

Molecular profiling of contact dermatitis skin identifies allergen-dependent differences in immune response

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Background: Allergic contact dermatitis (ACD) is the most common occupational disease. Although murine contact hypersensitivity provides a framework for understanding ACD, it carries important differences from its human counterpart. Unlike the contact hypersensitivity model, which is induced by potent sensitizers (ie, dinitrofluorobenzene), human ACD is induced by weak-to-moderate sensitizers (ie, nickel), which cannot induce reactions in mice. Distinct hapten-specific immune-polarizing responses to potent inducers were suggested in mice, with unclear relevance to human ACD.

Objective: We explored the possibility of distinct T-cell polarization responses in skin to common clinically relevant ACD allergens.

Methods: Gene-expression and cellular studies were performed on common allergens (ie, nickel, fragrance, and rubber) compared with petrolatum-occluded skin, using RT-PCR, gene arrays, and immunohistochemistry.

Results: Despite similar clinical reactions in all allergen groups, distinct immune polarizations characterized different allergens. Although the common ACD transcriptome consisted of 149 differentially expressed genes across all allergens versus petrolatum, a much larger gene set was uniquely altered by individual allergens. Nickel demonstrated the highest immune activation, with potent inductions of innate immunity, T_H1/T_H17

and a T_H22 component. Fragrance, and to a lesser extent rubber, demonstrated a strong T_H2 bias, some T_H22 polarization, and smaller T_H1/T_H17 contributions. **Conclusions:** Our study offers new insights into the pathogenesis of ACD, expanding the understanding of T-cell activation and associated cytokines in allergen-reactive tissues. It is the first study that defines the common transcriptome of clinically relevant sensitizers in human skin and identifies unique pathways preferentially activated by different allergens, suggesting that ACD cannot be considered a single entity. (*J Allergy Clin Immunol* 2014;■■■:■■■-■■■.)

Key words: Allergic contact dermatitis, patch testing, T-cell polarization, human skin, allergens, nickel, fragrance, rubber

Allergic contact dermatitis (ACD) is the most common occupational disease, with a prevalence of 15% to 25%.¹⁻³ It is characterized by intensely pruritic patches, with erythema, edema, and occasional vesicles. ACD represents a type IV delayed-type hypersensitivity response to antigens that come in contact with the skin. It is induced by allergens or haptens, which are small chemically reactive molecules (<500 Da). The most common clinically relevant sensitizers in occupational and nonoccupational exposure include metals (particularly nickel), fragrance, and rubber.¹⁻⁶ Two distinct phases are involved in ACD: the sensitization phase, which represents the first contact with the allergen and has no clinical manifestations, and the elicitation phase, which occurs on rechallenge, producing visible dermatitis with peak inflammatory reactions at 72 and 24 hours in human and mice, respectively.⁷⁻¹⁰ Patch testing is the criterion standard procedure to diagnose ACD.¹¹ A positive patch test result induces the elicitation phase, resulting in an eczematous rash, identifying the offending sensitizer.

Immune mechanisms of ACD are incompletely understood. In murine contact hypersensitivity (CHS), a model for human ACD, reactions to strong sensitizers (ie, dinitrochlorobenzene, diphenylcyclopropanone, dinitrofluorobenzene, and trinitrochlorobenzene) have been characterized at the molecular level, but mice do not have reactions to weak sensitizers (ie, metals, fragrance, and rubber) that are clinically relevant in human ACD.^{2,12,13} Hence, whether effector immune responses are similar between strong haptens and medically relevant antigens is unknown. Furthermore, most studies generalized ACD as a single phenomenon rather than investigating the differences among the many allergens.^{8,9,14-17} In murine CHS models, different potent sensitizers have been used to induce distinct polar T-cell responses,⁹ implying the possibility that effector immune activation pathways could also be allergen specific in human ACD.

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Abbreviations used

ACD:	Allergic contact dermatitis
AD:	Atopic dermatitis
CCL13:	Chemokine, CC motif, ligand 13
CCL17:	Chemokine, CC motif, ligand 17
CCL18:	Chemokine, CC motif, ligand 18
CCL26:	Chemokine, CC motif, ligand 26
CHS:	Contact hypersensitivity
CTLA4:	Cytotoxic T-lymphocyte antigen 4
CXCL1:	Chemokine, CXC motif, ligand 1
CXCL2:	Chemokine, CXC motif, ligand 2
CXCL9:	Chemokine, CXC motif, ligand 9
CXCL10:	Chemokine, CXC motif, ligand 10
CXCL11:	Chemokine, CXC motif, ligand 11
DC:	Dendritic cell
DEG:	Differentially expressed gene
IHC:	Immunohistochemistry
PI3:	Peptidase inhibitor 3
RT-PCR:	Real-time PCR
S100A:	S100-Calcium binding protein

Both CD4⁺ and CD8⁺ T cells can act as effectors in type IV delayed-type hypersensitivity and CHS reactions (although CD4⁺ T cells appear to dominate human effector responses).^{14,18} Effector responses involve a complex interplay between dendritic cells (DCs), Langerhans cells, keratinocytes, T-cell activation, and loss of regulatory T cell-mediated suppression. Although it was historically considered a T_H1-dominated or a mixed T_H1/T_H2 response, ACD is increasingly recognized as involving production of T_H17 and T_H22 cytokines.¹⁴⁻²¹

Most human studies that evaluated effector responses to common allergens have been performed on peripheral blood from patients with ACD using cytokine activation assays.^{17,22-28} Very few studies were performed directly on inflamed skin, and these studies were further restricted to analyses of a limited subset of immune pathways.^{14,18,29-32}

This study is the first to perform extensive molecular and cellular profiling for a range of clinically relevant allergens identified by patch tests to establish common denominators of ACD (irrespective of allergen) and identify potential allergen-specific differences. Our data suggest that allergens induce differential immune activation in patch-tested skin.

METHODS**Patients' characteristics and skin samples**

Patch testing was performed on 24 patients with patch-test proven ACD to common allergens using the 15 most common allergens of the North American Contact Dermatitis Group³³ and petrolatum-occluded skin (as control). At 72 hours, the patch test site was evaluated for reactivity and positive reactions were clinically graded as 1+, 2+, or 3+.^{34,35} Biopsies were taken from petrolatum and allergen-reactive patches of 24 patients (16 women/8 men, ages 20-63 years, median = 40.5 years) under institutional review board-approved protocols. Overall, 10 patients had positive reactions to nickel, 3 to fragrance, 7 to rubber, and 4 to metals other than nickel (for details, see Table E1 and the Methods section in this article's Online Repository at www.jacionline.org).

Histologic analysis

Immunohistochemistry (IHC) and immunofluorescence were performed on frozen tissue sections using antihuman mAbs (see Table E2 in this article's

Online Repository at www.jacionline.org). Epidermal thickness and positive cells/millimeter were quantified for IHC using ImageJ V1.42 (National Institutes of Health, Bethesda, Md) and immunofluorescence was imaged with MetaView Software (Visitron Systems, Puchheim, Germany).

Quantitative real-time PCR and gene-array analysis

RNA was extracted for real-time (RT)-PCR with EZ-PCR Core Reagents (Life Technologies, Grand Island, NY), and custom primers were generated.³⁶⁻⁴³ Expression values were normalized to hARP. Affymetrix U133Plus 2.0 arrays (Affymetrix, Santa Clara, Calif) were used for gene arrays, as previously described.³⁶⁻⁴³

Statistical analysis

Quality control of microarrays was carried out using standard QC metrics and R package Quality Control. Images were scrutinized for spatial artifacts using Harshlight. Expression values were obtained using the Guanine Cytosine Robust Multi-Array Analysis algorithm. Probe sets with 1 or more sample with expression values of more than 4, and standard deviation (SD) of more than 0.15, were kept for analyses. Expression values were modeled using linear mixed-effect models with allergen group as fixed factor and a random effect for each patient. Fold changes for the comparisons of interest were estimated, and hypothesis testing was conducted using contrasts with the linear models framework in limma package. *P* values from paired *t* tests were adjusted for multiple hypotheses using the false-discovery rate procedure. To clarify whether distinct tissue reactions were not due to clinical scoring differences between allergens, we adjusted for patch score using a linear model in limma package, without significant differences. RT-PCR and IHC data were also analyzed using the linear mixed-effect model. Statistical methodology is detailed in this article's Online Repository at www.jacionline.org.

RESULTS**Similar clinical reactions and cellular infiltrates characterize different allergens**

Similar clinical reactions with elevated erythematous patches and variable vesicles were observed with all allergens. The intensity of positive patch test results was graded as 1+, 2+, or 3+, as guided by erythema and vesiculation.^{34,35} Representative positive reactions to common allergens (nickel, fragrance, and rubber) and a negative reaction to petrolatum (as a control) are depicted in Fig 1, A-D. Small, nonsignificant increases in epidermal thickness were noted on hematoxylin and eosin only with select allergens compared with petrolatum (Fig 1, E-H). IHC revealed significantly larger infiltrates of T cells (CD3⁺ and CD8⁺) and DC subsets (myeloid/CD11c⁺ and mature/DC-lysosome-associated membrane glycoprotein⁺) across most allergens when compared with petrolatum-occluded skin (*P* < .02 for all except other metals) (Figs 1 and 2; see Table E3 in this article's Online Repository at www.jacionline.org). Higher infiltrates of resident/CD1c⁺ DCs and eosinophils (major basic protein⁺) were also observed in allergen-occluded compared with petrolatum-occluded biopsies (significant only for rubber, *P* < .01) (Fig 2; also see Fig E1 in this article's Online Repository at www.jacionline.org; Table E3).

Despite similar clinical and histologic manifestations among the allergens, some differences were also noted. Overall, nickel demonstrated the greatest magnitude of cellular infiltrates, particularly T cells (CD3⁺ and CD8⁺) and CD11c⁺ DCs (Fig 2; Table E3). Conversely, other metals (cobalt and potassium dichromate) demonstrated the smallest infiltrates, attaining significance when compared with petrolatum only for mature/DC-LAMP⁺ DCs (*P* = .04). Finally, Langerhans cells/Langerin⁺

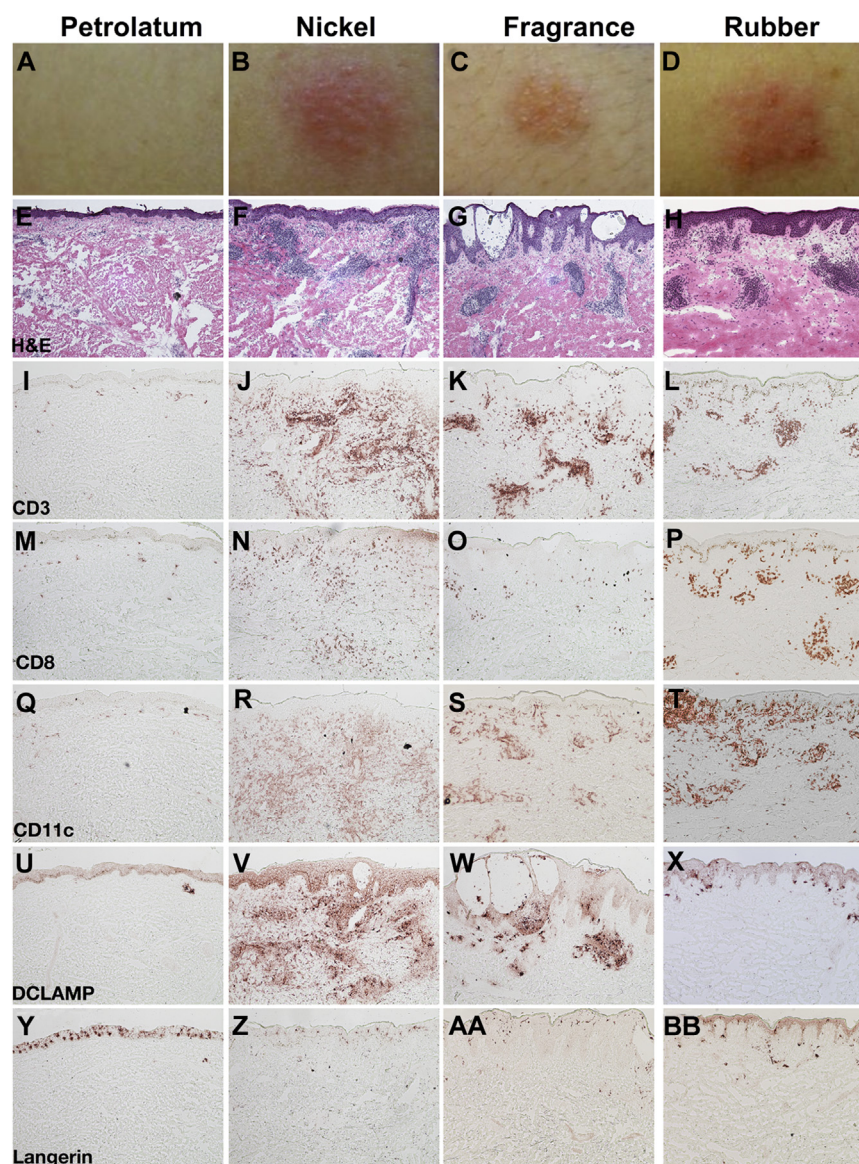


FIG 1. Representative positive patch reactions to nickel, fragrance, rubber (**B-D**) and a negative reaction to petrolatum (**A**). Hematoxylin and eosin (**E-H**) and staining for CD3 (**I-L**), CD8 (**M-P**), CD11c (**Q-T**), DCLAMP (**U-X**), and Langerin (**Y-BB**), demonstrating increased infiltrates of T cells, DCs, and eosinophils and exfiltration of Langerhans cells in biopsies of allergen- versus petrolatum-occluded skin (histology magnification, $\times 10$).

were increased in petrolatum compared with all allergen groups, consistent with their mobilization from the skin on allergen exposure (**Fig 1, Y-BB**).⁴⁴⁻⁴⁶

Clusters of T cells and DCs form close to vesicles in allergen-reactive skin by immunofluorescence

On closer histologic evaluation, we observed cellular clusters in the dermis underneath intraepidermal vesicles (**Fig 1, K; 1 W**). To further explore this observation, immunofluorescence was used to label CD3⁺ or CD8⁺ T cells (green) and DC-LAMP⁺ DCs (red) (see **Fig E2, A-D**, in this article's Online Repository at www.jacionline.org). Clumps of CD3⁺ T cells were intermixed with DC-LAMP⁺ DCs (red) just below the vesicles. This colocalization was seen only in allergen-reactive but not in petrolatum-occluded skin (**Fig E2**). A similar clustering pattern, though less pronounced compared with CD3, was observed with CD8⁺ T cells, and some double-positive CD8⁺/DC-LAMP⁺

staining was noted, accounting for a select subset of CD8⁺ DCs.⁴⁷ The visualized T-cell/DC aggregates potentially correspond to skin-associated lymphoid tissues, conceptualized as key players in allergen responses in mice.⁴⁸

Genomic profiling identifies common and distinct molecular responses to different allergens

Gene arrays were performed to define differentially expressed genes (DEGs) in nickel, rubber, and fragrance compared with petrolatum-occluded skin as represented in the Venn diagram in **Fig 3, A** (with criteria of fold change >2 and false-discovery rate $< .05$). Because other (non-nickel) metals induced only limited genomic changes, these were excluded from this analysis (see **Methods**). We identified a core set of 149 (145 upregulated and 4 downregulated) DEGs in all allergen groups versus petrolatum. This common gene set defines the ACD phenotype or transcriptome (**Fig 3, A and B**; see **Table E4** in this article's

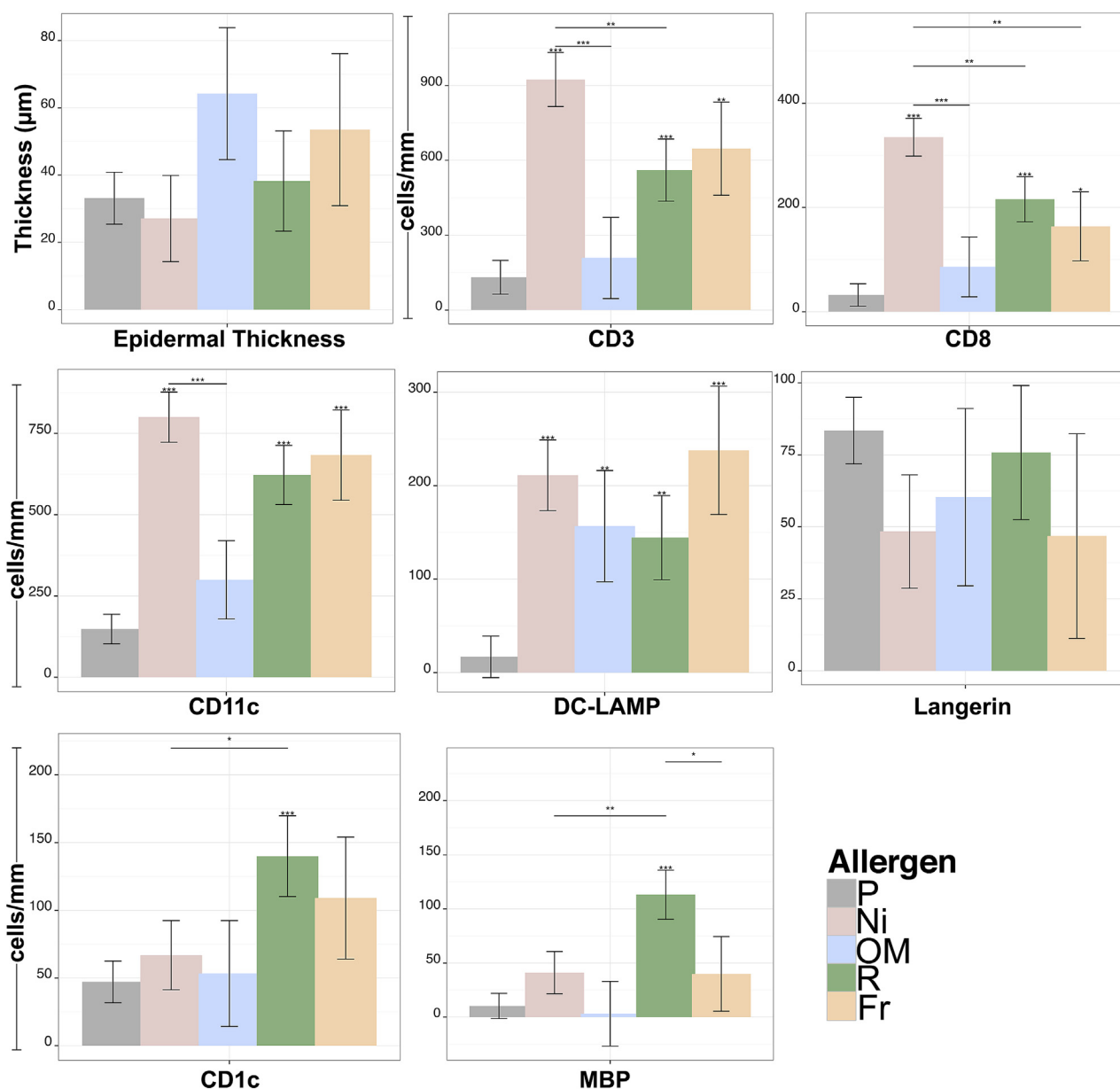


FIG 2. Epidermal thickness and cell counts for CD3, CD8, CD11c, DC-LAMP, Langerin, CD1c, and major basic protein in each allergen group were compared with petrolatum and nickel. Epidermal thickness was not significantly changed among allergens versus petrolatum. In general, nickel demonstrated the largest infiltrates whereas samples in contact with other metals exhibited much smaller cellular infiltrates. Fr, Fragrance; MBP, major basic protein; Ni, nickel; OM, other metals; P, petrolatum; R, rubber. Mean ± SEM. * $P < .05$, ** $P < .01$, and *** $P < .001$.

Online Repository at www.jacionline.org). The ACD transcriptome is presented as a heatmap in Fig 3, B, grouped by allergen type and stratified by clinical scoring (red: upregulated genes, blue: downregulated genes; Table E4). The ACD transcriptome consists of immune genes, including markers of T-cell-related genes (granzyme B, inducible T-cell costimulator, CD2, ITK, IL2RA, CCR7, CD80, and CD3D), immune regulators (cytotoxic T-lymphocyte antigen 4 [CTLA4], BATF3, and programmed cell death 1 ligand 1/CD274), DC antigens (CD11c/integrin alpha X/CD11c and CD1b), general inflammatory markers (matrix metalloproteinase 12), and several chemokines (chemokine, CXC motif, ligand 1 [CXCL1], chemokine, CC motif, ligand

17 [CCL17], CCL19, and chemokine, CC motif, ligand 26 [CCL26]) (Fig 3, A and B; Table E4). For these common genes, nickel generally exhibited the greatest differences from petrolatum followed closely by fragrance, while the latter demonstrated greater differential expression of major T_H2 -related chemokines (CCL17 and CCL26) (see Table E4). We also noted that the intensity gradient of the genomic signal loosely paralleled the clinical grading (Fig 3, B).

Beyond the 149 common genes, the genomic changes induced by individual allergen groups were notably different. These allergen-specific changes were most impressive with nickel, which generated 1899 distinct DEGs (2951 probes),

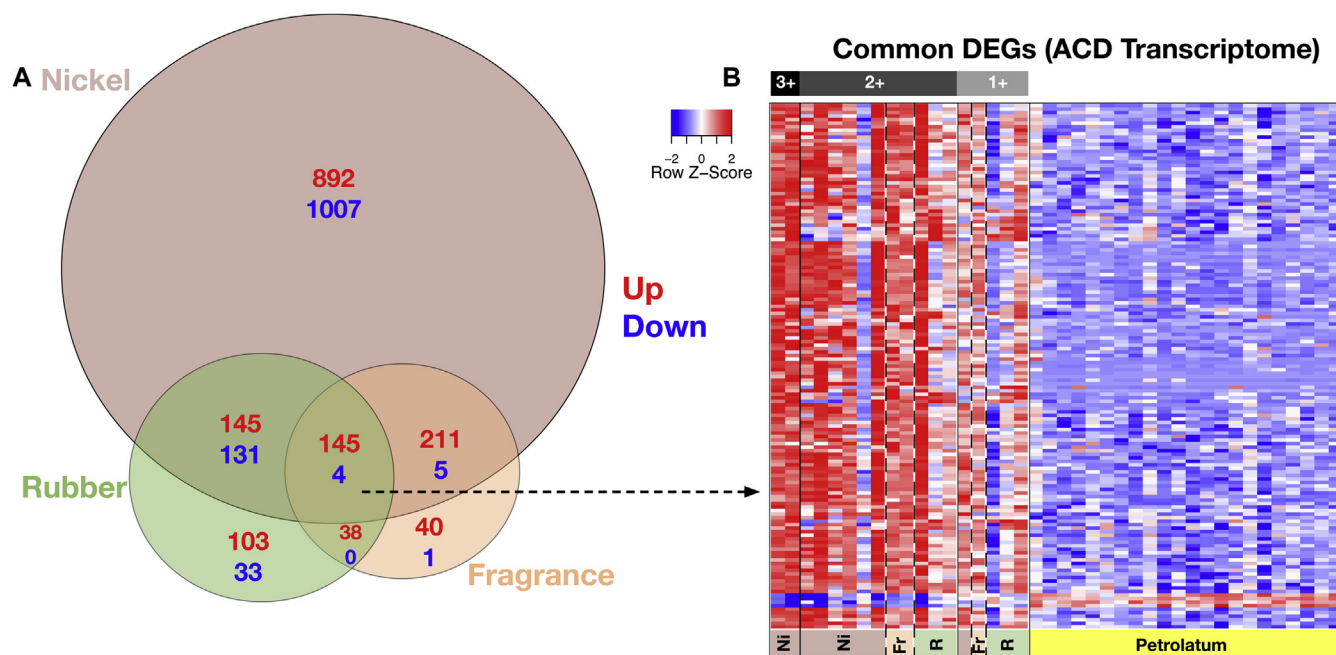


FIG 3. Common and unique DEGs for nickel, rubber, and fragrance compared with petrolatum with direction of dysregulation (*red*/upregulated; *blue*/downregulated; **A-B**). A core set of 149 DEGs in all allergen groups versus petrolatum (intersection of 3 circles, **A**) was used to define the allergic contact dermatitis transcriptome (depicted as a heatmap in **B**; *red*/upregulated; *blue*/downregulated; samples arranged by clinical scoring and allergen group). Allergen-dependent genomic changes were more impressive, particularly in nickel (**A**). *Fr*, Fragrance; *Ni*, nickel; *P*, petrolatum; *R*, rubber.

followed by rubber (136 genes, 174 probes) and fragrance (41 genes, 47 probes) (Fig 3, A; see Tables E5-E7 in this article's Online Repository at www.jacionline.org). Some genes were commonly activated by nickel and rubber (276 genes) but not with fragrance, while conversely 216 genes were activated in nickel and fragrance but not in rubber (Fig 3, A). To identify unique molecular changes to individual allergen groups, we analyzed DEGs in each group versus petrolatum, showing a similar pattern of correlation between molecular and clinical intensity (see Fig E3 and Table E8 in this article's Online Repository at www.jacionline.org). We noted that innate/IFN/ T_H1 -related (chemokine, CXC motif, ligand 9 [CXCL9], chemokine, CXC motif, ligand 10 [CXCL10], chemokine, CXC motif, ligand 11 [CXCL11], 2'-5' oligoadenylate synthetase like, IL-6, IL-1B, IL12RB2, myxovirus resistance 1, and Toll-like receptor 7) and T_H17 -related genes (chemokine, CXC motif, ligand 2 [CXCL2], IL23A, and peptidase inhibitor 3 [PI3]) were commonly induced by nickel, whereas T_H2 -related genes (tumor necrosis factor ligand superfamily, member 4 [OX40L]) were particularly induced by fragrance. Rubber notably induced proliferation-promoting genes (cyclin B1-2/CCNB1-2 and Cell division cycle associated 2) and IL-22, the T_H22 cytokine (Fig 3; Tables E5-E8).

Individual allergen groups show a trend toward differential immune skewing on gene arrays

To further evaluate for differential immune polarization between various allergen groups, we analyzed an inflammatory gene subset within the DEGs in individual allergen groups versus petrolatum. Major T-cell-, DC-, and cytokine and

chemokine-related genes were significantly upregulated in allergen-reactive samples, and gene induction seemed to parallel clinical scoring (Fig 4, A; Table E8). Nickel, representing the majority of 2+/3+ reactions, generally exhibited the highest induction of inflammatory markers than did other metals, fragrance, or rubber (Fig 4, A; Table E8). Particularly impressive increases were observed in nickel for T-cell markers (granzyme B, CD28, CD2, LCK, CCR7, and JAK3), T_H1 /IFN-related genes (CXCL10), and T_H17 -related genes (PI3, CXCL1, and CXCL2) ($P < .01$; Fig 4, A; Table E8).

Despite the overall stronger genomic signal with nickel, select immune pathways were more highly induced with other allergens (ie, T_H2 -inducing chemokines—chemokine, CC motif, ligand 13 [CCL13], CCL17, and chemokine, CC motif, ligand 18 [CCL18]—were highest in fragrance). We next assessed the extent to which individual allergens activated major inflammatory and regulatory pathways represented in the gene arrays using previously defined gene lists for these axes (Table E8).^{36,38,41,43} The box-plot graphs in Fig 4, B, demonstrate that different allergens appear to selectively upregulate certain immune pathways to a greater degree than do others. For example, nickel strongly induced TNF- (ie, IL-1B), T_H1 /IFN- (ie, CXCL9, CXCL10, CXCL11, and 2'-5' oligoadenylate synthetase like), T_H17 - (ie, CXCL1, CXCL2, IL-23A, and PI3/elfin), and T_H22 -related genes (ie, S100-calcium binding protein A9 [S100A9] and IL-32) as well as negative immune regulatory genes (ie, LILRB2, CTLA4, CD274/programmed cell death 1 ligand 1, and PTPRC/CD45) (Fig 4, B). Fragrance potently induced T_H2 chemokines (ie, CCL13, CCL17, CCL18, chemokine, CC motif, ligand 22, and CCL26) and negative regulators (CTLA4), and rubber generally resembled fragrance, albeit with smaller inductions. Finally, non-nickel metals generally showed

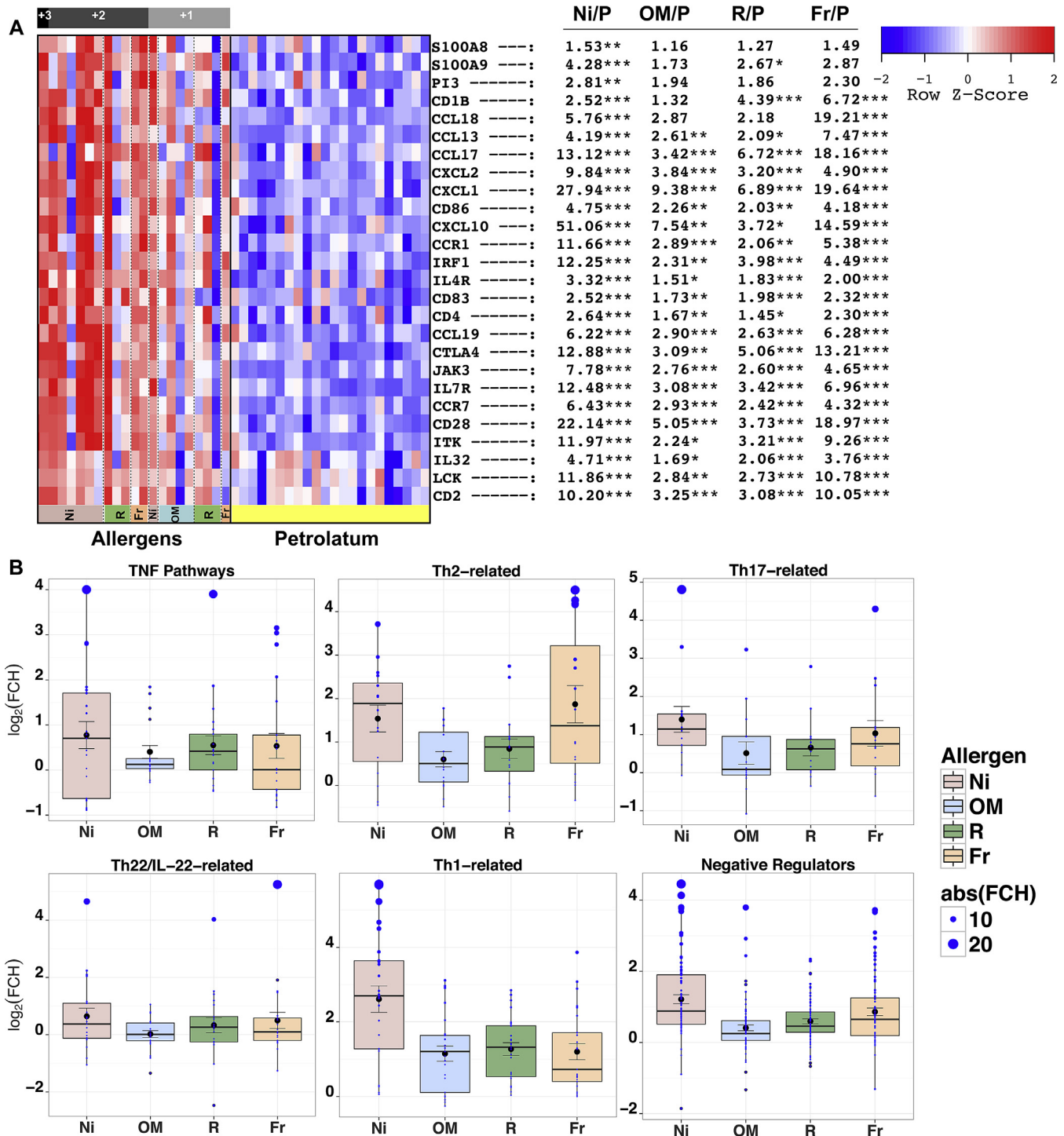


FIG 4. A, A subset of differentially expressed immune genes. Samples in the heatmap are grouped by allergen type and arranged by clinical scoring +1 to +3 (grey to black), red/upregulated, blue/downregulated. Fold changes (FCH) of allergen compared with petrolatum are shown. * $P < .05$, ** $P < .01$, and *** $P < .001$. **B,** Box plots of the individual allergen response across major immune pathways represented in gene arrays. Mean $\pm$ SEM (diameter of circle represents FCH). Fr, Fragrance; Ni, nickel; OM, other metals; P, petrolatum; R, rubber.

weaker immune activation. These findings imply that individual allergens can induce polar immune responses, suggesting that ACD should not be considered a single immunologic phenomenon (Fig 4, B; mean/median, and major representative pathways by allergen type). Because metals such as nickel

are recognized by T-cell receptors in conjunction with MHC-related proteins, we investigated whether there is an association between HLA haplotypes and the allergen groups by assessing all HLA markers present in our gene-array results. Consistent with recent publications, nickel and other

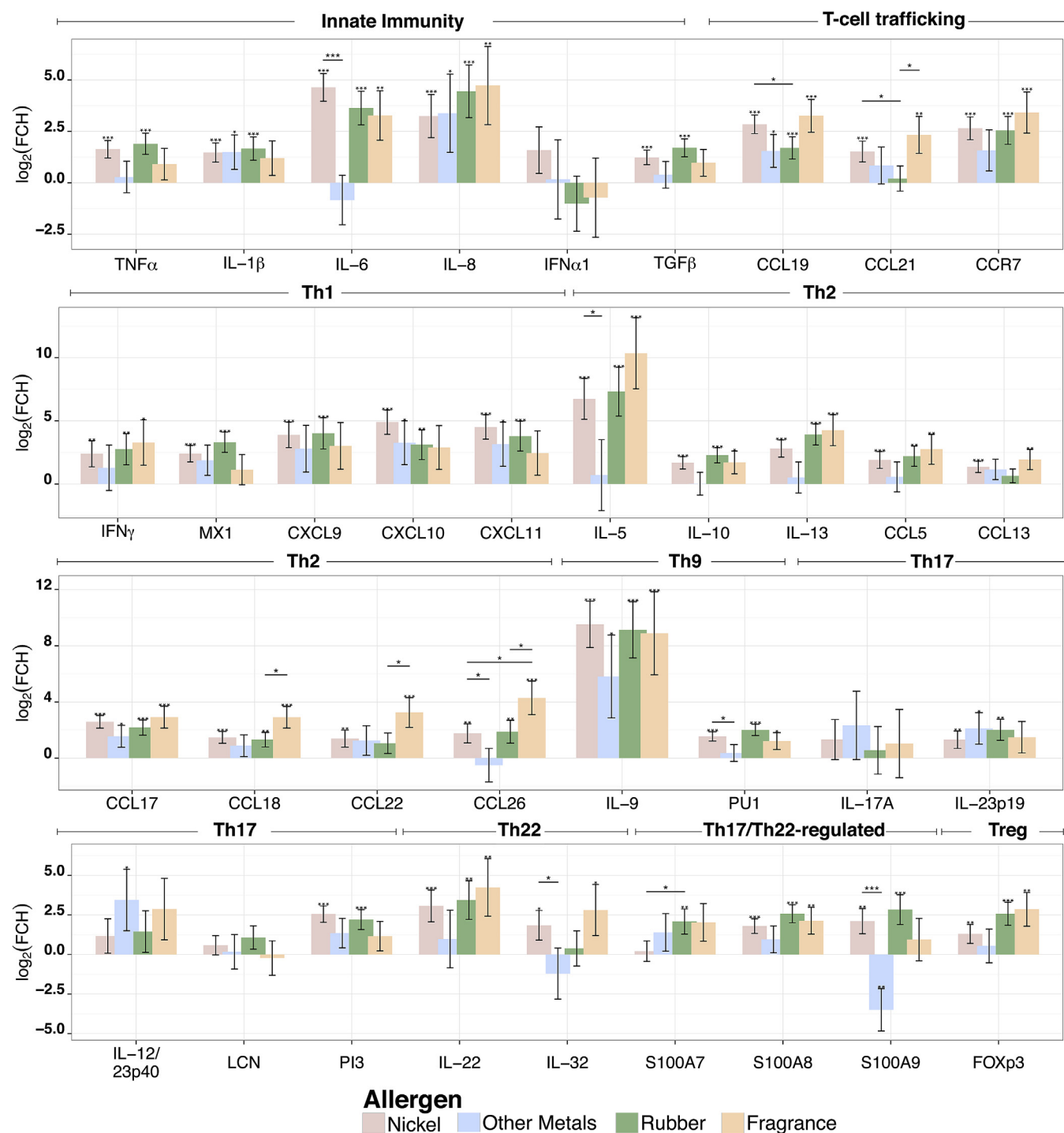


FIG 5. Real-time PCR was used to evaluate the expression of major cytokines and inflammatory markers; data were grouped according to allergen type and by the major inflammatory and regulatory pathways represented. Fold changes (FCH) and *P* value of allergen versus petrolatum (directly above the SEM bars) and nickel versus the other allergens are indicated if significant. **P* < .05, ***P* < .01, and ****P* < .001.

metals demonstrated increased expression of HLA-DPA1, HLA-DPB1/ILA-D⁴⁹⁻⁵¹ as well as HLA-DOB, HLA-DMB, and HLA-DMA. Interestingly, we also found significantly increased expression of HLA-DOB and HLA-DPA1 in rubber and HLA-DQB2 in fragrance (see Fig E4 in this article's Online Repository at www.jacionline.org).

Genomic profiling by RT-PCR confirms allergen-specific immune polarization

To confirm differential immune activation in an allergen-dependent manner and to extend the analysis to low-abundance cytokines that are poorly detected by gene arrays, mRNA expression of representative inflammatory, innate, and various

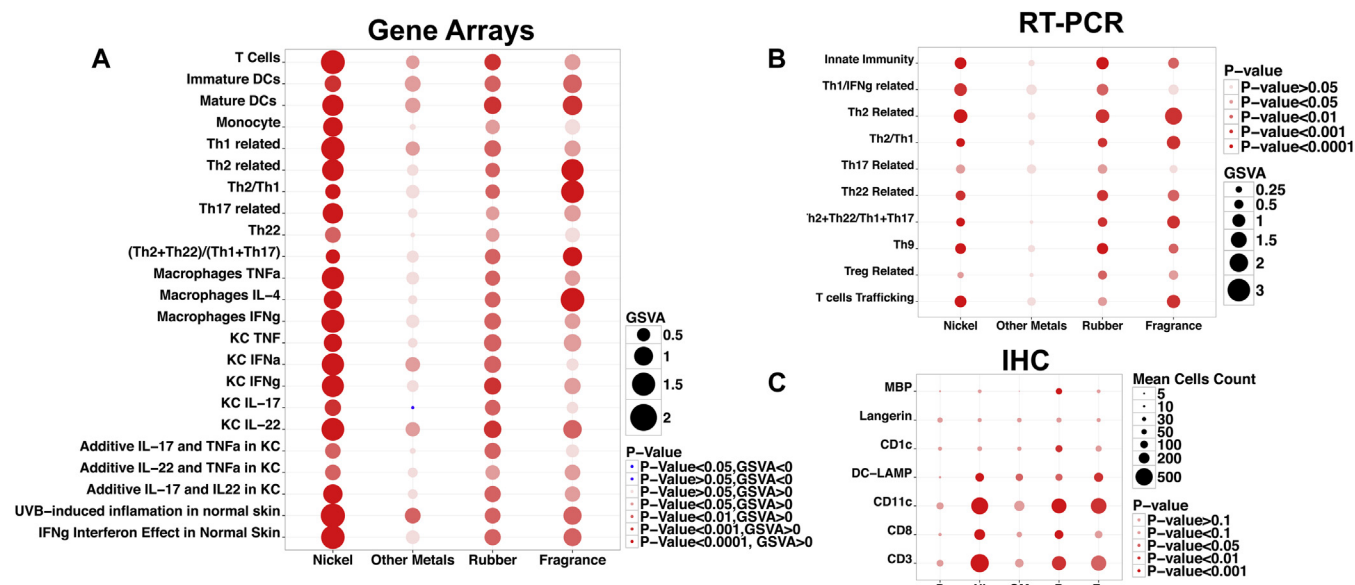


FIG 6. Bubble plots comparing different measures of the immune response across different allergens. Bubble diameter proportional to the gene-set variation analysis (GSVA) score for gene arrays (A) and RT-PCR (B) or mean cell counts for IHC (C). Larger bubbles reflect higher magnitudes of expression/cellular infiltrates, and the color of each bubble indicates the significance (P value). Fr, Fragrance; Ni, nickel; OM, other metals; P, petrolatum; R, rubber.

T_H-induced genes was measured (Fig 5; see Table E9 in this article's Online Repository at www.jacionline.org). Overall, very significant increases in many inflammatory products were observed in all allergen groups (although less evident for other metals) compared with petrolatum, with nickel and fragrance showing the greatest increases. Markers of innate immunity (IL-6 and IL-8) were significantly upregulated for most allergens. T-cell trafficking genes (CCL19 and CCR7) were upregulated in all allergens, with the greatest inductions in fragrance.

Mirroring the gene arrays, higher mRNA expression of some innate (IFN- α 1 and IL-6) and T_H1-related (ie, IFN- α 1, CXCL10, and CXCL11) genes was observed in nickel than in other allergen groups. Conversely, fragrance exhibited greater expression of most T_H2-related cytokines (IL-5 and IL-13) and chemokines (CCL5, CCL13, CCL17, CCL18, chemokine, CC motif, ligand 22, and CCL26), T_H22-related genes (IL-22 and IL-32), and regulatory T cells (FOXP3). T_H9-related genes (IL-9 and PU-1) were similarly increased in nickel, rubber, and fragrance. In line with gene-array data, metals other than nickel generated the weakest mRNA changes than did other allergens, with the exception of T_H17-regulated cytokines (IL-17A, IL-23p19, and IL-12/23p40), which were most elevated in other metals. Although rubber usually showed smaller mRNA inductions than did the more potent inducers, it exhibited similar or greater expression of select T_H17- (IL-23p19, lipocalin2/LCN, and PI3/elfafin), T_H22 (IL-22), and T_H17/T_H22-regulated genes (S100A7/A8/A9). As illustrated for IL-5 and IL-9 in Fig E5 (in this article's Online Repository at www.jacionline.org), activated T cells are the likely source of these polar cytokine responses.

Pathway analysis of genomic data suggests that different allergens induce distinct immune reactions

Fig 6 offers bubble-plot overviews of gene-array, RT-PCR, and IHC data, classified by allergen type. Bubble diameters are

proportional to the gene-set variation analysis score (for gene arrays and RT-PCR) or mean cell counts (for IHC). Larger bubbles reflect higher magnitudes of immune responses/cellular infiltrates; significance is indicated by color intensity (false-discovery rate/P value).

To dissect the effects of each allergen group on major cells or pathways, we evaluated their relative genomic scores for previously published lists of major T-cell, DC, monocyte, keratinocyte, T-cell axes, and immune gene sets (Fig 6, A).^{36,38,41,43} Overall, nickel consistently demonstrates the highest immune activation among all allergens, confirming it as a potent immune activator. As visualized by bubbles representing innate immunity (ie, TNF or IFN- α -treated keratinocytes or macrophages), T_H1, T_H17, and T_H2/T_H1 and T_H2+T_H22/T_H1+T_H17 ratios, nickel exhibits potent induction of innate immunity and T_H1/T_H17 polarization. In contrast, fragrance shows strong T_H2 skewing, a much smaller T_H1/T_H17 polarization, and a T_H22 polarization component. These findings are also supported by the RT-PCR bubble plot (Fig 6, B), where fragrance exhibits greater T_H2/T_H1 and T_H2+T_H22/T_H1+T_H17 ratios than does nickel. Similar to fragrance, but with a lesser magnitude, rubber shows T_H2+T_H22 immune deviation (Fig 6, A and B). Nickel's higher immune activation is also reflected by its IHC counts (Fig 6, C) with the greatest T-cell (CD3⁺ and CD8⁺) and DC (CD11c⁺ and DC-LAMP⁺) infiltrates; moderate infiltrates characterized fragrance and rubber, while other metals had only modest infiltrates (Fig 6, C).

To evaluate consistency between the clinician-dependent scoring and molecular markers of inflammation, we performed a Spearman rank correlation analysis between clinical scoring and cellular infiltrates and the immune gene sets listed in Fig 6, A-C.^{36,38,41,43} The most impressive correlations with clinical scoring were demonstrated by IHC with cellular infiltrates, particularly T cells ($r = 0.75/\text{CD3}^+$, $r = 0.68/\text{CD8}^+$), CD11c⁺ DCs ($r = 0.64$), and eosinophils/ major basic protein⁺ ($r = 0.56$)

($P < .01$; see Table E10 in this article's Online Repository at www.jacionline.org). The strongest correlations with clinical scoring on gene arrays were found with T-cell-related ($r = 0.71$), and T-cell pathways genes, including T_H17 -related ($r = 0.49$), T_H1 -related ($r = 0.47$), and T_H22 -related ($r = 0.43$). High correlations were also detected with IL-22 and IFN- γ -induced genes in keratinocytes ($r = 0.62$ and 0.53 , respectively; $P < .05$; see Table E11 in this article's Online Repository at www.jacionline.org). For RT-PCR, correlations with scoring were strongest for T_H2 - and T_H22 -related products ($r = 0.46$ and 0.45 , respectively; $P < .04$).

DISCUSSION

Distinct hapten-specific immune polarizations to potent sensitizers have been demonstrated in murine CHS models, including a T_H2 bias for fluorescein isothiocyanate⁵² and T_H1 /IFN- γ polarity for dinitrofluorobenzene and TNCB.^{2,10,53-55} Nevertheless, because mice do not mount inflammatory responses to clinically relevant allergens,^{2,9,12,13} and because the relevance of murine models to human ACD is unclear,^{8,9,12} we used patch testing to common allergens to evaluate for polar T-cell polarization in humans.

Past human ACD studies largely involved blood analyses of hapten-specific effector T cells. These studies predominantly focused on nickel and suggested polar responses on hapten re-exposure in PBMCs. Nickel-specific responses were shown to be predominantly T_H1 -polarized,^{22-24,27,56-59} with additional T_H2 and T_H17 components compared with controls.^{17,23,30,60} Characterization of specific effector responses to other allergens is limited.⁶¹ One study noted a T_H2 -biased response to paraphenylenediamine, while another described mixed polarity by chromium.⁶¹⁻⁶³ Attempts at using peripheral blood as an *in vitro* diagnostic tool for different allergens have also been mixed, suggesting inherent limitations to using circulating cells to assess cutaneous reactions to contact sensitizers.⁶³⁻⁶⁶ The pertinence of peripheral data is particularly uncertain considering that relevant DC populations are absent in blood.^{9,29}

Few human ACD studies include skin analyses. However, these evaluated a limited set of markers, grouped immune responses to different allergens as a single phenomenon, or focused on a single allergen (particularly nickel), discounting the potential for allergen-specific differences.^{8,9,14-17,59,67} Elevated IL-1 and IL-36 family cytokines and associated molecules were noted in biopsies of positive patch test results to multiple allergens.⁶⁸ Another study, characterizing tissue responses to 9 different allergens, detected high levels of IL-17, IL-23, IFN- γ , and IL-4,¹⁵ while CCL17 and CXCL10 were significantly elevated in collective samples of nickel- and fragrance-allergic skin.⁶⁷ A nickel-specific skin study identified selective increases in inflammatory markers by gene arrays and RT-PCR (ie, granzyme B, CCR7, S100A7, and CCL19), but did not address T-cell polarization, cytokine production, and clinical correlations.⁶⁹ Another skin-based nickel-specific study focused on CCL17 and CCL27, noting significantly increased CCL17.⁷⁰

Our study expands the understanding of T-cell activation and associated cytokines in allergen-reactive skin. It is the first human study to define shared and unique ACD transcriptomes for clinically relevant allergens, determine allergen-specific polar immune responses, and associate clinical intensity with immune activation. Although the common ACD transcriptome consists of

only 149 genes ($\sim 30\%$ immune-related), a much larger set was uniquely altered by different allergens, most notably nickel. Although marked immune responses were noted with all allergen groups, specific allergens more potently induced certain pathways. Nickel significantly increased T_H1 /IFN and innate immune responses, consistent with its selective binding to Toll-like receptor 4, which preferentially induces T_H1 activation by DCs.⁷¹⁻⁷³ It also induced significant T_H17 skewing, consistent with PBMCs data,¹⁷ and T_H22 . Fragrance, and to a lesser extent rubber, demonstrated a strong T_H2 bias and some T_H22 polarization, with much smaller T_H1 / T_H17 contributions. This differs from a report that noted significant T_H1 and minimal T_H2 effector responses in peripheral blood to fragrance,⁶¹ thus emphasizing the importance of studying effector responses to topical sensitizers directly in skin. Interestingly, induction of negative immune regulators paralleled the inflammatory responses, being highest with nickel, though regulatory T cell-related FOXP3 was highest in fragrance. In addition, although associations between several HLAs with nickel and other metals were previously reported,⁴⁹⁻⁵¹ our data associate the expression of other HLA genes with rubber and fragrance.

The polar immune responses seen with various allergens might serve as a model for atopic dermatitis (AD), another common inflammatory skin disease. These eczematous processes are closely related; patients with AD exhibit increased ACD reactions, possibly due to impaired barrier function, with allergens potentially triggering AD flares.⁷⁴⁻⁷⁶ Recent work demonstrated that patients with intrinsic AD were frequently sensitized to metals (nickel and cobalt),^{76,77} while our recently published study identified significantly higher T_H1 / T_H17 / T_H22 polarization in intrinsic AD than in extrinsic AD.³⁸ In this report, we identify high expression of T_H1 / T_H17 / T_H22 genes in nickel-inflamed skin and T_H17 markers in other metals, suggesting the possibility that these allergens may be potential triggers for intrinsic AD and may thus serve to simulate this subtype.

In contrast to metals, patch tests to dust mite and other environmental allergens induce T_H2 -polarized responses, simulating acute AD.⁷⁸⁻⁸⁰ Similarly, the T_H2 / T_H22 polarization we noted for fragrance might be of particular relevance to AD because fragrances are common sensitizers in atopics.^{76,81} In addition, because acute AD is a T_H2 / T_H22 -polarized process,⁴² fragrance might provide an experimental tool for simulating AD,⁸² potentially expanding our understanding of AD and its triggers.

Finally, although much of our study focused on effector T-cell functions in the elicitation phase, it also raises the concept of inflammatory skin-associated lymphoid tissue, which has recently reemerged following mice data.⁴⁸ This model showed that dermal DCs rather than Langerhans cells were central to CHS responses.^{2,83} Our histologic data, which revealed close aggregates of dermal DCs and T cells in reactive human skin, introduces the possibility for inflammatory skin-associated lymphoid tissue in which DCs might be able to stimulate T cells locally rather than in nearby lymph nodes, producing nearby vesicles as seen in our samples.

Our study offers new insights into the pathogenesis of human ACD. It defines the common transcriptome of clinically relevant sensitizers in human skin and identifies unique immune pathways preferentially activated by different allergens, suggesting that ACD cannot be considered a single entity. At the mechanistic

level, it suggests that only some sensitizers (fragrance) show classic “allergic” T_H2 polarization as might be implied by the term “allergic contact dermatitis,” while others (nickel) generate a T_H1/T_H17 polarity. Collectively, our findings provide a novel framework for understanding effector responses to clinically relevant sensitizers, and may help guide future human studies of ACD as well as intrinsic and extrinsic AD.

Clinical implications: The polar responses seen with nickel (T_H1/T_H17) and fragrance (T_H2/T_H22) could provide a model system to study immune polarizations in acute intrinsic and extrinsic atopic dermatitis.

REFERENCES

- Peiser M, Tralau T, Heidler J, Api AM, Arts JH, Basketter DA, et al. Allergic contact dermatitis: epidemiology, molecular mechanisms, in vitro methods and regulatory aspects. Current knowledge assembled at an international workshop at BfR, Germany. *Cell Mol Life Sci* 2012;69:763-81.
- Kaplan DH, Igyarto BZ, Gaspari AA. Early immune events in the induction of allergic contact dermatitis. *Nat Rev Immunol* 2012;12:114-24.
- Thyssen JP, Linneberg A, Menne T, Johansen JD. The epidemiology of contact allergy in the general population—prevalence and main findings. *Contact Dermatitis* 2007;57:287-99.
- Wentworth AB, Yiannias JA, Keeling JH, Hall MR, Camilleri MJ, Drage LA, et al. Trends in patch-test results and allergen changes in the standard series: a Mayo Clinic 5-year retrospective review (January 1, 2006, to December 31, 2010). *J Am Acad Dermatol* 2014;70:269-75.
- Saripalli YV, Achen F, Belsito DV. The detection of clinically relevant contact allergens using a standard screening tray of twenty-three allergens. *J Am Acad Dermatol* 2003;49:65-9.
- Belsito DV. Occupational contact dermatitis: etiology, prevalence, and resultant impairment/disability. *J Am Acad Dermatol* 2005;53:303-13.
- Nosbaum A, Vocanson M, Rozieres A, Hennino A, Nicolas JF. Allergic and irritant contact dermatitis. *Eur J Dermatol* 2009;19:325-32.
- Saint-Mezard P, Rosieres A, Krasteva M, Berard F, Dubois B, Kaiserlian D, et al. Allergic contact dermatitis. *Eur J Dermatol* 2004;14:284-95.
- Honda T, Egawa G, Grabbe S, Kabashima K. Update of immune events in the murine contact hypersensitivity model: toward the understanding of allergic contact dermatitis. *J Invest Dermatol* 2013;133:303-15.
- Christensen AD, Haase C. Immunological mechanisms of contact hypersensitivity in mice. *APMIS* 2012;120:1-27.
- Belsito DV. Patch testing: after 100 years, still the gold standard in diagnosing cutaneous delayed-type hypersensitivity. *Arb Paul Ehrlich Inst Bundesamt Sera Impfstoffe Frankf A M* 1997;91:195-202.
- Vocanson M, Hennino A, Cluzel-Tailhardat M, Saint-Mezard P, Benetiere J, Chavagnac C, et al. $CD8^+$ T cells are effector cells of contact dermatitis to common skin allergens in mice. *J Invest Dermatol* 2006;126:815-20.
- Saint-Mezard P, Krasteva M, Chavagnac C, Bosset S, Akiba H, Kehren J, et al. Afferent and efferent phases of allergic contact dermatitis (ACD) can be induced after a single skin contact with haptens: evidence using a mouse model of primary ACD. *J Invest Dermatol* 2003;120:641-7.
- Pennino D, Eyerich K, Scarponi C, Carbone T, Eyerich S, Nasorri F, et al. IL-17 amplifies human contact hypersensitivity by licensing hapten nonspecific $Th1$ cells to kill autologous keratinocytes. *J Immunol* 2010;184:4880-8.
- Zhao Y, Balato A, Fischelevich R, Chapoval A, Mann DL, Gaspari AA. $Th17/Tc17$ infiltration and associated cytokine gene expression in elicitation phase of allergic contact dermatitis. *Br J Dermatol* 2009;161:1301-6.
- Gober MD, Gaspari AA. Allergic contact dermatitis. *Curr Dir Autoimmun* 2008;10:1-26.
- Larsen JM, Bonefeld CM, Poulsen SS, Geisler C, Skov L. IL-23 and $T(H)17$ -mediated inflammation in human allergic contact dermatitis. *J Allergy Clin Immunol* 2009;123:486-92.
- Dyring-Andersen B, Skov L, Lovendorf MB, Bzorek M, Sondergaard K, Lauritsen JP, et al. $CD4(+) T$ cells producing interleukin (IL)-17, IL-22 and interferon-gamma are major effector T cells in nickel allergy. *Contact Dermatitis* 2013;68:339-47.
- Oboki K, Ohno T, Saito H, Nakae S. $Th17$ and allergy. *Allergol Int* 2008;57:121-34.
- Ricciardi L, Minciullo PL, Saitta S, Trombetta D, Saija A, Gangemi S. Increased serum levels of IL-22 in patients with nickel contact dermatitis. *Contact Dermatitis* 2009;60:57-8.
- Peiser M. Role of $Th17$ cells in skin inflammation of allergic contact dermatitis. *Clin Dev Immunol* 2013;2013:261037.
- Minang JT, Arestrom I, Troye-Blomberg M, Lundeberg L, Ahlborg N. Nickel, cobalt, chromium, palladium and gold induce a mixed $Th1$ - and $Th2$ -type cytokine response in vitro in subjects with contact allergy to the respective metals. *Clin Exp Immunol* 2006;146:417-26.
- Minang JT, Troye-Blomberg M, Lundeberg L, Ahlborg N. Nickel elicits concomitant and correlated in vitro production of $Th1$ -, $Th2$ -type and regulatory cytokines in subjects with contact allergy to nickel. *Scand J Immunol* 2005;62:289-96.
- Almogren A, Adam MH, Shakoor Z, Gadelrab MO, Musa HA. $Th1$ and $Th2$ cytokine profile of $CD4$ and $CD8$ positive peripheral blood lymphocytes in nickel contact dermatitis. *C Eur J Immunol* 2013;38:6.
- Hansen MB, Skov L, Menne T, Olsen J. Gene transcripts as potential diagnostic markers for allergic contact dermatitis. *Contact Dermatitis* 2005;53:100-6.
- Nasorri F, Sebastiani S, Mariani V, De Pita O, Puddu P, Girolomoni G, et al. Activation of nickel-specific $CD4^+$ T lymphocytes in the absence of professional antigen-presenting cells. *J Invest Dermatol* 2002;118:172-9.
- Sebastiani S, Albanesi C, Nasorri F, Girolomoni G, Cavani A. Nickel-specific $CD4(+) and CD8(+) T$ cells display distinct migratory responses to chemokines produced during allergic contact dermatitis. *J Invest Dermatol* 2002;118:1052-8.
- Buchvald D, Lundeberg L. Impaired responses of peripheral blood mononuclear cells to nickel in patients with nickel-allergic contact dermatitis and concomitant atopic dermatitis. *Br J Dermatol* 2004;150:484-92.
- Werfel T, Hentschel M, Renz H, Kapp A. Analysis of the phenotype and cytokine pattern of blood- and skin-derived nickel specific T cells in allergic contact dermatitis. *Int Arch Allergy Immunol* 1997;113:384-6.
- Werfel T, Hentschel M, Kapp A, Renz H. Dichotomy of blood- and skin-derived IL-4-producing allergen-specific T cells and restricted V beta repertoire in nickel-mediated contact dermatitis. *J Immunol* 1997;158:2500-5.
- Albanesi C, Cavani A, Girolomoni G. IL-17 is produced by nickel-specific T lymphocytes and regulates ICAM-1 expression and chemokine production in human keratinocytes: synergistic or antagonistic effects with IFN-gamma and TNF-alpha. *J Immunol* 1999;162:494-502.
- Kalish RS, Johnson KL. Enrichment and function of urushiol (poison ivy)-specific T lymphocytes in lesions of allergic contact dermatitis to urushiol. *J Immunol* 1990;145:3706-13.
- Zug KA, Warshaw EM, Fowler JF Jr, Maibach HI, Belsito DL, Pratt MD, et al. Patch-test results of the North American Contact Dermatitis Group 2005-2006. *Dermatitis* 2009;20:149-60.
- Marks JG, Belsito DV, DeLeo VA, Fowler JF Jr, Fransway AF, Maibach HI, et al. North American Contact Dermatitis Group patch test results for the detection of delayed-type hypersensitivity to topical allergens. *J Am Acad Dermatol* 1998;38:911-8.
- Pratt MD, Belsito DV, DeLeo VA, Fowler JF Jr, Fransway AF, Maibach HI, et al. North American Contact Dermatitis Group patch-test results, 2001-2002 study period. *Dermatitis* 2004;15:176-83.
- Guttman-Yassky E, Suarez-Farinas M, Chiricozzi A, Nogales KE, Shemer A, Fuentes-Duculan J, et al. Broad defects in epidermal cornification in atopic dermatitis identified through genomic analysis. *J Allergy Clin Immunol* 2009;124:1235-44.e58.
- Nogales KE, Zaba LC, Shemer A, Fuentes-Duculan J, Cardinale I, Kikuchi T, et al. IL-22-producing “ $T22$ ” T cells account for upregulated IL-22 in atopic dermatitis despite reduced IL-17-producing $Th17$ T cells. *J Allergy Clin Immunol* 2009;123:1244-52.e2.
- Suarez-Farinas M, Dhingra N, Gittler J, Shemer A, Cardinale I, de Guzman Strong C, et al. Intrinsic atopic dermatitis shows similar $Th2$ and higher $Th17$ immune activation compared with extrinsic atopic dermatitis. *J Allergy Clin Immunol* 2013;132:361-70.
- Guttman-Yassky E, Lowes MA, Fuentes-Duculan J, Zaba LC, Cardinale I, Nogales KE, et al. Low expression of the IL-23/ $Th17$ pathway in atopic dermatitis compared to psoriasis. *J Immunol* 2008;181:7420-7.
- Guttman-Yassky E, Lowes MA, Fuentes-Duculan J, Whynt J, Novitskaya I, Cardinale I, et al. Major differences in inflammatory dendritic cells and their products distinguish atopic dermatitis from psoriasis. *J Allergy Clin Immunol* 2007;119:1210-7.
- Suarez-Farinas M, Tintle SJ, Shemer A, Chiricozzi A, Nogales K, Cardinale I, et al. Nonlesional atopic dermatitis skin is characterized by broad terminal differentiation defects and variable immune abnormalities. *J Allergy Clin Immunol* 2011;127:954-64, e1-4.
- Gittler JK, Shemer A, Suarez-Farinas M, Fuentes-Duculan J, Gulewicz KJ, Wang CQ, et al. Progressive activation of $T(H)2/T(H)22$ cytokines and selective epidermal proteins characterizes acute and chronic atopic dermatitis. *J Allergy Clin Immunol* 2012;130:1344-54.

43. Tintle S, Shemer A, Suarez-Farinas M, Fujita H, Gilleaudeau P, Sullivan-Whalen M, et al. Reversal of atopic dermatitis with narrow-band UVB phototherapy and biomarkers for therapeutic response. *J Allergy Clin Immunol* 2011;128:583-93, e1-4.
44. Griffiths CE, Dearman RJ, Cumberbatch M, Kimber I. Cytokines and Langerhans cell mobilisation in mouse and man. *Cytokine* 2005;32:67-70.
45. Weinlich G, Heine M, Stossel H, Zanella M, Stoitzner P, Ortner U, et al. Entry into afferent lymphatics and maturation in situ of migrating murine cutaneous dendritic cells. *J Invest Dermatol* 1998;110:441-8.
46. Bergstresser PR, Toews GB, Streilein JW. Natural and perturbed distributions of Langerhans cells: responses to ultraviolet light, heterotopic skin grafting, and dinitrofluorobenzene sensitization. *J Invest Dermatol* 1980;75:73-7.
47. Shortman K, Heath WR. The CD8⁺ dendritic cell subset. *Immunol Rev* 2010;234:18-31.
48. Egawa G, Kabashima K. Skin as a peripheral lymphoid organ: revisiting the concept of skin-associated lymphoid tissues. *J Invest Dermatol* 2011;131:2178-85.
49. Wang Y, Dai S. Structural basis of metal hypersensitivity. *Immunol Res* 2013;55:83-90.
50. Yin L, Crawford F, Marrack P, Kappler JW, Dai S. T-cell receptor (TCR) interaction with peptides that mimic nickel offers insight into nickel contact allergy. *Proc Natl Acad Sci U S A* 2012;109:18517-22.
51. Liu J, Harberts E, Tammara A, Girardi N, Filler RB, Fischelevich R, et al. IL-9 regulates allergen-specific Th1 responses in allergic contact dermatitis. *J Invest Dermatol* 2014 [E-pub ahead of print].
52. Larson RP, Zimmerli SC, Comeau MR, Itano A, Omori M, Iseki M, et al. Dibutyl phthalate-induced thymic stromal lymphopoietin is required for Th2 contact hypersensitivity responses. *J Immunol* 2010;184:2974-84.
53. Dearman RJ, Basketter DA, Kimber I. Characterization of chemical allergens as a function of divergent cytokine secretion profiles induced in mice. *Toxicol Appl Pharmacol* 1996;138:308-16.
54. Dearman RJ, Kimber I. Cytokine fingerprinting: characterization of chemical allergens. *Methods* 1999;19:56-63.
55. Kimber I, Dearman RJ. Allergic contact dermatitis: the cellular effectors. *Contact Dermatitis* 2002;46:1-5.
56. Cavani A, Nasorri F, Prezzi C, Sebastiani S, Albanesi C, Girolomoni G. Human CD4⁺ T lymphocytes with remarkable regulatory functions on dendritic cells and nickel-specific Th1 immune responses. *J Invest Dermatol* 2000;114:295-302.
57. Cavani A, Mei D, Guerra E, Corinti S, Giani M, Pirrotta L, et al. Patients with allergic contact dermatitis to nickel and nonallergic individuals display different nickel-specific T cell responses: evidence for the presence of effector CD8⁺ and regulatory CD4⁺ T cells. *J Invest Dermatol* 1998;111:621-8.
58. Minang JT, Arestrom I, Zuber B, Jonsson G, Troye-Blomberg M, Ahlborg N. Nickel-induced IL-10 down-regulates Th1- but not Th2-type cytokine responses to the contact allergen nickel. *Clin Exp Immunol* 2006;143:494-502.
59. Martin A, Gallino N, Gagliardi J, Ortiz S, Lascano AR, Diller A, et al. Early inflammatory markers in elicitation of allergic contact dermatitis. *BMC Dermatol* 2002;2:9.
60. Czarnobilska E, Jenner B, Kaszuba-Zwoinska J, Kapusta M, Obtulowicz K, Thor P, et al. Contact allergy to nickel: patch test score correlates with IL-5, but not with IFN-gamma nickel-specific secretion by peripheral blood lymphocytes. *Ann Agric Environ Med* 2009;16:37-41.
61. Sieben S, Hertl M, Al Masaoudi T, Merk HF, Blomeke B. Characterization of T cell responses to fragrances. *Toxicol Appl Pharmacol* 2001;172:172-8.
62. Sieben S, Kawakubo Y, Al Masaoudi T, Merk HF, Blomeke B. Delayed-type hypersensitivity reaction to paraphenylenediamine is mediated by 2 different pathways of antigen recognition by specific alpha-beta human T-cell clones. *J Allergy Clin Immunol* 2002;109:1005-11.
63. Lindemann M, Rietschel F, Zabel M, Grosse-Wilde H. Detection of chromium allergy by cellular in vitro methods. *Clin Exp Allergy* 2008;38:1468-75.
64. Lindemann M, Bohmer J, Zabel M, Grosse-Wilde H. ELISpot: a new tool for the detection of nickel sensitization. *Clin Exp Allergy* 2003;33:992-8.
65. Traidl-Hoffmann C, Ring J. Is there an in vitro test for type IV allergy discriminating between sensitization and allergic disease? *Clin Exp Allergy* 2008;38:1412-5.
66. Moed H, von Blomberg M, Bruynzeel DP, Scheper R, Gibbs S, Rustemeyer T. Improved detection of allergen-specific T-cell responses in allergic contact dermatitis through the addition of 'cytokine cocktails'. *Exp Dermatol* 2005;14:634-40.
67. Kamsteeg M, Jansen PA, van Vlijmen-Willems IM, van Erp PE, Rodijk-Olthuis D, van der Valk PG, et al. Molecular diagnostics of psoriasis, atopic dermatitis, allergic contact dermatitis and irritant contact dermatitis. *Br J Dermatol* 2010;162:568-78.
68. Mattii M, Ayala F, Balato N, Filotico R, Lembo S, Schiattarella M, et al. The balance between pro- and anti-inflammatory cytokines is crucial in human allergic contact dermatitis pathogenesis: the role of IL-1 family members. *Exp Dermatol* 2013;22:813-9.
69. Pedersen MB, Skov L, Menne T, Johansen JD, Olsen J. Gene expression time course in the human skin during elicitation of allergic contact dermatitis. *J Invest Dermatol* 2007;127:2585-95.
70. Riis JL, Johansen C, Vestergaard C, Bech R, Kragballe K, Iversen L. Kinetics and differential expression of the skin-related chemokines CCL27 and CCL17 in psoriasis, atopic dermatitis and allergic contact dermatitis. *Exp Dermatol* 2011;20:789-94.
71. Agrawal S, Agrawal A, Dougherty B, Gerwitz A, Blenis J, Van Dyke T, et al. Cutting edge: different Toll-like receptor agonists instruct dendritic cells to induce distinct Th responses via differential modulation of extracellular signal-regulated kinase-mitogen-activated protein kinase and c-Fos. *J Immunol* 2003;171:4984-9.
72. Qi H, Denning TL, Soong L. Differential induction of interleukin-10 and interleukin-12 in dendritic cells by microbial Toll-like receptor activators and skewing of T-cell cytokine profiles. *Infect Immun* 2003;71:3337-42.
73. Re F, Strominger JL. IL-10 released by concomitant TLR2 stimulation blocks the induction of a subset of Th1 cytokines that are specifically induced by TLR4 or TLR3 in human dendritic cells. *J Immunol* 2004;173:7548-55.
74. Gittler JK, Krueger JG, Guttman-Yassky E. Atopic dermatitis results in intrinsic barrier and immune abnormalities: implications for contact dermatitis. *J Allergy Clin Immunol* 2013;131:300-13.
75. Dhingra N, Gulati N, Guttman-Yassky E. Mechanisms of contact sensitization offer insights into the role of barrier defects vs. intrinsic immune abnormalities as drivers of atopic dermatitis. *J Invest Dermatol* 2013;133:2311-4.
76. Giordano-Labadie F, Rance F, Pellegrin F, Bazex J, Dutau G, Schwarze HP. Frequency of contact allergy in children with atopic dermatitis: results of a prospective study of 137 cases. *Contact Dermatitis* 1999;40:192-5.
77. Yamaguchi H, Kabashima-Kubo R, Bito T, Sakabe J, Shimauchi T, Ito T, et al. High frequencies of positive nickel/cobalt patch tests and high sweat nickel concentration in patients with intrinsic atopic dermatitis. *J Dermatol Sci* 2013;72:240-5.
78. Tupker RA, De Monchy JG, Coenraads PJ, Homan A, van der Meer JB. Induction of atopic dermatitis by inhalation of house dust mite. *J Allergy Clin Immunol* 1996;97:1064-70.
79. Leung DY. Atopic dermatitis: new insights and opportunities for therapeutic intervention. *J Allergy Clin Immunol* 2000;105:860-76.
80. Brinkman L, Aslander MM, Raaijmakers JA, Lammers JW, Koenderman L, Bruijnzeel-Koomen CA. Bronchial and cutaneous responses in atopic dermatitis patients after allergen inhalation challenge. *Clin Exp Allergy* 1997;27:1043-51.
81. Lammintausta K, Kalimo K, Fagerlund VL. Patch test reactions in atopic patients. *Contact Dermatitis* 1992;26:234-40.
82. Landheer J, Giovannone B, Mattson JD, Tjabringa S, Bruijnzeel-Koomen CA, McClanahan T, et al. Epicutaneous application of house dust mite induces thymic stromal lymphopoietin in nonlesional skin of patients with atopic dermatitis. *J Allergy Clin Immunol* 2013;132:1252-4.
83. Kaplan DH, Jenison MC, Saeland S, Shlomchik WD, Shlomchik MJ. Epidermal langerhans cell-deficient mice develop enhanced contact hypersensitivity. *Immunity* 2005;23:611-20.

1 **Methods**

2 **Patients' Characteristics and skin samples**

3 Patch testing was performed, using the North American Contact Dermatitis
4 Group Standard (NACDG) for 15 common contact allergens, and at 72 hours the
5 patch test site was evaluated for positive reactions. A reaction score of 1+, 2+, or
6 3+ was assigned to any positive reactions, assessed via established patch test
7 guidelines.^{E1,E2} As a control, a patch of skin was also occluded with petrolatum
8 under the same conditions; petrolatum was chosen because all allergens except
9 fragrance in the patch testing kit are contained in a petrolatum vehicle. Punch
10 biopsies were taken from positive patch test reactions from 24 patients (16
11 females, 8 males, ages 20-63 years, median 40.5 years) under institutional
12 review board-approved protocols. Positive reactions were grouped into four
13 categories: nickel (n=10), fragrance (n=2 Balsam of Peru, 1 Fragrance Mix),
14 rubber (n=5 Carba Mix, 2 Thiuram), and other metals (n=3 Cobalt, 1 Potassium
15 Dichromate). Two patients had a reaction of 3+, eleven patients had a reaction of
16 2+, and nine patients had a reaction of 1+. See Table E1 in the Online
17 Repository for patient demographics and breakdown of allergen responses and
18 reaction level.

19 **Immunohistochemistry and Immunofluorescence**

20 Biopsy specimens were frozen in O.C.T. medium and cryostat tissue
21 sections of all patients were stained with hematoxylin (Fisher, Fair Lawn, NJ) and
22 eosin (Shandon, Pittsburgh, PA). Immunohistochemistry (IHC) and
23 immunofluorescence (IF) were performed by using purified anti-human

monoclonal antibodies (Table E2). For IHC, biotin-labeled horse anti-mouse secondary antibodies (Vector Laboratories) were used to detect the primary antibodies, and the staining signal was developed with chromogen 3-amino-9-ethylcarbazole (AEC, Sigma-Aldrich). Epidermal thickness and positive cells per millimeter were manually quantified by using computer-assisted image analysis software (ImageJ 1.42; National Institutes of Health, Bethesda, MD).

For immunofluorescence in Figures E2 and E5, frozen skin sections were fixed with acetone, blocked in 10% normal goat serum (Vector Laboratories) for 30 minutes, incubated in primary antibodies (See Table E2) overnight at 4°C and amplified with the appropriate secondary antibody (Rhodamine-conjugated goat anti-rabbit from Invitrogen Cat #54255A, Alexa 488-conjugated goat anti mouse from Invitrogen Cat #A21121) for 30 minutes. Sections were co-stained overnight with a second primary antibody and amplified with the appropriate secondary antibody for 30 minutes to identify co-localization. IF images were acquired using the appropriate filters of a Zeiss Axioplan 2 widefield fluorescence controlled by METAVUE software (MDS Analytical Technologies, Downingtown, PA). Images in all IF figures are presented with the green and red stains above a merged image; within merged images, yellow signal represents colocalized staining. Dermal collagen generated green auto-fluorescence and fluorochrome-conjugated antibodies produce background epidermal fluorescence.

Quantitative real-time (RT) PCR and gene-array analysis

RNA was extracted and Affymetrix Human U133Plus 2.0 arrays (Affymetrix,

Santa Clara, CA) were used as previously described.^{E3} Briefly, we used human HGU133Plus2.0 GeneChip probe arrays (Affymetrix Inc, Santa Clara, Calif) containing approximately 54,000 probe sets to assess expression of more than 47,000 transcripts, including approximately 38,500 genes and unigenes. Total RNA was extracted from tissues frozen in liquid nitrogen by the Qiagen RNeasy Mini Kit (Valencia, Calif). DNA was removed with on-column DNase digestion by the Qiagen RNase-free DNase Set. Total RNA (50 ng) was reverse transcribed and amplified with Ovation Whole Blood Solution from NuGen (San Carlos, Calif). The labeled target was fragmented and hybridized to probe arrays, using Encore Biotin Module from NuGen (San Carlos, Calif). The chips were washed, stained with streptavidin-phycoerythrin, and scanned (HP GeneArray Scanner; Hewlett-Packard Company, Palo Alto, Calif). On each chip, the human housekeeping genes β -actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as controls. Suite 5.0 software normalized the expression level values using these controls.

RT-PCR Analysis

The TaqMan RT-PCR reaction was performed with EZ-PCR Core Reagents (Life Technologies), according to the manufacturer's directions. The primers and probes for CCL13 (Hs00234646_m1), CCL17 (Hs00171074_m1), CCL18 (Hs00268113_m1), CCL19 (Hs00171149_m1), CCL20 (Hs01011368_m1), CCL21 (Hs00989654_g1), CCL22 (Hs01574247_m1), CCL26

69 (Hs00171146_m1), CCL5 (Hs00174575_m1), CCR7 (Hs01013469_m1),
 70 CXCL10 (Hs01124251_g1), CXCL11 (Hs04187682_g1), CXCL9
 71 (Hs00171065_m1), Foxp3 (Hs01085834_m1), GATA3 (Hs00231122_m1), IFNA1
 72 (Hs00256882_s1), IFNG (Hs00989291_m1), IL10 (Hs00961622_m1), IL13
 73 (Hs00174379_m1), IL17A (Hs00174383_m1), IL17F (Hs00369400_m1), IL18
 74 (Hs01038788_m1), IL1B (Hs01555410_m1), IL2 (Hs00174114_m1), IL22
 75 (Hs01574154_m1), IL12p19 (Hs00900828_g1), IL23p40 (Hs01011518_m1),
 76 IL2RA (Hs00907779_m1), IL31 (Hs01098710_m1), IL32 (Hs00992441_m1), IL33
 77 (Hs00369211_m1), IL4 (Hs00174122_m1), IL5 (Hs01548712_g1), IL6
 78 (Hs00985639_m1), IL7 (Hs00174202_m1), IL7R (Hs00902334_m1), IL8
 79 (Hs00174103_m1), IL9 (Hs00914237_m1), LCN2 (Hs01008571_m1), MX1
 80 (Hs00895608_m1), OX40 (Hs00937194_g1), OX40L (Hs00182411_m1), PI3
 81 (Hs00160066_m1), PU1 (Hs02786711_m1), RORG (Hs01076122_m1), S100A7
 82 (Hs01923188_u1), S100A8 (Hs00374264_g1), S100A9 (Hs00610058_m1),
 83 TBET (Hs00203436_m1), TGFB (Hs00998133_m1), TNFa (Hs01113624_g1),
 84 TSLP (Hs00263639_m1), TSLPR (Hs00845692_m1) were designed by Life
 85 Technologies. The human acidic ribosomal protein (hARP) gene, a
 86 housekeeping gene, was used to normalize each sample and each gene. The
 87 primer sequences used were generated with the Primer Express algorithm
 88 (Applied Biosystems, Foster City, Calif), version 1.0, by using published genetic
 89 sequences (National Center for Biotechnology Information–PubMed), as follows:
 90 hARP forward CGCTGCTGAACATGCTCAA, hARP reverse
 91 TGTCGAACACCTGCTGGATG, and hARP probe 6-FAM-

TCCCCCTTCTCCTTTGGGCTGG-TAMRA (Gene Bank accession no. NM-001002). The data were analyzed and quantified by using the software provided with the Applied Biosystems PRISM 7700 (Sequence Detection Systems, version 1.7).

Statistical Analysis

Statistical methods are used in this paper to gather evidence that gene expression, pathway activity and population cell counts differ among different allergens. Microarray, RT-PCR gene expression and immunohistochemistry cell counts were obtained from an initial sample of n=24 subjects (16 female and 8 male) with mean age of 42.38 years old (SD= 14.96).

In this paper, the main comparisons are planned to identify genes that are differentially expressed in specific allergens when compared to petrolatum. Limited comparisons between allergens were also performed where sample size allows. Quality control of microarrays was carried out using standard QC metrics and R package Quality Control and Harshlight.^{E4} One patient (#17) was classified as an outlier and removed from the analysis and another patient (#18) that did not have a petrolatum sample was also taken out of the analysis. The distributions of the included allergens and their corresponding patch test score distributions in the final sample of n=24 are displayed in Supplementary Table E1B.

113 Expression measures were obtained using GCRMA algorithm.^{E5}
114 Probesets with at least 1 sample with expression values larger than 4 and
115 standard deviation (SD)>0.15 were kept for further analyses. To follow normality
116 assumptions required by most of the statistical methods used in this paper, the
117 expressions were log2-transformed. Although data from immunohistochemistry
118 are measured as counts, we assumed normal distribution for statistical modeling
119 purposes.

120

121 ***Genomics Statistical Analysis:***

122 In Figure E3, a heatmap for gene expression was used to illustrate how allergens
123 expressions are different in a set of differentially expressed genes from
124 petrolatum. The criteria for gene selection were: fold-change higher than 2 and
125 FDR (False-Discovery Rate) lower than 0.05. The distance matrix was built
126 based on Euclidean distances and complete linkage (McQuitty, 1960) was used
127 as the clustering algorithm.

128 For microarray and RT-PCR data, log2-transformed expression values
129 were modeled using linear mixed-effects models with allergen group as a fixed
130 effect and a random effect for each patient. Comparisons of interest were
131 assessed using linear contrasts via restricted log-likelihood maximization
132 (REML). The contrasts of interest were designed to identify 3 kinds of
133 differences: between each type of allergen and petrolatum, between nickel and
134 other allergens, and between rubber and fragrance allergens. Analysis was
135 conducted under the general framework of *limma* package. P-values from

moderated (paired) t-test were adjusted for multiple hypotheses across genes using Benjamini-Hochberg procedure.

The ACD transcriptome is comprised of that set of common genes that were differentially expressed in all allergen groups. Genes that met the criteria for being considered differentially expressed compared with petrolatum were significant by adjusted p-values (FDR) with a fold-change difference greater than 2 versus petrolatum. The venn diagrams in Figure 3 (created with *venerable* package in R) include the number of differentially-expressed genes in each allergen group. The intersection point of rubber, fragrance, and nickel contains the core set of 149 common DEGs that fulfilled the above-delineated criteria. For this analysis, “Other metals” was not included because cobalt and potassium dichromate induced very small genomic changes compared to other allergens included in the analysis. Furthermore, earlier studies have implicated cobalt as an atypical allergen that generates an irritant poral reaction.^{E6}

To clarify whether distinct immune reactions were not due to clinical scoring differences between allergens (i.e. the score of the patch test), we added the patch score as a covariate in a linear model that regressed gene array $\log_2(\text{FCH})$ (from allergens vs. petrolatum). Patch score was found to be non-significant when included as a covariate in limma model. Specifically, for each probe-set (gene), we evaluated if the effect of the patch score covariate was significant. After adjusting for error multiplicity, we did not find this covariate to be significant in any of the probesets (minimum FDR $\approx$ 1). The allergen versus petrolatum fold-changes were then measured with and without patch score

included as a covariate. The mean and median differences were close to zero with a standard deviation as 0.44 in $\log_2(\text{FCH})$ scale. This outcome may be due to sample size given the unequal distribution among allergens and patch test score; this was an anticipated outcome given that the study was not explicitly designed to address the contribution of the patch-test score in the molecular analysis and thus our n-values for each reaction level were limited.

Pathway Analysis:

For Figure 4, box-plots were created using the distribution of the $\log_2(\text{FCH})$ of immune genes within each allergen vs. petrolatum gene array comparison, with genes assigned to specific immune pathways. This highlights major immune pathway differences between allergens. The size of the individual dots (representing distinct genes) is scaled to their FCH for each allergen.

Figure 5 represents bar plots of RT-PCR $\log_2(\text{FCH})$ of each allergen vs. petrolatum, with mean $\pm$ SEM displayed in a bar plot. Stars directly above the error bars indicate the significance for comparisons against petrolatum ($p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***)). Comparisons between allergens are also indicated, here with lines that link the two allergens being compared, again with stars to indicate significance.

Z-score quantifications of pathways among allergens were also obtained. The activity of entire signaling pathways for each patient/sample was quantified by using a per-patient GSEA-like method. GSVA (Gene Set Variation Analysis) is an unsupervised sample-wise enrichment method described in Hanzelmann et

al. (2013).^{E7} Enrichment scores were obtained by choosing the option z-score in *gsva* function available in this package. The formulation for scores evaluation is described in Lee et al. (2008).^{E8} The authors proposed the linear combination of normalized expressions with weights in the combination being defined as $1/\sqrt{k}$ for k being the number of genes in the pathway. This methodology was applied to obtain pathway enrichment scores in both microarray and RT-PCR analyses. These enrichment scores were used as inputs for the same linear mixed model framework, described previously for gene expressions, in order to evaluate significant differences in allergens vs. petrolatum. These estimated differences in allergen response are displayed in a bubble chart where the size of the bubble is proportional to the magnitude of the difference. Additionally, color intensity indicates the significance of the difference. After estimating the differences, the ratios Th2/Th1 and Th22+Th1/Th1+Th17 were also added to the plot to illustrate how differently those pathways were represented among the allergens.

Immunohistochemistry Statistical Analysis:

Biomarkers for IHC analysis were analyzed with linear mixed effect models as implemented in *lme* function that is part of *nlme* package in R. The high magnitude of the cell counts and inspection of the data distribution with histograms contributed to assumption of Gaussian distribution for the responses. Each allergen group was treated as fixed effect and a random intercept for each patient was included. Estimates and significance of linear contrasts representing differences between a specific allergen and petrolatum are represented in the

bubble plot displayed in Figure 6. Models are fit by maximization of restricted log-likelihood.

Determination of correlation between Patch Test Score and Biomarkers/Pathways:

In order to measure the degree of association between the level of reaction and IHC biomarkers/pathway activity, the Spearman correlation coefficient and its test of significance are presented in Table E10. Per-patient differences in cell counts between allergen and petrolatum were analyzed for each cell type/stain, and enrichment scores were obtained by z-score method implemented in GSVA R package.

References

- E1. Marks JG, Belsito DV, DeLeo VA, Fowler JF, Jr., Fransway AF, Maibach HI, et al. North American Contact Dermatitis Group patch test results for the detection of delayed-type hypersensitivity to topical allergens. *J Am Acad Dermatol* 1998; 38:911-8.
- E2. Pratt MD, Belsito DV, DeLeo VA, Fowler JF, Jr., Fransway AF, Maibach HI, et al. North American Contact Dermatitis Group patch-test results, 2001-2002 study period. *Dermatitis* 2004; 15:176-83.
- E3. Tintle S, Shemer A, Suarez-Farinas M, Fujita H, Gilleaudeau P, Sullivan-Whalen M, et al. Reversal of atopic dermatitis with narrow-band UVB phototherapy and biomarkers for therapeutic response. *J Allergy Clin Immunol* 2011 Sep;128(3):583-93 e1-4.
- E4. Suarez-Farinas M, Pellegrino M, Wittkowski KM, Magnasco MO. Harshlight: a "corrective make-up" program for microarray chips. *BMC bioinformatics*. 2005;6:294.
- E5. Wu Z, Irizarry R, Gentleman R, Murillo F, Spencer F. A model-based background adjustment for oligonucleotide expression arrays. *J Am Stat Assoc* 2004;99:909-17.
- E6. Storrs FJ, White CR, Jr. False-positive "poral" cobalt patch test reactions reside in the eccrine acrosyringium. *Cutis* 2000; 65:49-53.
- E7. Hänzelmann S, Castelo R, Guinney J. GSVA: gene set variation analysis for microarray and RNA-Seq data. *BMC Bioinformatics* 2013; 14(7)
- E8. Lee E, Chuang HY, Kim JW, et al. Inferring pathway activity toward

251 precise disease classification. PLoS Comput Biol 2008, **4**(11)

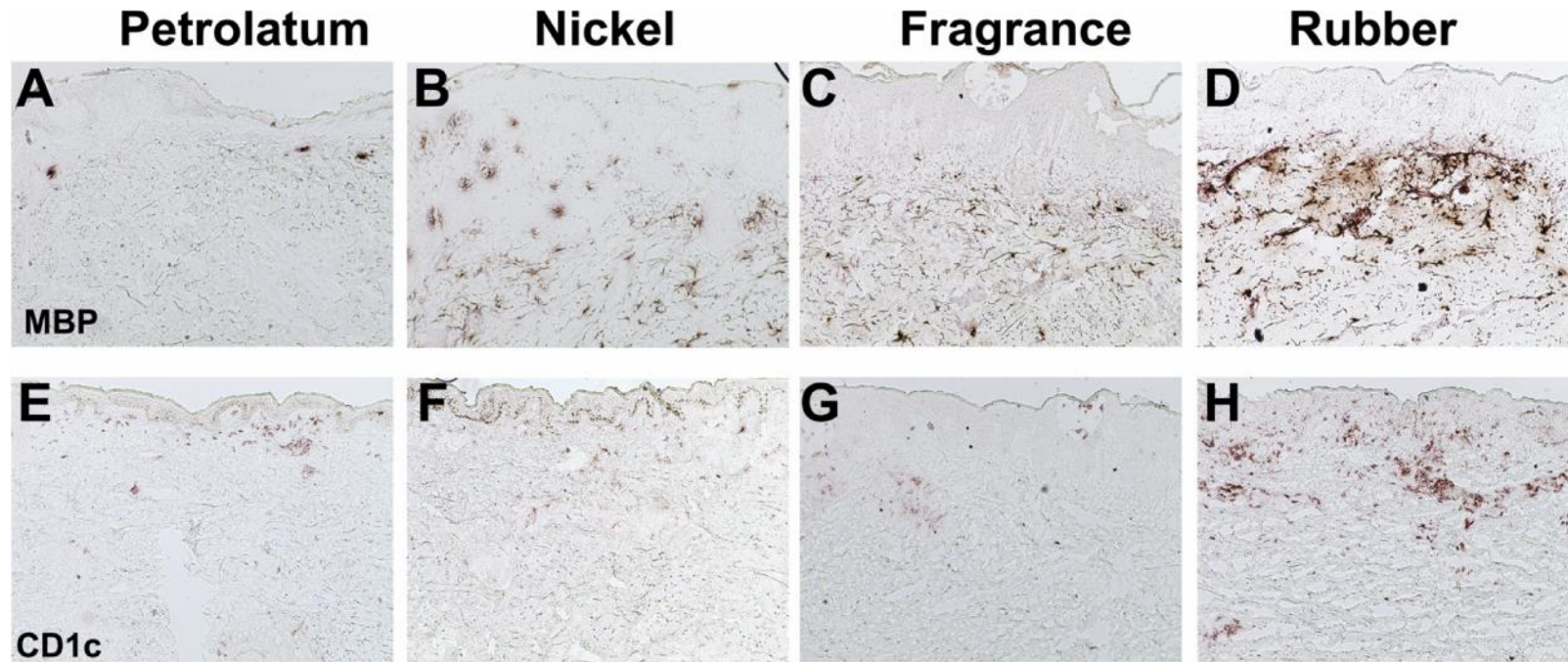


Fig E1

Nikhil Dhingra , Avner Shemer , Joel Correa da Rosa , Mariya Rozenblit , Mayte Suárez-Fariñas , Judilyn Fuentes-Du...

Molecular profiling of contact dermatitis skin identifies allergen-dependent differences in immune response

Journal of Allergy and Clinical Immunology, 2014

<http://dx.doi.org/10.1016/j.jaci.2014.03.009>

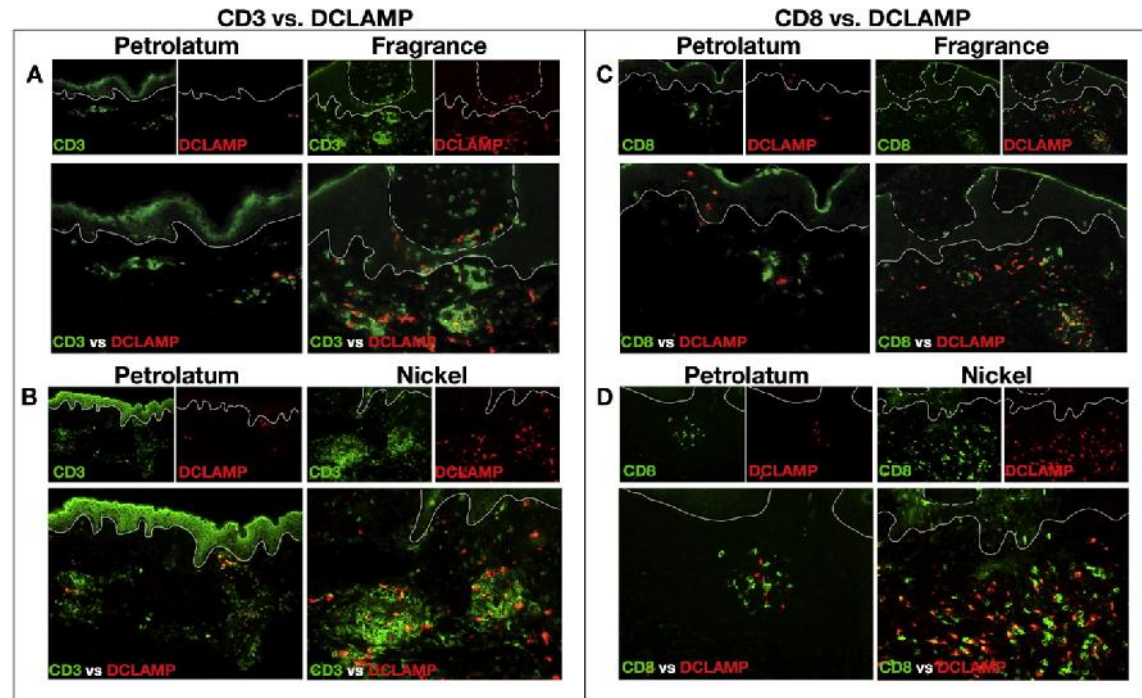


Fig E2

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Figure E3

DEGs (Allergen vs. Petrolatum)

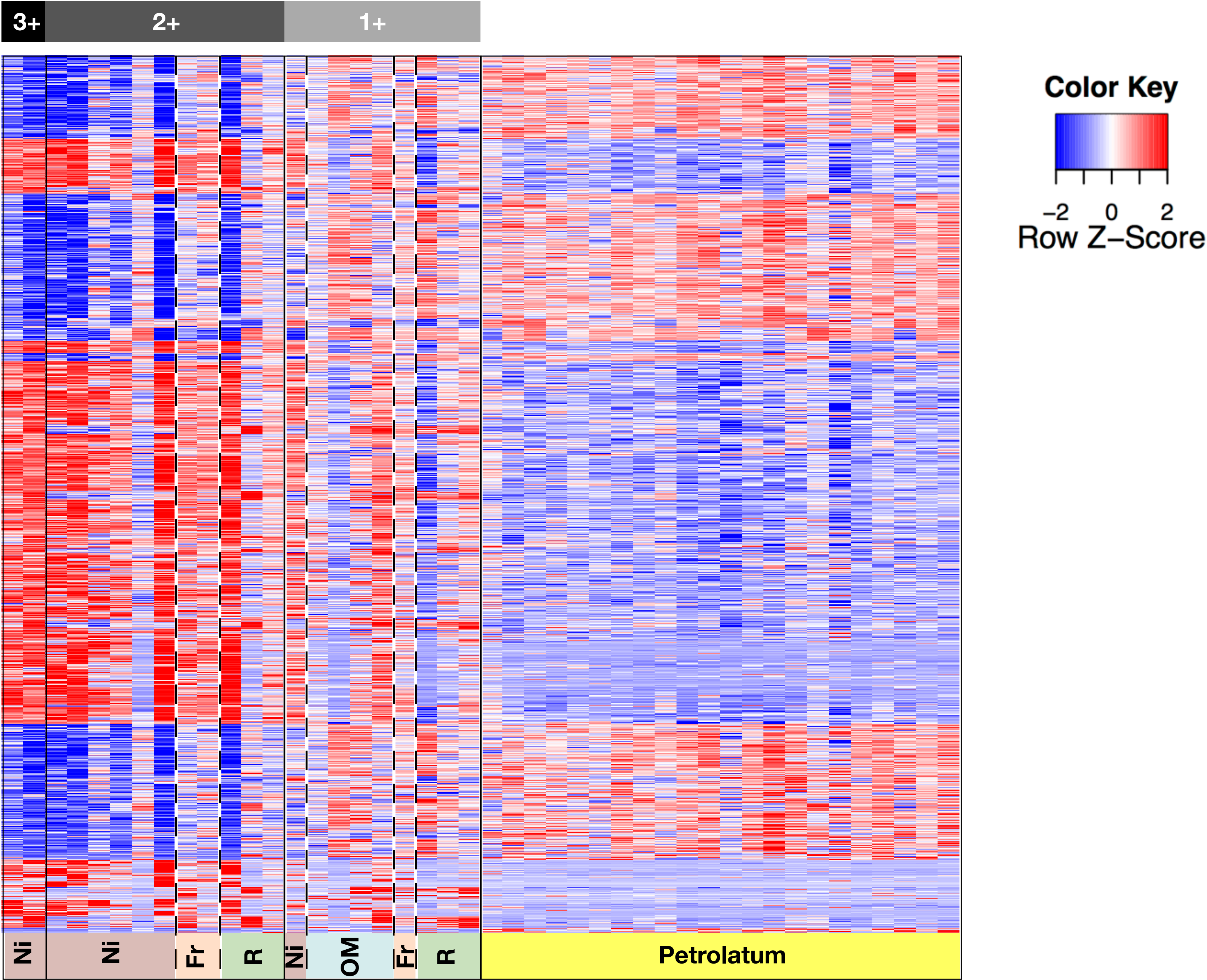
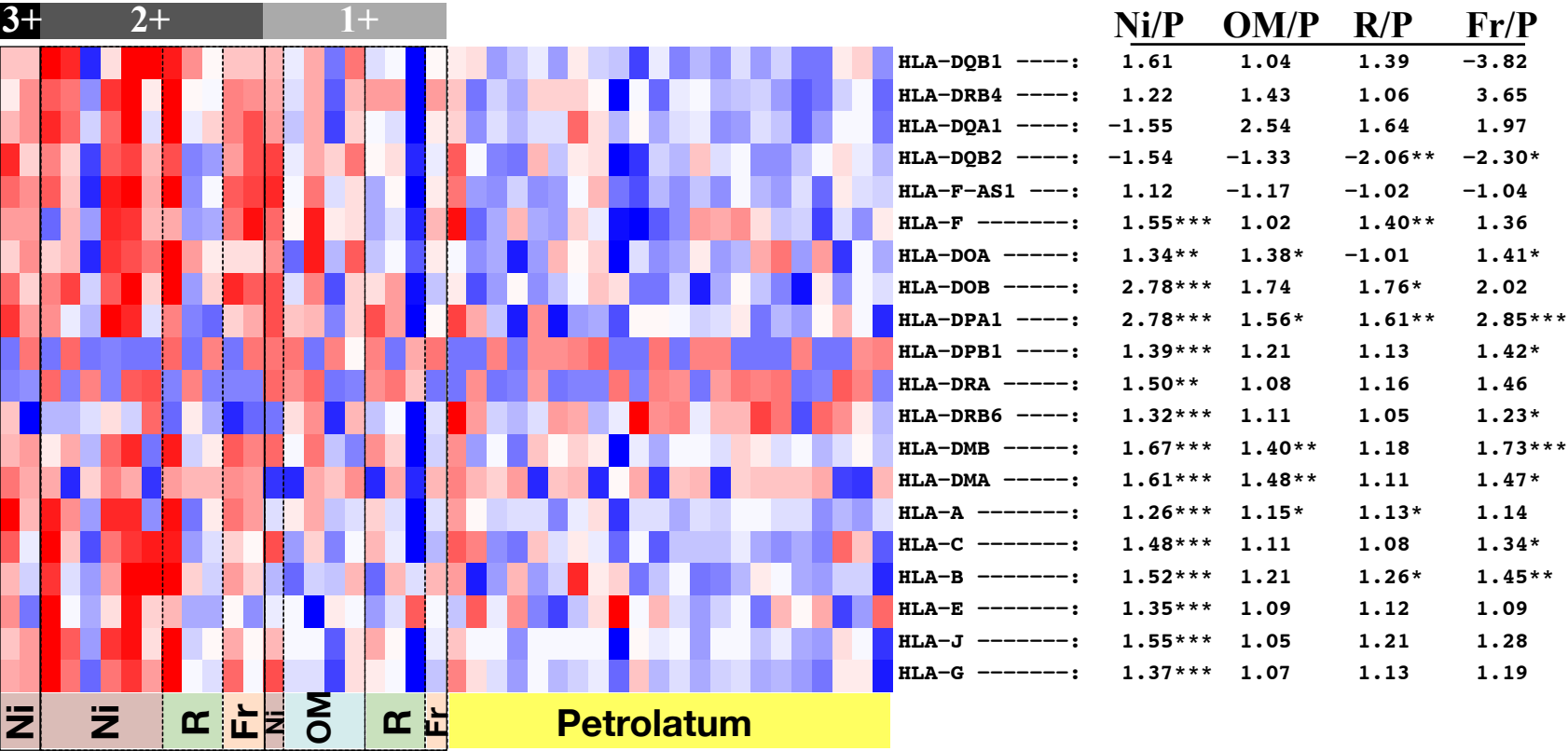
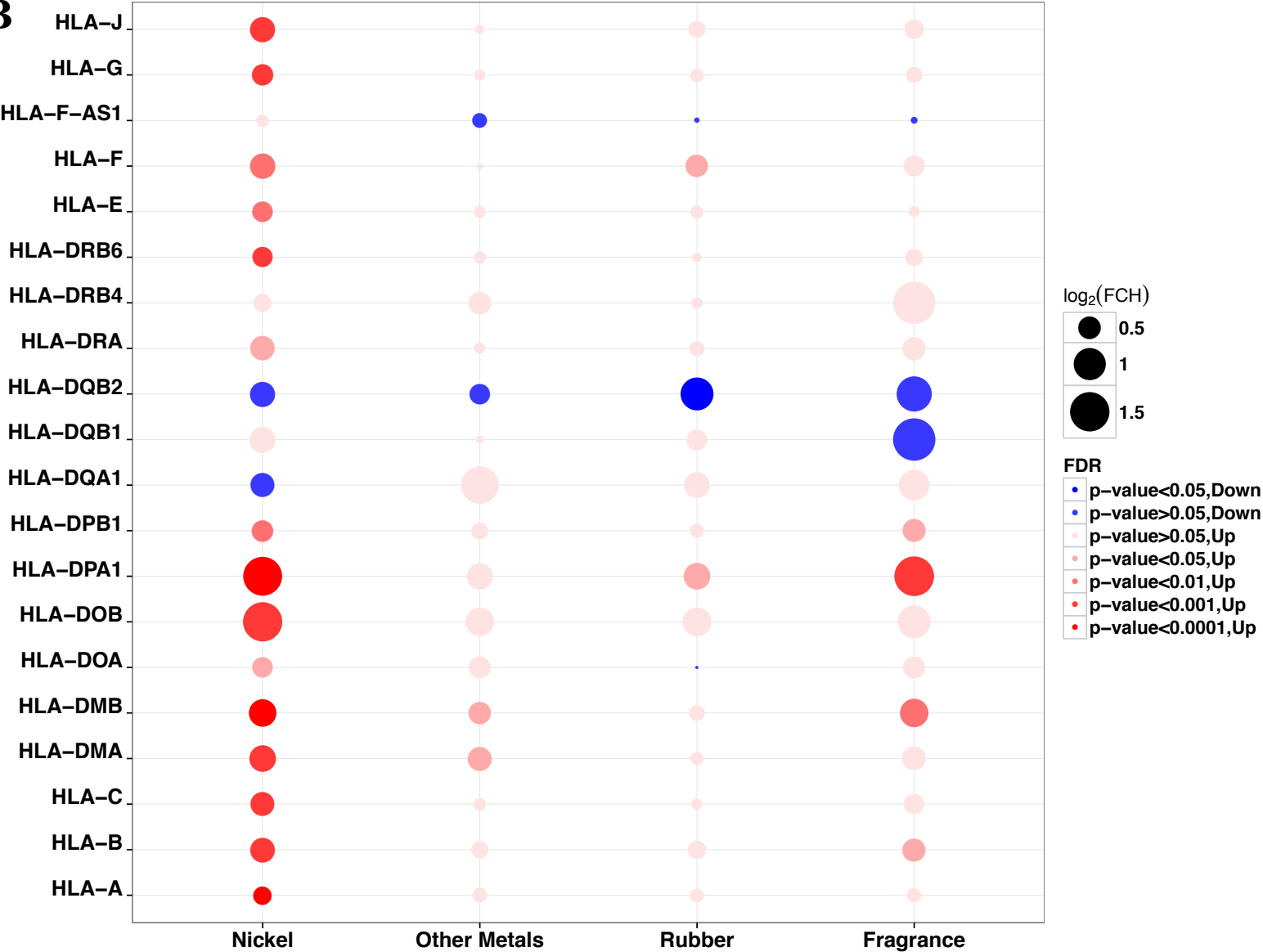


Figure E4

A



B



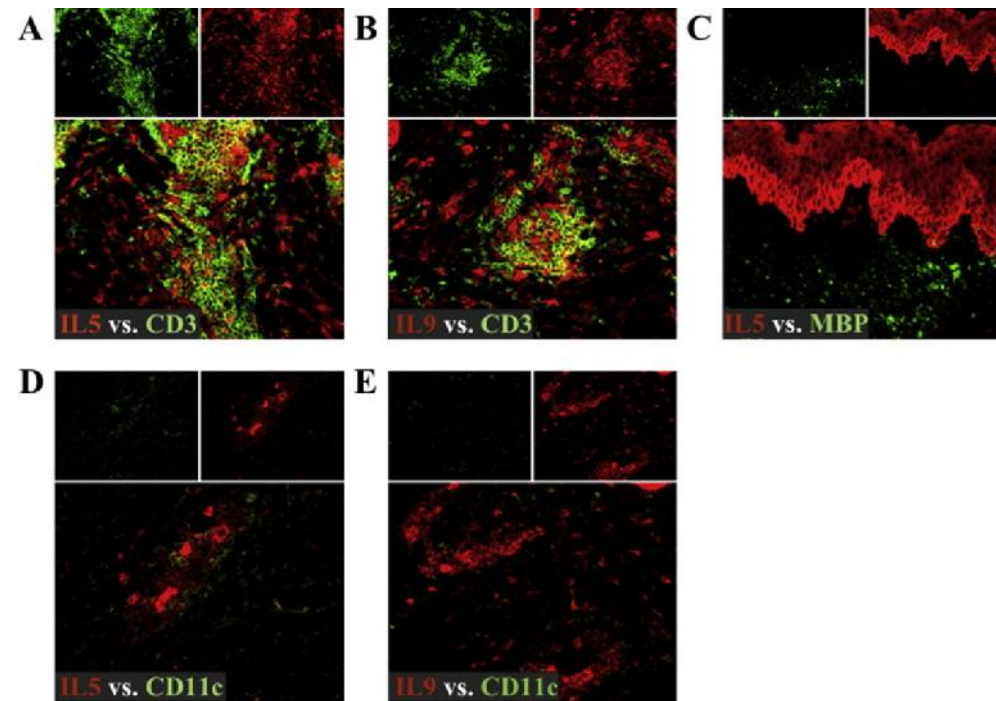


Fig E5

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Table E1: Characteristics of study patients

A)

Patient #	Gender	Age	Allergen group ^a	Reaction ^b
1	F	32	Other Metals	+1
2	F	45	Nickel	+2
3	M	31	Rubber	+1
4	F	33	Nickel	+2
5	M	42	Fragrance	+1
6	F	52	Nickel	+2
7	F	55	Rubber	+2
8	F	50	Fragrance	+2
9	M	34	Rubber	+1
10	F	48	Nickel	+2
11	F	51	Nickel	+2
12	F	55	Nickel	+2
13	F	37	Nickel	+3
14	F	54	Nickel	+1
15	M	36	Other Metals	+1
16	F	33	Other Metals	+1
17	M	23	Rubber	+1
18	F	39	Other Metals	+1
19	M	63	Rubber	+2
20	F	28	Nickel	+3
21	M	39	Rubber	+2
22	M	20	Fragrance	+2
23	F	57	Nickel	+2
24	F	60	Rubber	+2

a: Allergens were grouped into four categories: 10 nickel, fragrance (2 Balsam of Peru, 1 Fragrance Mix), rubber (5 Carba mix, 2 Thiuram), and other metals (3 Cobalt, 1 Potassium Dichromate) (see Supplementary methods)

b: Positive-patch tests were graded according to established guidelines (See Methods and Supplementary Methods)

B)

Allergen	Frequency	Percent	Level of Reaction		
			+1	+2	+3
Fragrance	3	13.04%	1	2	0
Nickel	10	39.13%	4	0	0
Other Metals	4	17.39%	1	7	2
Rubber	7	26.09%	3	4	0
Total	24	100	9	13	2

Table E2. Antibodies used for Immunohistochemistry and Immunofluorescence

Antibody	Vendor	Clone	Isotype	Dilution
CD3	BD Biosciences	SK7	IgG1	1:100
CD8	Abcam	144B	IgG1	1:25
CD11c	BD Biosciences	B-ly6	IgG1	1:100
DC-LAMP	Fisher Scientific	104.G4	IgG1	1:50
Langerin	Immunotech	DCGM4	IgG1	1:100
CD1c	Miltenyi Biotec	AD5-8E7	IgG2a	1:100
MBP	Thermo Scientific	BMK13	IgG1	1:15
CD11c*	BD Biosciences	B-ly6	IgG1	1:100
CD3*	BD Biosciences	SK7	IgG1	1:100
CD8*	BD Biosciences	SK1	IgG1	1:50
DCLAMP*	Immunotech	104.G4	IgG1	1:50

***Used for immunofluorescence**