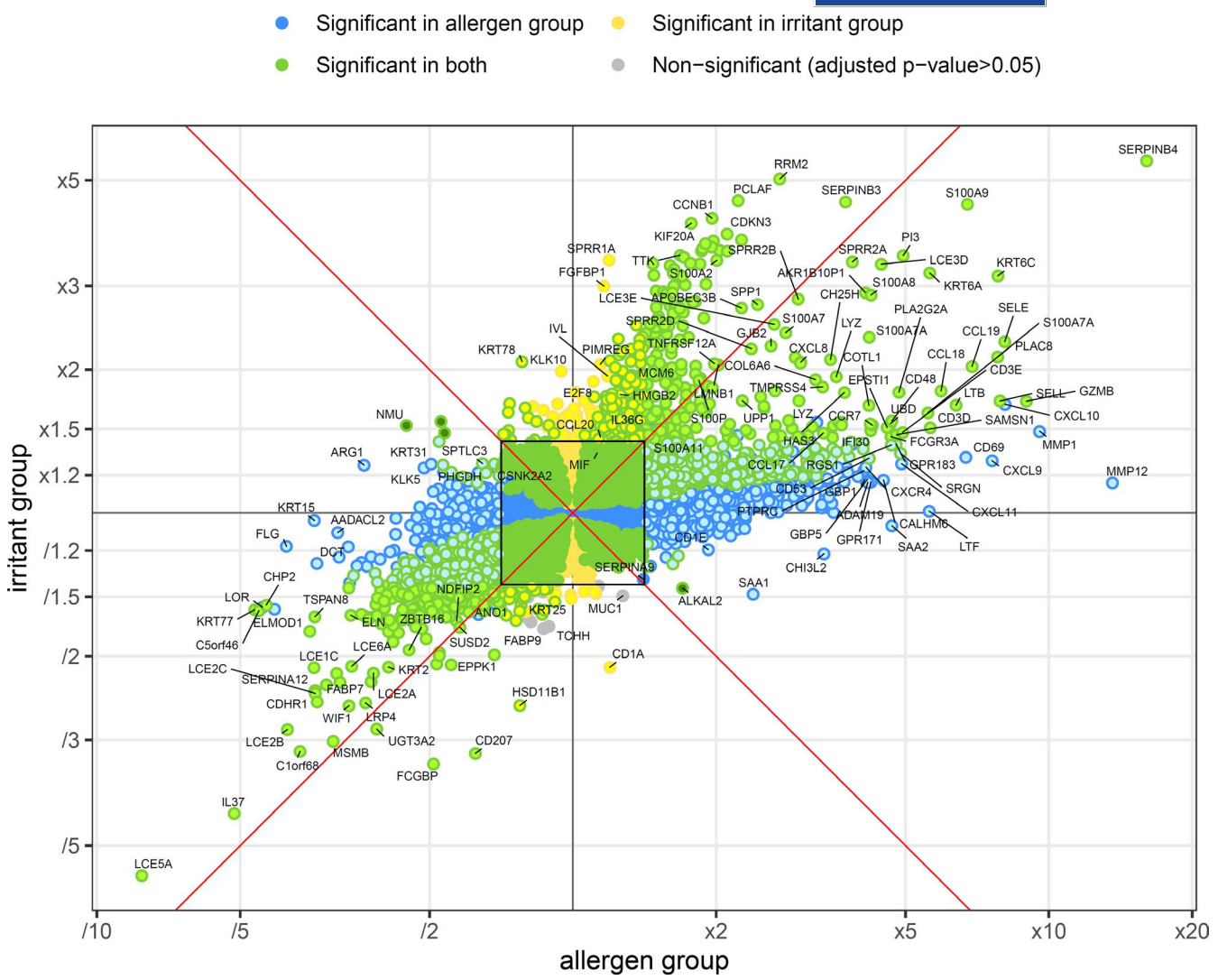


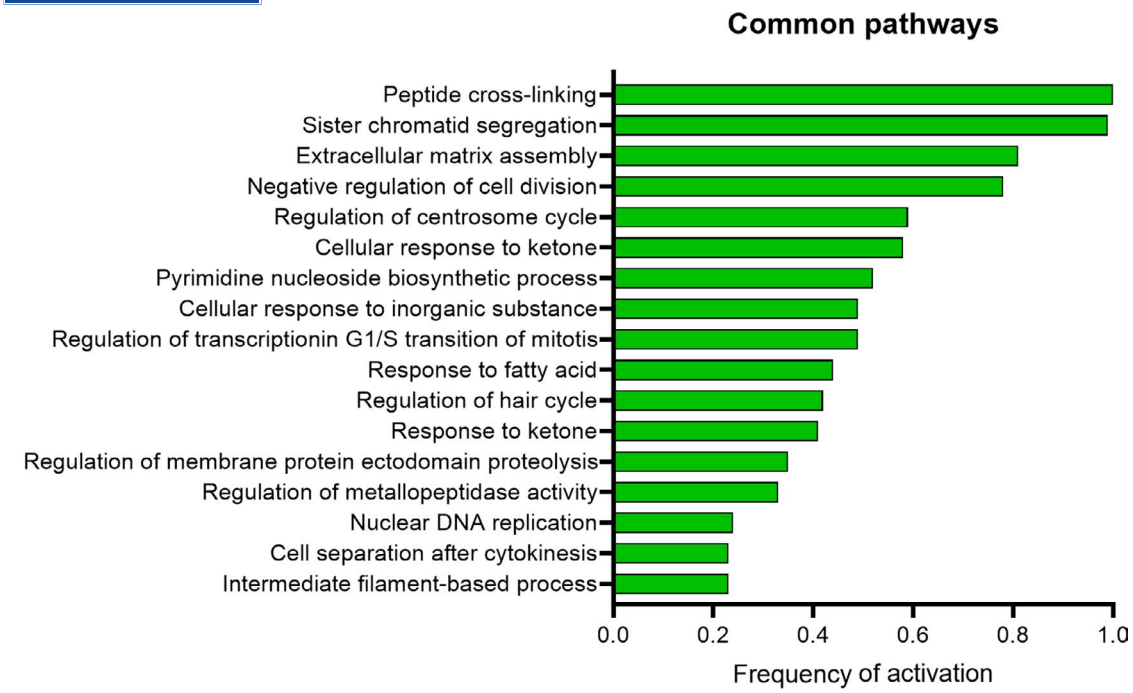
FIGURE 2 Common and distinct molecular responses induced by allergens and irritants. Each point of the graph illustrates the variation in the average expression levels of each gene in the allergen and irritant groups, after correction for the donor effect (mean fold changes). Green dots indicate genes (n = 457) significantly modulated in both groups. Blue dots indicate genes (n = 1956) predominantly modulated in the allergen group. Yellow dots indicate genes (n = 255) predominantly modulated in the irritant group. Gray dots indicate nonsignificant differences (adjusted P value <.05). The black square delimits fold changes >1.41 or <1/1.41

were excluded. The expression of 2371 and 699 genes was found to be modulated consistently among the 34 allergen and 17 irritant samples, respectively (Tables S1 and S2). Assuming a common response for all the tested molecules in a given group, we subsequently estimated the average expression level of each DEG, after correction for the donor effect, to establish whether this modulation was specific to the allergen group or the irritant group, or present in both groups. We detected 1956 DEGs predominantly modulated in the allergen group, 255 DEGs predominately modulated in the irritant group, and 457 DEGs that were modulated in both groups (Figure 2).

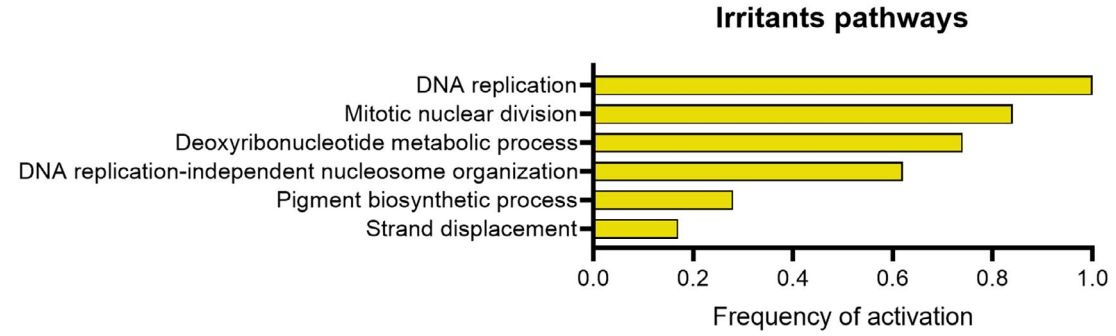
Gene ontology (GO) annotations revealed several commonly regulated pathways pertaining to the epidermal compartment ("peptide cross-linking" or "extracellular matrix assembly"), involved in metabolism ("cellular response to ketone" or "response to fatty acid") or implicated in cell proliferation ("sister chromatid segregation", "negative regulation of cell division", or "regulation of centrosome cycle") (Figure 3A and Table S3). Pathways predominantly modulated in the irritant group were mainly related to cell proliferation. Among the most significant hits were "DNA replication", "mitotic nuclear cell division", or "deoxyribonucleotide metabolic process" (Figure 3B and Table S3). Finally, GO terms most commonly associated with the allergen group were related to immune pathways ("inflammatory pathways", "leukocyte activation", "response to virus", "regulation of apoptotic signaling pathway", "regulation of type-I interferon", etc.), but GO terms associated with chemical detoxification and the epidermal compartment were also identified, with significant down-regulation of genes associated with the "cornification" pathway (Figure 3C and Table S3).

Thus, distinct signaling pathways are activated in allergen and irritant patch test responses.

(A)


Common pathways
Peptide cross-linking
Sister chromatid segregation
Extracellular matrix assembly
Negative regulation of cell division
Regulation of centrosome cycle
Cellular response to ketone
Pyrimidine nucleoside biosynthetic process
Cellular response to inorganic substance
Regulation of transcriptionin G1/S transition of mitotis
Response to fatty acid
Regulation of hair cycle
Response to ketone
Regulation of membrane protein ectodomain proteolysis
Regulation of metallopeptidase activity
Nuclear DNA replication
Cell separation after cytokinesis
Intermediate filament-based process
0.0
0.2
0.4
0.6
0.8
1.0
Frequency of activation

(B)


Irritants pathways
DNA replication
Mitotic nuclear division
Deoxyribonucleotide metabolic process
DNA replication-independent nucleosome organization
Pigment biosynthetic process
Strand displacement
0.0
0.2
0.4
0.6
0.8
1.0
Frequency of activation

(C)

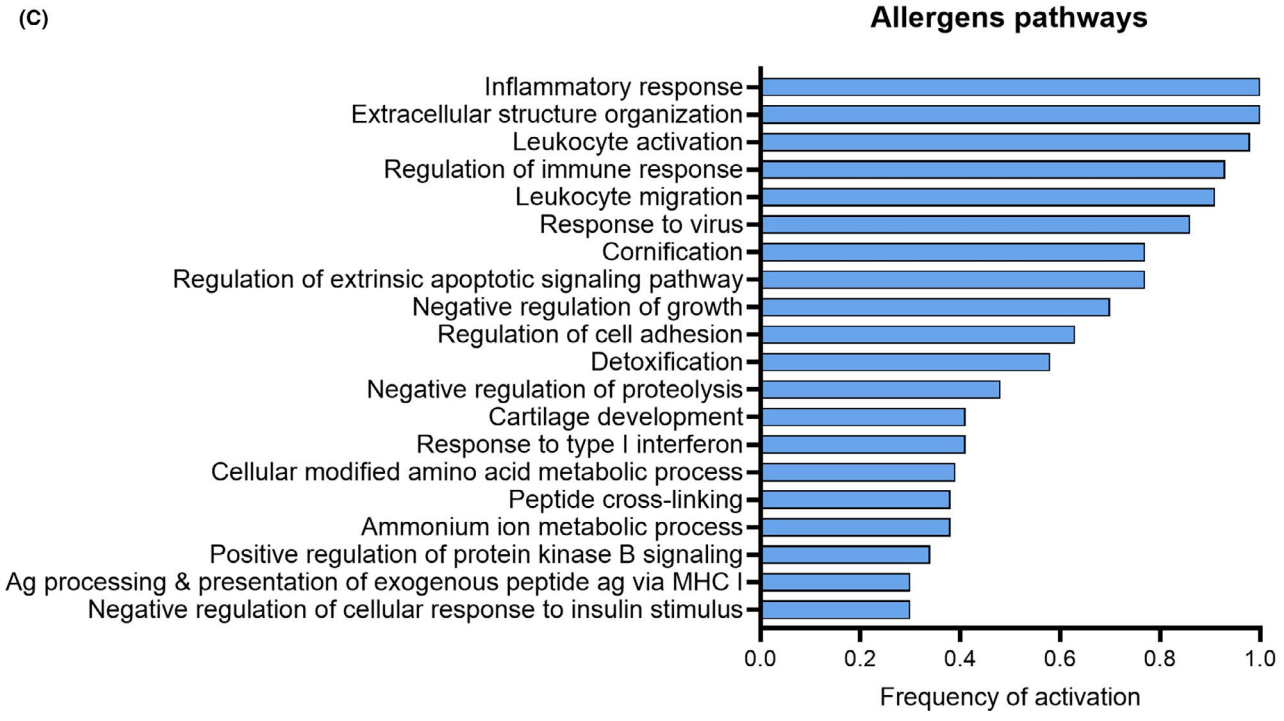

Allergens pathways
Inflammatory response
Extracellular structure organization
Leukocyte activation
Regulation of immune response
Leukocyte migration
Response to virus
Cornification
Regulation of extrinsic apoptotic signaling pathway
Negative regulation of growth
Regulation of cell adhesion
Detoxification
Negative regulation of proteolysis
Cartilage development
Response to type I interferon
Cellular modified amino acid metabolic process
Peptide cross-linking
Ammonium ion metabolic process
Positive regulation of protein kinase B signaling
Ag processing & presentation of exogenous peptide ag via MHC I
Negative regulation of cellular response to insulin stimulus
0.0
0.2
0.4
0.6
0.8
1.0
Frequency of activation

FIGURE 3 Signaling pathway analysis of allergen and irritant patch test responses. Shown are the most significant hits for the "biological process" GO terms for genes commonly (A) or predominantly regulated in the irritant (B) and allergen (C) groups. The size of the bar indicates the level of the estimated frequency of activation of the respective pathway (from 0 to 1)

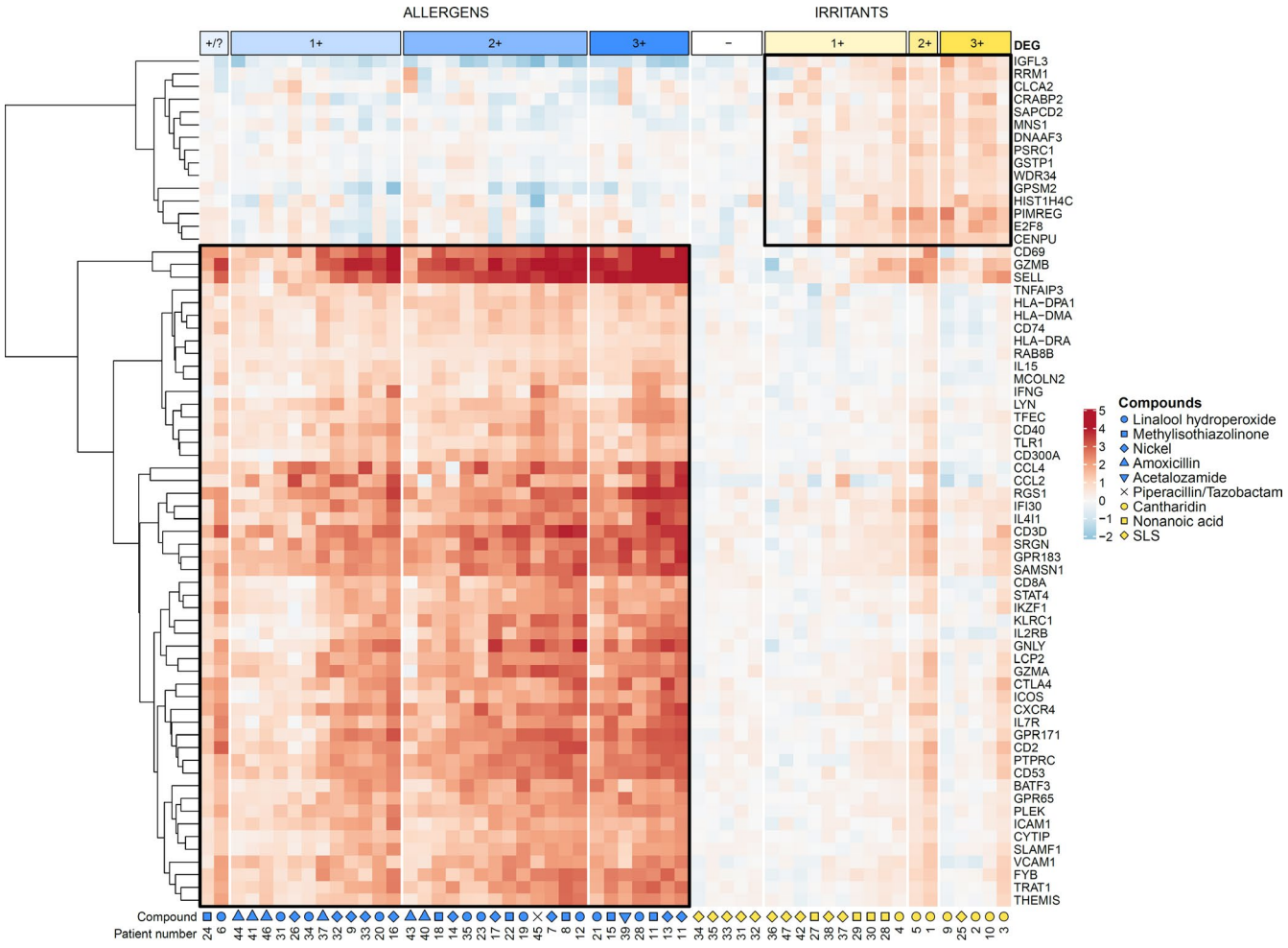


FIGURE 4 Unique molecular signatures for allergens and irritants. A set of 67 DEGs was used to illustrate the core transcriptomes of the 52 positive allergen and irritant-induced reactions. Results are depicted as a heatmap, in which samples are grouped by allergens and irritants and stratified by clinical reaction scores (+/? to 3+) (top of the figure). Blue, white, and red colors represent low, moderate, and high signal intensity, respectively. Chemical compounds and patient numbers are reported at the bottom of the figure

3.3 | The core transcriptome of allergen-induced reactions mainly consists of T-cell-related transcripts

By comparing DEGs from the allergen and irritant samples, we were then able to define a core transcriptome specific for each reaction. We first selected genes with the highest positive fold change (Fc) and false detection rate (FDR) responses, i.e., genes with the highest and most reproducible differential expression among all the samples from the respective groups. The allergen and irritant transcriptomes are illustrated in a heatmap (Figure 4).

The irritant transcriptome consisted of several cell cycle-related genes, such as E2F8, MNS1, PSCR1, MLF1IP, FAM64A, SAPCD2, or RRM1. It also included genes involved in epithelial cell differentiation, including GPSM2 or CLCA2. Among other irritant genes, we also detected genes associated with retinoic acid metabolism, such as CRABP2 and RDH12, and with cellular energy metabolism, like IGFL3 (Figure 4 and Table S2). Importantly, these markers were not upregulated in the five SLS-negative reactions.

The allergen transcriptome consisted of (a) several T-cell-related transcripts, including CD2, CD3D, CD3E, TRAC, or TCRBC1 (Figure 4 and Table S1); (b) DEGs down-stream of TCR signaling, such as LCK, THEMIS, FYB, TRAT1, or LCP2, and also (c) numerous DEGs involved in the activation and differentiation of type-1 cytotoxic CD8+ T cells (CTLs), comprising CD8A, IFNG, CCL5, IL2RB, GZMA, GZMB, GZMK, GNLY, PRF1, TBX21, EOMES, RUNX3, STAT1, STAT4, IKZF1, BAFT3, CD69, CD300A, ICOS, CTLA4, KLRC1, KLRD1, NKG7, CST7, PLEK, SLAMF1, or SRGN. Among other DEGs, we found (d) several markers known to be upregulated by IFN-γ cytokine on surrounding cells, like HLA-DMA, HLA-DPA1, HLA-DQB1, HLA-DRA, CD40, CD74, CD86, IL-15, CSF2RB, IL10RA, CCL2, CXCL9, CXCL10,

CXCL11, IDO1, ICAM1, VCAM1, IRF5, IRF8, PDE4B, TNFAIP3, or IFI30 (Figure 4; Table S1).

A previous gene-expression study associated the clinical intensity of patch test reactions to contact allergens with the immune response.[25] To confirm these results and extend our analysis to irritant triggers, we estimated the average level of variation in core gene expression for each sample independently. We observed that core marker expression correlated with the intensity of the clinical symptoms, for both allergens (Figure 5A) and irritants (Figure 5B). Of note, the correlation was not only detected at the global level (including the 52 and the 15 genes of the allergen and irritant core signatures, respectively), but also at the individual gene level (Figure S1).

Taken together, these data confirm that unique biomarkers characterize the inflammatory reactions induced by chemical allergens and irritants, and that the expression of these markers strongly correlates with the magnitude of the clinical responses.

3.4 | A small set of biomarkers allows for robust discrimination between allergen- and irritant-induced skin inflammation

Finally, we sought to identify a minimal set of biomarkers susceptible to discriminate between the two types of skin inflammation. To this end, we applied a biomarker discovery approach (using Boruta and Random Forest classification algorithms and k-fold cross validation) to select and assess the performance of different genes or gene combinations (here referred to as classifiers).[30] We identified more than 4000 classifiers capable of correctly classifying the different allergen or irritant samples with an accuracy ranging from 90% to 100%. The first 30 are shown in Table 2. Each classifier combined between 1 and 5 different genes, identified among 15 of the major DEGs of the core transcriptomes: GPR183, IGFL3, PLEK, IL15, HLA-DRA, GPR65, SAMSN1, HLA-DPA1, RAB8B, MCOLN2, CD69, HLA-DMA, CD74, and HLA-DPB1.

To confirm the predictive potential of the respective models, we next evaluated the best performance classifiers on a previously published[27] and newly generated (Ljungberg Silic L. *et al*, manuscript in preparation) gene array datasets. Majority of the classifiers achieved very good prediction results, correctly classifying the allergen (nickel[27] and amerchol AL-101 (Ljungberg Silic L. *et al*, manuscript in preparation) or the irritant (SLS[27]) origin in approximately 90% and 66% of patch-test samples respectively (Table 3). The predictive values of irritant samples from Wisgrill et al. were slightly lower, which could be explained by the fact that these investigators used different gene arrays (the sensitivity of fold change measures vary depending on gene arrays).[27] We then plotted ROC (receiver operator characteristic) curves, which compare the sensitivity and specificity metrics of the classifiers according to different, and not a unique, decision thresholds. The AUC values derived from ROC curves showed that nickel and SLS samples from Wisgrill et al. are in fact highly discriminated by most of the classifiers (Table 3), as illustrated for the GPR183/IGFL3 couple in Figure S2.

Importantly, several of the biomarker genes detected in this study were validated by quantitative RT-PCR, confirming the differential expression recorded in a large majority of our gene array samples (Figure 6 and Table S4).

Collectively, these results therefore indicate that a small set of biomarkers is sufficient to distinguish between allergen and irritant patch test responses.

4 | DISCUSSION

The main objective of our study was to compare the molecular responses induced by a range of chemicals with distinct proinflammatory properties, with the specific aim of identifying skin biomarkers for contact allergy. Our results clearly showed that the transcriptome of CD/skin inflammation induced by clinically relevant allergens differed from that induced by reference irritants, with core gene modules that were induced by both allergens and irritants. In addition, we confirmed that unique pathways are preferentially activated/inhibited depending on the nature of the tested molecules, with the magnitude of gene activation correlating with the intensity of the patch test-induced clinical inflammation. Finally, using the machine-learning approach, we identified and validated several minimal biomarker combinations, which demonstrated excellent performance in distinguishing contact allergy from irritation.

4.1 | Major hallmarks of the allergen signature

Skin allergy pathophysiology has been studied extensively over the past few decades, both in humans and in models. In contrast, far less is known about the skin inflammation induced by chemical irritants, especially in humans. Several studies had reported that transcripts from chemokines or innate sensors involved in antiviral responses and cell death, distinguished hapten- from irritant-induced responses.[26-28,31-33] Our results confirmed the potential of CXCL9, CXCL10, and ZBP1, and, more generally, of the antiviral and type I interferon-related pathways (Figure 3) as factors for discriminating between allergic- and irritant-induced skin inflammation. As expected, our results revealed that allergen, but not irritant, core signatures were enriched with numerous T-cell-related transcripts, which we attributed to the differentiation of type-1 cytotoxic CD8+ T cells. Indeed, we detected a significant increase in CD8A transcripts, and to a lesser extent CD8B transcripts, in the allergen reactions, but detected relatively few CD4 transcripts (Figures 2, 4 and 6). Moreover, we found no differential expression for several NK or gamma delta T-cell-specific markers (such as NCR1, NCAM1, STYK1, TCRD, or TCRG; **data not shown**), suggesting that the cytotoxic markers identified were mostly related to CD8+ T lymphocytes. These findings confirm those of previous studies performed in humans and mice, which showed that numerous cytotoxic CD8+ T cells infiltrate the skin of allergic individuals to instigate the formation of eczematous lesions.[34-37] In another recent study, we showed that these cells persist for months in previously affected skin sites, as resident memory T cells, precipitating eczema relapses.[38] Thus, the infiltration of cytotoxic CD8+ T cells represents a major hallmark

TABLE 2 Performance of the 30 best classifier models identified by Random Forest algorithm

Gene sets	Accuracy	Precision-Allergen	Precision-Irritant	Recall-Allergen	Recall-Irritant	F1-Score-Allergen	F1-Score-Irritant	AUC
IGFL3, HLA-DRA	1.00	1.00	1.00	1.00	0.99	1.00	1.00	1.00
IGFL3, HLA-DRA, HLA-DMA	0.99	0.98	1.00	1.00	0.96	0.99	0.98	1.00
IGFL3, PLEK	0.98	0.98	1.00	1.00	0.95	0.99	0.97	1.00
IGFL3, HLA-DRA, HLA-DPA1	0.98	0.98	1.00	1.00	0.95	0.99	0.97	1.00
IGFL3, HLA-DPA1	0.98	0.99	0.98	0.99	0.98	0.99	0.97	1.00
IGFL3, HLA-DRA, CD74	0.98	0.98	1.00	1.00	0.95	0.99	0.97	1.00
IGFL3, PLEK, HLA-DRA, MCOLN2	0.98	0.98	1.00	1.00	0.95	0.99	0.97	1.00
IGFL3, PLEK, HLA-DRA, HLA-DMA	0.98	0.98	1.00	1.00	0.95	0.99	0.97	1.00
IGFL3, PLEK, HLA-DPA1	0.98	0.98	1.00	1.00	0.94	0.99	0.97	1.00
IGFL3, HLA-DRA, MCOLN2	0.98	1.00	0.96	0.97	1.00	0.99	0.97	1.00
IGFL3, HLA-DRA, RAB8B	0.98	0.99	0.96	0.98	0.99	0.99	0.97	1.00
HLA-DRA, CD69	0.98	0.97	1.00	1.00	0.94	0.99	0.96	0.99
IGFL3, HLA-DRA, CD69	0.98	0.97	1.00	1.00	0.94	0.99	0.97	1.00
GPR183, IGFL3, HLA-DRA	0.98	0.97	1.00	1.00	0.94	0.99	0.97	1.00
GPR183, IGFL3, CD69	0.98	0.97	1.00	1.00	0.94	0.99	0.96	1.00
IL15, CD69	0.98	0.97	1.00	1.00	0.94	0.99	0.96	0.98
GPR183, IGFL3	0.98	0.97	1.00	1.00	0.94	0.99	0.97	1.00
IGFL3, PLEK, HLA-DRA, RAB8B	0.98	0.98	1.00	1.00	0.95	0.99	0.97	1.00
IGFL3, IL15, CD69	0.98	0.97	1.00	1.00	0.94	0.99	0.96	1.00
IGFL3, PLEK, CD69	0.98	0.97	1.00	1.00	0.94	0.99	0.96	1.00
IGFL3, IL15, HLA-DRA	0.98	0.97	1.00	1.00	0.94	0.99	0.96	1.00
GPR183, IL15, HLA-DRA	0.98	0.97	1.00	1.00	0.94	0.99	0.96	1.00
IGFL3, PLEK, IL15	0.98	0.97	1.00	1.00	0.94	0.99	0.96	1.00
GPR65	0.98	0.97	1.00	1.00	0.94	0.99	0.96	0.99
GPR183, IGFL3, PLEK	0.98	0.97	1.00	1.00	0.94	0.99	0.96	1.00
IL15, HLA-DRA	0.98	0.97	1.00	1.00	0.94	0.99	0.96	1.00
PLEK, IL15	0.98	0.97	1.00	1.00	0.94	0.98	0.96	0.99
PLEK, CD69	0.97	0.97	0.98	0.99	0.94	0.98	0.96	0.99
IGFL3, IL15	0.97	0.97	0.98	0.99	0.94	0.98	0.95	1.00
PLEK, HLA-DRA	0.96	0.97	0.96	0.97	0.94	0.97	0.94	1.00
GPR183, CD69	0.96	0.95	1.00	1.00	0.88	0.97	0.93	0.98

Note: Performance metrics: Accuracy is the rate of correct predictions among the total number of predictions the classifier performed with both allergen and irritant samples. Precision is the rate of correct predictions among the total number of positive predictions (considering both true positive and false positive predictions) for a given class sample (allergen or irritant). Recall is the rate of correct predictions among the total number of predictions (considering both true positive and false negative predictions) the classifier made with samples from a specific class (allergen or irritant). F1-Score for a given class is the harmonic mean of the Precision and Recall values. AUC is the area under the ROC curve calculated for a standard binary classifier. The ROC curve compares the sensitivity (here Recall Allergen) and the specificity (here Recall Irritant) of each binary classifier, when the decision threshold varies. The decision threshold is the value at which the binary classifier considers that a classification belongs to a specific class (allergen or irritant). An AUC value near to the 1.00 means an excellent discrimination of the two classes.

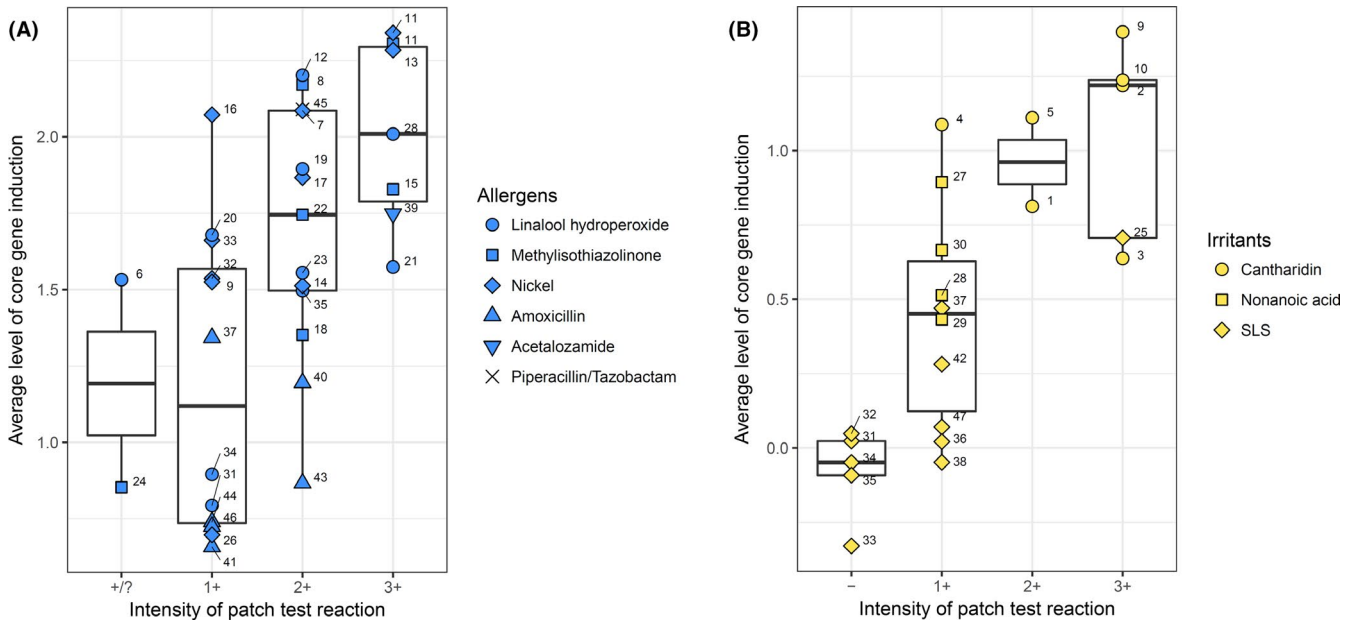


FIGURE 5 Gene expression correlates with the magnitude of the clinical response. Box-and-whisker plots depict the distribution of the average levels of variation in core gene expression for the allergen (A) and irritant (B) groups depending on the clinical score, i.e., the intensity of the PT reactions. Each point represents the average expression level (log2 Fc) of core genes (including 52 genes for the allergen signature and 15 genes for the irritant signature) for one sample, after correction for the donor effect. Patient numbers are reported next to each sample

TABLE 3 Validation of the best classifiers using external gene array datasets

			Wisgrill et al.[27]			Ljungberg Silic et al. (in preparation)
Gene sets			Recall Allergen (Nickel, *n* = 11)	Recall Irritant (SLS, *n* = 6)	AUC	Recall Allergen (Amerchol L-101, *n* = 8)
GPR65			0.91	0.67	0.83	1
GPR183	IGFL3		0.91	0.67	0.94	0.88
PLEK	IL15		0.91	0.67	0.85	1
PLEK	CD69		0.91	0.67	0.86	1
IL15	HL-DRA		0.91	0.67	0.79	1
GPR183	IGFL3	PLEK	0.91	0.67	0.92	0.88
GPR183	IGFL3	CD69	0.91	0.67	0.92	1
IGFL3	PLEK	CD69	0.91	0.67	0.88	1
IGFL3	IL15	CD69	0.91	0.67	0.86	1
IGFL3	PLEK	HLA-DPA1	0.91	0.67	0.88	1

Note: The classifiers shown in Table 2 were validated on two external gene array datasets, generated from biopsies of positive PT samples (i) to nickel and SLS (access code E-MTAB-8945) and (ii) to amerchol (access code GSE169573).

Different metrics, Recall and AUC of ROC curves, are shown for the 10 best performance classifiers.

of the allergen signature, which, in addition, correlates strongly with the magnitude of the clinical response (Figure 5A).

4.2 | Major hallmarks of the irritant signature

In contrast to allergic reactions, the most significant hits recorded for irritant-induced inflammation were associated with cell proliferation. This was expected as irritants trigger potent keratinocyte damage, followed by rapid and intensive tissue renewal.[17,39,40] The occurrence of intense tissue remodeling was supported by our observation of strong upregulation of genes associated with cell metabolism (RDH12, CRABP2, IGFL3, etc.), peptide crosslinking (LCE5A, LCE3D, SPPR2A, etc.), and extracellular matrix assembly (MFAP4, MYH11, FBLN5, etc.), with the later genes being upregulated in both irritant and allergen samples

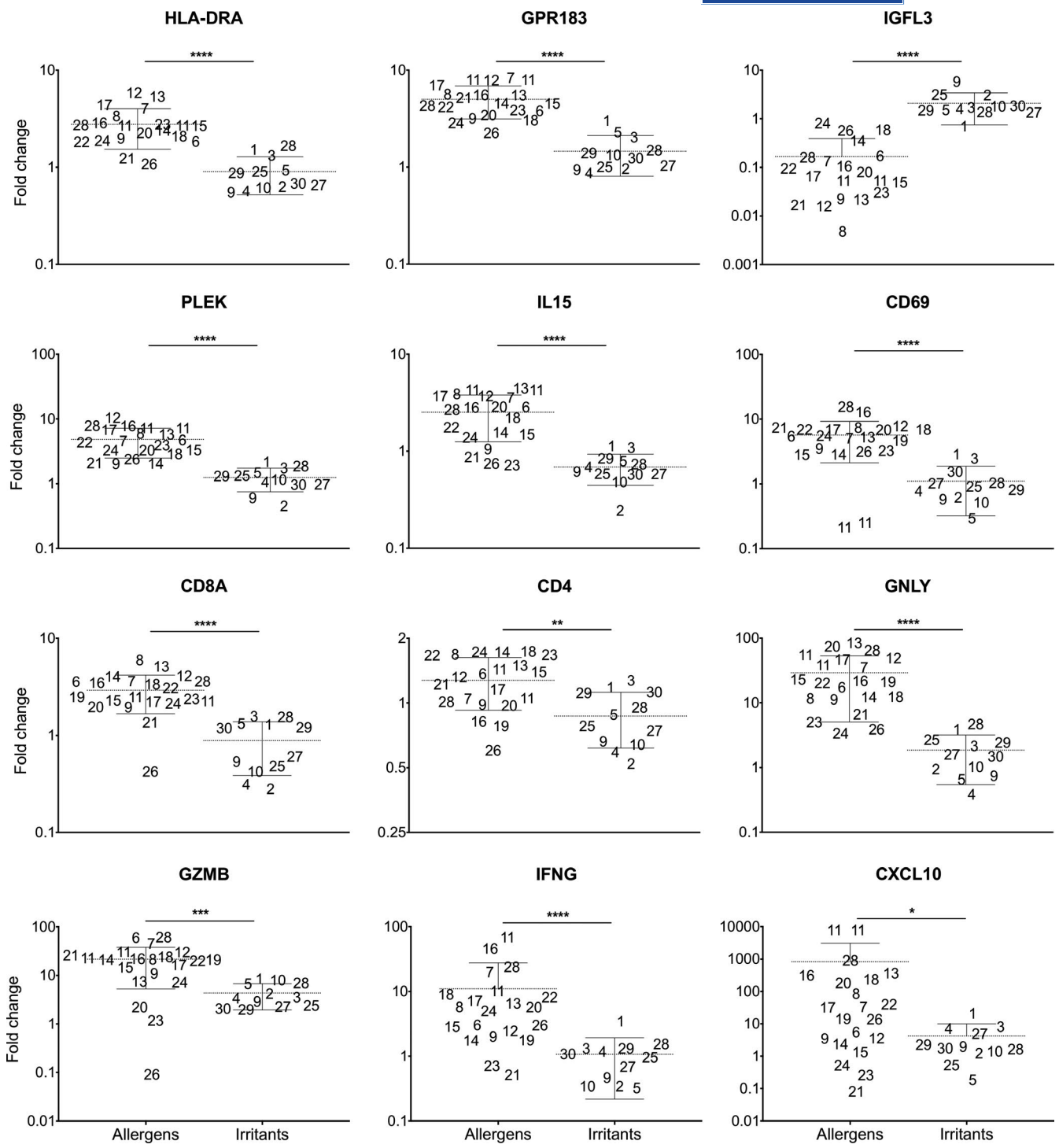


FIGURE 6 Validation of a small set of segregating biomarkers by qRT-PCR. Gene expression of HLA-DRA, GPR183, IGFL3, PLEK, IL15, CD69, CD8A, CD4, GZMB, GNLY, IFNG, and CXCL10 mRNA transcripts in allergen and irritant samples was analyzed by qRT-PCR. Results are expressed as fold changes versus control skin. Patient numbers are reported above each sample. **P* < .05, ***P* < .01, ****P* < .001, *****P* < .0001; Mann-Whitney *U* test

(Tables S1 and S2). In contrast, we detected relatively few GO terms related to "inflammatory pathways", which is surprising because SLS and, more commonly, cantharidin induced strong, and even bullous, reactions in some patients (Table 1). This finding may be explained by the long-time interval before biopsy collection (72 h post application). In the hours following exposure, irritants trigger keratinocyte release of many cytokines (IL-1β, IL-6, or TNF-α), chemokines (IL-8/CXCL8, CCL2, CCL3, CCL4, CCL17, and CCL20), and arachidonic acid derivatives (such as prostaglandin E2).[11,12,33,41] At 72 h, the molecular inflammation would be tightly regulated. Nevertheless, we detected upregulation of several DEGs, such as IL-36G, IL-8/CXCL8, CCL2, CCL17, CCL18, MIF, and

diverse HMGB or S100 proteins. All these mediators have been reported to exert crucial roles by stimulating leukocyte recruitment and cytokine production by keratinocytes,[42-45] suggesting that they participate actively in late irritant inflammation. Of note, few cantharidin samples (patients 1, 4, 5) also showed marks of allergen signature. It will be interesting to determine whether it is the result of bystander recruitment and activation of cytotoxic T cells by the inflammatory environment and cytokine signaling, as reported upon microbial infection.[46,47]

4.3 | The predictive value of molecular signatures to improve the diagnosis of ACD

Beyond gene profiling of allergic and irritant reactions, another objective of this work was to identify a set of predictive biomarkers susceptible to serve as future platforms to improve clinical diagnosis. Using random forest classifier methodology, we identified and validated several classifier models, including 1–5 different genes of the major DEGs of the core transcriptomes, as minimal biomarkers that distinguish allergic and irritant reactions in human skin. The performance of these classifiers confirms a recent study conducted by Fortino et al. on similar material (including 89 biopsies collected from positive and negative patch-test reactions to 4 different allergens and 2 irritants), and which identified 22 potential biomarkers (CD47, BATF, FASLG, RGS16, SYNPO, SELE, PTPN7, WARS, PRC1, EXO1, RRM2, PBK, RAD54L, KIFC1, SPC25, PKMYT, HISTH1A, TPX2, DLGAP5, TPX2, CH25H, and IL37). Interestingly, if none of the biomarkers identified by Fortino et al. were present among our top 50 of the most discriminant genes, possibly due to key differences in experimental setups, most of them were significantly upregulated in the two sample groups (Tables S1–S3 and data not shown). This illustrates that multiple biomarker combinations can be used to discriminate between the two types of skin inflammation.

Nevertheless, whatever the final combination chosen, it will be crucial to demonstrate in a future study that it is sufficiently efficient in detecting false positive patch-test reactions, which are triggered by the proinflammatory properties of allergens in nonallergic individuals, and which remains the main problem encountered in the clinic. Indeed, all the analyses performed in this study were based on the assumption that none of the collected data for chemical allergens were the results of false-positive reactions. Thus, complementary trials are needed to determine whether biomarkers identified in this study are robust and sensitive enough to detect patients with clinically relevant ACD.

Finally, it will also be important to determine if the same gene signatures characterize both 72-h patch test reactions and clinical lesions of active eczema. The detection of relevant allergy biomarkers in contact dermatitis lesions could help prevent patients from being sent for unnecessary allergology workup. Currently, it is estimated that only one in five patients consulting for ACD is truly allergic,[48] with the majority of these patients having ICD.

In conclusion, our study characterized the main features of allergic and irritant reactions to chemicals. We have identified several biomarkers that clearly distinguish the two types of inflammation. Our findings provide the basis for the development of new approaches to refine and improve the diagnosis of ACD.

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CONFLICT OF INTEREST

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AUTHOR CONTRIBUTIONS

MAL, AN, VL, AM, and AC carried out the experiments and analyzed the data. MP, SDB, JN, PEJ, and LB provide support for bioinformatic/biostatistics analysis. MAL, AN, AR, FH, MCFL, PP, CD, AH, and MB managed the clinical study, performed sample collections, obtained consents, and/or participated in the interpretation of the data. JFN and MV designed the experiments and supervised this study. JFN and MV wrote the manuscript. All authors approved the final submitted and published version.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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