

# Molecular Characterization of Human Skin Response to Diphenycprone at Peak and Resolution Phases: Therapeutic Insights

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Diphenycprone (DPCP) is a hapten that induces delayed-type hypersensitivity (DTH) reactions. It is used as an immune-modulating therapeutic, but its molecular effects in human skin are largely unknown. We studied cellular and molecular characteristics of a recall response to 0.04% DPCP at 3-day (peak) and 14-day (resolution) time points using immune markers, reverse-transcriptase-PCR (RT-PCR), and gene array approaches. A peak response showed modulation of ~7,500 mRNA transcripts, with high expression of cytokines that define all major effector T-cell subsets. Concomitant increases in T-cell and CD11c+ dendritic cell (DC) infiltrates were measured. The resolution reaction was characterized by unexpectedly high levels of T cells and mature (DC-lysosome-associated membrane glycoprotein positive (DC-LAMP+)) DCs, but with marked decreases in expression of IL-2, IFN $\gamma$ , and other T cell-derived cytokines. However, negative immune regulators such as IDO1 that were high in peak reactions, continued to have high expression in resolution reactions. In the resolution reaction, ~1,500 mRNA transcripts were significantly different from placebo-treated skin. These data suggest that the response to DPCP evolves from an inflammatory/effector peak at day 3 to a more regulated immune response after 14 days. This model system could be useful for further dissection of mechanisms of immune activation or negative immune regulation in human skin.

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## INTRODUCTION

Haptens such as dinitrochlorobenzene and diphenycprone (DPCP) induce strong delayed-type hypersensitivity (DTH) recall reactions associated with influx of T cells and dendritic cells (DCs) into skin as well as upregulation of an array of inflammatory cytokines during a “peak” response that ranges from 2 to 4 days in human skin (Stute *et al.*, 1981). The cellular and molecular events associated with DTH responses in human skin are incompletely understood, especially if one considers that agents such as dinitrochlorobenzene and DPCP have been used, somewhat paradoxically, to increase local immune responses for the resolution of warts (Upitis and Krol,

2002) and melanoma metastases (Damian *et al.*, 2014) and also to decrease pathogenic immunity for the restoration of hair growth in alopecia areata (Freyschmidt-Paul *et al.*, 2003). One might postulate from these various therapeutic applications that cytotoxic effector immune pathways are induced by topical haptens, although activation of negative regulatory pathways also occurs to eventually downregulate the inflammatory response. Available data from murine models suggest that resolution of cutaneous immune responses to haptens may be strongly dependent on regulatory T cells (Tregs) in the skin (Lehtimäki *et al.*, 2012), leading to active mechanisms of immune suppression during the resolution phase of a DTH response. In fact, negative regulatory immune mechanisms may be of more general importance for maintaining skin homeostasis as noninflammatory in the face of a large population of effector memory T cells that normally reside in the skin (Clark *et al.*, 2006). The elimination of Tregs from the skin has the consequence of increasing skin inflammation due to environmental or chemical allergens in animal models (Dudda *et al.*, 2008; Freyschmidt *et al.*, 2010). The absence of negative regulatory mechanisms likely contributes to inflammatory skin diseases such as psoriasis where chronic activation of T cells and DCs, with ongoing production of many inflammatory cytokines, leads to focal plaques of disease that rarely resolve spontaneously.

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Abbreviations: ARG1, arginase, liver; DC, dendritic cell; DC-LAMP, lysosome-associated membrane glycoprotein, dendritic cell-specific; DPCP, diphenycprone; DTH, delayed-type hypersensitivity; Foxp3, forkhead box P3; PPD, purified protein derivative; RT-PCR, reverse-transcriptase-PCR; Treg, regulatory T cell; XCR1, chemokine, C motif, receptor 1

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Cellular and molecular events associated with DTH responses to topical and intradermal antigens are much better understood for the sensitization and early elicitation phases (particularly the peak DTH reaction that occurs within 4 days of recall exposure) compared with later phases of the elicitation response when negative regulatory pathways might be dominant (Vocanson *et al.*, 2009). Very little is known about immune cellular elements and expression of negative regulatory immune molecules during this resolution phase in humans.

In this study, we performed a comprehensive analysis of immune cell types, cytokines defining distinct T-cell subsets in humans, and overall alterations in the skin transcriptome induced during a DTH response to a strong contact allergen (DPCP) in human skin. We defined a peak DTH response as that occurring 3 days after DPCP challenge application and a resolution phase reaction as that occurring 14 days after application, and profiled reactions at both time points to define cellular and molecular responses during these two phases of the DTH reaction. Our results identified immature myeloid DCs as a major cellular population in the peak response, along with increased synthesis of cytokine transcripts suggestive of pan-T cell activation. The resolution phase contained a large infiltrate of T cells and mature (lysosome-associated membrane glycoprotein, dendritic cell-specific (DC-LAMP)+) DCs, as well as dermal Langerin+ cells, along with an altered balance of positive-to-negative regulatory gene transcripts, suggestive of active immune regulation during this phase of the immune response. We believe the cutaneous DPCP reaction creates a tractable and reproducible system for the future study of positive and negative immune regulation in human skin.

## RESULTS

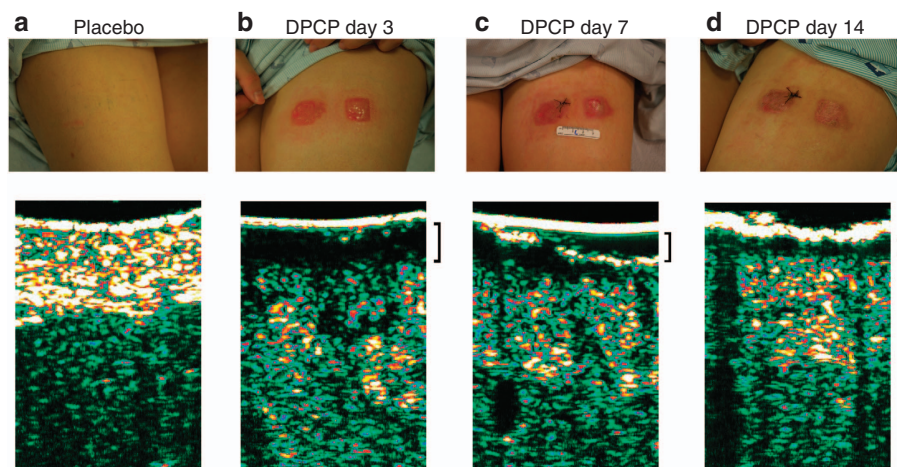
### Kinetics of skin inflammation induced by DPCP

Healthy individuals (demographics listed in Supplementary Table S1 online) were sensitized to DPCP and 2 weeks later

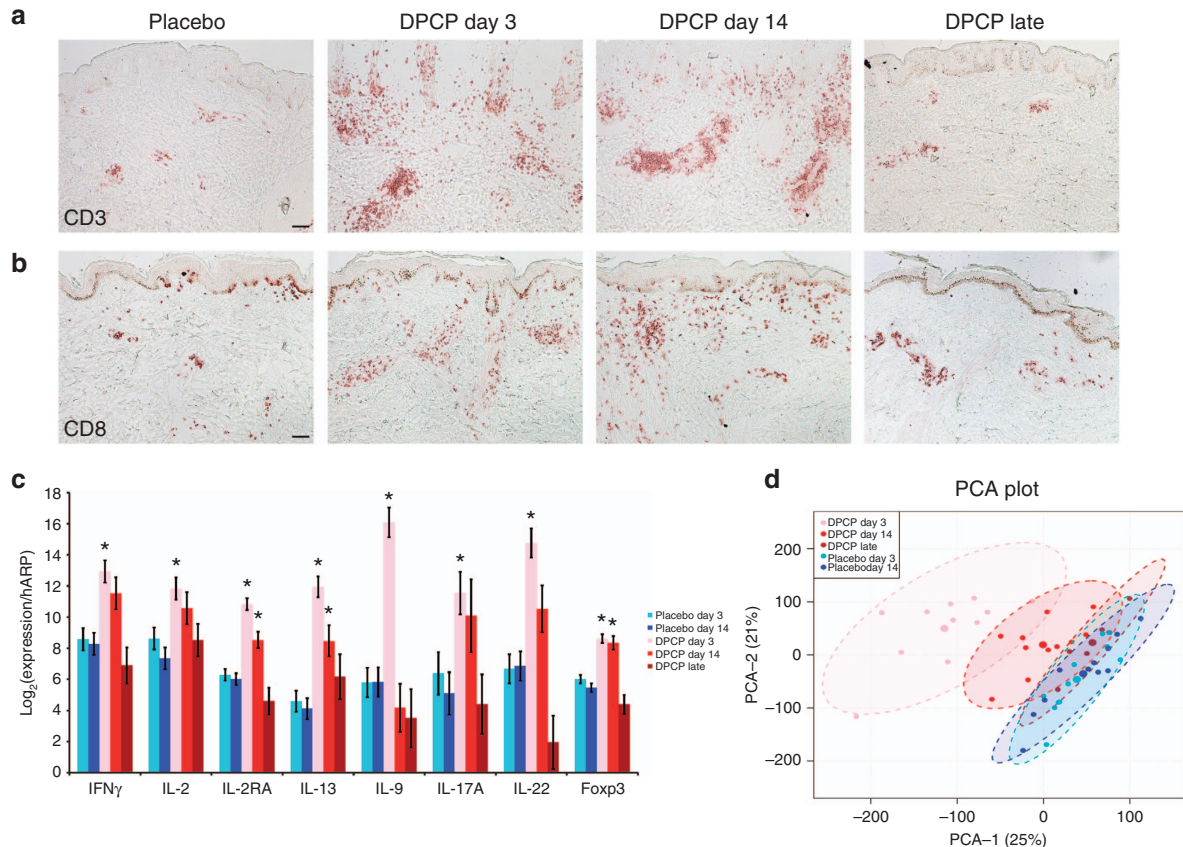
received two challenge applications on their left thigh and two placebo applications on their right thigh. At 3 days after challenge, all subjects exhibited strong clinical inflammation (erythema, induration, and epithelial vesiculation in some cases) only in DPCP-treated sites, whereas placebo-treated sites were indistinguishable from background skin (Figure 1). The clinical DPCP-induced reaction was clearly diminished by 14 days after the challenge dose. Erythema and induration were quantified using a 0–4 scale derived from the psoriasis area severity index. The mean response at day 3 was 5.5 (for sum of erythema and induration) and was reduced to 2.1 by day 14 ( $P < 10^{-7}$ ) (Supplementary Table S1 online). Ultrasound measurements further confirmed a decrease in dermal inflammation over time (Figure 1). Overall, these data support the classical view that 3 days after challenge represents the clinical peak of a DTH response.

### Kinetics of cellular and molecular immune response to DPCP in DTH reactions

Biopsies were collected from both DPCP- and placebo-treated sites at day 3 (clinical peak) and at day 14 (resolving inflammation). The first six patients also underwent skin biopsies of reaction sites 4–8 months after DPCP challenge (late biopsy). In the acute response, T cells appeared rapidly at DTH challenge sites with a mean of 1,113 CD3+ T cells per mm of skin. Overall, T-cell counts diminished slightly by day 14, with a mean of 946 cells per mm. In late biopsies, T cells were reduced to levels seen in placebo sites (<100 per mm). Relative to placebo and generally expected, increases were seen in CD3+ and CD8+ lymphocytes (Figure 2a and b), as well as in DC populations in the day 3 and day 14 biopsies, but two patterns of response were seen with cellular infiltrates as will be discussed in the next section. T cell activation in biopsies was assessed by reverse-transcriptase-PCR (RT-PCR) measures of mRNAs that are associated with specific T cell subsets, e.g., IFN $\gamma$  defining a T helper type 1 (Th1) response, IL-13 defining a T helper type 2 (Th2) response, or IL-17



**Figure 1. Visualization of inflammatory reactions induced by diphenylpicrylhydrazine (DPCP) over time.** Clinical photography with ultrasound imaging below of a skin site (a) treated with placebo, (b) 3 days after treatment with DPCP, (c) 7 days after treatment with DPCP, and (d) 14 days after treatment with DPCP. Brackets on ultrasound images indicate extents of inflammatory reactions as reflected by dermal thickness. Shown is a representative subject (subject 008).



**Figure 2. Reactions to diphencyprone (DPCP) include immune activation markers that are present at day 3 but diminish over time.** (a, b) Immunohistochemistry for (a) CD3 and (b) CD8 on placebo-treated samples, DPCP day 3 samples, DPCP day 14 samples, and DPCP late samples. Scale bar = 100  $\mu$ m. (c) Reverse-transcriptase-PCR (RT-PCR) analysis for IFN $\gamma$ , IL-2, IL-2RA, IL-13, IL-9, IL-17A, IL-22, and forkhead box P3 (Foxp3). Shown are average normalized expression values for placebo-treated day 3 samples (light blue bars,  $n = 11$ ), placebo-treated day 14 samples (dark blue bars,  $n = 11$ ), DPCP day 3 samples (pink bars,  $n = 11$ ), DPCP day 14 samples (red bars,  $n = 11$ ), and DPCP-treated samples 4–8 months after challenge (late) (brown bars,  $n = 6$ ). \* $P < 0.01$  when compared with placebo and error bars represent SEM. (d) Principal component analysis (PCA) of microarray data showing all samples as individual dots colored according to sample type. The larger dots are averages for each sample type.

defining a T helper type 17 (Th17) response (Figure 2c). Marked increases in cytokine mRNA levels occurred in day 3 DPCP-treated sites, with changes ranging from 9-fold to >1,000-fold ( $\log_2$  3.2 to >10 as graphed in Figure 2c). The day 14 reactions showed reduced expression of each T cell subset cytokine with the largest declines in IL-9 and IL-13. All cytokine levels normalized in late biopsies (Figure 2c). The day 3 DPCP response was also associated with a 6-fold increase in forkhead box P3 (Foxp3) mRNA, but this product did not diminish on average in day 14 biopsies. Overall, visible skin inflammation in the DPCP reaction at days 3 and 14 was better correlated with levels of proinflammatory cytokine mRNAs than with the number of infiltrating T cells or DCs. The diminishing production of inflammatory cytokine mRNAs at day 14 with persistence of Foxp3 expression suggests the possibility of an altered positive versus negative regulatory immune balance in day 3 versus day 14 reactions.

#### Molecular profiling of day 3 and day 14 DPCP responses

To profile the global set of gene expression changes in the DPCP reactions, skin biopsies were also hybridized to

Affymetrix HGU133 Plus 2.0 arrays. A principal component analysis of the skin responses is diagrammed in Figure 2d. Day 3 DPCP reactions were widely separated from placebo responses in the major axis of variation (principal component analysis-1), whereas the day 14 reactions approached the placebo sites and late biopsies overlapped completely with placebo responses. Using cutoffs of false discovery rate of <0.05 and fold change of >2, the day 3 DTH responses had 3,670 upregulated and 3,884 downregulated probe sets compared with placebo-treated skin. Tables 1 and 2 list the top 10 upregulated and downregulated, respectively, genes in day 3 and day 14 reactions. As the “top” gene sets are largely different at day 3 versus day 14, the tables are constructed with a listing of gene expressions for both sets of genes at both day 3 and day 14. Among genes with the highest induction at day 3, *granzyme B* (119-fold increase) and *IL-24* (107-fold increase) are molecules with identified roles in tissue rejection or antineoplastic control of melanomas or other cancers. Strong expression of *CXCL10* (104-fold increase) and *CXCL9* (85-fold increase) suggests activation of signal transducer and activator of transcription 1-regulated genes by IFN $\gamma$



**Table 1. Top 10 upregulated genes in diphenylprone (DPCP) day 3 and DPCP day 14 versus placebo samples**

Gene	Description	Fold change at day 3	Fold change at day 14
(a) Top upregulated transcripts at day 3			
<i>SERPINB4</i>	Serpin peptidase inhibitor, clade B (ovalbumin), member 4	340.5	10.6
<i>S100A7A</i>	S100 calcium binding protein A7A	170.9	6.1
<i>DEFB4A</i>	Defensin, $\beta$ 4A	169.5	17.0
<i>MMP1</i>	Matrix metalloproteinase 1 (interstitial collagenase)	167.3	4.9
<i>MMP12</i>	Matrix metalloproteinase 12 (macrophage elastase)	147.9	8.4
<i>GZMB</i>	Granzyme B (granzyme 2, cytotoxic T lymphocyte-associated serine esterase 1)	118.8	11.2
<i>IL24</i>	Interleukin 24	106.8	1.3
<i>CXCL10</i>	Chemokine (C-X-C motif) ligand 10	104.2	25.5
<i>S100A9</i>	S100 calcium binding protein A9	97.7	15.7
<i>APOBEC3A</i>	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3A	95.0	1.2
(b) Top upregulated transcripts at day 14			
<i>CXCL9</i>	Chemokine (C-X-C motif) ligand 9	84.7	37.3
<i>COL6A6</i>	Collagen, type VI, $\alpha$ 6	11.0	36.1
<i>CCL18</i>	Chemokine (C-C motif) ligand 18 (pulmonary and activation-regulated)	15.0	32.1
<i>CXCL10</i>	Chemokine (C-X-C motif) ligand 10	104.2	25.5
<i>SERPINB3</i>	Serpin peptidase inhibitor, clade B (ovalbumin), member 3	68.0	23.3
<i>COL6A5</i>	Collagen, type VI, $\alpha$ 5	15.8	20.1
<i>CD1B</i>	CD1b molecule	10.5	19.6
<i>DEFB4A</i>	Defensin, $\beta$ 4A	169.5	17.0
<i>S100A9</i>	S100 calcium binding protein A9	97.7	15.7
<i>OASL</i>	2'-5'-Oligoadenylate synthetase-like	54.5	12.1
(c) Regulatory genes with selective expression in day 14 samples			
<i>ARG1</i>	Arginase, liver	0.2	11.6
<i>FLT3</i>	Fms-related tyrosine kinase 3	2.5	11.5
<i>XCR1</i>	Chemokine (C motif) receptor 1	0.3	6.1

production. At day 14, the genes with highest expression at day 3 were strongly downregulated (on average >10-fold), with IL-24 expression reduced to background levels in placebo-treated skin. In the day 14 biopsies, CCL18 was amplified from the day 3 response and CD1b also became more strongly expressed, likely corresponding to increased DCs at this time point.

The only genes common in top upregulated products between day 3 and day 14 were *defensin  $\beta$ 4*, *CXCL10*, and *S100A9*. On a more global level, of the 793 upregulated and 726 downregulated probe sets at day 14, 317 were uniquely expressed at day 14 and not day 3 (Supplementary Figure S2a online), illustrating that the overall transcriptomic response is distinct between these two time points and that day 14 reactions are not simply an intermediate between day 3 and placebo responses. These unique genes at day 14 included *arginase, liver* (*ARG1*), *FLT3*, and *chemokine, C motif, receptor 1* (*XCR1*) (Table 1c, confirmatory immunohistochemistry and immunofluorescence to colocalize with CD11c+ DCs in Supplementary Figure S2b and c online), all of which may have roles in immune response resolution. In terms of genes that were downregulated in the DPCP response

(Table 2), we noted that the day 3 and day 14 responses were largely distinct, as with upregulated genes. The top down-regulated gene at day 3 was *IL-37*, which is known to negatively regulate inflammatory responses and where ~20-fold increased expression at day 14, relative to day 3, could represent an active regulatory mechanism. A full list of genes that are upregulated or downregulated in day 3 and day 14 biopsies is available on request and all microarray data have been deposited in a public database. *Granulysin* was also highly upregulated (47-fold) and appeared in mononuclear leukocytes in the dermis at days 3 and 14. To localize cells producing granulysin, two-color immunofluorescence was performed. The strongest colocalization with granulysin was CD3+ T cells, with some but not all CD8+ cells staining for this cytolytic effector molecule. A few CD11c+ DCs also appeared to produce granulysin (Supplementary Figure S3 online).

#### Immune infiltrates follow two distinct patterns after a DPCP challenge

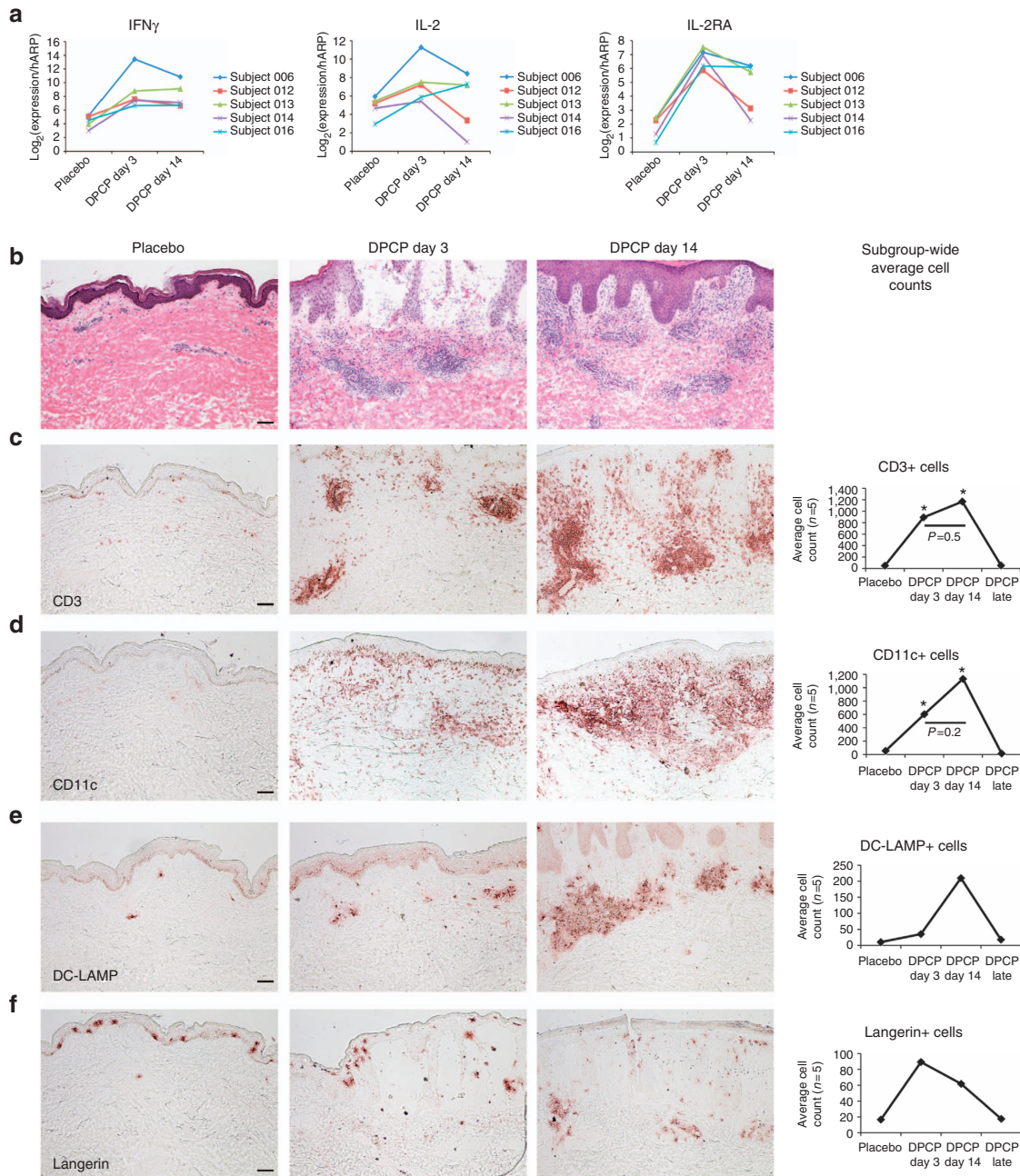
In all patients, we measured marked increases in CD3+ T cells and CD11c+ DCs in day 3 "peak" reactions. However,

**Table 2. Top 10 downregulated genes in diphencyprone (DPCP) day 3 and DPCP day 14 versus placebo samples**

Gene	Description	Fold change at day 3	Fold change at day 14
<i>(a) Top downregulated transcripts at day 3</i>			
IL37	Interleukin 37	– 46.0	– 2.3
ATP1A2	ATPase, Na <sup>+</sup> /K <sup>+</sup> transporting, $\alpha$ 2 polypeptide	– 38.3	– 3.2
GDF10	Growth differentiation factor 10	– 36.4	– 3.2
SGCG	Sarcoglycan, $\gamma$ (35 kDa dystrophin-associated glycoprotein)	– 29.8	– 4.4
MYOC	Myocilin, trabecular meshwork inducible glucocorticoid response	– 29.2	– 7.1
VIT	Vitrin	– 26.8	– 2.7
DPP6	Dipeptidyl-peptidase 6	– 25.6	– 3.2
THRSP	Thyroid hormone responsive	– 24.9	– 3.4
DLG2	Discs, large homolog 2 ( <i>Drosophila</i> )	– 23.7	– 1.4
FIBIN	Fin bud initiation factor homolog (zebrafish)	– 23.4	– 4.7
<i>(b) Top downregulated transcripts at day 14</i>			
WIF1	WNT inhibitory factor 1	– 6.2	– 27.1
GALNTL2	UDP-N-acetyl- $\alpha$ -D-galactosamine:polypeptide N-acetylglucosaminyltransferase-like 2	– 2.1	– 9.9
RBP4	Retinol binding protein 4, plasma	– 10.8	– 8.2
BTC	Betacellulin	– 19.8	– 8.1
LEP	Leptin	– 7.2	– 8.0
TNMD	Tenomodulin	– 10.2	– 7.8
MYOC	Myocilin, trabecular meshwork inducible glucocorticoid response	– 29.2	– 7.1
MUC7	Mucin 7, secreted	– 5.6	– 6.1
IL17D	Interleukin 17D	– 13.6	– 6.1
SPINK1	Serine peptidase inhibitor, Kazal type 1	– 3.4	– 5.9

we detected two distinct reaction patterns for the day 14 “resolution” reaction. One pattern seen in 5/11 patients (subgroup A, Figure 3) showed increasing numbers of T cells and/or DCs in the day 14 biopsies compared with the day 3 reactions, a response that was entirely unexpected (Figure 3c and d). This subset of patients also showed even larger increases in mature (DC-LAMP<sup>+</sup>) DCs in day 14 biopsies compared with day 3 biopsies, and this subgroup had the most pronounced dermal infiltrates of Langerin<sup>+</sup> cells (Figure 3e and f). However, the expression of inflammation-associated cytokines and CD25 (IL-2RA) usually decreased in day 14 biopsies compared with day 3 reactions, and hence we detected a disassociation between cellular immune infiltrates and production of inflammatory cytokines/activation molecules (Figure 3a). As further evidence for this, both CD3<sup>+</sup> and CD11c<sup>+</sup> cell counts did not significantly correlate with gene expression levels of any of the following immune activation markers: IFN $\gamma$ , IL-2, and IL-2RA (Supplementary Figure S4 online). The other response pattern seen in 6/11 patients (subgroup B, Supplementary Figure S5 online) fit the expected response kinetics for a DTH reaction: T-cell and DC infiltrates peaked at day 3 and were significantly reduced at day 14 ( $P=0.01$  and  $0.001$ , respectively). As in subgroup A, expression of inflammatory cytokines and activation molecules was decreased in resolution biopsies.

**Subjects whose infiltrates decrease from day 3 to day 14 have higher levels of negative regulatory cells and molecules at day 3**  
We performed immunofluorescence for Foxp3 along with CD3 in order to more specifically identify Tregs that were then quantified as Foxp3<sup>+</sup>CD3<sup>+</sup> cells. On average, subjects in subgroup B had more Tregs than those in subgroup A at day 3 (21.25 vs. 9; Figure 4a and b). We also performed RT-PCR for several known downregulatory molecules. For all molecules tested (IL-10, lymphocyte activation gene 3, programmed cell death 1, programmed cell death ligand 1, programmed cell death ligand 2, IDO1, and CTLA4), mRNA levels were higher at day 3 in subgroup B than subgroup A. In addition, there were nonsignificant but consistent trends for subjects in subgroup B to have higher levels of these molecules even in placebo-treated skin, suggesting baseline immunological differences between subjects in the two subgroups (Figure 4c–i). On a more global level, when looking at a comprehensive list of negative regulator genes (Supplementary Table S2 online has “negative regulator” list, curated through Gene Ontology term 0002683 “negative regulation of immune system process” and literature review, and expression values for DPCP day 3), subjects in subgroup B had higher expression of many of these molecules at day 3 (Supplementary Figure S6 online). These data may help explain why subgroup B had decreased cellular infiltrates at day 14 and suggest that different individuals resolve their inflammatory responses to DPCP with



**Figure 3. Reverse-transcriptase-PCR (RT-PCR) and histological analysis of subjects whose CD3+ or CD11c+ infiltrates increase from 3 days to 14 days after diphencyprone (DPCP) challenge (subgroup A, n = 5).** (a) RT-PCR analysis for IFN $\gamma$  (left panel), IL-2 (middle panel), and IL-2RA (right panel). Shown are normalized expression values for each subject individually to highlight that almost all samples have decreased expression of these genes at day 14 compared with day 3. (b–f) Hematoxylin and eosin (H&E; b) and immunohistochemical analysis of samples for (c) CD3, (d) CD11c, (e) lysosome-associated membrane glycoprotein, dendritic cell-specific (DC-LAMP), and (f) Langerin. For all histological images, left panels show placebo reactions, middle panels show DPCP day 3 reactions, and right panels show DPCP day 14 reactions. Shown is a representative subject (subject 013). For immunohistochemical stains, line graphs indicate subgroup-wide average cell counts for placebo, DPCP day 3, DPCP day 14, and DPCP late samples. \*P < 0.05 when compared with placebo. Scale bar = 100  $\mu$ m.

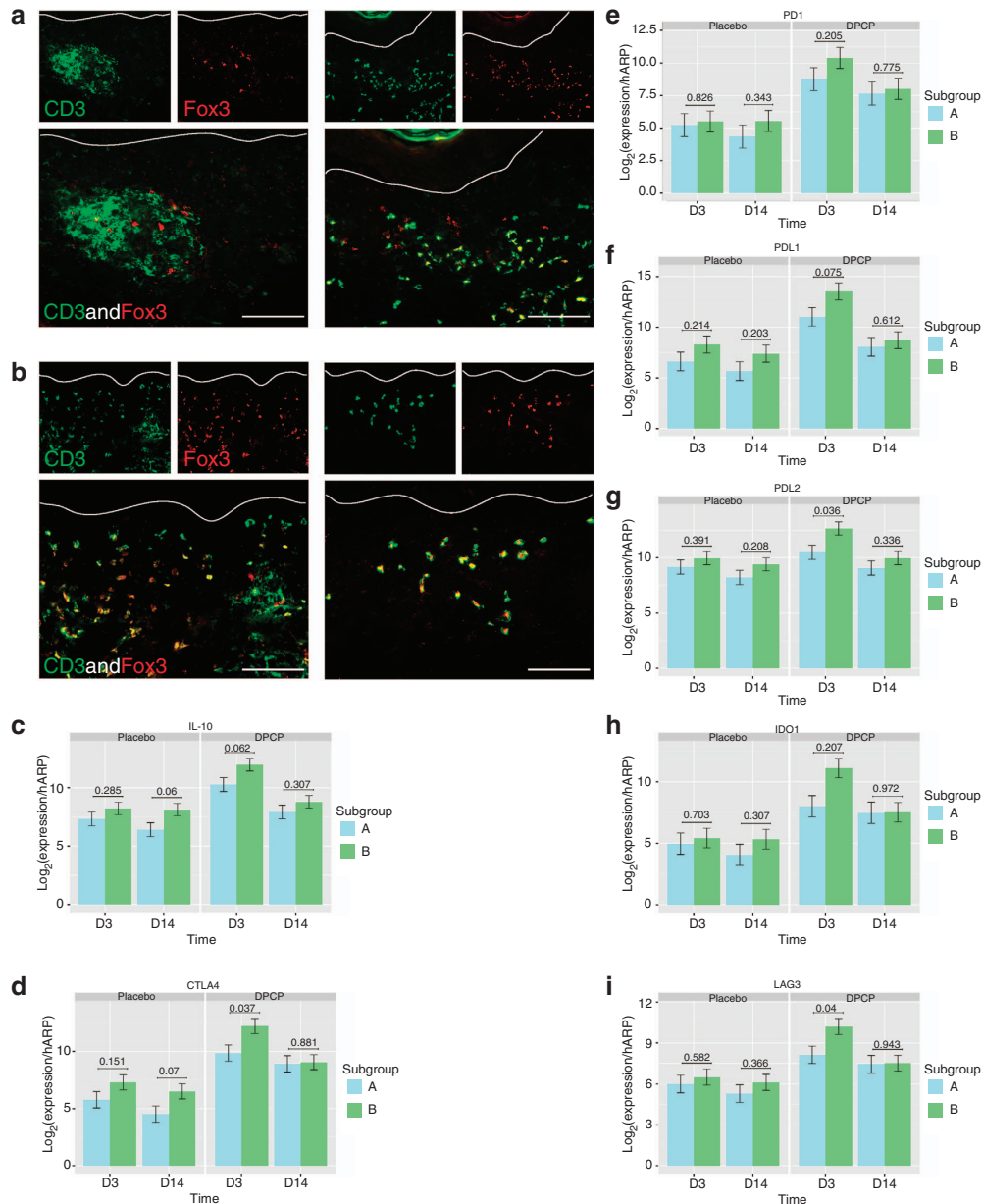
different kinetics (inflammation resolution was faster in subgroup B, based on ultrasound measures at days 7 and 14, see Supplementary Figure S7 online).

## DISCUSSION

This study with DPCP provides the detailed cellular and molecular phenotype of a DTH response at both peak and

resolution stages. The strong clinical response at day 3 was accompanied by large increases in infiltrating T cells and myeloid DCs, as well as by differential regulation (>2-fold change) of 7,555 gene transcripts. The resolution phase was characterized by variable changes in infiltrating leukocytes, overall increased in subgroup A and decreased in subgroup B compared with the peak reaction. In molecular terms, the





**Figure 4. Negative regulatory immune cells and molecules are increased in diphenylprone (DPCP) day 3 reactions of subgroup B compared with subgroup A.** (a, b) Forkhead box P3 (Foxp3; red)–CD3 (green) immunofluorescence for (a) a representative subgroup A member (subject 012) and (b) a representative subgroup B member (subject 021). Left panels are DPCP day 3 samples and right panels are DPCP day 14 samples. Regulatory T cells (Tregs) were quantified as double positive for Foxp3 and CD3. Subject 012 had 4 double-positive cells at day 3 (subgroup-wide average of 9) and 24 at day 14 (subgroup-wide average of 20.33), whereas subject 021 had 37 at day 3 (subgroup-wide average of 21.25) and 19 at day 14 (subgroup-wide average of 12). (c–i) Reverse-transcriptase–PCR (RT–PCR) analysis for (c) IL-10, (d) cytotoxic T-lymphocyte antigen 4 (CTLA4), (e) programmed cell death 1 (PD1), (f) programmed cell death ligand 1 (PDL1), (g) programmed cell death ligand 2 (PDL2), (h) IDO1, and (i) lymphocyte activation gene 3 (LAG3). Shown are average normalized expression values of placebo day 3, placebo day 14, DPCP day 3, and DPCP day 14 skin for both subgroups ( $n = 5$  for subgroup A (blue bars),  $n = 6$  for subgroup B (green bars)).  $P$ -values are shown to compare subgroup A with subgroup B for each sample type. Scale bar = 100  $\mu$ m.

resolution phase showed decreased expression of T cell effector cytokines and molecules associated with the activation phase of immune responses, but relatively higher levels of transcripts associated with negative regulation of immune responses. In addition, the resolution phase included expression of certain genes including ARG1, XCR1, and FLT3 that were not present during the peak response. ARG1 has been

shown to lead to reversible blocks in T cell proliferation (Bronte *et al.*, 2003), XCR1 contributes to Treg development (Lei *et al.*, 2011), and FLT3 is involved in maintaining the balance between DC and Treg numbers (Liu and Nussenzweig, 2010). Although we chose to characterize the immune response to DPCP in normal human skin, our results identify immune mechanisms that may relate to the use of

DPCP as an immune modulator in treatment of melanoma or alopecia areata.

In a recent report, 50 patients with cutaneous metastases of melanoma were treated with repeated application of DPCP. Of these patients 46% had complete regression of their lesions and a further 38% had partial responses, but corresponding study of the immune response to DPCP was not conducted (Damian *et al.*, 2014). The potential of DPCP as an adjuvant to improve immunotherapy has been demonstrated in mice (von Moos *et al.*, 2012). Several immune pathways that we found to be induced by DPCP in normal skin might mediate this antimelanoma response. First, we identified significant upregulation of IL-24 mRNA, a cytokine with established anti-melanoma activity (Jiang *et al.*, 1995). As this cytokine was upregulated only transiently (peak at day 3, but resolved at day 14), it could indicate the need for chronic/repeated application of DPCP to maintain effector cytokines for tumor responses. Furthermore, we observed strong increases in IFN $\gamma$  expression and this cytokine enhances antigen presentation potential, is antiproliferative for many cell types (Braumüller *et al.*, 2013), and its induced molecule CXCL10 has anti-melanoma activity (Antonicelli *et al.*, 2011). Another cytokine that we found to be upregulated by DPCP, IL-9, has recently been linked to melanoma regression (Purwar *et al.*, 2012). In addition to these cytokines with potential anti-neoplastic effects, DPCP use resulted in many infiltrating CD8+ cells, along with increased granzyme B and granulysin expression, and these would be probable cytotoxic effectors. Granulysin has been shown to promote chemotaxis of CD4+ T cells in addition to its cytolytic properties (Deng *et al.*, 2005), and both of these functions may contribute to the peak DTH response considering that we observed granulysin expression on both CD8+ and CD8- T cells. We also noted a marked increase in DCs and especially DC-LAMP+ mature DCs that could provide a local environment to increase antigen presentation of tumor antigens (immature DCs, in the absence of appropriate costimulation, present antigens in a tolerogenic manner), as well as XCR1+ DCs that have a role in antigen cross-presentation (Kroczeck and Henn, 2012). Overall, this work forms a basis for investigating specific immune elements that may be induced in melanoma lesions that are treated with DPCP as well as the baseline immune competence of cancer patients.

Many different reports have shown that 50–60% of patients with patchy alopecia areata have meaningful hair regrowth when treated with chronic application of DPCP (Freyschmidt-Paul *et al.*, 2003; Ohlmeier *et al.*, 2012). A smaller number of studies have been conducted to investigate immune alterations induced in scalp that are associated with hair regrowth. Identified effects of DPCP include decreasing CD4+ T cell but increasing CD8+ T cell infiltrates (Simonetti *et al.*, 2004). In addition, major histocompatibility complex class II expression has been shown to be altered by DPCP treatment in different studies, with both increases (Hunter *et al.*, 2011) and decreases (Bröcker *et al.*, 1987) having been demonstrated. Furthermore, DPCP alters the cytokine profile in treated alopecic scalp, in particular, increasing IL-2 and IL-10 expression (Hoffmann *et al.*, 1994).

This increased IL-10 expression has been hypothesized to inhibit the lesional T cells of alopecia areata, and our data confirm an upregulation of IL-10 expression, as well as increases in CD8+ T cells, in normal human skin treated with DPCP. Our data also suggested roles for various other regulatory molecules in the response to DPCP whose potential relevance to alopecia areata treatment have not yet been elucidated. However, our data do not directly inform on the potential effects of DPCP on scalp tissue as well as on alterations that may arise from repeated applications.

We compared our findings on DPCP with those published on a DTH reaction to purified protein derivative (PPD) (Tomlinson *et al.*, 2011). The reaction to DPCP at 3 days compared with placebo-treated skin largely encompasses genes that are induced by a specific antigen (PPD in this case, 48 hours after exposure compared with 6 hours) (Supplementary Figure S8 online). Of the genes that were upregulated in the reactions, 780 were common to PPD and DPCP, but each agent also had unique upregulated genes. Overall, there was significant correlation with differentially expressed genes ( $r=0.61$ ,  $P<10^{-16}$ ) and a gene set enrichment analysis for genes regulated in the PPD reaction had a normalized enrichment score of  $>4.0$ , indicating highly similar modulation of gene sets. The analysis of the PPD data by Tomlinson *et al.* (2011) emphasized the polar Th1 nature of immune reactions that is supported by measured increases in IFN $\gamma$  mRNA, signal transducer and activator of transcription 1, and many other IFN-regulated gene products. Without measurement of cytokine production by more sensitive techniques, it is a bit uncertain if other “polar” T cell subsets are significantly activated by PPD. However, the DPCP response clearly showed activation of all defined “polar” T cell subsets, i.e., Th1, Th2, Th9, Th17, and Th22, based on cytokine mRNA measures by RT-PCR. Ingenuity Pathway Analysis significantly linked the canonical pathways of “IL-10 Signaling,” “IFN Signaling,” “IL-6 Signaling,” and “IL-17 Signaling” to the set of genes upregulated in both DPCP and PPD reactions. All of these pathways except for “IFN Signaling” were also significantly linked to the set of genes uniquely upregulated in DPCP reactions. We feel that the system provided here provides a valuable human model for studying DTH reactions, particularly as one does not need to select a subset of antigen-reactive individuals from a larger subset of nonimmunized or nonreactive individuals. In addition, our system with DPCP controls sensitization and therefore fixes the amount of time between initial antigen exposure and challenge reaction, unlike many alternative antigens.

In contrast to soluble protein antigens, haptens derivatize with proteins that are present in the skin and may thus be present for longer time periods. Hence, different response kinetics may be expected for hapten-induced DTH reactions than PPD reactions. In this study, we did identify an expected inflammatory reaction in the skin that was strong at 3 days after DPCP exposure and T cell activation, as judged by specific markers (IFN $\gamma$ , IL-2, and IL-2RA) as well as other activation-associated molecules, was highest at this time point. However, much to our surprise, T cell infiltrates



persisted for much longer than expected, based on the PPD model, and 5/11 patients showed increased numbers of T cells or DCs at day 14 compared with day 3. In addition, the number of mature (DC-LAMP+) DCs peaked at day 14 in 7/11 patients. Certainly, these data establish a disassociation between T cell activation/cytokine production and the magnitude of the cellular immune response defined by the number of T cells and DCs in the skin. Therefore, the possibility of active negative regulatory mechanisms suppressing T cell activation to a potentially long-lived cutaneous antigen is suggested by these data.

Overall, understanding the active mechanisms present during the resolution of responses to DPCP could have implications not only for cell-mediated immunity in general, but also for other skin pathologies of either chronic immune activation or ineffective immune responses. Most importantly, this system could provide a tractable human antigen-specific model system for primary discovery of new pathways that may be involved specifically in immune regulation in peripheral tissues. As all T cell subsets become activated by *in vivo* DPCP, this system might also have utility to test the mechanism/pharmacodynamic actions of new immune modulators designed to selectively inhibit “polar” T cell subsets or activated T cells. For example, there has recently been great interest in selective blockade of IL-17 for the treatment of psoriasis and IL-4 for the treatment of atopic dermatitis. As these and many other key cytokines are activated by DPCP, this system can be used to test a variety of pharmacologic agents targeted against pathogenic immune molecules.

## MATERIALS AND METHODS

The detailed protocols and statistical analysis are described in Supplementary Materials and Methods online.

### Study subjects and skin samples

Skin biopsies of DPCP challenge reactions and placebo-treated sites were obtained from 11 volunteers under a protocol approved by The Rockefeller University's Institutional Review Board (Supplementary Figure S1 online). Written, informed consent was obtained from all subjects and the study adhered to the Declaration of Helsinki Principles.

### RNA extraction, quantification, and microarray

Total RNA was extracted using the miRNeasy Mini Kit (Qiagen, Valencia, CA) and the amount was assessed by NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). The quality of extracted RNA was examined using Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA). RNA was hybridized to HGU133 Plus 2.0 chips (Affymetrix, Santa Clara, CA).

### Statistical analysis

Microarray data were analyzed using R/Bioconductor packages (<http://www.r-project.org>). The data discussed in this publication have been deposited in the National Center for Biotechnology Information's Gene Expression Omnibus (GSE accession number GSE52360, <http://www.ncbi.nlm.nih.gov/geo/>). Ingenuity Pathway Analysis ([www.ingenuity.com](http://www.ingenuity.com)) was used to determine the canonical pathways significantly linked to various gene sets.

## Quantitative RT-PCR

The pre-amplification quantitative RT-PCR technique was used for measuring various genes in total RNA extracted from skin biopsy samples according to the company's instructions.

## Immunohistochemistry and immunofluorescence

Frozen skin sections were prepared and standard procedures were used.

## CONFLICT OF INTEREST

The authors state no conflict of interest.

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## SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/jid>

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