Modulation of Inflammatory Proteins in Serum May Reflect Cutaneous Immune Responses in Cancer Immunotherapy

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Diphencyprone (DPCP), a topical contact sensitizer, has shown efficacy in treating cutaneous melanoma metastases, including at times beyond the directly treated sites, but biomarkers indicative of treatment response have not been characterized. Thus, we performed a proteomic analysis of the skin and serum of five patients with cutaneous melanoma metastases treated with DPCP on days 0, 63, and 112 of the treatment course. In the serum, we found a significant upregulation ($P < 0.05$) in 13 of 96 assessed immuno-oncology proteins after DPCP treatment. Upregulated proteins included those of the T helper 1 axis (CXCL9, CXCL10), immune checkpoint proteins (PD-1), and various proteins with roles in promoting tumor immunity such as CD80 and TNFRSF4/9. Given the positive clinical response to topical treatment noted in the five patients studied, these proteins may represent prognostic biomarkers in the serum for evaluating the efficacy of DPCP treatment of cutaneous melanoma metastases. Because DPCP does not lead to nonspecific immune-related adverse events seen with immune checkpoint inhibitors, our study provides evidence for potential tumor-specific systemic immune activation and systemic antitumor effectors elicited by topical DPCP.

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INTRODUCTION

Diphencyprone (DPCP), a topical hapten that causes delayed-type hypersensitivity reactions, has shown a 65% objective response rate in treating cutaneous metastases in patients with melanoma (Read et al., 2019). However, the molecular mechanisms mediating this effect are unknown, and no biomarkers of treatment response have been identified. To better understand these pathways, we performed a proteomic analysis of the skin and serum of patients with cutaneous melanoma metastases treated with DPCP.

Our cohort comprised six patients with melanoma with skin metastases. Written, informed consent was obtained from all subjects, and ethics approval was granted by The Rockefeller University's Institutional Review Board. Topical DPCP ointment was applied twice weekly to the skin metastases of patients with melanoma for 14 weeks (until day 112), with the intent of inducing tolerable inflammation. Biopsies of skin metastases and serum samples were taken before (day 0), during (day 63), and at the end (day 112) of the DPCP treatment course. Five of six patients showed at least partial skin metastasis regression in response to DPCP, and treatment was well-tolerated without systemic side effects. One nonresponder left the trial before a successful delayed-type hypersensitivity response could be induced and so was excluded from further analysis (Table 1). Also of note, we included one patient with concurrent imiquimod treatment who was treating only a select area of skin metastases, but this patient was included in a previous transcriptomic analysis of tissue (Gulati et al., 2016b), and his serum immune profile did not differ substantially from that of other patients. We quantified the expression of 96 proteins using the Olink immuno-oncology panel.

RESULTS

Our patient cohort included three males and three females, with ages ranging from 52 to 98 years (Table 1). Clinically, all patients who completed the treatment course exhibited partial or complete skin metastasis regression in response to DPCP. Partial or complete regression was observed by day 63 in all patients except for patient 01, who did not have a delayed-type hypersensitivity response to DPCP and so left the trial before day 63. Patients 02 and 05 exhibited robust inflammation and partial metastasis regression in response to DPCP. Patient 03 exhibited complete clinical skin metastasis regression on DPCP treatment. Patient 04 exhibited few areas of melanoma metastasis regression on DPCP treatment, but new lesions also developed. Patient 06 exhibited nearly complete clinical skin metastasis regression on DPCP treatment (Figure 1).

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Abbreviations: DPCP, diphencyprone; ICI, immune checkpoint inhibitor; Th, T helper

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Table 1. Patient Information

ID	Sex	Age	Concurrent Treatment at Initiation of Trial	Previous Treatments	Primary Site	Cutaneous Metastases Sites
01[1]	F	52	None	Excision, radiation therapy, ipilimumab, imiquimod, IL-2, cryotherapy, temozolomide	Right temple	Face, scalp
02	M	98	None	Excision, imiquimod	Chest	Chest
03	F	91	None	Excision, imiquimod	Pretibial	Pretibial
04	M	93	None	Excision, imatinib, ipilimumab	Right foot	Right lower extremity
05	M	66	Imiquimod	Excision, radiation therapy, cryotherapy	Left forehead	Scalp
06	F	81	None	Excision	Right calf	Right calf

Abbreviations: DTH, delayed-type hypersensitivity; F, female; ID, identification; M, male.
[1]Patient 01 was excluded from analysis because she left the trial before a DTH response could be induced.

In serum, 13 proteins (ADGRG1, matrix metalloproteinase 12, CXCL9, CXCL10, TNF, CCL20, TNFRSF4, TNFRSF9, PD-1, IL-12, IL-33, CD83, PGF) were significantly upregulated on day 63 compared with that on day 0 ($P < 0.05$), and five proteins (matrix metalloproteinase 12, CXCL9, CXCL10, PD-1, and TNFRSF9) were significantly upregulated

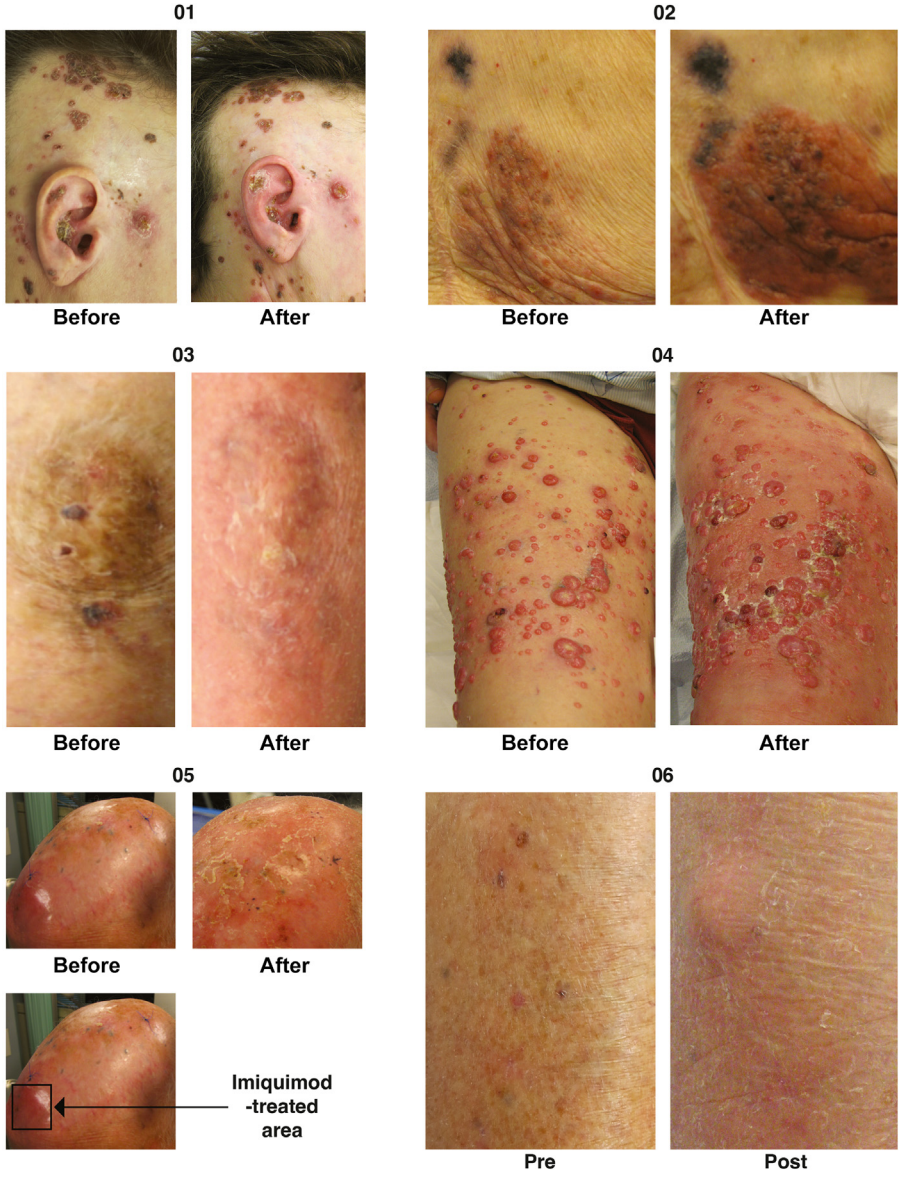


Figure 1. Clinical photographs. Clinical photographs before and after DPCP treatment. For patient 05, an additional photograph is included to indicate the site of concurrent imiquimod treatment at the start of DPCP treatment. All patients consented to the publication of clinical images taken during the study. DPCP, diphencyprone.

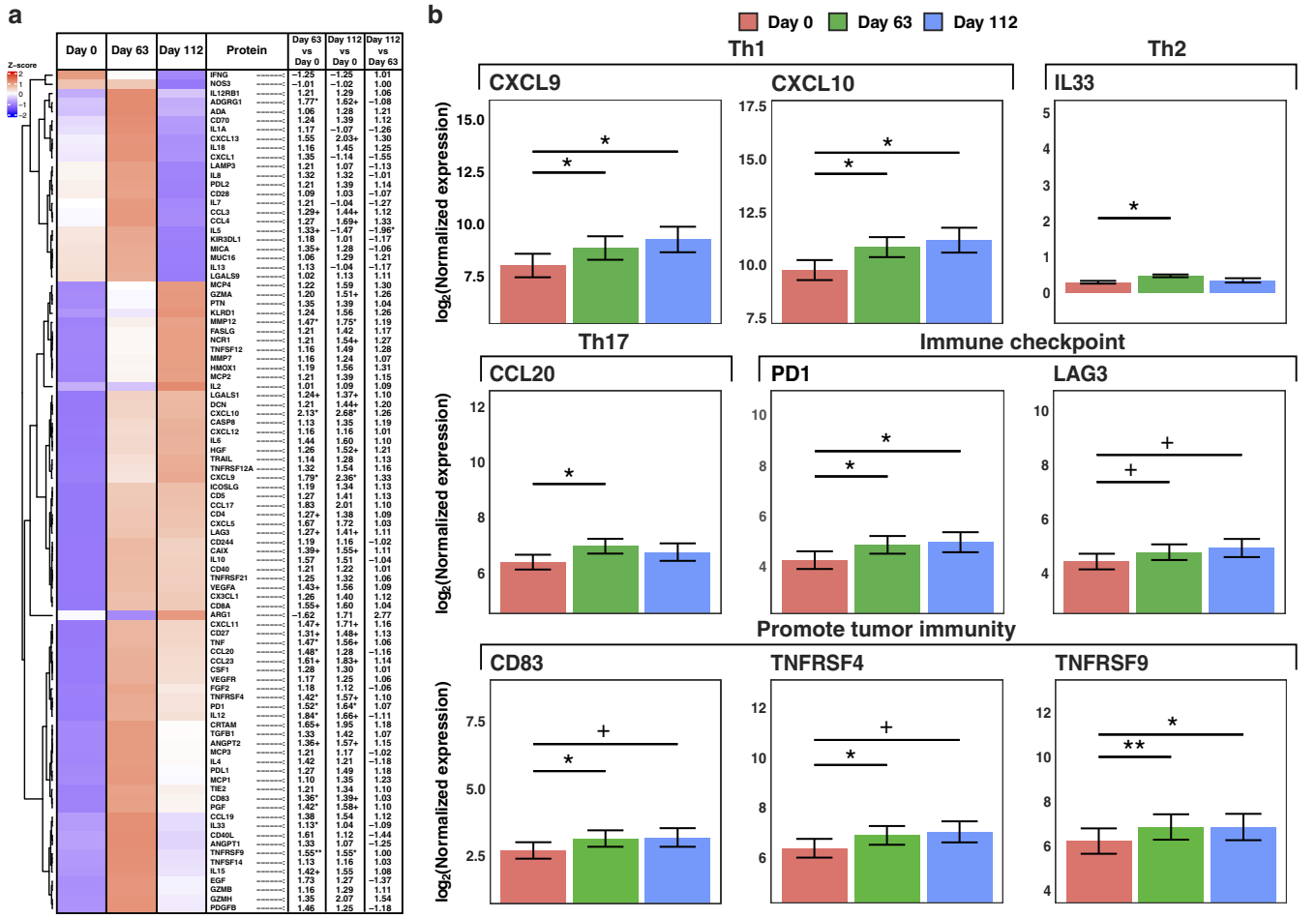


Figure 2. Heatmap and bar plots of immuno-oncology markers in serum. Heatmap and bar plots of immuno-oncology markers in serum of five patients with melanoma after twice weekly application of topical DPCP to skin metastases. The heatmap shows all 96 immuno-oncology proteins assessed through Olink proteomics, with grouping by unsupervised hierarchical clustering. Red and blue boxes correspond to upregulated and downregulated protein expression values, respectively. (**a**) The corresponding table lists the markers and respective fold changes in the serum on days 0, 63, and 112 compared with each other. Bar plots represent the $\log_2$ normalized expression of selected proteins in the serum of five patients with cutaneous melanoma metastases treated with DPCP on days 0, 63, and 112. Line segments indicate comparison between respective groups. Error bars represent one standard error interval around the estimated mean. (**b**) *P*-values were derived from *t*-statistics obtained upon fitted mixed-effects models. $+P < 0.1$, $*P < 0.05$, and $**P < 0.01$. DPCP, diphencyprone; Th, T helper.

on day 112 compared with that on day 0 ($P < 0.05$) (Figure 2a). There was a significantly upregulated T helper (Th) 1 response, including in CXCL9 on both day 63 ($P = 0.014$) and day 112 ($P = 0.013$) and in CXCL10 on both day 63 ($P = 0.024$) and day 112 ($P = 0.033$) compared with that on day 0. However, there was only a significant upregulation in Th2 (IL-33; $P = 0.021$) and Th17 (CCL20; $P = 0.016$) markers on day 63 but not on day 112 (Figure 2b). This polarization toward the Th1 axis in serum correlates with our previous gene expression findings in the skin, which showed elevated *CXCL10* and *CXCL11* mRNA expression in cutaneous metastases that regressed after DPCP application, thus suggesting a role for the Th1 axis in immune-mediated tumor regression (Gulati et al., 2016b). Systemic inflammatory activity may predict changes in leukocyte populations, previously shown by immunohistochemistry that showed extensive immune cell infiltration into skin sites treated with DPCP, including T cells marked by CD3, myeloid dendritic cells marked by CD11c, and macrophages marked by CD163 (Gulati et al., 2016b).

There was also significant upregulation in the serum of proteins involved in promoting tumor immunity, including CD83 on day 63 ($P = 0.035$), TNFRSF4 on day 63 ($P = 0.038$), and TNFRSF9 on day 63 ($P = 0.008$) and day 112 ($P = 0.034$). In addition, there was progressive upregulation of immune checkpoint proteins, including PD-1 on day 63 ($P = 0.019$) and day 112 ($P = 0.040$) and LAG3 on day 63 ($P = 0.086$) and day 112 ($P = 0.086$) that was trending toward significance (Figure 2b). When patients were ranked according to the degree of clinical improvement, a weak positive correlation was found, with median (%) molecular improvement in serum biomarkers. The biomarkers selected were the ones that changed significantly ($P < 0.1$) when comparing day 112 with day 0 or day 63 with day 0 (Figure 3).

Figure 4 depicts the correlation between the changes in protein levels of the serum and skin after treatment with DPCP after filtering within two standard errors of the perfect correlation line ($r = 0.74$, $P < 0.001$). Correlated proteins included TNF, IL-8, IL-12RB1, CXCL10, and CD83, which

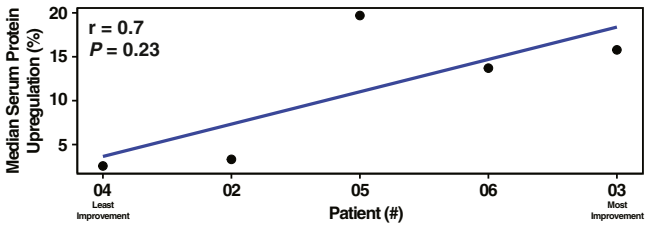


Figure 3. Scatterplot of median serum protein upregulation by patients. A scatterplot depicts the association between the median percentage upregulation of serum proteins with expression levels that changed significantly ($P < 0.1$) when comparing day 112 with day 0 or day 63 with day 0 (y-axis) and patients, ranked according to the degree of clinical improvement on completing DPCP treatment (x-axis). r is Spearman correlation. DPCP, diphencyprone.

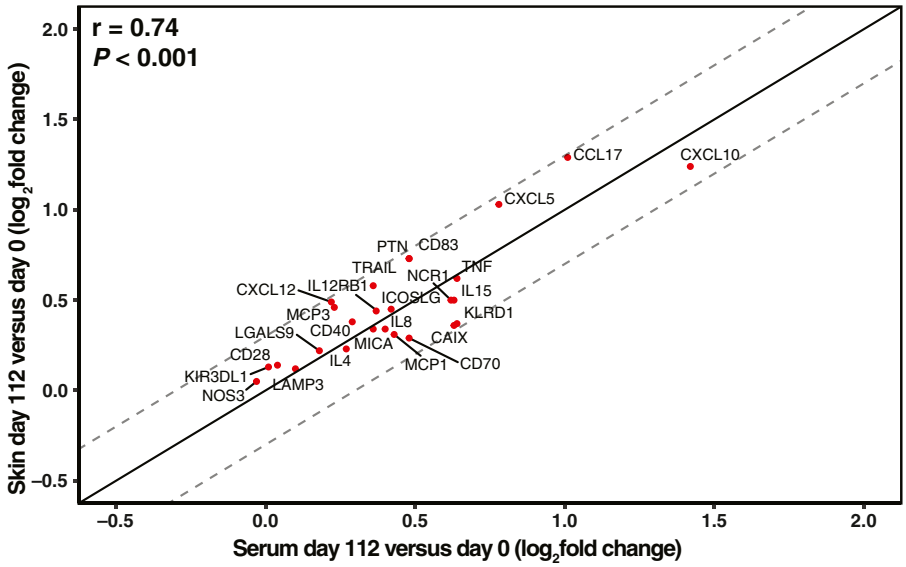
have roles in leukocyte chemotaxis, immune activation, or tumor suppression (Choi and Lee, 2020; Grosche et al., 2020; Gulati et al., 2016b; Liu et al., 2011; Montfort et al., 2019).

DISCUSSION

Serum profiling is of particular interest because it can provide a prognostic barometer of treatment efficacy by following changes in biomarker levels in a way that can be carried out with greater frequency than tissue sampling through biopsy. As such, studies with more serum collection time points exploring the correlation between the timing and degree of change in biomarkers with clinical improvement are warranted. Our analysis comparing median serum protein upregulation and degree of clinical improvement yielded a weak positive correlation. A limitation of this analysis is the difficulty of precisely assessing clinical improvement given the challenging nature of measuring skin metastases. Moreover, given the growing interest in liquid biopsies for noninvasive treatment monitoring, it is important to investigate the relationships between protein biomarkers in both serum and tissue to identify those of significance (Marrugo-Ramírez et al., 2018), in addition to transcriptomic analyses with and without treatment, to elucidate the mechanism of action of DPCP.

In the case of DPCP treatment of cutaneous melanoma metastases, CXCL10, CD83, and TNFRSF4/9 protein monitoring in serum may provide insight into clinical responses during treatment. CD83 is a member of the Ig family and is present on the surface of activated dendritic cells, therefore exhibiting the potential to serve as a marker of immune activation (Grosche et al., 2020). Elevation of TNFRSF4 in tissue has been positively correlated with tumor-infiltrating immune cells in other solid tumors, and greater *TNFRSF9* mRNA expression in tissue is associated with higher immune cell infiltrates; both are linked to positive clinical outcomes and considered prognostic biomarker candidates in melanoma and other cancers (Fröhlich et al., 2020; Ma et al., 2022), highlighting the potential for these proteins to serve as similar biomarkers for melanoma response to DPCP. In addition, DPCP does not cause systemic immune-related adverse events as seen with immune checkpoint inhibitors (ICIs) that nonspecifically activate the immune system (Choi and Lee, 2020), but there have been observed beneficial systemic effects of DPCP beyond its application site, such as lymph node metastasis regression (Damian et al., 2014). TNFRSF9 may represent a biomarker of distant melanoma response, given its roles in mediating leukocyte extravasation and facilitating the migration of tumor-specific lymphocytes into malignant tissue, thereby inferring a tumor-specific T-cell response (Eiva et al., 2022; Fröhlich et al., 2020). In addition, TNFRSF9 contributes to the clonal expansion of T cells and regulates CD28 costimulation to promote a Th1 response, which correlates with the response to immunotherapy in melanoma (Eiva et al., 2022; Gulati et al., 2016a). Although the biological role of serum TNFRSF4 in patients is still unclear, the expression levels of serum TNFRSF4 have correlated with the efficacy of anti−PD-1 therapy in the treatment of advanced gastric cancer (Ohmura et al., 2020). Higher levels of serum TNFRSF9 have been associated with longer progression-free survival in certain solid tumors, and there is

Figure 4. Scatterplot comparing the $\log_2$ fold change in protein expression in the skin and serum after DPCP treatment. A scatterplot depicts the association between $\log_2$ fold change in the skin (y-axis) and serum (x-axis) protein expression on day 112 compared with that on day 0. The perfect fit line (slope = 1, intercept = 0) is drawn with two standard error confidence intervals to filter a set of proteins that are similarly regulated in both skin and serum. r is Spearman correlation. DPCP, diphencyprone.



also preclinical support for antitumor activity when both the TNFRSF9 pathway is active, and a PD-1 inhibitor is used (Zhang et al., 2022).

The specific role of serum PD-1 and its prognostic value are matters of much debate as well (Chang et al., 2019). For example, pretherapeutic serum PD-1 levels have been reported to be predictive of active disease and worse prognosis (Khan et al., 2020). In addition, patients with melanoma with high baseline serum PD-1 levels have shown poor responses to PD-1 inhibition therapy, possibly owing to circulating PD-1—neutralizing anti—PD-1 antibodies (Ugurel et al., 2020). On the other hand, stable or increasing PD-1 levels after initiating cancer therapy, such as with an epidermal growth factor receptor tyrosine kinase inhibitor, have been associated with favorable outcomes (Sorensen et al., 2016). In our study, levels of immune checkpoint proteins such as PD-1 and LAG3 both increased in serum after initiating DPCP treatment, suggesting potential synergy between DPCP and ICIs as a future cancer therapy regimen to explore for patients with cutaneous melanoma metastases. This hypothesis is gaining support owing to the promising outcomes of such topical and systemic combination immunotherapy approaches (Fujimura et al., 2016; Gulati et al., 2016a).

Although the serum biomarkers were specific to topical DPCP in our study, successful serum monitoring has been achieved in systemic ICI therapy (An et al., 2021); therefore, further studies are needed to evaluate the potential prognostic value of proteins that correlate between skin and serum when combining DPCP with ICIs or other systemic treatments. Given DPCP's well-established safety profile, with minimal systemic adverse effects, unlike as seen with ICIs, our study provides evidence for potential tumor-specific systemic immune activation and systemic antitumor effectors elicited by topical DPCP.

MATERIALS AND METHODS

We enrolled six patients under a protocol approved by The Rockefeller University's Institutional Review Board (ClinicalTrials.gov Identifier NCT01711684). Written, informed consent was obtained from all subjects, and the study adhered to the Declaration of Helsinki Principles. The patients all underwent rigorous screening processes, including medical history, physical examination, and point-of-care HIV test to ensure that they did not have any conditions and were not on any medications that could interfere with immune reactions. While enrolled in our DPCP trial at The Rockefeller University (New York, NY), all patients continued to receive their standard oncologic follow-up and monitoring visits. All patients consented to the publication of clinical images taken during the study.

The DPCP ointment preparation was dissolved in a vehicle of an emollient, isopropyl myristate, and a surfactant polysorbate 80. Polyoxyl stearate was added as a gelling agent, and methyl and propyl paraben were added as preservatives. All nonactive ingredients were of United States Pharmacopeia/National Formulary grade and are commonly used in the formulations of lotions and cosmetics.

Patients were sensitized to 0.4% DPCP on one of their cutaneous metastases and their right upper arm as well as to 0.04% DPCP (also in a topical ointment preparation) on their left lower arm. Two weeks later, effective sensitization was confirmed by noting induration at the application sites, and then challenge applications were applied to the subject's cutaneous metastases. Also at this visit, one 0.2 ml application of 0.4% DPCP was applied to one area of nonmelanoma skin, and another 0.2 ml application of 0.04% DPCP was applied to a different area of nonmelanoma skin (both areas were on the upper thigh). These two applications were completed to determine the concentration of DPCP that would induce tolerable inflammation in each patient so that the appropriate concentration could be used for challenge (treatment) applications. Each application of DPCP (occurring twice weekly) was self-administered by the patient such that all cutaneous metastases were covered with a thin layer of ointment (the patient was asked to return the tube containing the DPCP ointment at each clinic visit for weighing to ensure compliance) and then covered with Tegaderm for at least 2 hours.

Patients had their blood drawn before DPCP treatment (day 0), during DPCP treatment (day 63), and on completion of DPCP treatment (day 112). Skin biopsies of melanoma metastasis sites treated with DPCP were also performed on days 0 and 112. All skin biopsies (6 mm full-thickness punch) were bisected. A 10 μg protein from each skin and serum sample was used for Olink Proseek multiplex ultrasensitive platform using the immuno-oncology panel (96 biomarkers). Patients were deemed to have partial regression if either decreased size or decreased number of metastases was observed, but still, some metastases remained. Complete regression was determined when no metastatic lesions were visible after treatment.

Statistical analysis

Heatmap. The heatmap shows the z-score obtained after scaling the normalized protein expression across time points (columns). The proteins (rows) are hierarchically clustered using the euclidean distance and the complete-linkage method (Defays, 1977) as implemented in the ComplexHeatmap R package. Next to the heatmap, we added a table with estimated fold changes between time points and symbols associated with their statistical significance. The fold changes originate from back-transformed differences between $\log_2$ expressions estimated in a linear mixed-effects model. The symbols describing the level of significance are ** ($P < 0.01$) and * ($P < 0.05$) + ($P < 0.1$).

Linear-mixed models. We used the lme R package to fit linear mixed-effects models (Laird and Ware, 1982) that describe each protein expression over time. Because protein expression levels from multiple samples of the same individual are expected to correlate, this approach allows us to systematically account for intrasubject variability, similar to a paired t-test. Our model is specified by setting the time point as a fixed factor and including a random intercept for each subject. We fitted a mixed-effects model with a random intercept accounting for variability between subjects. We did not consider including baseline as a covariate. We considered our approach to be equivalent to fitting a repeated measures model with the Compound Symmetry structure for the covariance matrix. The marginal means in $\log_2$ scale at each time point and 95% confidence intervals are estimated and displayed in bar plots. The treatment effects over time are obtained from pairwise differences between time points estimated by restricted maximum likelihood. Statistical significance is obtained through a *t*-statistic, with degrees of freedom estimated by the containment method. Inference is performed with the package emmeans in R software. Owing to the small sample size and exploratory nature of the study, we show unadjusted *P*-values.

Tissue versus serum correlation. A scatterplot and the Spearman correlation coefficient illustrate the association between log fold changes in skin tissue and serum. We overlaid the perfect fit line (slope = 1, intercept = 0) onto the scatterplot and added a band

on the basis of the standard error for the intercept in the least-squares fitted line. Proteins within the intervals created by this band were selected as the ones with the closest relationship between serum and skin tissue.

Serum protein upregulation versus degree of clinical improvement correlation. A scatterplot and the Spearman correlation coefficient illustrate the association between the median serum protein upregulation and the degree of clinical improvement by patients, ranked in order from least to most clinical improvement. Selected proteins included in this analysis were those with expression levels that changed significantly ($P < 0.1$) when comparing day 112 with day 0 or day 63 with day 0.

Ethics committee approval

This study was approved by The Rockefeller University's Institutional Review Board (approval number JKR-0788, ClinicalTrials.gov listing: NCT01711684).

Data availability statement

The datasets related to this article can be found at https://data.mendeley.com/datasets/4v8vsjdzp8/draft?a=f415785c-6dc1-4dde-bdf0-dc8d2edcee8a, an open-source online data repository hosted at Mendeley Data. All other supporting data are available on written request to the corresponding author.

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CONFLICT OF INTEREST

JGK is a consultant to and receives honoraria from AbbVie, Aclaris, Allergan, Almirall, Amgen, Artax Biopharma, Arena, Aristea, Asana, Aurigene, Biogen Idec, Boehringer Ingelheim, Bristol-Myers Squibb, Escalier, Galapagos, Janssen, Kyowa Kirin, Lilly, MoonLake Immunotherapeutics, Nimbus, Novartis, Pfizer, Sanofi, Sienna Biopharmaceuticals, Sun Pharma, Target-Derm, UCB, Valeant, and Ventyx and receives grant support (to The Rockefeller University) from AbbVie, Akros, Allergan, Amgen, Avillion, Biogen, Botanix, Boehringer Ingelheim, Bristol-Myers Squibb, Exicure, Innovaderm, Incyte, Janssen, Kyowa Kirin, Lilly, Nimbus Lackshmi, Novan, Novartis, PAREXEL, Pfizer, Regeneron, UCB, and Vitae Pharmaceuticals. The remaining authors state no conflict of interest.

AUTHOR CONTRIBUTIONS

Conceptualization: JH, NG, JGK; Data Curation: JH, JCdR; Formal Analysis: JCdR; Investigation: JH, NG; Methodology: JH, NG, JGK; Project Administration: JH, NG; Resources: YE, NG; Software: JCdR; Supervision: KYS, NG, JGK; Visualization: JH, JCdR; Writing - Original Draft Preparation: JH, AA, NG; Writing - Review and Editing: JH, JCdR, AA, SO, DY, YL, AS, JU, KYS, JGK, NG

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