Psoriasis is characterized by deficient negative immune regulation compared to transient delayed-type hypersensitivity reactions [version 1; peer review: 4 approved]

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Abstract
Diphencyprone (DPCP) is a hapten that causes delayed-type hypersensitivity (DTH) reactions in human skin, and is used as a topical therapeutic for alopecia areata, warts, and cutaneous melanoma metastases. We examined peak DTH reactions induced by DPCP (3 days post-challenge) by comprehensive gene expression and histological analysis. To better understand how these DTH reactions naturally resolve, we compared our DPCP biopsies to those from patients with psoriasis vulgaris, a chronic inflammatory disease that does not resolve. By both microarray and qRT-PCR, we found that psoriasis lesional skin has significantly lower expression of many negative immune regulators compared to peak DPCP reactions. These regulators include: interleukin-10, cytotoxic T lymphocyte-associated 4 (CTLA4), programmed cell death 1 (PD1), programmed cell death 1 ligand 1 (PDL1), programmed cell death 1 ligand 2 (PDL2), and indoleamine 2,3-dioxygenase (IDO1). Their decreased expression was confirmed at the protein level by immunohistochemistry. To more completely determine the balance of positive vs. negative immune regulators in both DPCP reactions and psoriasis, we developed one comprehensive gene list for positive regulatory (inflammatory) genes, and another for negative regulatory (immunosuppressive) genes, through Gene Ontology terms and literature review. With this approach, we found that DPCP reactions have a higher ratio of negative to positive regulatory genes (both in terms of quantity and expression levels) than psoriasis lesional skin. These data suggest that the disease chronicity that distinguishes psoriasis from transient DTH reactions may be related to absence of negative immune regulatory pathways, and induction of these is therefore of therapeutic interest. Further study of these negative regulatory mechanisms that are present in DPCP reactions, but not in psoriasis, could reveal novel players in the pathogenesis of chronic inflammation. The DPCP system used here thus provides a tractable model for primary discovery of pathways potentially involved in...
immune regulation in peripheral tissues.

Keywords
psoriasis, delayed-type hypersensitivity, immune regulation, diphencyprone

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Competing interests: No competing interests were disclosed.
Grant information: This research was supported by National Institutes of Health (NIH) grant UL1 RR024143 from the National Center for Research Resources and the Milstein Medical Program. NG was supported by a Medical Scientist Training Program grant from the National Institute of General Medical Sciences of the NIH under award number T32GM07739 to the Weill Cornell/Rockefeller/Sloan-Kettering Tri-Institutional MD-PhD Program. The content of this study is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.
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How to cite this article: Gulati N, Suárez-Fariñas M, Correa da Rosa J and Krueger JG. Psoriasis is characterized by deficient negative immune regulation compared to transient delayed-type hypersensitivity reactions [version 1; peer review: 4 approved]
F1000Research 2015, 4:149 https://doi.org/10.12688/f1000research.6581.1
First published: 11 Jun 2015, 4:149 https://doi.org/10.12688/f1000research.6581.1
Introduction

Diphenycyprone (DPCP) is a hapten that induces delayed-type hypersensitivity (DTH) reactions in human skin, and is used therapeutically for alopecia areata\(^1\), warts\(^2\), and cutaneous melanoma metastases\(^3\). The mechanisms by which DPCP decreases pathogenic immunity for the promotion of hair growth in alopecia areata are incompletely understood. DPCP has been shown to alter the cytokine profile in treated alopecic scalp, in particular increasing interleukin (IL)-2 and IL-10 expression\(^4\). This increased IL-10 expression has been hypothesized to inhibit the lesional T cells of alopecia areata, but a comprehensive evaluation of other negative immune regulators induced by DPCP is lacking. We have previously shown that human skin responses to DPCP evolve from an inflammatory/effector peak at 3 days post-challenge to a more regulated immune response, with diminished markers of T cell activation, at 14 days. This study included comprehensive gene expression profiling, by microarray and qRT-PCR approaches, of biopsies from DPCP-challenged healthy volunteer skin at 3 days (peak reaction), 14 days (actively resolving reaction), and 120 days (4–8 months; fully resolved reaction) compared to placebo-treated skin\(^5\). We have also previously performed similar transcriptomic profiling of psoriasis vulgaris lesional vs. non-lesional skin. This resulted in a meta-analysis derived transcriptome (MAD3) which combined the results of 3 individual microarray experiments, in an effort to address the variability in differentially expressed genes observed between experiments\(^6\). In this study, we expand our previous characterizations of transient DTH reactions and chronic psoriasis biopsies by directly comparing them to each other, particularly in relation to positive and negative immune regulation.

Methods

Study subjects and skin samples/Consent

For diphenycyprone (DPCP) reaction microarray, qRT-PCR, and immunohistochemistry studies, skin biopsies were obtained from 11 volunteers under a protocol approved by The Rockefeller University’s Institutional Review Board (IRB Number JKR-0742). Written, informed consent was obtained from all subjects and the study adhered to the Declaration of Helsinki principles. This trial is registered at clinicaltrials.gov under NCT01452594 (https://clinicaltrials.gov/ct2/show/NCT01452594). For each volunteer, biopsies were taken of placebo-treated skin as well as DPCP reactions 3, 14, and 120 days after challenge, as previously described\(^1\).

For psoriatic lesional vs. non-lesional skin microarray data, we used the meta-analysis derived (MAD3) transcriptome described in 6. Psoriatic lesional tissue for qRT-PCR and immunohistochemistry studies were from deidentified residual samples of plaque-type psoriasis vulgaris from previous studies for whom no clinical characteristics are available; a psoriasis area severity index of more than 12 (moderate-to-severe psoriasis vulgaris with >10% body surface area involvement) was required for entry into these trials.

RNA extraction, quantification, and microarray

Total RNA was extracted using the miRNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s protocol with on-column DNase digestion. The amount of RNA was assessed by NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc., 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) to measure relative gene expression.

Statistical analysis.

Microarray data were analyzed using R/Bioconductor packages (http://www.r-project.org). The Harshlight package\(^7\) was used to scan Affymetrix chips for spatial artifacts. Expression values were normalized using the GeneChip Robust Multi-array Average (GCRMA) algorithm. Genes with low variation and low expression in most samples were filtered out prior to the analysis. Batch effect due to hybridization date was adjusted using ComBat\(^8\), To identify differentially expressed genes, we fitted by REML (Restricted Maximum Likelihood) a linear mixed effect model with treatment (placebo/DPCP) and day (3/14) as fixed effects and a random intercept for each patient. Hypotheses of interest were tested using contrasts in R’s limma package framework. The p-values resultant from the moderated paired Student’s t-tests were adjusted for multiple hypotheses using the Benjamini-Hochberg procedure, which controls for the false discovery rate. The DPCP 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/). Psoriasis data were derived from\(^5\).

Quantitative RT-PCR

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. Briefly, 5 ng of total RNA was subjected to first-strand cDNA synthesis using High Capacity cDNA Reverse Transcription kits (Applied Biosystems, Carlsbad, CA). The resulting cDNA was subjected to 14 cycles of pre-amplification using TaqMan PreAmp Master Mix Kit (Applied Biosystems) with desired pooled assay mix. The Gene Amp PCR System 9700 (Applied Biosystems) was used for the pre-amplification reaction with the following thermal cycler conditions: 10 min at 95°C and 14 cycles of 15 seconds at 95°C followed by 4 min at 60°C. 12.5 μl of pre-amplified cDNA was then used for quantitative RT-PCR reaction using TaqMan Gene Expression Master Mix (Applied Biosystems). The 7900HT Fast Real-Time PCR System was used for PCR reactions, and the thermal cycler conditions were as follows: 2 minutes at 50°C, 5 minutes at 95°C, and 40 cycles of 15 seconds at 95°C followed by 60 seconds at 60°C. Data were analyzed by the Applied Biosystems PRISM 7700 software (Sequence Detection Systems, ver. 1.7) and normalized to human acidic ribosomal protein (hARP) housekeeping gene.

All assays were from Applied Biosystems and inventoried assays used in this study were as follows: IL10 (Hs00961622_m1), CTLA4 (Hs03044418_m1), PDCD1 (PD1) (Hs01550088_m1), CD274 (PDL1) (Hs0125301_m1), PDCD1LG2 (PDL2) (Hs01057777_m1), IDO1 (Hs00984148_m1), and LAG3 (Hs01558563_m1). For RPLP0/ hARP, a custom primer/probe set was used (Forward: CGCTGCT-GAACATGCTCAA, Reverse: TGGTGAAACACCTGTGGGATG, Probe: 6-FAM-TCCCCCTTCTCCTTTGGGCTGG-TAMRA).

Immunohistochemistry

Frozen sections of skin biopsies were dried at room temperature and then fixed for 2 minutes in acetone. Next, the samples were blocked

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with 10% normal serum of the species in which the secondary antibody was made and then the samples were incubated overnight at 4°C with the appropriate primary antibody. Biotin-labeled secondary antibodies (Vector Laboratories, Burlingame, CA) were amplified with avidin-biotin complex (Vector Laboratories) and developed with chromogen 3-aminobenzidine (Sigma Aldrich, St. Louis, MO) to produce a red color indicative of positive staining.

Primary antibodies used in this study are as follows (all are mouse monoclonal): IL10 (Life Technologies, Clone 945A2A5, IgG1, 1:50 dilution), CD95 (FAS) (BD Biosciences, Clone DX2, IgG1, 1:50), LAG3 (Enzo Life Sciences, Clone 1B7B4, IgG1, 1:50), PD1 (eBioscience, Clone MIH4, IgG1, 1:50), PDL1 (eBioscience, Clone MIH1, IgG1, 1:50), PDL2 (eBioscience, Clone MIHI8, IgG1, 1:100), IDO1 (LifeSpan Biosciences, Inc., Clone 10.1, IgG3, 1:100), and CTLA4 (abcam, Clone BNI3, IgG2a, 1:100).

Results

Since DTH reactions naturally resolve, we sought to compare our DPCP biopsies (from 5) to those taken from patients with psoriasis vulgaris (from 6), a chronic T cell-mediated inflammatory disease that does not resolve and which, in many ways, represents amplifications of background immune circuits that exist in normal human skin. To globally assess the balance of positive vs. negative immune regulators in both DPCP reactions and psoriasis using our microarray data, we developed one comprehensive gene list for positive regulatory or inflammatory genes and another gene list for negative regulatory or immunosuppressive genes (through Gene Ontology terms and literature review previously discussed in 5). Table 1 has “negative regulator” list and fold change values for DPCP day 3 and psoriasis transcriptomes. “Positive regulator” list is derived from GO term 0002684 “positive regulation of immune system process” but with genes removed that are in common with GO term 0002683 “negative regulation of immune system process”. Our microarray data showed increased fold changes of many negative regulators in DPCP day 3 biopsies vs placebo-treated skin (DPCP day 3 transcriptome) compared to psoriasis lesional vs non-lesional skin (psoriasis transcriptome). For instance, CTLA4 expression was significantly increased 21.6-fold in the DPCP day 3 transcriptome, but non-significantly increased 3.7-fold in the psoriasis transcriptome. Venn diagrams show that the psoriasis transcriptome only has seven genes from the negative regulator list, while the DPCP day 3 transcriptome has 52 (Figure 1a). Although the DPCP day 3 transcriptome also has more genes from the positive regulator list than psoriasis, the odds ratio for the positive regulator list was not significantly different between these two transcriptomes. The odds ratio for the negative regulator list, however, was significantly different (Figure 1b). The altered balance between positive vs. negative regulatory transcripts in psoriasis compared to DPCP reactions can also be seen in Figure 1c which shows that DPCP transcriptomes at all time points (days 3, 14, and 120) have a higher ratio of negative to positive regulator genes than

Table 1. Expression of negative regulator genes in DPCP day 3 vs. placebo and psoriasis lesional vs. non-lesional skin samples.

| Probe     | Symbol | Description | DPCP 3 day   | psoriasis |
|-----------|--------|-------------|--------------|-----------|
|           |        |             | FCH          | FDR       | p        | FDR |
| 207526_s_at | IL1RL1 | interleukin 1 receptor-like 1 | 42.8 | 5.3E-11 | 2.1E-09 | 1.1 | 3.6E-03 | 1.3E-02 |
| 227458_s_at | CD274 | CD274 molecule | 34.6 | 1.9E-12 | 1.3E-10 | 23.7 | 0.0E+00 | 0.0E+00 |
| 236341_s_at | CTLA4 | cytotoxic T-lymphocyte-associated protein 4 | 21.6 | 3.7E-11 | 1.5E-09 | 3.7 | 6.0E-02 | 1.4E-01 |
| 207238_s_at | PTPRC | protein tyrosine phosphatase, receptor type, C | 18.0 | 1.6E-12 | 1.2E-10 | 2.6 | 0.0E+00 | 0.0E+00 |
| 206341_s_at | IL2RA | interleukin 2 receptor, alpha | 17.9 | 4.7E-14 | 6.3E-12 | 1.3 | 1.6E-01 | 2.9E-01 |
| 210146_x_at | LILRB2 | leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 2 | 12.5 | 3.6E-08 | 5.4E-07 | 2.9 | 0.0E+00 | 0.0E+00 |
| 222062_s_at | IL27RA | interleukin 27 receptor, alpha | 12.3 | 9.9E-13 | 7.6E-11 | 1.3 | 1.7E-03 | 6.7E-03 |
| 217192_s_at | PRDM1 | PR domain containing 1, with ZNF domain | 11.1 | 8.7E-12 | 4.6E-10 | 3.0 | 0.0E+00 | 0.0E+00 |
| 215719_x_at | FAS | Fas (TNF receptor superfamily, member 6) | 9.3 | 7.8E-07 | 7.6E-06 | 1.1 | 2.8E-02 | 7.6E-02 |
| 205926_s_at | IL27RA | interleukin 27 receptor, alpha | 8.3 | 5.9E-11 | 2.3E-09 | 1.2 | 1.3E-01 | 2.4E-01 |
| 204780_s_at | FAS | Fas (TNF receptor superfamily, member 6) | 8.0 | 2.6E-05 | 1.6E-04 | 1.1 | 1.3E-01 | 2.4E-01 |
| 242743_at | IL4R | interleukin 4 receptor | 8.0 | 7.6E-13 | 6.2E-11 | 1.1 | 6.0E-02 | 1.4E-01 |
| 242809_at | IL1RL1 | interleukin 1 receptor-like 1 | 6.6 | 1.5E-06 | 1.3E-05 | 1.0 | 7.6E-01 | 8.3E-01 |
| 207697_x_at | LILRB2 | leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 2 | 6.6 | 1.2E-13 | 1.4E-11 | 1.5 | 0.0E+00 | 0.0E+00 |
| 212588_at | PTPRC | protein tyrosine phosphatase, receptor type, C | 6.5 | 2.8E-08 | 4.3E-07 | 2.4 | 0.0E+00 | 0.0E+00 |
| Probe        | Symbol   | Description                                                                 | DPCP day 3 | psoriasis |
|--------------|----------|-----------------------------------------------------------------------------|------------|-----------|
|              |          |                                                                             | FCH        | p         | FDR     | FCH        | p         | FDR     |
| 216252_x_at  | FAS      | Fas (TNF receptor superfamily, member 6)                                    | 6.3        | 9.4E-08   | 1.2E-06 | 1.1        | 7.0E-04   | 2.9E-03  |
| 230052_s_at  | NFKBID   | nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, delta | 6.2        | 1.0E-07   | 1.3E-06 | 1.4        | 1.4E-02   | 4.3E-02  |
| 211336_x_at  | LILRB1   | leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 1 | 5.2        | 4.8E-16   | 1.5E-13 | 1.3        | 2.3E-03   | 8.7E-03  |
| 212587_s_at  | PTPRC    | protein tyrosine phosphatase, receptor type, C                             | 5.1        | 6.5E-13   | 5.5E-11 | 1.8        | 2.2E-03   | 8.3E-03  |
| 207104_x_at  | LILRB1   | leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 1 | 5.1        | 3.0E-17   | 1.6E-14 | 1.2        | 0.0E+00   | 0.0E+00  |
| 211269_s_at  | IL2RA    | interleukin 2 receptor                                                     | 5.1        | 2.8E-08   | 4.4E-07 | 1.1        | 1.8E-01   | 3.1E-01  |
| 204781_s_at  | FAS      | Fas (TNF receptor superfamily, member 6)                                    | 4.7        | 3.4E-06   | 2.7E-05 | 1.2        | 3.6E-02   | 9.3E-02  |
| 1552480_s_at | PTPRC    | protein tyrosine phosphatase, receptor type, C                             | 4.5        | 9.0E-08   | 1.2E-06 | 1.0        | 3.3E-01   | 4.7E-01  |
| 203233_at    | IL4R     | interleukin 4 receptor                                                      | 4.4        | 1.2E-10   | 4.0E-09 | 3.9        | 0.0E+00   | 0.0E+00  |
| 206060_s_at  | PTPN22   | protein tyrosine phosphatase, non-receptor type 22 (lymphoid)              | 4.3        | 7.6E-06   | 5.5E-05 | 2.3        | 1.0E-04   | 4.0E-04  |
| 223834_at    | CD274    | CD274 molecule                                                              | 3.8        | 5.7E-09   | 1.1E-07 | 1.5        | 3.6E-03   | 1.3E-02  |
| 235458_at    | CD274    | CD274 molecule                                                              | 3.8        | 6.6E-07   | 6.6E-06 | 1.5        | 1.4E-03   | 5.5E-03  |
| 231794_at    | CTLA4    | cytotoxic T-lymphocyte-associated protein 4                                | 3.4        | 1.6E-09   | 3.7E-08 | 1.2        | 6.4E-02   | 1.4E-01  |
| 227900_at    | CBLB     | Cbl proto-oncogene, E3 ubiquitin protein ligase B                           | 3.3        | 1.8E-04   | 8.3E-04 | 0.8        | 9.6E-03   | 3.1E-02  |
| 220418_at    | UBASH3A  | ubiquitin associated and SH3 domain containing A                            | 3.3        | 3.0E-05   | 1.8E-04 | 1.1        | 2.7E-02   | 7.3E-02  |
| 202643_s_at  | TNFAIP3  | tumor necrosis factor, alpha-induced protein 3                              | 3.2        | 1.0E-08   | 1.8E-07 | 1.2        | 1.9E-01   | 3.2E-01  |
| 240070_at    | NFKBID   | nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, delta | 2.6        | 6.5E-06   | 4.8E-05 | 1.0        | 8.8E-02   | 1.9E-01  |
| 223506_at    | ZC3H8    | zinc finger CCCH-type containing B                                          | 2.6        | 5.6E-04   | 2.2E-03 | 1.6        | 0.0E+00   | 0.0E+00  |
| 209744_x_at  | ITCH     | itchyc E3 ubiquitin protein ligase                                          | 2.6        | 3.8E-07   | 4.1E-06 | 2.8        | 1.4E-02   | 4.1E-02  |
| 225622_at    | PAG1     | phosphoprotein associated with glycosphingolipid microdomains 1            | 2.6        | 3.4E-05   | 2.0E-04 | 1.9        | 0.0E+00   | 0.0E+00  |
| 217094_s_at  | ITCH     | itchyc E3 ubiquitin protein ligase                                          | 2.4        | 1.1E-05   | 7.8E-05 | 2.4        | 6.9E-02   | 1.5E-01  |
| 224211_at    | FOX3     | forkhead box P3                                                             | 2.4        | 1.2E-05   | 7.9E-05 | 1.0        | 0.0E+00   | 1.0E-04  |
| 243196_s_at  | TRAFD1   | TRAF-type zinc finger domain containing 1                                   | 2.3        | 4.0E-05   | 2.3E-04 | 0.9        | 4.8E-01   | 6.1E-01  |
| 228996_at    | RC3H1    | ring finger and CCCH-type domains 1                                         | 2.3        | 5.5E-05   | 3.0E-04 | 1.8        | 2.5E-01   | 3.9E-01  |
| 219364_at    | DHX58    | DEXH (Asp-Glu-X-His) box polypeptide 58                                     | 2.3        | 5.3E-07   | 5.4E-06 | 1.3        | 0.0E+00   | 0.0E+00  |
| 202763_at    | CASP3    | caspase 3, apoptosis-related cysteine peptidase                              | 2.2        | 1.8E-05   | 1.1E-04 | 1.1        | 1.1E-01   | 2.2E-01  |
| Probe          | Symbol | Description                                                                 | FCH | p      | FDR       | p      | FDR       |
|----------------|--------|------------------------------------------------------------------------------|-----|--------|-----------|--------|-----------|
| 236539_at      | PTPN22 | protein tyrosine phosphatase, non-receptor type 22 (lymphoid)               | 2.2 | 6.9E-05| 3.7E-04   | 2.0   | 0.0E+00   |
| 202644_s_at    | TNFAIP3| tumor necrosis factor, alpha-induced protein 3                              | 2.2 | 1.4E-06| 1.3E-05   | 1.1   | 2.8E-01   |
| 205298_s_at    | BTN2A2 | butyrophilin, subfamily 2, member A2                                        | 2.2 | 3.7E-04| 1.6E-03   | 1.1   | 1.9E-01   |
| 217513_at      | MILR1  | mast cell immunoglobulin-like receptor 1                                    | 2.1 | 6.0E-06| 4.4E-05   | 1.2   | 5.9E-02   |
| 205299_s_at    | BTN2A2 | butyrophilin, subfamily 2, member A2                                        | 2.0 | 3.9E-05| 2.2E-04   | 1.1   | 1.1E-01   |
| 242497_at      | TRAFD1 | TRAF-type zinc finger domain containing 1                                    | 1.8 | 4.1E-04| 1.7E-03   | 1.0   | 6.3E-01   |
| 234066_at      | IL1RL1 | interleukin 1 receptor-like 1                                               | 1.8 | 7.5E-03| 2.0E-02   | 1.0   | 3.6E-01   |
| 235668_at      | PRDM1  | PR domain containing 1, with ZNF domain                                     | 1.8 | 3.0E-06| 2.4E-05   | 2.2   | 0.0E+00   |
| 1555628_a_at   | HAVCR2 | hepatitis A virus cellular receptor 2                                        | 1.8 | 6.7E-04| 2.6E-03   | 1.0   | 4.3E-01   |
| 209682_at      | CBLB   | Cbl proto-oncogene, E3 ubiquitin protein ligase B                           | 1.8 | 6.1E-05| 3.3E-04   | 0.8   | 0.0E+00   |
| 209354_at      | TNFRSF14| tumor necrosis factor receptor superfamily, member 14                      | 1.8 | 3.2E-06| 2.6E-05   | 1.0   | 7.1E-01   |
| 227354_at      | PAG1   | phosphoprotein associated with glycosphingolipid microdomains 1             | 1.7 | 7.0E-02| 1.3E-01   | 1.3   | 3.7E-02   |
| 209743_s_at    | ITCH   | itchy E3 ubiquitin protein ligase                                            | 1.7 | 4.4E-03| 1.3E-02   | 1.2   | 0.0E+00   |
| 35254_at       | TRAFD1 | TRAF-type zinc finger domain containing 1                                    | 1.7 | 1.7E-05| 1.1E-04   | 0.9   | 2.8E-01   |
| 1555629_at     | HAVCR2 | hepatitis A virus cellular receptor 2                                        | 1.7 | 2.4E-04| 1.1E-03   | 1.0   | 7.6E-01   |
| 202837_at      | TRAFD1 | TRAF-type zinc finger domain containing 1                                    | 1.6 | 5.5E-05| 3.0E-04   | 1.0   | 9.1E-01   |
| 235057_at      | ITCH   | itchy E3 ubiquitin protein ligase                                            | 1.6 | 2.7E-02| 6.0E-02   | 0.9   | 2.0E-01   |
| 1554285_at     | HAVCR2 | hepatitis A virus cellular receptor 2                                        | 1.6 | 5.4E-04| 2.2E-03   | 1.0   | 1.5E-01   |
| 225626_at      | PAG1   | phosphoprotein associated with glycosphingolipid microdomains 1             | 1.5 | 2.3E-02| 5.2E-02   | 1.3   | 6.0E-04   |
| 201536_at      | DUSP3  | dual specificity phosphatase 3                                              | 1.5 | 3.2E-04| 1.4E-03   | 0.8   | 0.0E+00   |
| 1553042_a_at   | NFKBID | nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, delta | 1.5 | 3.0E-03| 9.4E-03   | 1.1   | 2.6E-02   |
| 220049_s_at    | PDCD1LG2| programmed cell death 1 ligand 2                                            | 1.4 | 5.4E-03| 1.5E-02   | 1.0   | 4.3E-01   |
| 221331_x_at    | CTLA4  | cytotoxic T-lymphocyte-associated protein 4                                 | 1.3 | 4.8E-04| 2.0E-03   | 1.1   | 4.7E-02   |
| 234362_s_at    | CTLA4  | cytotoxic T-lymphocyte-associated protein 4                                 | 1.3 | 4.6E-03| 1.3E-02   | 1.1   | 1.2E-01   |
| 208010_s_at    | PTPN22 | protein tyrosine phosphatase, non-receptor type 22 (lymphoid)               | 1.3 | 2.4E-03| 7.8E-03   | 1.1   | 4.0E-04   |
| 234895_at      | CTLA4  | cytotoxic T-lymphocyte-associated protein 4                                 | 1.1 | 4.9E-02| 9.7E-02   | 1.0   | 2.0E-04   |
| 225893_at      | RC3H1  | ring finger and CCYH-type domains 1                                         | 1.1 | 7.0E-01| 7.8E-01   | 1.0   | 3.6E-01   |
| 224859_at      | CD276  | CD276 molecule                                                              | 0.9 | 4.4E-01| 5.6E-01   | 0.9   | 2.0E-04   |
| 236235_at      | ITCH   | itchy E3 ubiquitin protein ligase                                            | 0.8 | 2.6E-01| 3.7E-01   | 0.7   | 3.6E-03   |
| 239101_at      | ITCH   | itchy E3 ubiquitin protein ligase                                            | 0.7 | 1.5E-02| 3.6E-02   | 0.7   | 0.0E+00   |
| 1559583_at     | CD276  | CD276 molecule                                                              | 0.6 | 8.5E-02| 1.5E-01   | 1.0   | 3.2E-01   |
| 219768_at      | VTCN1  | V-set domain containing T cell activation inhibitor 1                      | 0.3 | 8.1E-05| 4.2E-04   | 0.6   | 2.2E-03   |
| 204472_at      | GEM    | GTP binding protein overexpressed in skeletal muscle                        | 0.3 | 1.1E-05| 7.6E-05   | 1.0   | 3.0E-01   |

FCH, fold change; FDR, false discovery rate.
Figure 1. Psoriasis lesional skin has an altered global balance of positive vs. negative regulatory gene transcripts compared to DPCP reactions. (a) Venn diagrams showing overlap of MAD3 psoriasis transcriptome (left) and DPCP day 3 transcriptome (right) with both positive regulatory (Pos) and negative regulatory (Neg) gene lists (common gene lists applied to both transcriptomes). The percentages of the MAD3 and DPCP day 3 transcriptomes comprised of the positive regulatory gene list are 5.7% and 7.3%, respectively. On the other hand, the percentages comprised of the negative regulatory gene list are 0.7% and 1.5%, respectively. (b) Odds ratios (OR) of negative regulatory (red bars) and positive regulatory (blue bars) gene lists in DPCP day 3 and psoriasis transcriptomes. (c) Black bars represent negative regulator genes, gray bars represent positive regulator genes, and white bars represent all MAD3 psoriasis transcriptome genes. The y-axis shows log$_2$ (fold change) of all genes in the given gene set. DPCP day 3 and MAD3 samples have comparable MAD3 transcriptome expression levels but there is a substantial difference between all DPCP time points (days 3, 14, and 120 or “late”) and MAD3 samples in terms of the relative levels of negative and positive regulator gene list expression. This is quantified as the “ratio of negative to positive regulator genes.”
the psoriasis transcriptome in terms of expression levels for each gene set as a whole (as opposed to number of genes as indicated in the Venn diagrams). This is despite the fact that the DPCP day 3 transcriptome has comparable expression levels of the MAD3 psoriasis transcriptome genes to actual psoriasis samples, and therefore highlights the negative regulator expression that is unique to DPCP reactions.

To confirm some of our microarray findings, we performed qRT-PCR and found that psoriasis lesional skin biopsies have significantly lower expression of many negative immune regulators compared to peak DPCP biopsies. These regulators include lymphocyte activation gene 3 (LAG3), cytotoxic T lymphocyte-associated 4 (CTLA4), indoleamine 2,3-dioxygenase (IDO1), programmed cell death 1 (PD1), programmed cell death 1 ligand 1 (PDL1), programmed cell death 1 ligand 2 (PDL2), and IL-10 (Figure 2a). We confirmed the decreased expression of these and FAS (which by gene expression had 9.3- and 1.1-fold changes in the DPCP day 3 and psoriasis transcriptomes, respectively) at the protein level by immunohistochemistry (Figure 2b).

Figure 2. Psoriasis lesional skin has lower expression of various negative immune regulators than DPCP day 3 reactions by both qRT-PCR and immunohistochemical approaches. (a) qRT-PCR analysis for negative regulators LAG3, CTLA4, IDO1, PD1, PDL1, PDL2, and IL-10. Shown are average normalized expression values for DPCP day 3 samples (n=11, purple bars) and psoriasis lesional skin (LS) samples (n=11, brown bars). All except PDL1 are p<0.05 by unpaired two-tailed t-test assuming equal variance. Error bars represent standard errors of the mean. (b) Immunohistochemistry showing increased protein expression of negative regulators in DPCP day 3 samples compared to psoriasis LS. Shown are stains with antibodies specific to the indicated targets. Scale bar = 100 μm (applies to all images).
Discussion/conclusions
These data suggest that disease chronicity in psoriasis could be related to absence of several negative immune regulatory pathways, with the implication that strategies to obtain stable clearance/restore tolerance in skin lesions may need to focus on increasing these negative pathways. These negative immune mechanisms may be of more general importance for maintaining skin homeostasis as non-inflammatory in the presence of a large population of effector memory T cells that normally reside in skin\(^{10}\). In addition, these negative immune regulators are likely involved in the therapeutic applications of DPCP, particularly alopecia areata where IL-10 has already been implicated\(^{1}\). Further study of these regulatory mechanisms that are present in DPCP reactions, but not in psoriasis, could reveal novel factors in the pathogenesis of chronic inflammation. The DPCP system used here provides a tractable model for primary discovery of pathways potentially involved in immune regulation in peripheral tissues.

Author contributions
NG and JGK conceived the study and designed the experiments. NG carried out the research. MS-F and JCR contributed to the design of experiments and provided expertise in genomics. NG prepared the first draft of the manuscript. All authors were involved in the revision of the draft manuscript and have agreed to the final content.

Competing interests
No competing interests were disclosed.

Grant information
This research was supported by National Institutes of Health (NIH) grant UL1 RR024143 from the National Center for Research Resources and the Milstein Medical Program. NG was supported by a Medical Scientist Training Program grant from the National Institute of General Medical Sciences of the NIH under award number T32GM07739 to the Weill Cornell/Rockefeller/Sloan-Kettering Tri-Institutional MD-PhD Program. The content of this study is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

I confirm that the funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Version 1

Reviewer Report 30 July 2015

https://doi.org/10.5256/f1000research.7066.r9716

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The paper under review provides interesting research data with immunopathogenic implications for both delayed-type hypersensitivity (DTH) reactions and psoriasis. Using both microarray and quantitative RT-PCR the authors found that peak DTH reactions induced by diphencyprone (DPCP) 3 days post-challenge has significantly higher expression of negative immune regulators than psoriasis lesional skin. These regulators include interleukin-10 (IL-10), cytotoxic T lymphocyte-associated 4 (CTLA4), programmed cell death 1 (PD1), programmed cell death 1 ligand 1 (PDL1), programmed cell death 1 ligand 2 (PDL2), and indoleamine 2,3-dioxygenase (IDO1), and their increased expression was confirmed at protein level by immunohistochemistry. According to the authors, these findings might explain at least in part the disease chronicity in psoriasis and might provide new therapeutic avenues.

Of course, further studies are required to extricate the dynamics of immune activation in DTH vs psoriasis lesions; even data on DPCP reactions at 14 days can be found in another paper by the same authors (Gulati et al., 2014) detailed microarray and quantitative RT-PCR data on the time course of evolving lesions of psoriasis is lacking. Koebner phenomenon, guttate psoriasis and rebounds following clobetasol propionate suppression of plaque psoriasis might provide interesting models in this respect. The authors discuss the potential role of IL-10 regulation in DPCP immunotherapy of alopecia areata, and it might be interesting in this context to recall “Renbök phenomenon” or inverse Koebner phenomenon, namely the observation of normal hair growth in psoriatic plaques in patients with co-existing psoriasis and alopecia areata (Mirmirani, 2015).

Pathogenic differences between DTH and psoriasis are not limited to the expression of genes involved in negative immune regulation, as illustrated by the lack of effect of blocking Th1/Th17 pathways with ustekinumab on the elicitation of ACD by patch testing (Nosbaum et al., 2012), even though IL-1 β activation of dendritic epidermal T cells and production of IL-17 seems to be important in experimental contact hypersensitivity (Nielsen et al., 2014). On the other hand, nickel-sensitized psoriasis patients develop a delayed but otherwise typical allergic contact dermatitis (ACD) even in close proximity to pre-existing psoriasis plaques, with a predominantly Th17...
response in psoriasis and a stronger Th2 and cytotoxic immune response in ACD (Quaranta et al., 2014). Th9 cells are skin homing or skin resident cells which have been identified both in psoriasis and ACD, but IL-9 can have pro-inflammatory or regulatory activities depending on context (Liu et al., 2014). The innate immune responses to damage associated molecular patterns and reactive oxygen species are of foremost importance for the elicitation of ACD in vivo (Martin, 2015).

Furthermore, the gene expression signatures and pathomechanisms of DTH reactions may differ according to the antigen (Dhingra et al., 2014) and duration of exposure. Nickel and other metal ions are able to elicitate ACD through the activation of toll-like receptor 4 (TLR4) (Schmidt et al., 2010), direct activation of the NLRP-inflammasome with production of IL-1β (Li et al., 2014) and other mechanisms (Vennegaard et al., 2014).

Negative immune regulators include cytokines such as IL-10, IL-35 and transforming growth factor β (TGF β) and membrane proteins which are greatly interdependent and subject to cross-regulation, and several cell types, including Foxp3+ regulatory T cells, monocytes and B cells can be involved in down-regulation of inflammatory and autoimmune processes. Some of these cell types are characterized by their plasticity, so their phenotype might be subject to therapeutic modulation.

In conclusion, the paper by Gulati and coworkers represents a seminal work in the study of the comparative dynamics of immune activation and downregulation in several skin disorders, with potential therapeutic implications.

**Competing Interests:** No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

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In this paper, Gulati and colleagues have tested the hypothesis that psoriatic skin lesions have significantly lower expression of negative immune regulators compared to peak DTH reactions from diphencyprone (DPCP). This study was done using two previously generated data sets described in previous publications from this group: 1) a study in 11 healthy human volunteers of recall responses to DPCP at 3-day (peak) and 14-day (resolution) using immunohistochemical techniques to visualize the infiltrating immune cells and gene expression (Affymetrix HGU133 Plus 2.0 Arrays) to perform a molecular profiling of the day 3 and day 14 DPCP responses, and in 6
cases 4-8 months later (reference 5); and 2) a meta-analysis derived (MAD) transcriptome of psoriatic skin from 3 different studies using Affymetrix HGU133 Plus 2.0 arrays in which microarray raw data were available through public databases (reference 6), termed the MAD-3 transcriptome.

The results show an imbalance in the expression of negative regulatory genes in DPCP day-3 peak reactions compared to psoriatic skin lesions, favoring the expression of more negative immune regulators in the former vs. the latter group (52 genes vs 7 genes). They generated odds ratios for the positive and negative immune regulators from a list of negative regulatory and positive regulatory genes derived from a GO search. The odds ratio for negative immune regulators was significantly higher in the DPCP day-3 (peak) group than the psoriatic skin group, while the odds ratios for the positive immune regulators were not statistically significantly different between the two groups. The enrichment for negative immune regulators held true also at day-14 (resolving) and day 120 (late). These findings were confirmed by qRT-PCR of several key genes and by immunohistochemistry.

This study is technically sound and the paper clearly written, with acceptable figures and tables. The title and the abstract are appropriate for the research described in the article. The methods are described in sufficient detail for the experiments to be replicated, although it would be useful to provide more detail about the methods underlying the calculations of the odds ratios. The interpretation of the results are reasonable based on the data and the conclusions provide a novel conceptual framework for understanding the fundamental mechanisms of skin homeostasis in psoriasis.

I had one question about the results and their interpretation. In the study from reference 5, the results suggested different patterns of immune responses after DPCP challenge, referred to as subgroups A and B, where subgroup B had higher levels of negative immune regulators at day-3 than subgroup A. Interestingly, subgroup B fit the typical kinetics of a DTH response in which T cell and DC infiltrates peaked at day-3 and were diminished at day-14; whereas, subgroup A showed an unexpected increase in the numbers of T cells and DC's at day-14 compared to day-3. It might be useful to know if the higher expression of negative immune regulators in DPCP skin compared with psoriatic skin was driven by the higher levels of these genes in subgroup B, or if it was generally true across both subgroups.

**Competing Interests:** No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.
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In this welcome addition to the field of psoriasis transcriptomics, Gulati and colleagues sought to test whether expression of genes involved in immune regulation in transient condition, diphencyprone (DPCP) induced delayed-type hypersensitivity (DTH), was significantly different from expression of genes involved in immune regulation of a chronic T cell-mediated disease, psoriasis vulgaris. Employing microarray and qRT-PCR based approaches to profile the transcriptomes from biopsied samples, the authors found that negative immune regulatory genes were significantly overexpressed in the DPCP vs. healthy controls comparison, while such a pattern of overexpression was not observed in the psoriasis vs healthy controls comparison. This lack of overexpression of key negative regulatory genes was found in both the microarray and the qRT-PCR analyses. The authors conclude that the relative dearth of negative immune regulatory genes in psoriasis may be associated with the persistent inflammation found in psoriasis and that strategies to restore negative regulators may have therapeutic implications.  

This paper is technically sound, with well accepted protocol employed in all wet experiments. The statistical analysis was also performed with well accepted and sufficiently rigorous methodology. The evidence supports the authors’ central claims. P-values for differential expression analysis were adjusted for multiple hypotheses by the Benjamini-Hochberg method to control for the FDR.  

We believe that this paper will be of interest to researchers in the field of psoriasis transcriptomics and genetics, as well as the broader fields of autoimmunity and dermatology. The article could be strengthened by addressing the following comments and questions:  

1. The authors note that “genes with low variation and low expression in most samples were filtered out prior to the analysis”. We think that the filtering threshold for variance and expression should be reported as well.  

2. Odds ratios are reported in the results but it is not clear how they were estimated in the methods. Furthermore, interpretation of the odds ratios is unclear as well.  

3. In Table 1, we have questions regarding the FCH and the p and FDR values. Is the FCH the absolute FC? Is it log2? We think that the labeling should be clearer. What do p and FDR values of “0E+00” indicate? Is it for p and FDR values that are high or low?  

4. Could the authors’ results be confounded by the fact that psoriatic skin has a larger proportion of keratinocytes relative to DTH skin? If the negative regulators were primarily expressed in non-keratinocyte cell types, then biopsy of psoriatic skin compared to DTH skin might show a lower proportion of negative regulators based simply on cell proportions rather than intrinsic immunologic differences.  

5. We feel that the biology of psoriasis and DTH (or contact dermatitis) can be expanded upon in the discussion section. For instance, a number of the genes listed in Table 1 are known T regulatory cell signature genes (including CTLA4 and IL2RA; please see Ferraro et al., 2014). Is the transcriptional profile of the observed DTH reaction consistent with the effects of a T
regulatory cell effect, for example as described in Rosenblum et al. (2011)?

6. An apparent difference between the DTH model and psoriasis is that in the DTH model, antigen was transiently given, whereas in psoriasis it is possible that self-antigens are continually expressed. Might this contribute to the observed transcriptional differences? Have the authors’ examined the transcriptional profile of a DTH model in which antigen is chronically given?

Competing Interests: No competing interests were disclosed.

We confirm that we have read this submission and believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.
To achieve meaningful results regarding the posed question, the authors define two groups of genes for comparison. One comprehensive gene list includes positive regulatory or inflammatory genes that enhance inflammation, the other genes particularly related to negative regulatory or immunosuppressive functions. The composition of the gene lists is based on scientifically verified Gene ontology terms. In addition to the microarray data, for select genes expression levels are verified by quantitative RT-PCR and by immunohistochemistry analyses of protein expression in skin samples.

As a result the authors report, that at the time of the peak inflammatory response DPCP-induced DTH reactions contain significantly more genes related to suppression of inflammation than psoriasis lesions. This results in an altered balance between positive vs. negative regulatory transcripts in psoriasis compared to DTH reactions, which is maintained throughout DTH healing.

The authors conclude that in psoriasis, but also beyond this disease the lack of negative immune regulatory genes may be related to inflammatory disease chronicity. They furthermore propose that DPCP reactions may serve to examine regulatory immune pathways.

The study addresses a highly relevant question. The scientific approach is novel, original and represents a truly innovative solution to obtain novel insights into immune regulation. The comparison of psoriasis and DCPC reactions is reasonable because in both of them inflammatory reactions result from T-cell driven mechanisms. The methods are sound and appropriate for the analysis. The underlying data are extensive and due to the large sample size create a reliable and credible basis for the study. Accordingly, results and conclusions are well supported. The abstract provides an adequate summary of the study. The results are clearly presented. The discussion is short, draws a clear and valid conclusion and avoids unnecessary speculations. Indeed, the insights from the study provide a novel approach to understand the chronicity of inflammation.

My personal assessment is that of an intriguing approach, which by itself is revealing and at the same time opens novel access paths into the investigation of chronic immune-mediated inflammation.
I support the publication of this article in the present form without any reservation. There are no changes necessary.

**Competing Interests:** No competing interests were disclosed.

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