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The gene expression and immunohistochemical time-course of diphenylcyclopropenone induced contact allergy in healthy humans following repeated epicutaneous challenges

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Key words: de novo sensitization, rechallenge, microarray, time-course analysis, transcriptional profiling.

ABSTRACT

The gene expression time-course of repeated challenge of contact allergy (CA) remains largely unknown. Therefore, using diphenylcyclopropanone (DPCP) as model allergen in healthy humans we set out to examine: i) the monotonous and complex gene expression time-course trajectories following repeated challenges with DPCP to find the predominant gene expression pattern, ii) the time-course of cell infiltration following repeated DPCP challenges, and iii) the transcriptome of a repeated CA exposure model.

We obtained punch biopsies from control and DPCP exposed skin from ten DPCP sensitized individuals at 5-6 monthly elicitation challenges. Biopsies were used for microarray gene expression profiling, histopathology and immunohistochemical staining. Validation of microarray data by qRT-PCR was performed on 15 selected genes. Early gene expression time points were also validated in an independent dataset.

An increasing and decreasing trend in gene expression followed by a plateau was predominantly observed during repeated DPCP challenges. Immune responses reached a plateau after two challenges histopathologically, immunohistochemically and in the time-course gene expression analysis. Transcriptional responses over time revealed a Th1/Th17 polarization as three upstream regulators (IFN- γ , IL-1 and IL-17) activated most of the top upregulated genes. Of the latter genes, 9 out of 10 were the same throughout the time-course. Excellent correlations between array and PCR data were observed.

The transcriptional responses to DPCP over time followed a monotonous pattern. This response pattern confirms and supports the newly reported clinical time-course observations in de novo sensitized individuals showing a plateau response, and thus, there is concordance between clinical response, histopathology, immunohistochemistry, and microarray gene expression in volunteers de novo sensitized to DPCP.

INTRODUCTION

Time-course designs allow examination of responses to treatments or biochemical stimulations during in vitro or in vivo experiments (1). The results of such experiments provide insights into the time-dependent development of human skin disorders and can facilitate identification of novel biomarkers. Although gene expression experiments are increasingly feasible, few such studies have explored the time-course in vivo in human skin during elicitation of contact allergy (CA). Thus far, the early gene expression time-course after an epicutaneous challenge with nickel has been studied (at 0/7/48/96h) (2), and fewer than 100 differentially expressed genes were identified in nickel-allergic individuals. Most of the differentially expressed genes were increased at the late time points (48/96h) compared

with the early time points (0/7h). Not surprisingly, the differential gene expression corresponded well with the clinical time-course that showed visible allergic reactions at 48 and 96 hours as opposed to 0 and 7 hours, which were clinically silent (i.e. no visible eczema). Further, the early time-course response to another strong hapten, diphenylcyclopropanone (DPCP), has been examined after a single challenge on Day 3 and Day 14 in de novo sensitized individuals (3), who reached an inflammatory/effector peak on Day 3 and a more regulated immune response on Day 14. The peak response showed high expression of cytokines that defined all major effector T-cell subsets, whereas the resolution reaction was characterized by marked decreases in expression of interleukin-2 (IL-2), IFN γ , and other T-cell derived cytokines. Combined, these few studies have provided a glimpse of the early gene expression time-course events of CA. However, the gene expression kinetics and time-course during repeated skin exposures to a contact allergen remain unknown. Hitherto, no time-course studies have been conducted in which de novo sensitized individuals are repeatedly exposed to a potent, experimental contact allergen such as DPCP. This type of repeated CA exposure model may shed light on the alterations that take place in human skin over time and may also provide a useful method to study CA reactions de novo. Accordingly, we evaluated the kinetics and time-course of the developing cutaneous reactions to DPCP in 10 de novo sensitized individuals by transcriptomics analysis. In addition, we examined the histopathological changes over time and performed immunohistochemical staining with antibodies specific for T cells and other immune markers.

MATERIALS AND METHODS

Volunteers

Ten healthy volunteers (1 woman and 9 men) between 20 and 41 years old (mean age: 24.5 years) were included. Volunteers who had been diagnosed with atopic dermatitis or received any active or prior treatment, 30 days before study inclusion, with ultraviolet radiation, topical corticosteroids or with other immunosuppressive medication were excluded from the study. The study was conducted from October to May to reduce any possible influence of UV exposure leading to an impaired immune responsiveness (4,5). Written informed consent was obtained from all participants. The study

was approved by The Regional Scientific Ethical Committees for Southern Denmark (Study-ID: S-20120085) and was conducted in accordance with the Declaration of Helsinki Principles.

Sensitization with DPCP

Contact sensitivity to DPCP was induced by the application of a single dose of DPCP of $30\text{ }\mu\text{g}\cdot\text{cm}^{-2}$ in a 12 mm Finn chamber (SmartPractice, Phoenix, AZ, USA) to the upper buttock for 48 h (6). The sensitizing dose was applied to the 11 mm Finn chamber filter paper as $22.8\text{ }\mu\text{l}$ of 0.125% DPCP in acetone. Preparation of DPCP (CAS no. 886-38-4; Alfa Aesar, Karlsruhe, Germany) solutions for sensitization, initial elicitation challenge and repeat challenges were performed by the Central Pharmacy, Odense University Hospital.

Initial elicitation challenge

The presence of contact sensitivity to DPCP was confirmed after 4 weeks, when the volunteers responded to an elicitation challenge on the inner upper-arm, consisting of 2 panels, each comprising a dose-series of 5 DPCP-loaded patches. Panel 1 was used for visual scoring and skinfold thickness measurements (7), while panel 2 was intended for biopsies. The DPCP doses were applied to filter-paper-lined 8 mm Finn Chambers as $10\text{ }\mu\text{l}$ of the appropriate concentration of DPCP in acetone to give the required dose range, increasing by 60 % increments. The chambers were loaded immediately before attachment to the skin and the doses were 1.24 , 1.98 , 3.17 , 5.1 and $8.12\text{ }\mu\text{g}\cdot\text{cm}^{-2}$. In addition, two vehicle control patches, each loaded with $10\text{ }\mu\text{l}$ of acetone, were applied. Elicitation patches were removed exactly 6 h after application (6). The responses were assessed visually in panel 2, 24 h after application and graded as negative (-), doubtful (?+), 1+, 2+, 3+ or 4+ using a modified version of the International Contact Dermatitis Research Group (ICDRG) clinical scoring system (Table S1). In panel 2, four 4 mm punch biopsies, two from a vehicle control site and two from a DPCP challenged site, were taken from each volunteer 24 h after application of patches. A patch test reaction of 1+ (corresponding to a weak positive reaction) was the intended target reaction used for selecting the

DPCP biopsy site in each participant. If such a challenge reaction was not obtained a doubtful or negative reaction was selected instead (for more details, see Figure S1).

Repeat challenges

Each individual received a series of four or five repeated challenges at 4-weekly intervals. Each challenge comprised two groups of challenge patches, one for clinical assessment and skinfold thickness measurements and one for biopsies. All challenges were applied with filter-paper-lined 8 mm Finn Chambers and removed after 6 h.

For biopsy, the same dose, as was biopsied at the initial elicitation challenge was also applied in an extra chamber and at 24 h two 4mm punch biopsies were taken from the challenge site. The biopsies taken from DPCP reactions in the initial elicitation challenge and repeat challenges were used for examining histopathological changes and the gene expression and immunohistochemical time-course of CA.

In order to avoid increased rechallenge reactivity at previously DPCP-challenged sites (8), patches were applied on previous non-challenged sites by using placement templates. Repeat challenges were also carried out alternately on the inner aspect of the left and right upper arm. A schematic of DPCP sensitization, the initial elicitation challenge and repeat challenges is provided in Figure S2.

RNA extraction, amplification and microarray hybridization

Full-thickness biopsy specimens were homogenized in a TissueLyser (Qiagen, Valencia, CA, USA). Total RNA was purified and extracted with the RNeasy Micro Kit (Qiagen), according to the manufacturer's instructions. RNA quantity was measured on a NanoDrop 1000 (Thermo Fisher Scientific, Wilmington, DE, USA) spectrophotometer, while the RNA integrity was assessed using Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). Synthesis of first- and second strand cDNA and biotin-modified aRNA followed by aRNA purification and fragmentation were performed using the GeneChip 3' IVT Express Kit (Affymetrix, Santa Clara, CA, USA). The fragmented and labeled aRNA was hybridized to Affymetrix Human Genome U133 Plus 2.0 Arrays.

Microarray gene expression data analysis

The mRNA expression data have been deposited at NCBI's Gene Expression Omnibus (GEO Series accession number GSE71996, <http://www.ncbi.nlm.nih.gov/geo/>). Probe cell intensity data was analyzed in the Expression Console software (Affymetrix, 2013) using the RMA algorithm to create CHP files. The RMA workflow includes quantile normalization and general background correction. Considering the time-course design and data structure, we applied the linear mixed-effect model with fractional polynomials as described by Tan and colleagues (1). Sub-models with effect for one treatment were also fitted for specific time points. Prior to this time-course analysis, probe-sets detecting a low expression variation across samples were filtered out (variance < 0.20) leaving 8602 probe-sets for further analysis. Statistical analyses were conducted on a probe-set level. The linear mixed-effect models were fitted using the R-package *lme4* using FDR to adjust for multiple testing. The genes that displayed a significant 1st order (monotonous) expression pattern were visualized in a plot with vehicle control to rechallenge 5 on the X-axis and the standardized gene expression measure on the Y-axis.

Differential gene expression analysis between vehicle controls and rechallenges 1-5 were performed by a paired t-test and by applying a cut-off filter ($FDR \leq 0.05$ and $FC \geq 2$). Expression data was visualized by heat maps and unsupervised hierarchical clustering in Qlucore Omics Explorer 3.1 (Qlucore AB, Lund, Sweden). The differentially expressed genes between vehicle control and rechallenge 1 were validated by comparison to and clustering of an independent dataset (3).

Network representation of differentially expressed genes was performed in Ingenuity Pathway Analysis (IPA, Ingenuity Systems). Gene expression levels are indicated in the IPA networks as red (upregulated) or green (downregulated).

Quantitative RT-PCR

Quantitative real-time RT-PCR analysis (qRT-PCR) of fifteen selected genes was performed by AROS Applied Biotechnology A/S (Aarhus, Denmark). RNA samples from seven complete volunteer series were included in the qRT-PCR analysis. cDNA was synthesized from 100 ng of RNA per

sample using High Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific, Waltham, MA, USA). The qPCR reaction was performed with TaqMan Universal PCR Master Mix (Applied Biosystems, USA) and GE Sample Loading Reagent (Fluidigm, San Francisco, CA, USA). The real-time PCR data was analysed using the Fluidigm BioMark software ver. 4.1.3 and normalized to *PPIA* (Hs03045993_gH), *B2M* (Hs99999907_m1), *RPLP0* (Hs00420895_gH) and *TBP* (Hs00920498_m1) reference genes. The following TaqMan assays (Thermo Fisher Scientific, Waltham, MA, USA) were used: *CXCL10* (Hs01124252_g1), *S100A9* (Hs00610058_m1), *MMP12* (Hs00159178_m1), *SERPINB4* (Hs01691258_g1), *CA6* (Hs01547831_m1), *AQP5* (Hs00387048_m1), *ID4* (Hs02912975_g1), *KRT15* (Hs00267035_m1), *GZMB* (Hs00188051_m1), *IL6* (Hs00985639_m1), *CCL13* (Hs00234646_m1), *CXCL1* (Hs00236937_m1), *CD274* (Hs01125301_m1), *TNFAIP2* (Hs00969305_m1) and *CTLA4* (Hs00175480_m1). Samples were analyzed in triplicates. Δ CT-values (gene expression relative to reference genes) and $\Delta\Delta$ CT-values (gene expression relative to positive control) were determined. FC ($2^{\Delta\Delta\text{CT}}$) was calculated using $\Delta\Delta$ CT-values.

Histopathology

Sections were cut from the formalin-fixed, paraffin-embedded blocks and stained with haematoxylin and eosin (H&E) and immunohistochemically with five different antibodies. The antibodies used in this study are listed in Table S2. H&E stained sections were visually graded (0-3) for inflammatory intensity, eosinophils and spongiosis. The IL5 stained sections (epidermal compartment only, because the dermal staining was more disperse and focal and thus difficult to grade) were also scored semiquantitatively using the same range. All evaluations were blinded. Evaluation of H&E and IL5 were performed by the same observer (OC).

Computer-assisted image analysis

Slides immunostained with antibodies to CD4⁺, CD8⁺, CD1a⁺ and Foxp3⁺ were scanned using a digital NanoZoomer slide scanner (NanoZoomer 2.0-HT C9600, Hamamatsu Photonics, Hamamatsu, Japan). Visiormorph software package (Visiopharm Integrator System (VIS) program, software

version 4.6.1.630, Visiopharm, Hørsholm, Denmark) was used for classifier training (i.e. software classifiers designed and trained by the observer to recognize nuclei with different morphology), manual outlining and labelling of positively stained cells and regions of interest (ROI).

CD4⁺ and CD8⁺ T cells were counted in an epidermal and dermal area of 1 mm², whereas CD1a⁺ and Foxp3⁺ cells were counted in the entire epidermal compartment and in a dermal area of 1 mm², respectively. In general, 2 sections were evaluated at each time point. However, when hair follicles, sebaceous glands and sweat glands were detected, that part of the dermal compartment was excluded from evaluation. These epithelial structures express an inflammatory reaction different, less or more intense, from that in the surrounding dermis, excluding comparison between different biopsies with varying representation of adnexal structures.

Statistical analyses

Statistical analyses were performed with GraphPad InStat, v.3.00 (GraphPad Software, San Diego, CA). Data are presented as mean $\pm$ standard error of the mean (SEM). Histopathological and immunohistochemical data were analyzed using the Friedman Test due to the repeated measures design. Dunn's multiple comparisons test was applied when significant differences were detected. $P < 0.05$ was considered statistically significant.

RESULTS

Minor histopathological and immunohistochemical changes at the initial elicitation challenge compared with subsequent challenges

Only minor histopathological changes emerged at the initial elicitation challenge to DPCP compared with vehicle control. DPCP induced slight spongiosis, a sparse infiltration with lymphocytes, mostly perivascular, and small numbers of eosinophils, perivascular and interstitially. A more pronounced spongiosis, lymphocytic inflammation, as well as an increased infiltration of eosinophils were observed at rechallenge 1 and subsequent rechallenges (Fig. 1a). Clear predominance of CD4⁺ over CD8⁺ T cells was observed throughout the time-course. The infiltration of these lymphocyte subsets

exhibited the same pattern until rechallenge 3 where the number of CD4⁺ cells remained stable (Fig. 1b), whereas CD8⁺ cells decreased (Fig. 1c). The expression level of IL-5, a member of the cytokine profile of the CD4 Th2-subpopulation, did also increase at rechallenge 1 and remained stable during the remaining challenges (Fig. 1d). Regulatory T cells (Tregs) expressing the signature marker, forkhead box P3 (Foxp3), were present in low numbers in the vehicle control and at the initial elicitation challenge to DPCP. An increase was detected at rechallenge 1. Nevertheless, this increase did not change significantly from rechallenges 2-5 (Fig. 1e). Langerhans cells expressing the CD1a protein were, as expected, almost exclusively found in the stratum spinosum layer of the epidermis. The number of Langerhans cells did not change significantly during the time-course of the study (Fig. 1f).

Time-course analysis revealed a predominant monotonous gene expression pattern

Prior to the time-course analysis, probe-sets displaying a low expression variation across samples were filtered out (variance < 0.20) leaving 8602 probe-sets for further analysis. These probe-sets were fitted to a linear mixed-effect model with the purpose of profiling the gene expression pattern in the DPCP reactions. This analysis investigated whether a monotonous or complex mixed-effect model could adequately fit the data. Among the 8602 variance filtered probe-sets, 1556 probe-sets (corresponding to 1116 genes) displayed a significant monotonous pattern (false discovery rate (FDR) ≤ 0.05) while only a small number of probe-sets (41) displayed a complex pattern. Among probe-sets displaying a monotonous time-course pattern, two subgroups containing 614 and 940 probe-sets (corresponding to 486 and 630 genes, respectively) exhibited an increasing or decreasing expression pattern, respectively (see Table S3 for a selected list of probe-sets and genes displaying a monotonous expression pattern; a complete list of genes/probe-sets is available upon request). Four genes (*C5orf24*, *LINC00908*, *PPA2* and *ZNF230*; each detected by more than one probe-set) apparently demonstrated both up- and downregulation in the time-course pattern, which may be explained by their expression signal being close to the background noise. Genes displaying the increasing and decreasing expression patterns in a plot — beginning with the vehicle control and ending with

rechallenge 5 — are shown in Figs. 2a and b. Only minor gene expression changes were observed between vehicle control and initial elicitation challenge. In contrast, major gene expression changes were found between the initial elicitation challenge and rechallenge 1. The gene expression level remained relatively constant from rechallenge 1 to rechallenge 5 suggesting that a plateau had been reached.

Comparison of gene expression following initial DPCP elicitation challenge and rechallenges 1-5

Using a paired t-test ($FDR \leq 0.05$ and $FC \geq 2$ ($\log_2 FC \geq 1$)) to compare gene expression between vehicle control and initial DPCP elicitation challenge revealed no significant differentially expressed genes. However, comparing the vehicle control with the first DPCP rechallenge showed significant changes in expression of 2941 genes. These 2941 genes were followed across all samples from vehicle control, initial elicitation challenge to rechallenges 1-5 in order to determine the expression pattern over time. A clear separation of upregulated and downregulated genes could be seen in all 10 volunteers (Fig. 3a). The vast majority of the differentially expressed genes between vehicle control and rechallenges 1-5, were the same (see Table 1 for a list of the top 10 upregulated and downregulated genes across rechallenges). A few of these consistent gene markers were associated with specific T cell subsets (Th1-related: *CXCL10* and *CXCL11*; Th22-related: *S100A9*). Genes encoding pro-inflammatory proteins such as *MMP12*, *HAS3* and *SERPINB4* were also among the top 10 upregulated genes. Other significantly upregulated gene transcripts further down the lists (present at all rechallenges), include markers of T cell related genes (*CD2*, *ITK*, *CD3D* and *JAK3*), immunosuppressive immune regulators (*LILRB2*, *FAS*, *PTPRC*, *PRDM1*, *HAVCR2* and *CD274*) and chemokines (*CXCL1*, *CCL17* and *CCL19*). Further, molecules involved in Treg function and/or immunosuppressive immune regulation (upregulated at one or more rechallenges) include *granzyme B*, *TGF- β 1/TGF- β 2*, *IL-6*, *CTLA4*, *CD80* and *CD86*. Differential immune skewing towards an IL1-, IL17- (Th17) and IFN γ - (Th1) response was demonstrated using Ingenuity Pathway Analysis (IPA). The interaction networks of these three upstream regulators contain most of the differentially

expressed genes listed in Table 1. The genes in the respective networks are shown in Fig. S3. A full list of genes that were upregulated or downregulated between vehicle control and rechallenges 1-5 is available on request.

Validation of microarray data by quantitative RT-PCR

The expression pattern of fifteen differentially expressed genes was verified by qRT-PCR, which showed that all samples clustered according to challenge. Further, co-clustering of genes and assays suggests strong agreement between array and PCR data (Fig. S4). The gene expression levels of the fifteen genes assessed by qRT-PCR strongly correlated ($R^2 \approx 0.82-0.98$) with the corresponding microarray data.

DISCUSSION

We have previously reported that repeated challenges with DPCP in newly sensitized volunteers result in a plateau response after second challenge evaluated by clinical scoring and measurement of skin fold thickness (7). Here, we confirm this novel clinical finding by histopathological, immunohistochemical and global gene expression analysis of skin biopsies from challenge sites collected 24 hours after challenge patch test. That reactivity reaches a plateau indicates that a regulatory control mechanism is operating that prevents an unrestricted increase in reactivity. One such inhibitory mechanism can be an inhibition of the continued expansion of relevant T effector cells, while another may be mediated by Tregs, which could suppress the activities of T effector cells through inhibitory cytokines, by cytolysis, or modulate the maturation and/or function of dendritic cells (9-11). We found an increase followed by a plateau level of FoxP3⁺CD4⁺ Tregs in the sites of allergen challenge, in addition to significantly upregulated genes encoding inhibitory cytokines. Collectively, the increased expression of *granzyme B*, *TGF- β 1/TGF- β 2*, *IL-6*, *CTLA4*, *CD80* and *CD86* indicate that Tregs are involved in regulating the inflammatory response at the plateau level of CA in humans. The above microarray-based findings were confirmed by qRT-PCR. The immunosuppressive immune regulators (*CTLA4*, *LILRB2*, *FAS*, *PTPRC*, *PRDM1*, *HAVCR2* and

CD274) significantly expressed in our study also correspond with the findings in a recent DPCP study in humans (12). The pro-inflammatory genes listed in Table 1 (*MMP12*, *SERPINB4*, *CXCL10* and *S100A9*) were also among the top 10 upregulated genes at Day 3 after a single challenge with DPCP in an independent dataset from Gulati et al (3). The differentially expressed genes between vehicle control and rechallenge 1 samples from our study were compared with placebo samples (Day 3) and treated samples (Day 3) from the Gulati dataset by heat map visualization (Fig. 3b) using the same order of genes as in our data set in Fig. 3a. The heat map shows that the differentially expressed genes identified in our study follow the same pattern of expression in the Gulati et al. data; i.e. genes that are induced at rechallenge 1 are also induced at Day 3, and vice versa. The large overlap of differentially expressed genes (probe level) between Day 3 vs. placebo in Gulati et al. and rechallenge 1 and vehicle control is also illustrated in the Venn diagram in Fig. 3c (FDR<0.05). Approximately two-thirds of the upregulated genes and half of the downregulated genes at rechallenge 1 were contained in the Gulati Day 3 signature. These results suggest that the gene expression signature not only remains constant at the plateau level of the CA response to DPCP, but also that the early (24 h) and peak (72 h) inflammatory stages are somewhat similar. The large overlap of genes between the first repeat challenge and the Gulati Day 3 data confirms this latter observation.

We found that as the strength of the inflammatory response increased to a plateau level following repeated challenges, the degree of infiltration by helper, cytotoxic and regulatory T cells (CD4+, CD8+ and Foxp3+) increased accordingly. Technological constraints prevented us from quantifying the numbers of DPCP-specific T cells in the tissues; such estimates could be made by in vitro limiting dilution analyses of peripheral blood mononuclear cells.

This is the hitherto first time-course analysis of genome-wide gene expression data obtained from repeated exposure to a newly encountered potent contact allergen. The results may be different for a weak allergen. The time-course mRNA expression profiling of serial samples collected from cutaneous reactions to DPCP have revealed a yet unknown ‘molecular picture’ of the cell state at the transcriptional level. It appears that gene activity and expression over time predominantly follow a monotonous time-course pattern. Thus, this study supports the previously reported clinical observations that repeated DPCP exposure leads to a plateau level of immune reactivity and not

boosting or down regulation of immune reactivity over a 5-6 month time period. The plateau level of reactivity following repeated DPCP exposure in volunteers in this experimental design supports that the volunteers may participate in repeated clinical experiments where DPCP is applied.

In conclusion, we have shown by use of global gene expression analysis, histopathology and immunohistochemical staining for helper, cytotoxic and regulatory T cells (CD4+, CD8+ and Foxp3+) that 4-weekly DPCP challenges in de novo sensitized individuals result in immune responses attaining a plateau level after 2nd challenge.

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CONFLICTS OF INTEREST

M.A.R. and T.L. are LEO Pharma employees, the pharmaceutical company that partially financed this study. The remaining authors have no conflict of interest to declare.

AUTHOR CONTRIBUTIONS

KFM and KEA initiated the study and drafted the protocol with support from LS and PSF. All authors contributed to design details. KFM performed all clinical experiments and collected biopsy material. KFM, OC and BWK established the immunohistochemical classifier and KFM performed the

immunohistochemical examination, OC performed the histopathological examination. KFM, QT, TK, MB and MT were involved in acquisition of mRNA data. QT, MB, MT, TK, TL and KFM analyzed and interpreted the mRNA data. KFM drafted the manuscript. All authors critically revised the manuscript. All authors have read and approved the last version of the manuscript.

REFERENCES

1. Tan Q, Thomassen M, Hjelmberg JV *et al.* A growth curve model with fractional polynomials for analysing incomplete time-course data in microarray gene expression studies. *Advances in Bioinformatics* 2011; Article ID 261514:1-6.
2. Pedersen MB, Skov L, Menné T *et al.* Gene expression time course in the human skin during elicitation of allergic contact dermatitis. *Journal of Investigative Dermatology* 2007;127:2585-2595.
3. Gulati N, Suárez-Fariñas M, Fuentes-Duculan J *et al.* Molecular characterization of human skin response to diphencyprone at peak and resolution phases: therapeutic insights. *Journal of Investigative Dermatology* 2014;134:2531-2540.
4. Bruze M. Seasonal influence on routine patch test results. *Contact Dermatitis* 1986; 14: 184.
5. Cooper KD, Oberhelman L, Hamilton TA, *et al.* UV exposure reduces immunization rates and promotes tolerance to epicutaneous antigens in humans: relationship to dose, CD1a-DR+ epidermal macrophage induction, and langerhans cell depletion. *Proc Natl Acad Sci* 1992; 89: 8497-8501.
6. Harper Smith AD, Coakley SL, Ward MD, *et al.* Exercise-induced stress inhibits both the induction and elicitation phases of in vivo T-cell-mediated immune responses in humans. *Brain Behav Immun* 2011; 25: 1136-1142.

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7. Mose KF, Andersen F, Skov L, *et al.* Repeated monthly epicutaneous challenges result in a clinically reproducible level of contact allergy in de novo sensitized individuals. *British Journal of Dermatology* 2017; DOI: 10.1111/bjd.14949
 8. Hindsén M, Bruze M, Christensen OB. The significance of previous allergic contact dermatitis for elicitation of delayed hypersensitivity to nickel. *Contact Dermatitis* 1997; 37: 101-106.
 9. Vignali DAA, Collison LW, Workman CJ. How regulatory T cells work. *Nature Reviews Immunology* 2008;8:523-532.
 10. Shevach EM. From vanilla to 28 flavors: multiple varieties of T regulatory cells. *Immunity* 2008;25:195–201.
 11. Shevach EM. Biological Functions of Regulatory T Cells. *Advances in Immunology* 2011;112:137-176.
 12. Gulati N, Suárez-Fariñas M, Correa da Rosa J *et al.* Psoriasis is characterized by deficient negative immune regulation compared to transient delayed-type hypersensitivity reactions. *F1000 Research* 2015;4:149.

Table 1. Top 10 upregulated and downregulated genes in diphenylcyclopropenone (DPCP) rechallenges 1 through 5 versus vehicle control. Gene expression changes among top 10 upregulated and downregulated genes at rechallenge 1 were monitored at rechallenges 2-5.

Transcripts marked with * were among top 10 upregulated or downregulated genes at the specified time points.

			Rechallenge 1		Rechallenge 2		Rechallenge 3		Rechallenge 4		Rechallenge 5	
Affymetrix ID	Gene symbol	Gene title	q-value	Fold change (Log2)	q-value	Fold change (Log2)	q-value	Fold change (Log2)	q-value	Fold change (Log2)	q-value	Fold change (Log2)
a) Top upregulated gene transcripts												
211906_s_at	SERPINB4	serpin peptidase inhibitor, clade B (ovalbumin), member 4	9,39E-04	7,17	4,72E-03	5,65 *	3,86E-05	7,33 *	1,88E-04	7,88 *	1,64E-02	8,87 *
204580_at	MMP12	matrix metalloproteinase 12 (macrophage elastase)	1,37E-06	6,90	2,96E-03	5,62 *	2,69E-06	6,74 *	9,21E-06	6,60 *	1,24E-02	6,60 *
204533_at	CXCL10	chemokine (C-X-C motif) ligand 10	5,41E-04	6,68	2,96E-03	5,78 *	7,61E-05	6,50 *	1,12E-04	5,64 *	3,38E-02	6,14 *
210163_at	CXCL11	chemokine (C-X-C motif) ligand 11	1,02E-03	6,38	4,63E-03	4,87 *	1,16E-04	5,92 *	3,03E-04	5,07 *	5,68E-02	5,73 *
203535_at	S100A9	S100 calcium binding protein A9	1,98E-03	5,53	3,58E-03	4,72 *	9,21E-05	5,89 *	1,40E-04	6,08 *	2,60E-02	6,42 *
209720_s_at	SERPINB3	serpin peptidase inhibitor, clade B (ovalbumin), member 3	2,70E-03	5,26	3,60E-03	4,41 *	2,53E-04	5,34 *	6,19E-04	5,82 *	3,13E-02	7,01 *
206211_at	SELE	selectin E	3,28E-05	5,26	8,16E-04	5,09 *	5,70E-07	5,45 *	3,21E-06	5,21 *	1,56E-02	5,06
206932_at	CH25H	cholesterol 25-hydroxylase	4,32E-04	5,15	2,96E-03	4,71 *	1,06E-06	5,12 *	9,49E-06	5,09 *	1,25E-02	5,45 *
227697_at	SOCS3	suppressor of cytokine signaling 3	5,41E-04	4,93	2,96E-03	4,54 *	2,64E-06	5,29 *	1,24E-05	5,36 *	1,56E-02	5,52 *
223541_at	HAS3	hyaluronan synthase 3	1,11E-03	4,91	2,99E-03	4,51 *	2,09E-05	5,31 *	4,29E-05	5,56 *	1,95E-02	5,82 *
b) Top downregulated gene transcripts												
205856_at	SLC14A1	solute carrier family 14, member 1	5,36E-03	-3,43	7,15E-03	-2,88 *	3,94E-04	-4,06 *	1,38E-03	-3,59 *	2,56E-02	-5,15 *
204734_at	KRT15	keratin 15	4,07E-03	-3,01	7,35E-03	-2,45 *	4,52E-05	-3,44 *	5,89E-04	-4,01 *	1,75E-02	-4,55 *
210576_at	CYP4F8	cytochrome P450, family 4, subfamily F, polypeptide 8	1,52E-02	-2,97	8,18E-03	-2,33 *	3,34E-02	-2,25	3,45E-03	-2,69	1,79E-02	-4,06 *
213456_at	SOSTDC1	sclerostin domain containing 1	2,69E-03	-2,86	5,76E-03	-1,92	5,77E-05	-2,87 *	5,50E-04	-3,48 *	2,71E-02	-3,61
209292_at	ID4	inhibitor of DNA binding 4, dominant negative helix-loop-helix protein	3,82E-03	-2,77	5,82E-03	-1,97	6,25E-05	-2,76	5,63E-04	-3,33 *	2,46E-02	-3,58
206393_at	TNNI2	troponin I type 2	2,61E-03	-2,65	4,88E-03	-2,25 *	1,00E-04	-2,96 *	2,22E-04	-2,83	1,68E-02	-3,62
203697_at	FRZB	frizzled-related protein	4,62E-03	-2,64	7,01E-03	-1,80	2,72E-04	-2,65	7,35E-04	-3,13 *	2,07E-02	-3,83 *
213611_at	AQP5	aquaporin 5	7,96E-03	-2,64	1,36E-02	-1,72	1,75E-03	-2,45	2,19E-03	-2,50	3,27E-02	-3,81 *
232481_s_at	SLITRK6	SLIT and NTRK-like family, member 6	1,77E-03	-2,61	3,08E-03	-2,27 *	5,92E-05	-2,73	7,04E-05	-2,93	1,29E-02	-3,47
206873_at	CA6	carbonic anhydrase VI	3,64E-03	-2,59	6,88E-03	-1,64	9,77E-04	-2,22	2,76E-03	-2,45	2,25E-02	-3,53

FIGURE LEGENDS

Figure 1. Histopathological and immunohistochemical changes over time for DPCP. (a)

Spongiosis, intensity of perivascular/interstitial lymphocytic infiltration and infiltration with eosinophils were significantly induced at rechallenge 1, whereafter a constant level was maintained throughout the time-course. **(b,c)** A brisk infiltration of CD4⁺ and CD8⁺ T cells was observed at rechallenge 1 followed by a plateau response. **(d)** High levels of expression of IL-5 and **(e)** a rich infiltration of forkhead box P3 (Foxp3) was seen at rechallenge 1 and remained stable in the following challenges. **(f)** Expression of CD1a was unchanged during the time-course of the study. Error bars denote SEM ($n=10$; $^{\vee}\text{Rc5}$: $n=5$). Significant changes from the vehicle control are designated by: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. VC: vehicle control; IEC: initial elicitation challenge; Rc1-5: rechallenge 1-5.

Figures 2a and b. Time-course analysis of (a) 614 and (b) 940 probe-sets (corresponding to 486 and 630 genes, respectively) displaying a monotonous expression pattern.

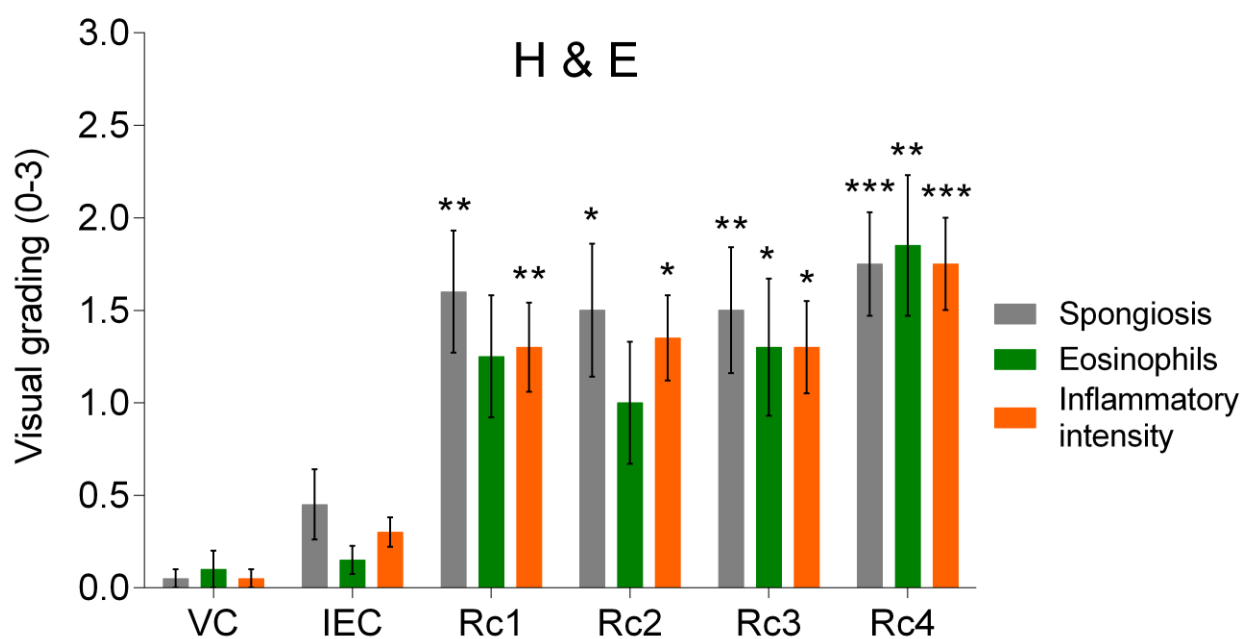
Genes displaying a significant increasing ($n=486$) and decreasing ($n=630$) trend followed by a plateau are visualized in plots with vehicle control to rechallenge 5 on the X-axes and the standardized gene expression measure on the Y-axes.

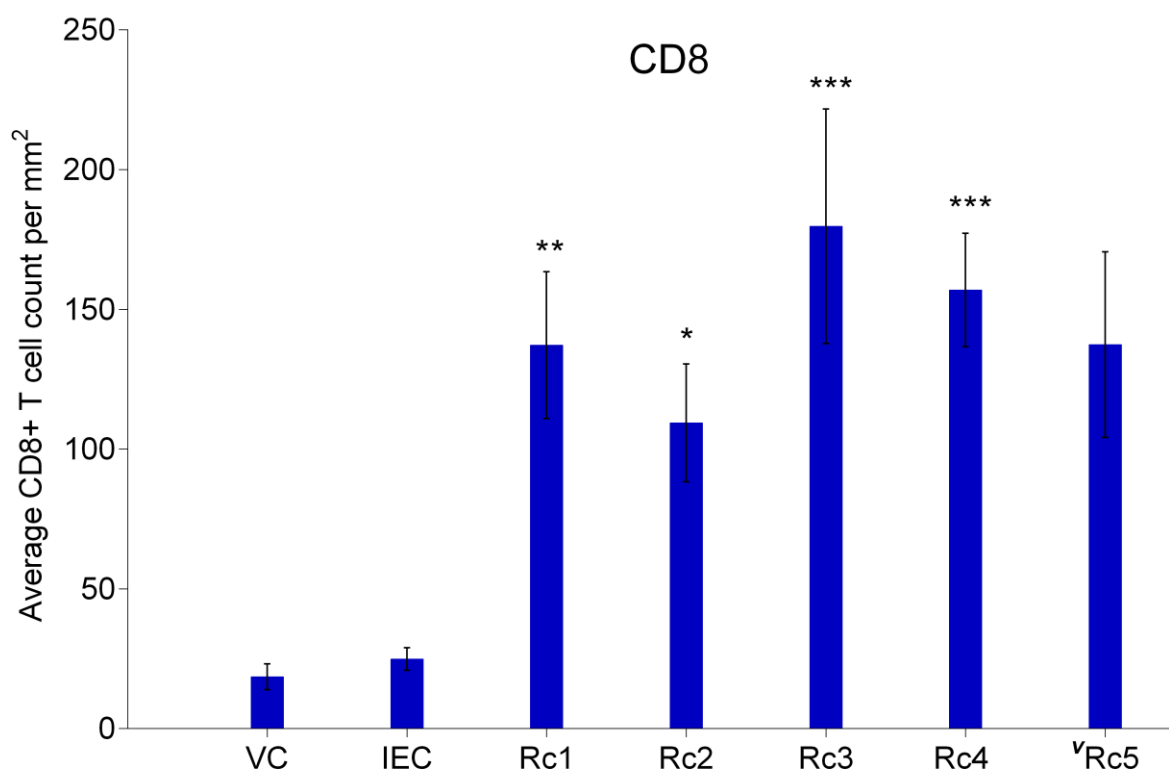
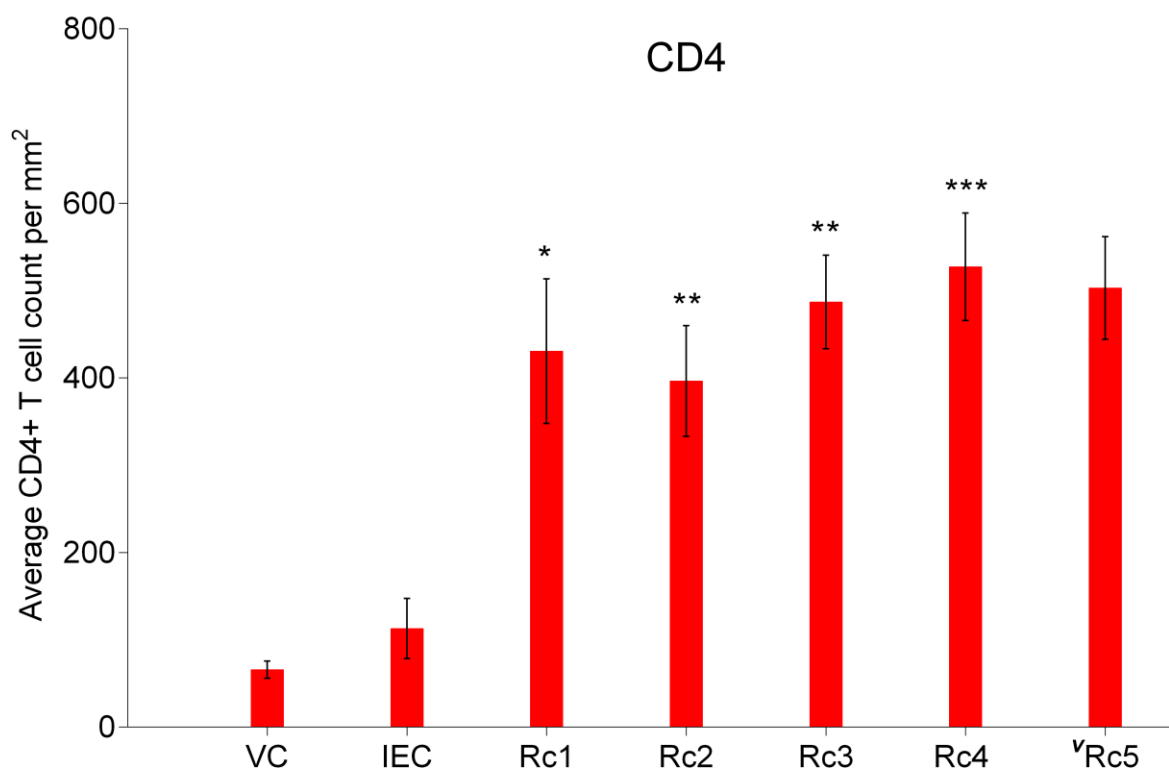
Figure 3a. Differential gene expression induced by diphenylcyclopropanone (DPCP).

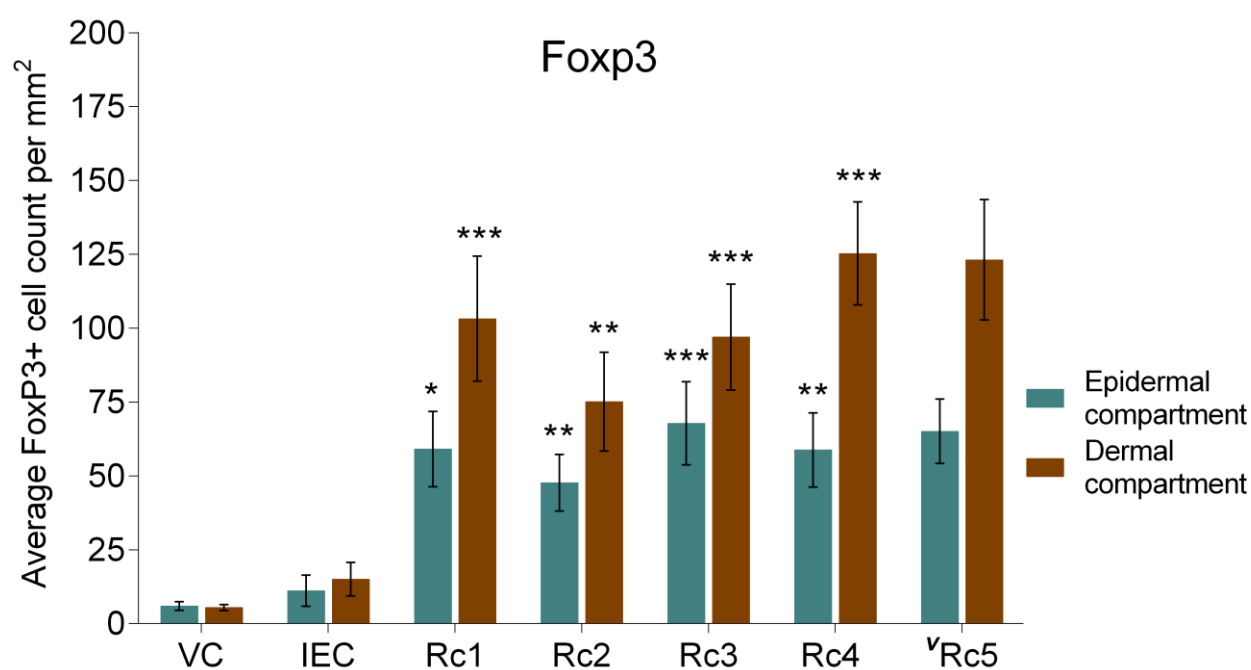
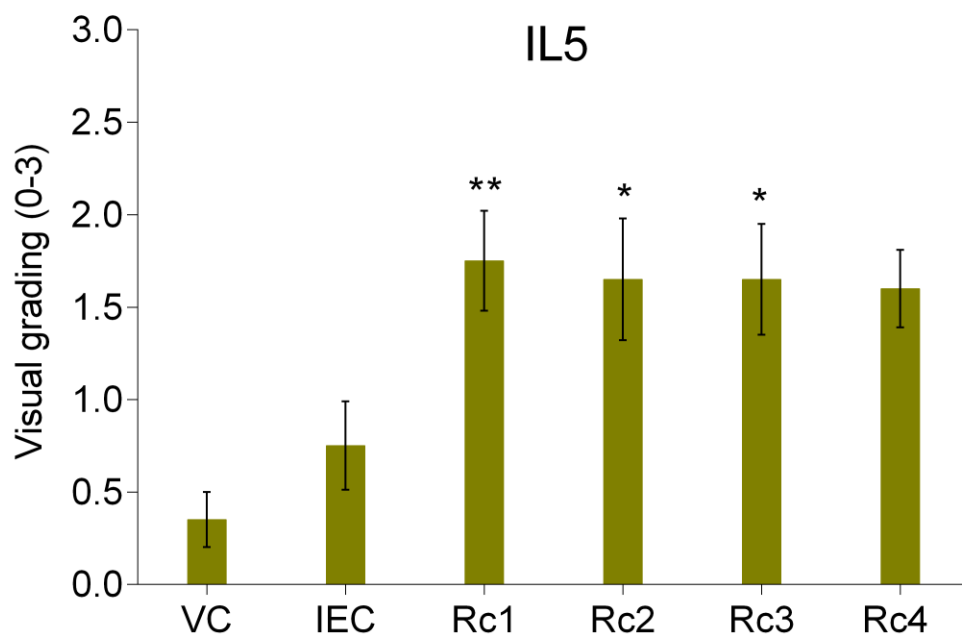
Heat map based on 2941 differentially expressed genes between vehicle control (v) and rechallenge 1 (Rc1). Filters: variance ($s/s_{\max}$) > 0.1 , $q = 0.05$, 2-fold change. The heat map is ordered according to treatment in the x-direction (color coded top row), each column representing one sample, and according to PC1 (Principal Component 1) in the y-direction, each row representing one gene. All expression values have been z-scaled (Mean = 0, Var = 1). Gene expression levels are indicated in the heat map as red (upregulated) or green (downregulated).

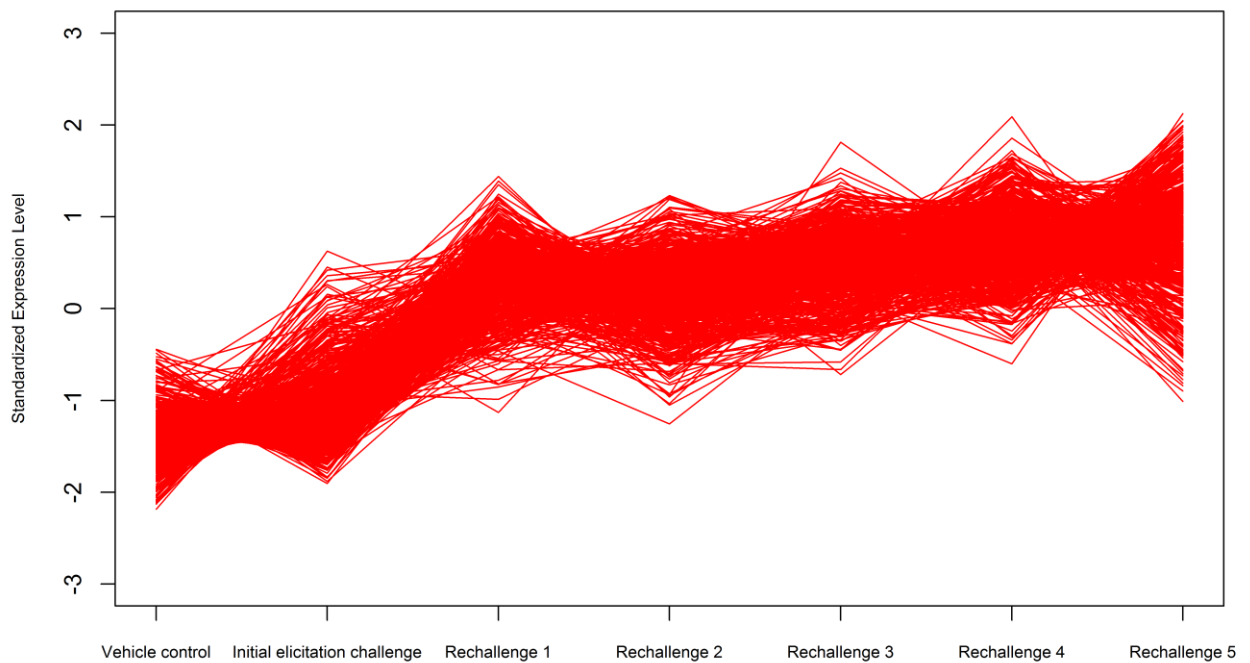
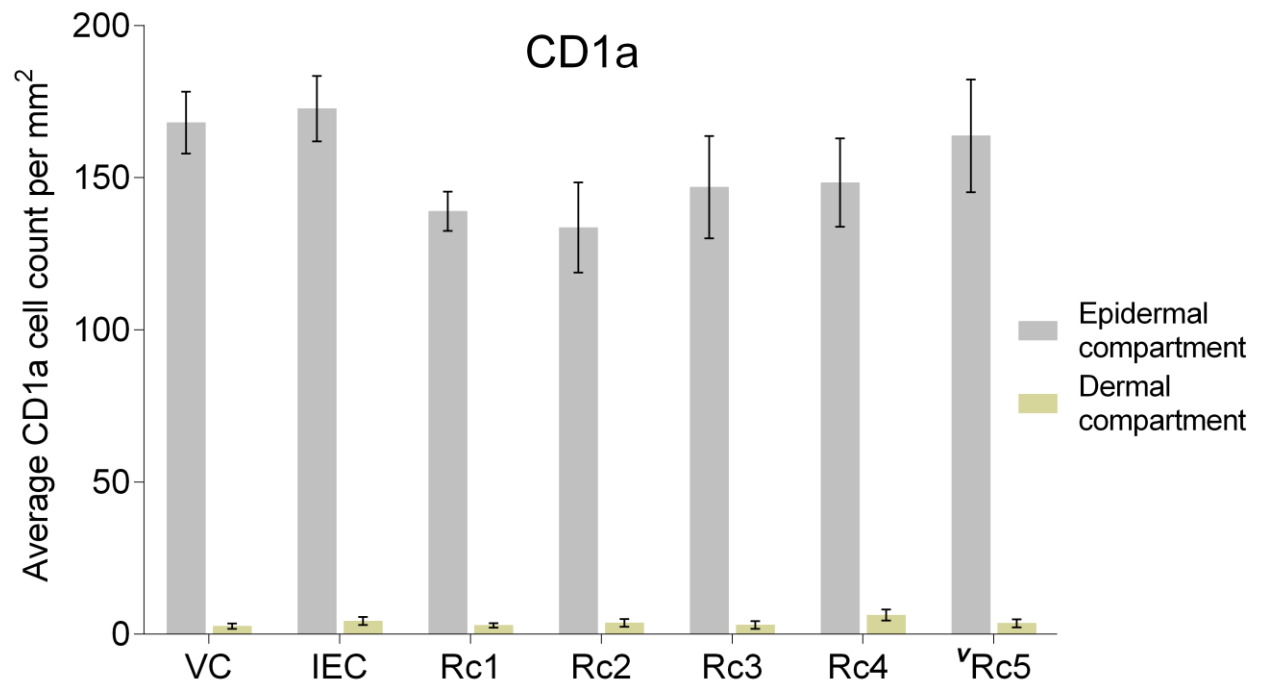
Figure 3b. Correlation between early time points in gene expression datasets. Heat map based on the 2941 differentially expressed genes presented in Figure 3a, but applied on the Gulati et al. Day 3 data: DPCP (green) vs. placebo control (black). The heat map is ordered according to treatment in the x-direction (placebo or DPCP), each column representing one sample (following the original numbering from Gulati et al.) and according to PC1 (principal component 1) in the y-direction, each row representing one gene, and in the same order as in Fig. 3a. The subject ID numbers ($n=11$) from the Gulati et al. data are shown in the bottom x-direction. Gene expression levels are indicated in the heat map as red (upregulated) or green (downregulated).

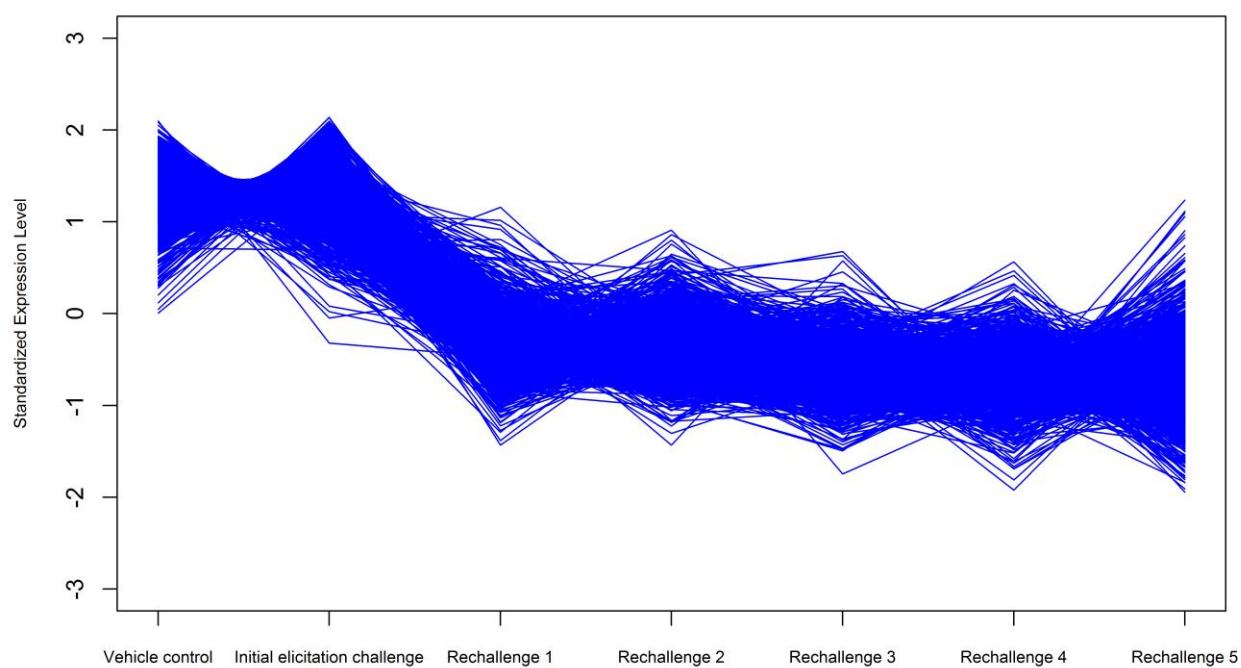
Figure 3c. Venn diagram of early time points in gene expression datasets. Venn diagram showing the overlap in differentially expressed genes (probe level) between Day 3 vs. placebo in Gulati et al. and rechallenge 1 and vehicle control in the current study (cut-off: FDR=0.05).

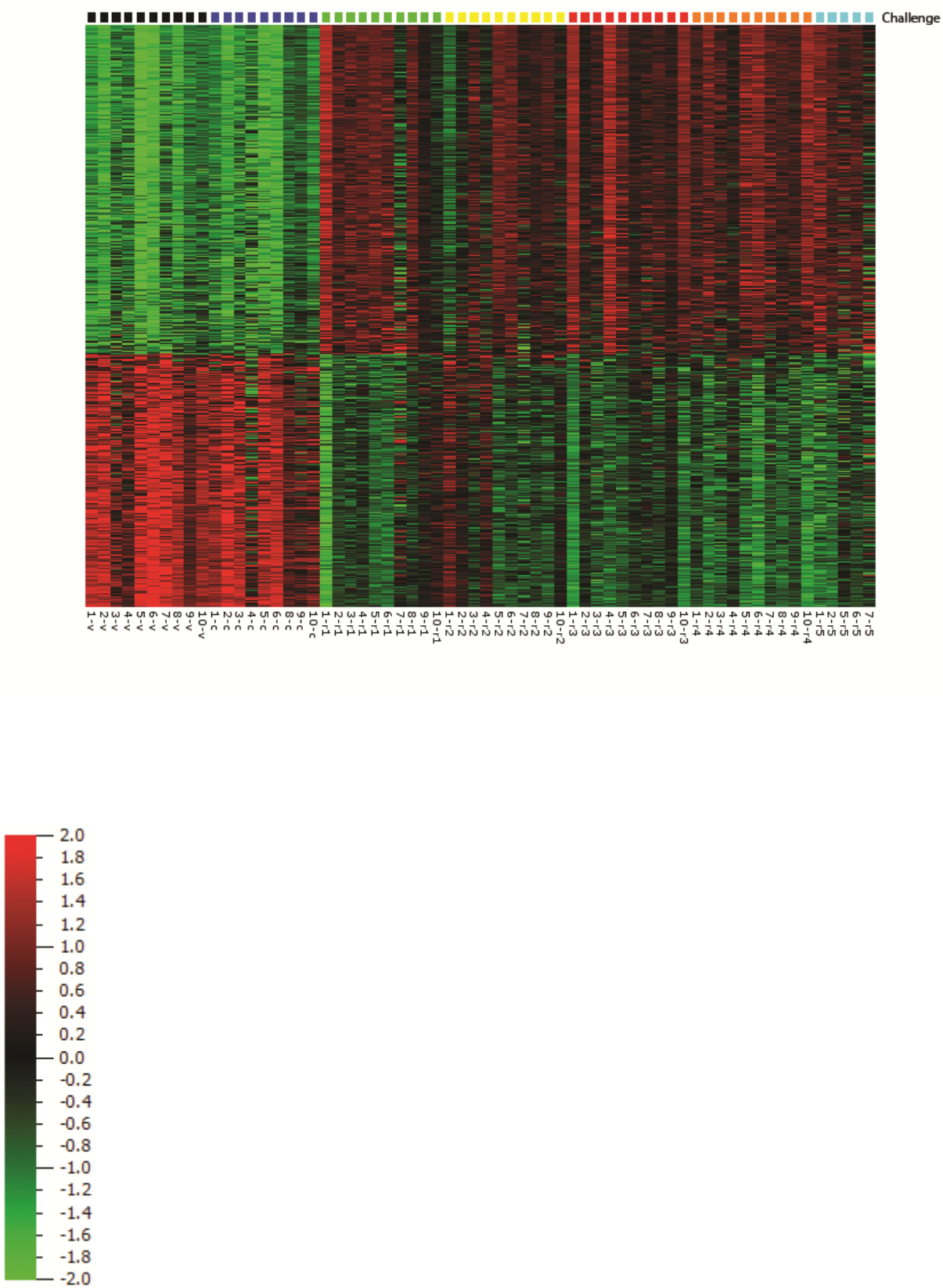


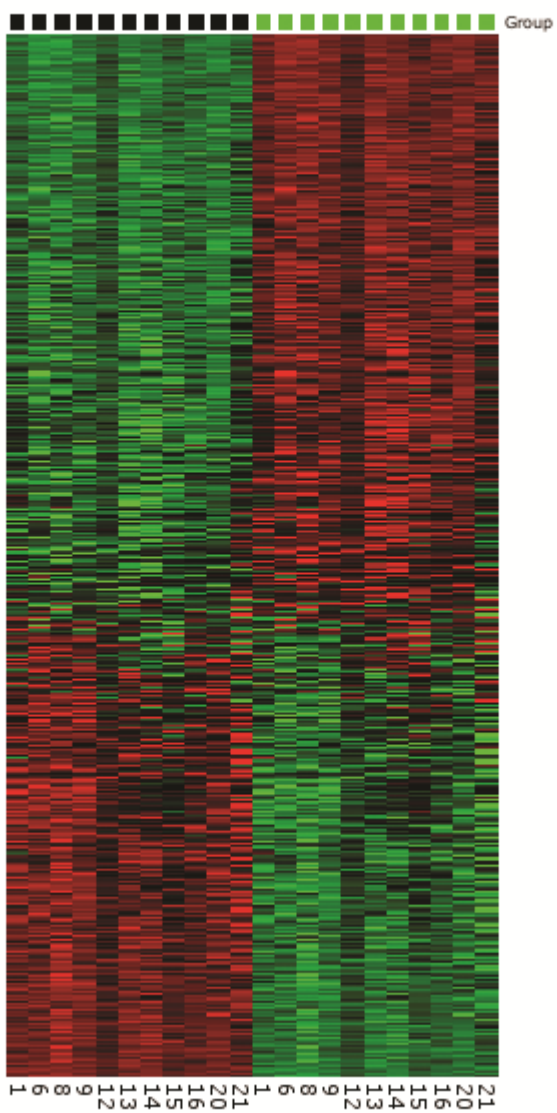




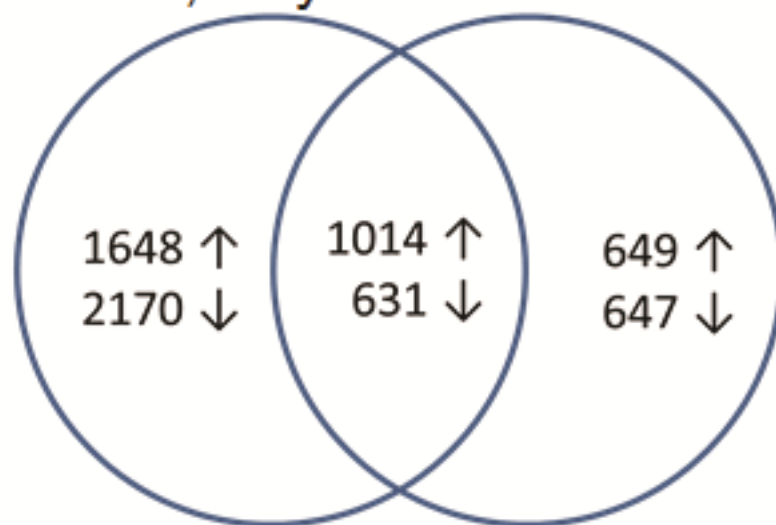








Gulati, Day 3



Rechallenge 1