

Gene expression is differently affected by pimecrolimus and betamethasone in lesional skin of atopic dermatitis

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Abstract

Background: Topical corticosteroids and calcineurin inhibitors are well-known treatments of atopic dermatitis (AD) but differ in their efficacy and side effects. We recently showed that betamethasone valerate (BM) although clinically more efficient impaired skin barrier repair in contrast to pimecrolimus in AD.

Objective: This study elucidates the mode of action of topical BM and pimecrolimus cream in AD.

Methods: Lesional AD skin samples after topical treatment with either BM or pimecrolimus were subjected to gene expression profile analysis.

Results: Betamethasone valerate resulted in a significant reduction in mRNA levels of genes encoding markers of immune cells and inflammation, dendritic cells, T cells, cytokines, chemokines, and serine proteases, whereas pimecrolimus exerted minor effects only. This corroborates the clinical finding that BM reduces inflammation more effectively than pimecrolimus. Genes encoding molecules important for skin barrier function were differently affected. Both BM and pimecrolimus normalized the expression of filaggrin and loricrin. BM, but not pimecrolimus, significantly reduced the expression of rate-limiting enzymes for lipid synthesis and the expression of involucrin and small proline-rich proteins, which covalently bind ceramides. This may explain the lack of restoration of functional stratum corneum layers observed after BM treatment.

Conclusion: The gene expression profiles are consistent with our previous findings that corticosteroids may exert a more potent anti-inflammatory effect but may impair the restoration of the skin barrier. Corticosteroids are still the main treatment for severe and acutely exacerbated AD; pimecrolimus may be preferable for long-term treatment and stabilization.

Abbreviations

ABC1, ATP-binding cassette, subfamily A; ABCA12, ATP-binding cassette, subfamily A member 12; ABCC1, ATP-binding cassette, subfamily C member 1; ABCG1, ATP-binding cassette, subfamily G member 1; AD, atopic dermatitis; ALOX5AP, arachidonate 5-lipoxygenase-activating protein; ANCOVA, Analysis of covariance; APC, antigen-presenting cells; BM, betamethasone valerate; BSL, baseline; CCL, chemokine (C-C motif) ligand; CFTR/MRP, ATP-binding cassette, subfamily C; COL, collagen; DC, dendritic cells; DHCR7, 7-dehydrocholesterol reductase; EASE, expression analysis systematic explorer; FADS1, fatty acid desaturase; FASN, fatty acid synthase; FDR, false discovery rate; HMGCR, 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase); HMGCS2, 3-hydroxy-3-methylglutaryl-coenzyme A synthase 2; IDEC, inflammatory dendritic epidermal cells; K (protein) or KRT (gene), keratin; LC, Langerhans cells; MHC, major histocompatibility complex; MMP, metalloproteinase; NFAT, nuclear factor of activated T cells; OPLS-DA, orthogonal partial least-squares to latent structures discriminant analysis; PC, pimecrolimus; pEASI, partial eczema area and severity index; PUVA, polyunsaturated fatty acids; RNA, ribonucleic acid; SELE, E-selectin; SERPIN, serine peptidase inhibitors; SMPD1, sphingomyelin phosphodiesterase 1; SMPD2, neutral sphingomyelinase 2; SPRL, small proline-rich-like molecules; SPRR, small proline-rich proteins; SPTLC2, serine palmitoyltransferase, long-chain base subunit 2; TGFB1, transforming growth factor beta 1; TGF- β , transforming growth factor beta; TNF-R, tumor necrosis factor receptor; TNFRS, tumor necrosis factor receptor superfamily; VIP, variable importance; WHITE, ATP-binding cassette, subfamily G

Current treatment strategies for atopic dermatitis (AD) involve the use of topical and systemically applied corticosteroids or calcineurin inhibitors and topical emollients. Systemic application of corticosteroids and the calcineurin inhibitor cyclosporin is used in extremely severe and recalcitrant disease manifestation, because of significant side effects. Topical corticosteroids have been the mainstay of treatment for AD since many decades. They act through cytosolic glucocorticoid receptors, which, once bound to the ligand, translocate to the nucleus and regulate expression of multiple genes including those of immune cells and of structural proteins in the skin (1). Side effects after topical treatment is less compared to those of systemic treatment. However, chronic use may lead to multiple side effects including skin atrophy, telangiectasia, perioral dermatitis, hemorrhage, and an increased rate of bacterial infection (2).

The need to efficiently manage inflammatory processes in AD in other skin diseases while minimizing side effects led to the development of topical calcineurin inhibitors (3). Calcineurin is a serine/threonine protein phosphatase that is essential for coordinating calcium signaling with nuclear action. Calcium-dependent dephosphorylation of the transcription factor nuclear factor of activated T cells (NFAT) by calcineurin is required for its translocation to the nucleus and the stimulation of cytokine gene expression, such as interleukin (IL)-2 (4). Calcineurin inhibitors block the phosphatase activity of calcineurin, leading to a reduced dephosphorylation of NFAT, and hence impair the expression of critical cytokines.

When administered systemically, the calcineurin inhibitor cyclosporine is highly effective in the treatment for recalcitrant AD and psoriasis and is approved for both indications. Systemic administration of cyclosporine may lead to side effects including hypertension, renal failure, and increased risk of cancer. This led to the development of topical calcineurin inhibitors tacrolimus and pimecrolimus (3). Because of their low penetration rates, therapeutic effects and side effects of calcineurin inhibitors are limited to the skin. The main targets of calcineurin inhibitors in the skin are thought to be keratinocytes, Langerhans cells (LC), and T cells, as these cells exhibit a strong expression of calcineurin receptors (3, 5, 6). Dendritic cell (DC) subtypes, such as LC and inflammatory dendritic epidermal cells (IDEC), play key roles in AD and influence the recruitment of inflammatory cells, T-cell priming, and cytokine and chemokine release (reviewed in Ref. 7, 8). Pimecrolimus was shown to affect T-cell proliferation and cytokine production as well as mast cell degranulation (9, 10).

The pathophysiological process of AD involves disruption of skin barrier function. A defective permeability barrier caused by changes in keratinocyte differentiation owing to altered expression of involucrin, loricrin, and filaggrin (in particular, by filaggrin mutations) and changes in lipid composition enables enhanced penetration of environmental allergens (cat dander, grass pollen, and house dust mites) into the skin. This initiates immunological reactions and inflammation (11–15). We recently observed that corticosteroids, although reducing the inflammatory response very effectively, delay the restoration of the epidermal barrier in patients with AD (16). In contrast, pimecrolimus did not exert the latter effect. To

learn more about the mode of action of these two compounds and to possibly identify additional differences between them, gene expression profile analysis was performed in skin biopsies obtained from patients with AD, which were treated topically with either betamethasone valerate (BM) or pimecrolimus.

Material and methods

Study design

Fifteen patients with mild-to-moderate AD (according to Hanifin and Rajka criteria) and a target lesion score (pEASI) of 3–8 (on a scale of 0–12) for both right and left target lesions, and symmetric AD lesions (not differing >1 point between the right and left sides) affecting the upper limbs by at least 10% were treated twice daily for 3 weeks on 1 upper limb with 1% pimecrolimus cream and on the other upper limb with 0.1% BM cream in a double-blind manner. The clinical, biophysical, immunohistological, and electron-microscopic results have already been published (16). Biopsy samples have been obtained before and after the end of the treatments for gene expression profiling measurement from ten patients of the group of 15. Of the ten patients, there were seven women and three men. The average age was 23.8 ± 3.6 years. After 3 weeks of treatment with pimecrolimus and BM cream, the clinical score (pEASI) revealed a reduction of 0.9 ± 2.1 and 2.9 ± 3.1 , respectively. The study was approved by the local ethics commission and the German Federal Institute for Pharmaceuticals and Medical Products (EudraCT-Nr. 2004-004824-11).

RNA extraction and purification

Total RNA from each frozen tissue section was obtained by acid guanidinium thiocyanate–phenol–chloroform extraction (17), Trizol, Invitrogen Life Technologies, Carlsbad, CA, USA), and the total RNA was then purified on an affinity resin (RNeasy, Qiagen Benelux B.V., Venlo, the Netherlands) according to the manufacturer's instructions. Total RNA was then quantified by measuring the absorbance at $\lambda = 260$ nm ($A_{260\text{nm}}$), and its purity was determined by estimating the ratio $A_{260\text{nm}}/A_{280\text{nm}}$. Integrity of the RNA molecules was confirmed by nondenaturing agarose gel electrophoresis. RNA was stored at approximately -80°C until analysis.

GeneChip experiment

RNA was amplified according to the manufacturer's instructions (Affymetrix, Palo Alto, CA, USA). Human GenomeU133Plus 2 expression probe arrays (Affymetrix) were used, comprising more than 54 000 probe sets, analyzing more than 30 000 transcripts and variants of more than 28 000 human genes. One GeneChip per sample per patient was used. The resultant image files (.dat files) were processed using the Microarray Analysis Suite 5 (MAS5) software (Affymetrix). Tab-delimited files containing data regarding signal intensity (Signal) and categorical expression level measure-

Table 1 Gene expression changes induced by three weeks of treatment with pimecrolimus of betamethasone. Significance filter was $P < 0.01$, and fold change > 1.3 .

Probe set	Name	Symbol	Unigene cluster	Chromosomal location	Mean (BSL)	P-value PC vs BSL	P-value BM vs BSL	Fold change PC/BSL	Fold change BM/BSL
Pimecrolimus and Betamethasone (common)									
211906_s_at	serpin peptidase inhibitor, clade B (ovalbumin), member 4	SERPINB4	Hs.123035	18q21.3	11.9	0.0024	0.0002	0.28	0.04
210413_x_at	serpin peptidase inhibitor, clade B (ovalbumin), member 3	SERPINB3	Hs.227948	18q21.3	12.3	0.0019	0.0001	0.33	0.06
232170_at	S100 calcium binding protein A7A	S100A7A	Hs.442337	1q21.3	10.1	0.0015	0.0001	0.25	0.07
209719_x_at	serpin peptidase inhibitor, clade B (ovalbumin), member 3	SERPINB3	Hs.227948	18q21.3	12.5	0.0094	0.0002	0.40	0.10
204580_at	matrix metalloproteinase 12 (macro phage elastase)	MMP12	Hs.1695	11q22.3	10.3	0.0000	0.0004	0.27	0.10
202018_s_at	lactotransferrin	LTF	Hs.529517	3p21.31	9.9	0.0071	0.0001	0.41	0.18
223541_at	hyaluronan synthase 3	HAS3	Hs.592069	16q22.1	9.9	0.0005	0.0008	0.34	0.20
204858_s_at	thymidine phosphorylase	TYMP	Hs.592212	22q13	8.1	0.0022	0.0004	0.47	0.26
207861_at	chemokine (C-C motif) ligand 22	CCL22	Hs.534347	16q13	8.4	0.0004	0.0016	0.45	0.26
203747_at	aquaporin 3 (Gill blood group)	AQP3	Hs.234642	9p13	10.5	0.0016	0.0004	0.55	0.29
213060_s_at	chitinase 3-like 2	CHI3L2	Hs.514840	1p13.3	8.2	0.0042	0.0016	0.43	0.30
217497_at	thymidine phosphorylase	TYMP	Hs.592212	22q13	8.5	0.0001	0.0007	0.49	0.31
221009_s_at	angiopoietin-like 4	ANGPTL4	Hs.9613	19p13.3	8.5	0.0031	0.0005	0.50	0.33
209803_s_at	pleckstrin homology-like domain, family A, member 2	PHLDA2	Hs.154036	11p15.5	9.1	0.0073	0.0038	0.55	0.33
39249_at	aquaporin 3 (Gill blood group)	AQP3	Hs.234642	9p13	11.7	0.0090	0.0002	0.64	0.33
218810_at	zinc finger CCCH-type containing 12A	ZC3H12A	Hs.656294	1p34.3	8.7	0.0035	0.0005	0.61	0.33
38037_at	heparin-binding EGF-like growth factor	HBEGF	Hs.799	5q23	7.6	0.0094	0.0043	0.62	0.33
216834_at	regulator of G-protein signaling 1	RGS1	Hs.75256	1q31	7.3	0.0005	0.0019	0.45	0.34
211361_s_at	serpin peptidase inhibitor, clade B (ovalbumin), member 13	SERPINB13	Hs.241407	18q21.3-q22	8.7	0.0046	0.0018	0.59	0.34
214226_at	protease, serine, 53	PRSS53	Hs.569575	16p11.2	8.2	0.0000	0.0001	0.46	0.35

Table 1 continued

Probe set	Name	Symbol	Unigene cluster	Chromosomal location	Mean (BSL)	P-value	qFDR	Fold change PC/BSL	Fold change BM/BSL
Betamethasone-treatment specific									
203535_at	S100 calcium binding protein A9	S100A9	Hs.112405	1q21	13.5	0.0007	0.0085	0.45	0.04
203691_at	peptidase inhibitor 3, skin-derived	PI3	Hs.112341	20q13.12	10.7	0.0001	0.0072	0.41	0.05
41469_at	peptidase inhibitor 3, skin-derived	PI3	Hs.112341	20q13.12	10.5	0.0002	0.0072	0.43	0.05
207356_at	defensin, beta 4A	DEFB4A	Hs.105924	8p23.1	10.2	0.0003	0.0074	0.39	0.08
209720_s_at	serpin peptidase inhibitor, clade B (ovalbumin), member 3	SERPINB3	Hs.227948	18q21.3	12.0	0.0003	0.0074	0.39	0.08
232082_x_at	small proline-rich protein 3	SPRR3	Hs.139322	1q21-q22	11.0	0.0004	0.0076	0.58	0.09
209800_at	keratin 16	KRT16	Hs.655160	17q21.2	13.1	0.0002	0.0072	0.47	0.09
202917_s_at	S100 calcium binding protein A8	S100A8	Hs.416073	1q21	14.5	0.0026	0.0112	0.58	0.10
208539_x_at	small proline-rich protein 2B	SPRR2B	Hs.568239	1q21-q22	11.6	0.0018	0.0101	0.68	0.12
214580_x_at	keratin 6A	KRT6A	Hs.700779	12q12-q13	13.3	0.0003	0.0075	0.53	0.15
1553835_a_at	collagen, type VI, alpha 5	COL6A5	Hs.205403	3q22.1	9.5	0.0007	0.0083	0.65	0.16
213780_at	trichohyalin	TCHH	Hs.432416	1q21.3	7.1	0.0084	0.0171	3.41	5.78
237905_at	keratin 25	KRT25	Hs.55412	17q21.2	7.1	0.0083	0.0170	2.81	5.72
209125_at	keratin 6A	KRT6A	Hs.700779	12q12-q13	13.4	0.0003	0.0073	0.58	0.18
229476_s_at	thyroid hormone responsive	THRSP	Hs.591969	11q13.5	6.0	0.0027	0.0113	1.57	5.36
205916_at	S100 calcium binding protein A7	S100A7	Hs.112408	1q21	14.2	0.0061	0.0150	0.75	0.19
239929_at	peptidase M20 domain containing 1	PM20D1	Hs.177744	1q32.1	5.4	0.0017	0.0101	1.07	5.05
224555_x_at	interleukin 1 family, member 7 (zeta)	IL1F7	Hs.166371	2q12-q14.1	6.3	0.0006	0.0081	2.18	4.96
205030_at	fatty acid binding protein 7, brain	FABP7	Hs.26770	6q22-q23	6.3	0.0005	0.0077	1.93	4.90
214549_x_at	small proline-rich protein 1A	SPRR1A	Hs.46320	1q21-q22	12.4	0.0004	0.0075	0.65	0.21
Pimecrolimus-treatment specific									
241722_x_at	Data not found				8.0	0.0079	0.1660	0.60	0.73
211713_x_at	KIAA0101	KIAA0101	Hs.81892	15q22.31	7.2	0.0030	0.1408	0.60	0.77
1555725_a_at	regulator of G-protein signaling 5	RGS5	Hs.24950	1q23.1	6.3	0.0048	0.1492	1.63	1.63
234552_at	epiplakin 1	EPK1	Hs.200412	8q24.3	8.9	0.0094	0.1722	0.63	0.52
213244_at	secretory carrier membrane protein 4	SCAMP4	Hs.144980	19p13.3	7.3	0.0023	0.1334	0.67	0.69
208549_x_at	prothymosin, alpha	PTMA	Hs.459927	2q37.1	12.1	0.0022	0.1334	0.67	0.77
1560500_at	Data not found				6.6	0.0019	0.1305	1.47	1.52
230141_at	AT rich interactive domain 4A (RBP1-like)	ARID4A	Hs.161000	14q23.1	6.4	0.0023	0.1334	1.46	1.38
212285_s_at	agrin	AGRN	Hs.273330	1p36.33	7.4	0.0015	0.1272	0.69	0.77
225305_at	solute carrier family 25, member 29	SLC25A29	Hs.578109	14q32.2	6.4	0.0089	0.1698	1.45	1.75
1554084_a_at	nucleolar protein 9	NOL9	Hs.59425	1p36.31	7.0	0.0065	0.1608	0.70	0.98

Table 1 continued

Probe set	Name	Symbol	Unigene cluster	Chromosomal location	Mean (BSL)	P-value	qFDR	Fold change PC/BSL	Fold change BM/BSL
207535_s_at	nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)	NFKB2	Hs.73090	10q24	7.3	0.0013	0.1202	0.70	0.47
223276_at	chromosome 5 open reading frame 62	C5orf62	Hs.29444	5q33.1	6.9	0.0055	0.1528	1.43	1.37
209508_x_at	CASP8 and FADD-like apoptosis regulator	CFLAR	Hs.390736	2q33-q34	8.3	0.0074	0.1627	0.70	0.90
201728_s_at	KIAA0100	KIAA0100	Hs.151761	17q11.2	7.5	0.0030	0.1399	0.70	0.91
216222_s_at	myosin X	MYO10	Hs.481720	5p15.1-p14.3	6.9	0.0032	0.1408	0.70	0.88
201861_s_at	leucine rich repeat (in FLII) interacting protein 1	LRRFIP1	Hs.471779	2q37.3	9.9	0.0022	0.1334	0.71	0.89
209370_s_at	SH3-domain binding protein 2	SH3BP2	Hs.167679	4p16.3	7.1	0.0033	0.1408	0.71	0.82
217650_x_at	ST3 beta-galactoside alpha-2, 3-sialyltransferase 2	ST3GAL2	Hs.368611	16q22.1	8.0	0.0066	0.1611	0.71	0.75
211012_s_at	golgin A6 family-like 4 promyelocytic leukemia	GOLGA6L4PML	Hs.534573	15q25.2	7.4	0.0074	0.1627	0.71	0.64

ment (Absolute Call) were obtained. Raw data were converted to expression levels using a 'target intensity' of 150. All DNA microarray experiments were conducted in the Genomics Factory EU, Basel, Switzerland, following the instructions of the manufacturer of the GeneChip system (Affymetrix) and as previously described (18).

Data analysis

Data used in our manuscript have been accepted and uploaded into the public microarray repository Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>) under the code GSE32473.

Data quality assessment and analysis were made using Partek Pro 6.5 (Partek Inc., St. Louis, MI, USA) and SIMCA -P+ (version 12) Umetrics AB, Umeå, Sweden.

Each array was normalized by dividing the signal intensities by the 50th percentile of that array. Analyses were conducted for pimecrolimus and for BM (both versus BSL) separately. Only probe sets with present or marginal calls in at least 70% of samples per analysis group were considered, which resulted in a list of 17 104 probe sets for the comparison of BM and baseline and 18 356 probe sets for the comparison of pimecrolimus and baseline.

Probe sets were annotated in Source, provided by the Genetics Department, Stanford University, <http://source.stanford.edu>. Categorization of gene lists in biological ontologies was performed with NIH Database for Annotation, Visualization and Integrated Discovery [DAVID, v. 6.7, (<http://david.abcc.ncifcrf.gov>)]. Significant enrichment of ontologies is reported with P-value, Benjamini-Hochberg multiple testing correction, and fold enrichment.

Orthogonal partial least-squares to latent structures discriminant analysis (OPLS-DA) (orthogonal partial least-squares to latent structures discriminant analysis) was applied to the probe set lists, which had been filtered as described above. Probe sets with $|P| \geq 0.015$ and $|P(\text{corr})| \geq 0.6$ were considered to have significant contribution to the model. P are the loadings of the X -part of the model, representing the importance of the variables in approximating X in a component of an OPLS model, and $P(\text{corr})$ is the correlation coefficient between X and the t scores as a measure to approximate the X and predicted Y (19).

Graphs display mean ratios, i.e., fold changes, for genes affected by treatment. Genes that are considered significant in paired t -test analysis and/or OPLS-DA models are marked with a star (*). Genes not marked are considered important by the authors as they reflect biological changes of interest. Some of those genes may not pass one or more filters but could be considered significant in terms of statistics, e.g., if a different threshold for significance would be applied.

Results

Gene expression analysis

To explore the effects of pimecrolimus and betamethasone on reduction in pEASI score using gene expression profiling, we

followed two different routes: A paired *t*-test was employed to assess gene expression changes from day 1 (before treatment) to the end of the study for each patient, using the day 1 sample for each patient as individual baseline, thus reducing noise and increasing the analytical power. This approach addresses the question whether there are significant gene expression changes after 3 weeks of treatment with either of the compounds. Probe sets with an unadjusted *P*-value smaller than 0.01 and an absolute fold change larger than 1.3 were considered significant. This resulted in 2378 probe sets that were affected by betamethasone treatment; for pimecrolimus treatment, 281 probe sets passed this filter. Table 1 lists 20 probe sets with the smallest *P*-value and highest fold change for each of the three groups.

Another exploration approach assumed that the samples are independent from each other and addressed whether gene expression could model the clinical endpoint of pEASI reduction and hence whether there is a causal relationship between pEASI score reduction and gene expression measurements. The degree of pEASI reduction was calculated for each sample. Multivariate regression models for baseline and endpoint samples were applied to assess the effect of the compounds on the pEASI reduction (Fig. 1A). One model was calculated for baseline and pimecrolimus-treated samples and another one for baseline and betamethasone-treated samples. As model algorithm, we chose orthogonal partial least-squares to latent structures (OPLS, 19). Regression models with $R^2 = 0.9909$ and $R^2 = 0.9821$ for PC treatment (586 probe sets) and BM treatment (549 probe sets), respectively, were obtained, when probe sets with loadings $|P| > 0.015$ and correlation coefficient $|P(\text{corr})| > 0.6$ were selected as having significant contribution to the models (see Supporting information for complete lists of probe sets and model contributions). As shown in Fig. 1, the two lists shared 132 probe sets, 454 probe sets were specific to pimecrolimus treatment, and 417 probe sets were important only in the betamethasone model (Fig. 1, upper left). Functional categorization of the probe sets that are common to both treatment regimens reveals their involvement in immune and inflammatory response, cytokine and chemokine activity, chemotaxis, and epidermal differentiation (Fig. 1, upper right). While probe sets unique to the pimecrolimus model are largely involved in cell proliferation and cell homeostasis (Fig. 1, lower left), probe sets unique to the BM model are further grouped in processes of immune and defense response, collagen, and MHC protein binding (Fig. 1, lower right).

Gene expression changes in paired *t*-test and regression models

Genes involved in inflammation and immune response

Betamethasone valerate treatment strongly down-regulated markers of inflammatory cells, in particular specific DC marker genes (i.e., CD1a, CD11b, CD11c), and T-cell markers, whereas only no-to-moderate effects on these genes were observed for pimecrolimus (Fig 2A,B). Both treatment regimens influenced the expression of genes coding for skin inflammatory processes and reduced the expression of chemo-

tactic chemokines [e.g., chemokine (C-C motif) ligand (CCL2, CCL19, CCL26) and E-selectin (SELE) (Fig. 2C)]. The effect of BM was stronger than that of pimecrolimus. Expression levels of the members of the TNF receptor superfamily (TNFRS) and transforming growth factor beta 1 (TGFB1) were only weakly affected by BM and pimecrolimus. In contrast, the expression of elafin, metalloproteinase 12 (MMP12), and serine peptidase inhibitors B3 and B4 (SERPIN B3, B4) were down-regulated by both regimens (Fig. 2D). Irrespective of the degree of suppression, down-regulation of these genes was always more pronounced after treatment with BM than with pimecrolimus.

Genes involved in dermal and epidermal integrity

In general, BM treatment caused a detectable reduction in levels of genes coding for collagens (COL1A1, COL6A2, COL3A1, COL6A1, COL5A1, COL3A1, COL1A2, and COL5A1), whereas pimecrolimus treatment had no significant effect. COL4A5 and COL4A6 were slightly overrepresented after betamethasone treatment (Fig. 3A).

Genes involved in the maintenance of epidermal integrity were differently affected by the treatments. For the keratin variants KRT1B, related to epidermal differentiation, a slight increase was noticed after pimecrolimus treatment, which was even more pronounced after BM treatment. Expression of the inflammation- and proliferation-associated keratins KRT17, KRT6A, KRT6B, and KRT16 was reduced by pimecrolimus and significantly reduced by betamethasone. In accordance, gene expression of the proliferation marker Ki67 (MKI67) was also significantly reduced by betamethasone treatment (Fig. 3B). Expression of lorcin (LOR) was slightly increased after both treatments. Effects of both treatments on filaggrin (FLG) and small proline-rich-like molecules (SPRL) 2A and 1B were minimal. Significant reductions were noted for involucrin (IVL) and small proline-rich proteins SPRR1A, SPRR1B, and SPRR3 after treatment with BM; a moderate reduction occurred after pimecrolimus treatment (Fig. 3C).

Effects of both BM and pimecrolimus on genes regulating lipid metabolism were varied. Significantly increased expression after betamethasone treatment was noted for fatty acid-binding protein 7 (FABP7) and fatty acid desaturase 1 (FADS1). Genes with lower expression following BM treatment included serine palmitoyl transferase, long-chain base subunit 1 and 2 (SPTLC1 und SPTLC2), neutral sphingomyelinase 2 (SMPD2), ATP-binding cassette, subfamily C (CFTR/MRP), member 1 (ABCC1), 3-hydroxy-3-methylglutaryl-coenzyme A reductase [HMG-CoA reductase (HMGCR)], and arachidonate 5-lipoxygenase-activating protein (ALOX5AP). Effects of pimecrolimus on genes regulating lipid metabolism were always in the same direction as with BM but were less pronounced (Fig. 3D).

Discussion

We recently found that BM, although producing good clinical results, does not lead to restoration of the skin barrier in AD. In contrast, pimecrolimus leads to restoration of the skin barrier but is clinically less effective (16). Here, we inves-

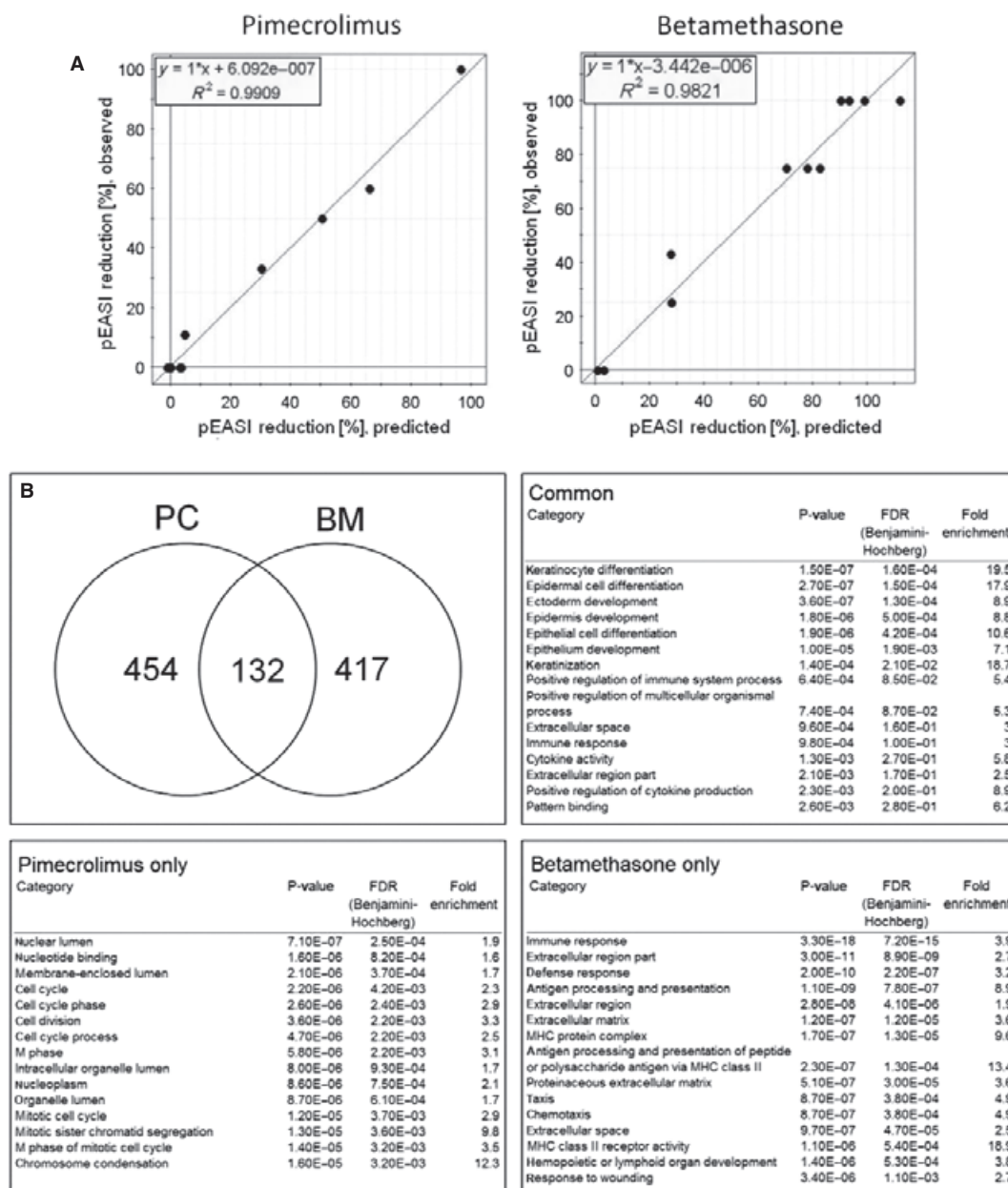


Figure 1 Genes affected by the treatments. Comparison of probe set lists from orthogonal partial least-squares to latent structures discriminant analysis regression analyses that model pEASI score reduction as calculated for each patient for each treatment regimen. (A), regression analysis for each compound separately, using probe sets with largest contribution. (B), the number of genes most significantly regulated by the treatments with either pimecrolimus (PC) or betamethasone (BV) is shown

in the circles. The overlapping part shows number of genes influenced by both treatments (upper left). These most commonly affected gene ontologies reflect changes in epidermal integrity, regulation of the immune system, and cytokine activity (upper right). Pimecrolimus-specific effects influence mitosis at large (lower left), and betamethasone-specific changes are relevant to immune system function (lower right). Categories are sorted by *P*-value.

titigated the mechanisms of action of both substances at the molecular level. Following two approaches in which we focused on treatment effect (paired *t*-test) and on modeling clinical endpoints, we found that some genes were affected similarly by BM and pimecrolimus, whereas others were

affected differently. Both drugs influenced keratinocyte and epidermal differentiation as well as positive regulation of the immune system process and cytokine production. This is in accordance with the well-known changes in epidermal differentiation, activation of the immune system and cytokines in

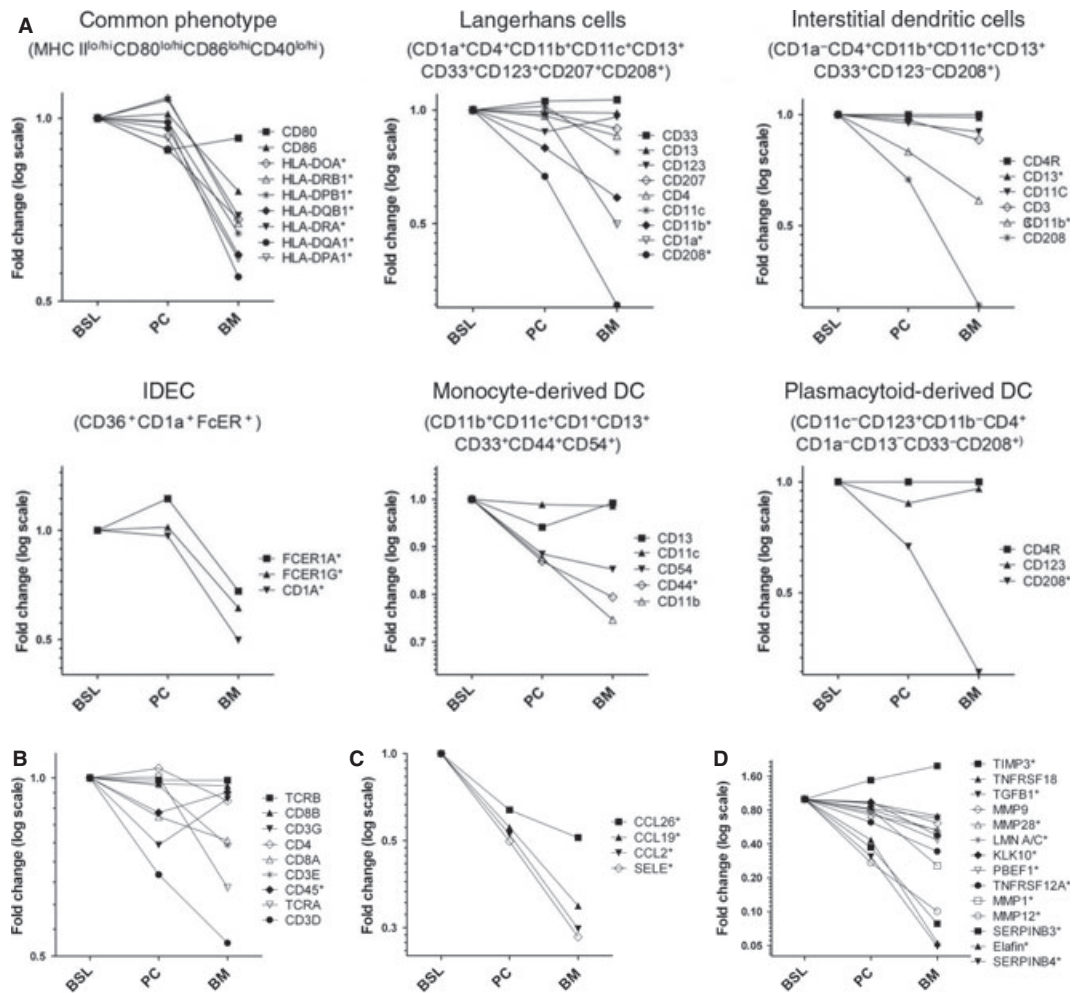


Figure 2 Immune cells. Influence of pimecrolimus (PC) and beta-methasone valerate (BM) on gene expression for specific dendritic cell (DC) markers, APCs (A), T-cell markers (B) on chemokines (C), on proteases (D). BSL, Baseline. Samples from patients were normalized to the individual baseline sample. Each line represents a single gene, connecting normalized signal intensities for the conditions BSL, PC, and BM. In the case where multiple probe sets of a

single gene were detected, the mean signal intensity value for those probe sets was plotted for visualization simplicity. The mean of expression value per group is shown; Y-axis: Normalized signal intensity; i.e., fold change. Genes that are considered significant in paired *t*-test analysis and/or orthogonal partial least-squares to latent structures discriminant analysis models are marked with a star (*).

AD, and normalization (at least partially) under corticosteroid and calcineurin inhibitors (16).

Pimecrolimus regulated additional genes involved in cell proliferation and cell homeostasis, whereas BM regulated additional genes involved in epidermal inflammation, chemotaxis, and immune response (Fig. 1B); this is shown in the detailed studies using paired *t*-test and multivariate regression analysis. BM treatment led to a pronounced reduction in markers of immune cells, DCs, MHC molecules, and T-cell markers, whereas PC treatment had only minor effects (Fig. 2A,B). Accordingly, a previous flow cytometry study showed that BM, but not pimecrolimus, depletes LC in patients with AD (20).

Expression of chemoattractant molecules CCL2, CCL19, and CCL26 (21) as well as selectin E (SELE), elafin, and the

serine proteases SERBINB3 and SERBINB4 was reduced by both treatments (Fig. 2C,D). Elafin and serine proteases SERBINB3 and SERBINB4 are related to inflammation in several organs (22). Mutations in the SPINK5 gene encoding the serine protease (SP) inhibitor, lymphoepithelial-Kazal-type 5 inhibitor (LEKTI), cause Netherton syndrome (NS). Netherton syndrome clinically resembles AD, and therefore, several authors suggested a role of serine protease in AD (21–23).

Inflammation-related expression of genes for growth factor TGF-β and TNF-Rs was not or only slightly affected (Fig. 2 D). It has been proposed that TGF-β is important for the stimulation of regulatory T cells (24), but according to our data, regulation of the gene expression of this growth factor does seem to be important for skin improvement in our treat-

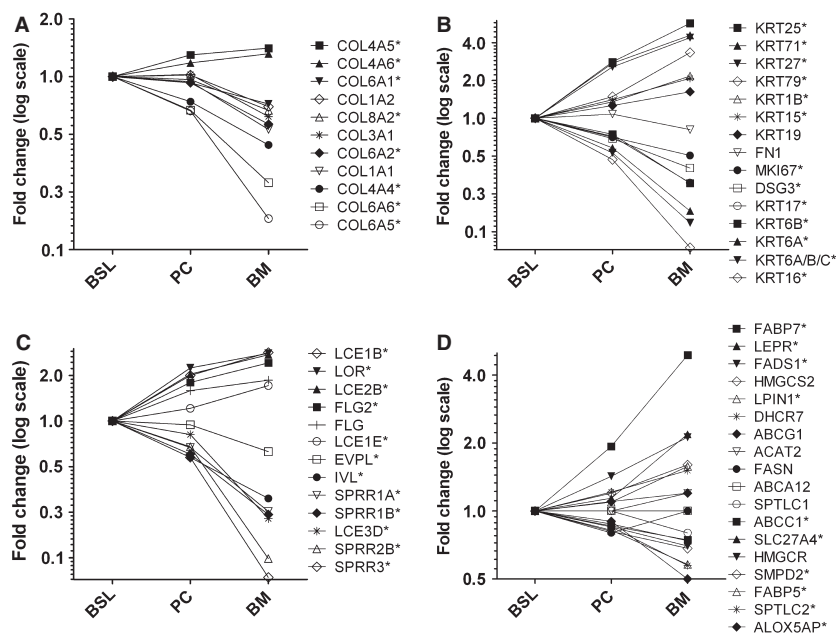


Figure 3 Collagens, epidermal integrity, and lipid metabolism. (A) Betamethasone valerate (BM) but not pimecrolimus (PC) decreased the expression of collagens in skin of patients with AD. (B, C) BM treatment resulted in a stronger dysregulation of genes involved in epidermal integrity (proliferation-associated keratins, involucrin) compared with PC. In particular, late markers of epidermal differentiation were slightly reduced after pimecrolimus

but severely reduced after BM treatment. (D) BM treatment had a more pronounced effect on the expression of genes (either increase or decrease) involved in lipid metabolism than PC. Genes that are considered significant in paired *t*-test analysis and orthogonal partial least-squares to latent structures discriminant analysis (OPLS-DA) or only in OPLS-DA models are marked with a star (*).

ments. Also, regulation of the expression of TNF-Rs does not seem to be a crucial step in AD treatment; anti-TNF therapy, which has been successfully used in psoriasis, seldom produces significant positive clinical effects in AD (25).

Structural proteins are influenced by the treatments. BM, but not pimecrolimus, significantly decreased the expression of genes encoding different collagens (Fig. 3A). Thinning of the dermis is a known side effect of corticosteroids leading to skin atrophy (26). In contrast, long-term treatment with pimecrolimus does not result in skin atrophy (27).

Proliferation-related keratins KRT6A, KRT6B, and KRT16, which are expressed in AD but not in skin of healthy controls, were profoundly reduced after BM treatment (Fig. 3B). This is consistent with the report of a pronounced reduction in epidermal proliferation after BM, which may ultimately lead to skin atrophy including the epidermal compartment with long-term use (16). Also, this is in accordance with the normalization of keratin expression after treatment with betamethasone (11, 16).

Expression of genes important for skin barrier function and epidermal differentiation was either increased or decreased after treatment. Both treatments influenced gene expression in the same direction, but the effects were more pronounced with BM than with PC. A slight increase in gene expression was noted for filaggrin and loricrin, and SPRL2A and SPRL1B (small proline-rich molecules) (Fig. 3C). Mutations in the filaggrin gene have been shown in about 20% of the patients with AD (13, 21, 28). Independent of the muta-

tion, filaggrin protein levels are reduced in nonlesional and lesional skin (11), and this may be a key factor in the defective skin barrier and dry skin in AD. Loricrin (a cornified envelope molecule) and filaggrin show gaps in the immunohistological staining band in lesional skin of AD that normalizes during treatment with both drugs (16). We suggest that the increased expression of filaggrin and loricrin may not be sufficient to normalize skin barrier function during treatment with BM because of the pronounced reduction in involucrin, small proline-rich proteins, and lipid-synthesizing enzymes caused by this treatment.

Involucrin and the late markers of epidermal differentiation, also called small proline-rich proteins SPRR1A, SPRR1B, and SPRR3, were slightly reduced after pimecrolimus but severely reduced after BM treatment (Fig. 3C). Very recently, reduced gene expression of SPRR1A in AD has been described (29). Sphingolipids are covalently attached to involucrin and SPRRs in the stratum corneum. The covalently attached sphingolipids serve as a backbone for the subsequent attachment of free sphingolipids, cholesterol, and free fatty acids in the epidermal skin barrier (reviewed in Ref. 30). Reduction in involucrin and SPRRs after BM treatment may result in reduction in protein-bound omega-hydroxyceramides and thus may result in ongoing disruption of the skin barrier after BM treatment (16).

Numerous studies showed a disturbance in stratum corneum epidermal lipid composition in AD, and it has been thought that this may be the main cause for the defect in skin

barrier function (reviewed in Ref. 12). We recently showed that BM treatment in AD leads to incompletely filled epidermal lamellar bodies and impaired barrier restoration, because lamellar bodies provide the lipids for the stratum corneum extracellular layers (16). Corroborating these findings, we found reduced expression of genes encoding the rate-limiting genes for lipid synthesis serine palmitoyl transferase (SPTLC2), HMG Co reductase (HMGCR), and fatty acid synthase (FASN). Lipid efflux pathways, expression of leptin receptor 1 (LEPR), lipin 1 (LPIN1), 3-hydroxy-3-methylglutaryl-CoA synthase 2 (HMGCS2), and ATP-binding cassette, subfamily G (ABCG1) were slightly increased after BM treatment (Fig. 3D). Function of these proteins in AD has not been examined yet. Expression of FADP5 was detected at a high level in AD skin lesions (31). Down-regulation of the expression may be a sign of normalization.

Genes related to unsaturated fatty acid metabolism were either up-regulated [fatty acid desaturase, (FADS1)] or down-regulated [arachidonate 5-lipoxygenase-activating protein (ALOX5AP)] after BM treatment (Fig. 3D). Fatty acid desaturase is related to the amount of n-6, whereas arachidonate metabolism is related to n-3 polyunsaturated fatty acids (PUFAs). The importance of polyunsaturated fatty acids in AD has been a matter of discussion for many years (32, 33).

In conclusion, gene expression profiles obtained in this study might help to explain at the molecular level the clinical observation that corticosteroids, while exerting a more potent anti-inflammatory effect, impair the restoration of the skin barrier and can induce skin atrophy. This confirms the hypothesis that topical corticosteroids are suitable for severe acutely exacerbated AD but that topical calcineurin inhibitors may be suitable for long-term and intermittent treatment and stabilization of the disease (34).

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