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Molecular Profiling of Immune Activation Associated with Regression of Melanoma Metastases Induced by Diphencyprone

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TO THE EDITOR

Diphencyprone (DPCP), a hapten that causes delayed-type hypersensitivity reactions, has been used to treat cutaneous metastases in melanoma patients. An 84% regression rate was observed in a case series of 50 patients who had cutaneous melanoma metastases treated with multiple topical DPCP applications (Damian et al., 2014). However, the immunological mechanisms underlying these cases of regression are not well understood. Previously, our group characterized normal skin reactions in healthy volunteers upon a single application of topical DPCP gel (Gulati et al., 2014). Several immune pathways that we found to be induced by DPCP in normal skin, including IFN-γ and granulysin, may mediate the antimelanoma responses that have been observed clinically. In this study, we used the same topical DPCP formulation, which we previously applied only once to healthy volunteers, and applied it multiple times to patients with cutaneous melanoma metastases. Our cohort included six melanoma patients, of whom five exhibited partial or complete melanoma metastasis regression in response to DPCP treatment. Written informed consent was obtained from all subjects, and the study adhered to the Declaration of Helsinki Principles. By both immunohistochemical and gene expression approaches, we comprehensively characterized the immune reactions induced by DPCP in these patients. We present results from five of six patients treated with DPCP, excluding patient 001, who left the trial before a delayed-type hypersensitivity reaction could be successfully induced (see Supplementary Materials and Methods and Supplementary Table S1 online). All five patients showed partial or complete regression of their cutaneous metastases upon DPCP treatment (see Figure 1 for an example of complete clinical and MLANA immunohistochemical response). Further evidence of successful melanoma regression came from profiling of the global set of gene expression changes in these reactions by microarray analysis (data deposited in the NCBI's Gene Expression Omnibus, GSE accession number GSE82105). Because the five patients received different numbers of repeated applications of DPCP, we defined each patient's final biopsy sample as "chronic." When comparing the chronic biopsy samples with the pre-DPCP metastasis biopsy samples, many of the most significantly down-regulated genes were hallmark melanoma or melanocyte genes, such as *PRAME, TYR, OCA2, DCT,* and *MLANA,* which were down-regulated 12- to 22-fold (Table 1).

To better ascertain the mechanisms involved in immune-mediated tumor regression induced by DPCP, we studied biopsy tissue samples from the patients at various time points. In line with our healthy volunteer data (Gulati et al., 2014), DPCP applications in melanoma patients led to extensive immune cell infiltrates, including $CD3^+$ T cells, $CD11c^+$ myeloid dendritic cells, and $CD163^+$ macrophages, both after a single and repeated applications. These infiltrates persisted in follow-up biopsies performed 30 days after cessation of DPCP treatment (see Supplementary Figure S1a online). In addition to these cells, which are presumably integral to immune-mediated antimelanoma responses, we found that DPCP application led to increases in granulysin. By two-color immunofluorescence, granulysin co-localized with $NKp46^+$ natural killer cells more than $CD8^+$ cytotoxic T cells (see Supplementary Figure S1b). However, based on quantitative reverse transcriptase–PCR analysis, the levels of induction of various immune effectors, including IFNG and GNLY, after one application of DPCP were significantly lower in the melanoma patients than in the corresponding skin sites of healthy volunteers previously studied (Figure 1b), thus suggesting background immune suppression in the setting of melanoma, along with possible age-related changes because the melanoma patients tended to be older than the healthy volunteers.

Because the down-regulation of the five "melanoma signature" genes found

Abbreviations: DPCP, diphencyprone; PD-1, programmed cell death protein 1; Th, T helper

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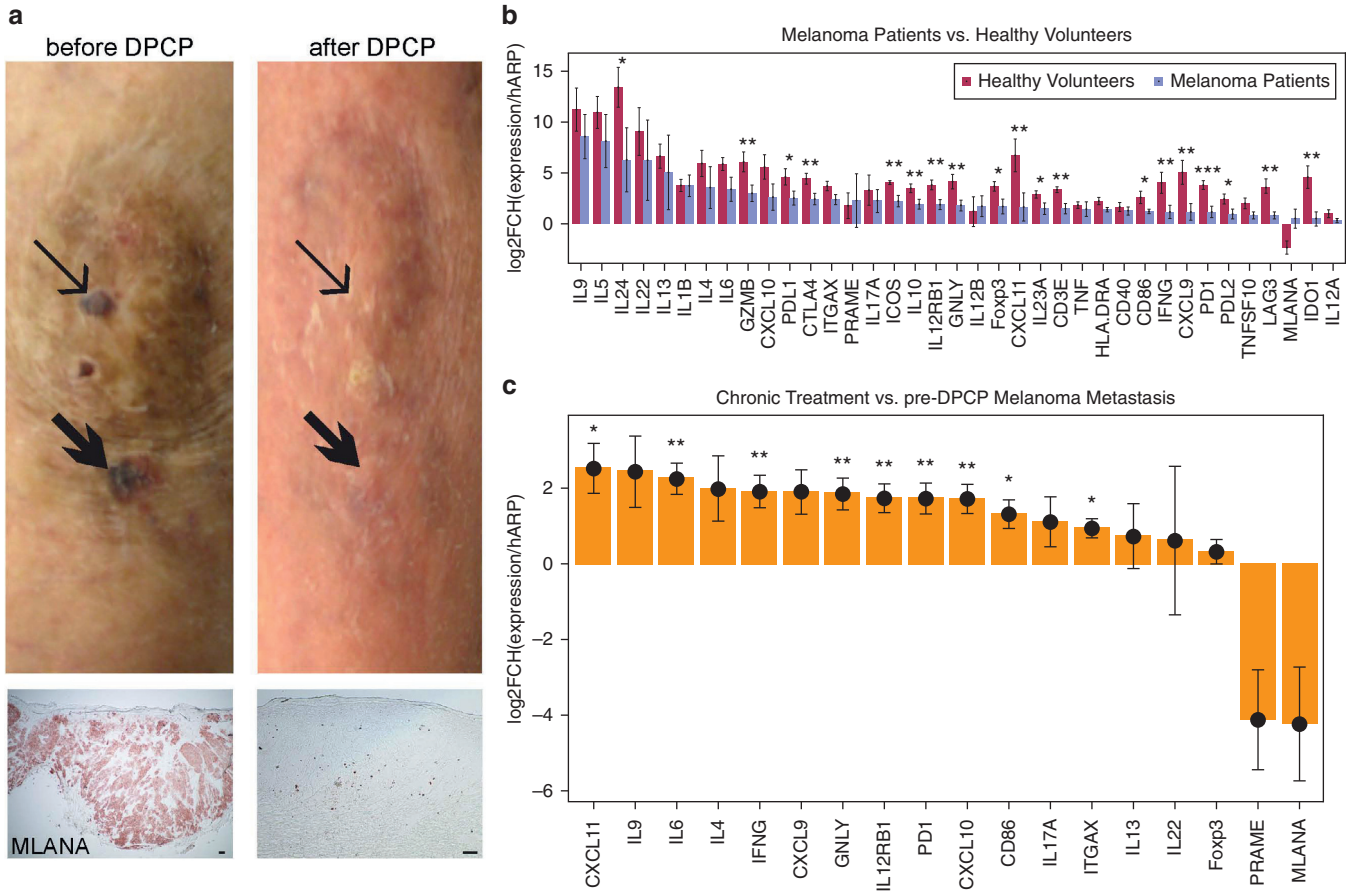

Figure 1. Patient 003 exhibited full clinical and histological melanoma metastasis regression upon DPCP treatment; quantitative reverse transcriptase–PCR analysis shows decreased immune responses induced by DPCP in melanoma patients compared with healthy volunteers, and significant shifts toward Th1 polarization upon chronic treatment. (**a**) Skin photography, with immunohistochemistry for melanocyte marker MLANA below, of patient 003's left lower leg (left) before DPCP application and (right) after 14 weeks of twice-weekly DPCP applications. Brown staining in histological image at right likely represents melanophages. There were two melanoma metastases visible before DPCP application, and their locations are indicated by arrows (the thick arrow shows the site that was biopsied). Scale bar = 100 μm. (**b**) Average normalized (to housekeeping gene *hARP*) inductions of expression in healthy volunteers treated with DPCP compared with placebo treatment of the same volunteers (red bars, n = 11) and in nonlesional skin of melanoma patients treated with DPCP compared with untreated skin (blue bars, n = 5). (**c**) Average normalized inductions of expression in chronically treated melanoma metastases compared with pre-DPCP melanoma metastases (n = 5). $*P < 0.1$, $**P < 0.05$, $***P < 0.01$ (by two-tailed Student *t* test—unpaired for **b** and paired for **c**); error bars represent standard error of the mean. Healthy volunteer samples taken from the study described in Gulati et al., 2014. DPCP, diphencyprone; Th, T helper.

in Table 1, combined together in one pathway, suggest a successful anti-melanoma response, we aimed to determine what other pathways correlate with it. For a curated collection of immune-related gene sets, gene set enrichment analysis was used in the classical manner, as previously described (Ruano et al., 2016). As expected, pathways with a significant positive correlation included those related to melanocytes and pigmentation, such as "Human-SkinPigmentation-melanin synthesis" ($r = 0.984$) (Table 1). Pathways with significant negative correlations (i.e., pathways that increased with chronic DPCP applications while the melanoma signature gene expression decreased) included ones related to immature dendritic cells, T helper (Th) 1, Th2, vitiligo, and allergic contact dermatitis (Table 1).

Our work with healthy volunteers showed that gene expression markers of all major T cell subsets (Th1, Th2, Th9, Th17, Th22, and regulatory T cells) significantly increased at 3 days after a single application of DPCP (Gulati et al., 2014). With our melanoma patients, we similarly performed biopsies of a normal skin site 3 days after a single application of DPCP. However, with these patients, we also performed biopsies of skin sites after repeated (twice weekly) DPCP applications, which is what is traditionally required when DPCP is used as a treatment. Quantitative reverse transcriptase–PCR analysis of paired baseline melanoma metastasis and nonlesional skin biopsy samples showed no evidence of significantly increased production of T-cell cytokines in untreated melanomas, but there was the expected significant increase in MLANA expression in the metastasis biopsy samples (see Supplementary Figure S2 online). These data suggest a lack of baseline immune activation in the melanoma metastases. By quantitative reverse transcriptase–PCR analysis of paired pre-DPCP metastasis and chronic DPCP application biopsy sites, we found that expression of Th1-related genes (*IFNG* and *CXCL10*) significantly increased (both $P < 0.05$) with repeated applications, whereas markers of all other major T-cell subsets (Foxp3,

Table 1. List of melanoma signature genes and correlated pathways

List of genes comprised by melanoma signature (from chronic vs. pre-DPCP melanoma metastasis gene list)

Probe	Gene	Symbol	FCH	FDR
204086_at	preferentially expressed antigen in melanoma	*PRAME*	−22.61	0.05
206630_at	tyrosinase	*TYR*	−20.02	0.02
206498_at	oculocutaneous albinism II	*OCA2*	−15.35	0.00
205337_at	dopachrome tautomerase	*DCT*	−12.43	0.04
206426_at	melan-A	*MLANA*	−12.02	0.03

Selected pathways that positively correlate with down-regulation of melanoma signature genes

Pathway	*r*	*P*
HumanSkinPigmentation-melanin synthesis	0.984	0.002
HumanSkinPigmentation-transcriptional factors	0.872	0.054
MITF targets	0.854	0.066
Melanocytes up	0.849	0.069

Selected pathways that significantly negatively correlate with down-regulation of melanoma signature genes

Pathway	*r*	*P*
immatureDC_AHCellMaps Up	−0.993	0.001
SuperEnhancers_Th1_Vahedi2015	−0.951	0.013
SuperEnhancers_Th2_Vahedi2015	−0.951	0.013
Vitiligo (LS) down	−0.908	0.033
NickelSignature up	−0.880	0.049

Abbreviations: FCH, fold change; FDR, false discovery rate; LS, lesional skin; MITF, microphthalmia-associated transcription factor.

IL-4, IL-13, IL-9, IL-17A, and IL-22) were not significantly increased. Also, granulysin and programmed cell death protein 1 (PD-1) were both significantly increased (Figure 1c). These findings, along with the increasing numbers of natural killer cells (see Supplementary Figure S1b), show the ability of DPCP to modulate cellular immunity, even on a background that may be relatively immunosuppressed.

Our finding that DPCP applications significantly increased PD-1 expression is potentially of great clinical relevance, because inhibitors of PD-1 are currently the standard-of-care treatment for melanoma (Ribas et al., 2015). We have previously shown that DPCP leads to increases in $CD8^+$ T cells, as well as PD-1, PD-L1, and PD-L2 in healthy volunteers (Gulati et al., 2014). Therefore, there is the potential for synergy between DPCP and PD-1 checkpoint inhibition. Patient 004 was having minimal regression of his bulky cutaneous metastases while solely receiving either DPCP as part of our trial or the PD-1 inhibitor pembrolizumab, but showed dramatic regression when they were combined. This supports the hypothesis that these two therapies might complement each other, but a larger trial with more patients is needed.

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 www.jidonline.org, and at http://dx.doi.org/10.1016/j.jid.2016.06.611.

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