IL-36γ (IL-1F9) Is a Biomarker for Psoriasis Skin Lesions

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In recent years, different genes and proteins have been highlighted as potential biomarkers for psoriasis, one of the most common inflammatory skin diseases worldwide. However, most of these markers are not only psoriasis-specific but also found in other inflammatory disorders. We performed an unsupervised cluster analysis of gene expression profiles in 150 psoriasis patients and other inflammatory skin diseases (atopic dermatitis, lichen planus, contact eczema, and healthy controls). We identified a cluster of IL-17/tumor necrosis factor-α (TNFα)-associated genes specifically expressed in psoriasis, among which IL-36γ was the most outstanding marker. In subsequent immunohistological analyses, IL-36γ was confirmed to be expressed in psoriasis lesions only. IL-36γ peripheral blood serum levels were found to be closely associated with disease activity, and they decreased after anti-TNFα-treatment. Furthermore, IL-36γ immunohistochemistry was found to be a helpful marker in the histological differential diagnosis between psoriasis and eczema in diagnostically challenging cases. These features highlight IL-36γ as a valuable biomarker in psoriasis patients, both for diagnostic purposes and measurement of disease activity during the clinical course. Furthermore, IL-36γ might also provide a future drug target, because of its potential amplifier role in TNFα- and IL-17 pathways in psoriatic skin inflammation.

Journal of Investigative Dermatology (2015) **135,** 1025–1032; doi:10.1038/jid.2014.532; published online 22 January 2015

INTRODUCTION

Psoriasis vulgaris (Pso), atopic dermatitis (AD), contact eczema (CE), and lichen planus (LP) all represent common chronic inflammatory skin diseases where differential diagnosis based on clinical features and histology can, in some cases, be difficult. They are associated with variable concomitant illnesses, low health-related quality of life, and collectively have a considerable economic burden. Mechanistically, these entities share different genetic and environmental influences and are orchestrated by a complex network of common and specific pro-inflammatory mediators that result in the expressed clinical phenotypes.

Despite their at times similar clinical appearance to erythrosquamous plaques, these skin diseases are driven by strictly different pathomechanisms, thus offering the possibility for accurate molecular diagnostics. Psoriasis is thought to be mainly mediated by T helper (Th) type 1/Th17 cytokines with a central role of tumor necrosis factor-α (TNF-α), whereas Th2 cytokines are supposed to have a key role in AD (Guttman-Yassky *et al.*, 2011; Bieber *et al.*, 2012; Perera *et al.*, 2012). CE lesions are characterized by Th1-mediated CCL chemokines, whereas LP is most probably driven by a type I IFN-mediated inflammation (Pedersen *et al.*, 2007; Wenzel *et al.*, 2008).

As the exact diagnosis of these diseases is a matter of great importance for patient-specific therapy, several earlier studies investigated the value of a number of genes and proteins as potential specific biomarkers (Bieber *et al.*, 2012; Villanova *et al.*, 2013). Unfortunately, most of these markers, like the S100 proteins A7, A8, and A9, are common inflammatory mediators that are highly expressed in psoriasis (Semprini *et al.*, 1999; Liu *et al.*, 2007) but also in other inflammatory skin diseases (Glaser *et al.*, 2009; Kerkhoff *et al.*, 2012).

Here we present data of 150 patients with different inflammatory skin disorders, demonstrating that IL-36γ, an IL-1F cytokine formerly known as IL-1F9, is specifically expressed in psoriasis skin lesions and closely associated with disease activity.

RESULTS

Chronic skin diseases are characterized by the expression of common pro-inflammatory mediators

Initially, an unsupervised hierarchical gene cluster analysis to visualize the average linkage of all genes specifically expressed within the different subsets was performed (Figure 1a). This identified a set of genes that were commonly

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Received 6 March 2014; revised 5 December 2014; accepted 8 December 2014; accepted article preview online 19 December 2014; published online 22 January 2015

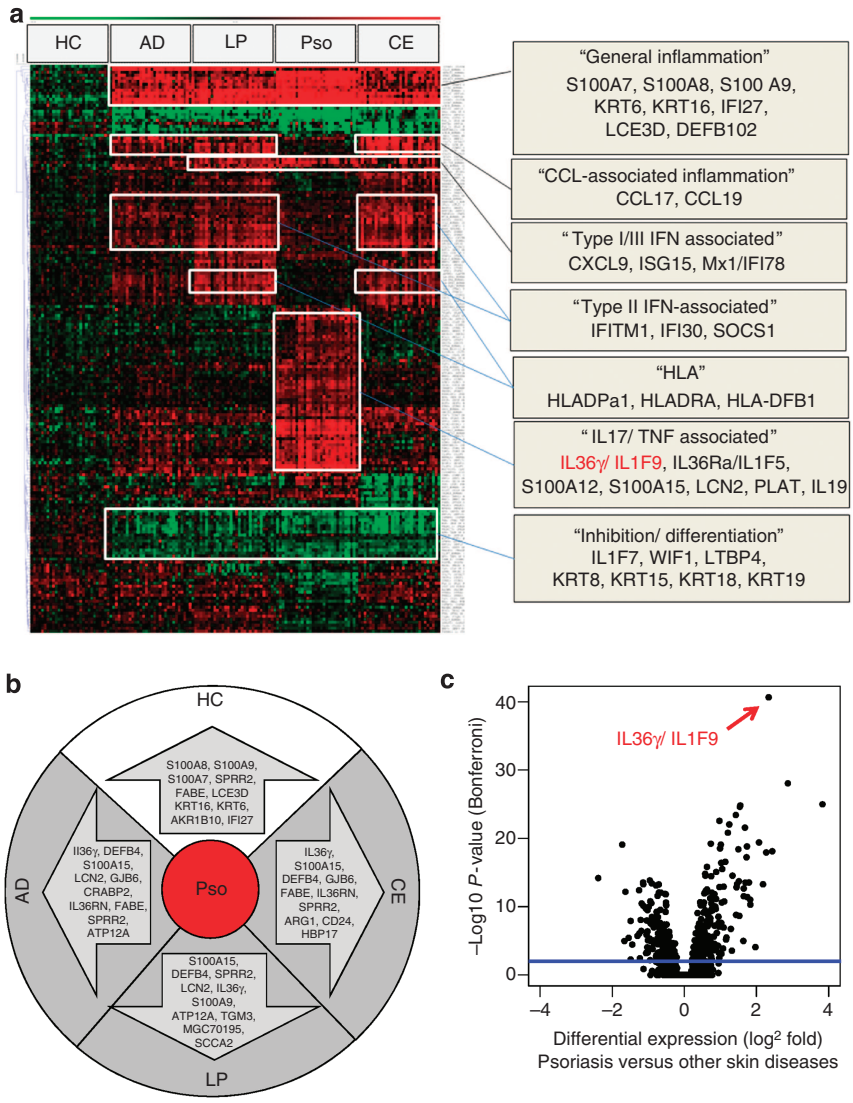


Figure 1. Gene expression analyses of lesional skin (psoriasis and other inflammatory skin diseases). (**a**) Unsupervised hierarchical gene cluster analysis including all 189 specifically expressed genes in one of the diseases as identified by Significance Analysis for Microarrays (SAM) (>2-fold expressed, $P<0.01$). (**b**) Top 10 upregulated genes in psoriasis when compared with HC, CE, LP, and AD, identified via SAM. (**c**) Volcano plot including all 1,539 genes of the original data set. Higher *y*-axis values (− log10 transformed *P*-values) correspond to a higher significance level of differentially expressed genes in a two-group comparison (psoriasis versus other skin diseases). The *x*-axis indicates the difference in gene expression between the two groups. Gene expression values were log2 transformed to ensure normal distribution. Positive values indicate higher expression in psoriasis samples versus other skin diseases and vice versa for negative values. AD, atopic dermatitis; CE, contact eczema; HC, healthy controls; LP, lichen planus; Pso, psoriasis vulgaris. $n=150$.

upregulated in all inflammatory skin diseases ("general inflammation") but not in healthy controls (HCs). Importantly, this gene-set encompassed nearly all of the top-regulated genes found in the different skin diseases as given in Table 1, including the S100 proteins A7, A8, A9, keratins KRT6 and KRT16, the IFN-regulated IFI27, as well as LCE3 and DEFB4. The mean expression rates of all significantly regulated genes are provided in Supplementary Table 1 online.

Downregulation of genes involved in regulation and differentiation of inflammatory skin diseases

Inversely to the pro-inflammatory factors commonly expressed in all inflammatory skin diseases, we found a set of jointly downregulated genes, reflecting the diminished immune regulation and differentiation in skin inflammation. These genes included WIF1, which has a role in tissue differentiation, LTBP4, which is involved in the regulation of TGF beta bioavailability, the keratins KRT8 and KRT18, and the anti-inflammatory IL-37, which is a natural suppressor of innate inflammatory and immune responses (Nold *et al.*, 2010).

Expression of specific gene clusters in different inflammatory skin diseases

As depicted in Figure 1a, the unsupervised cluster analysis highlighted the expression of specific gene clusters in different skin diseases. Genes associated with CCL chemokines (CCL17 and CCL19) and those reflecting a type II IFN-driven inflammation (IFITM1, IFI30, and SOCS1) were found in AD and LP, as

Table 1. Top 10 expressed genes in inflammatory skin diseases versus healthy controls

	AD		LP		Pso		CE	
	Gene	Count[1]	Gene	Count	Gene	Count	Gene	Count
1	S100A8	4.64	S100A8	4.23	S100A8	6.37	S100A8	4.76
2	LCE3D	3.73	KRT16	3.35	S100A9	5.70	S100A9	3.87
3	S100A9	3.53	S100A9	3.17	DEFB4	5.01	S100A7	3.02
4	CCL18	3.45	CCL18	3.05	S100A7	4.68	CCL18	3.01
5	S100A7	3.17	IFI27	2.92	LCE3D	4.36	KRT16	2.87
6	KRT16	3.07	KRT6	2.86	KRT6	4.01	CCL17	2.66
7	KRT6	2.34	LCE3D	2.85	SPRR2	3.94	LCE3D	2.56
8	AKR1B10	2.19	CXCL9	2.75	KRT16	3.77	MMP12	2.55
9	IFI27	2.10	S100A7	2.63	AKR1B10	3.22	TNFC	2.46
10	SPRR1A/B	1.92	BST2	1.99	FABE	3.09	CCL19	2.25

Abbreviations: AD, atopic dermatitis; CE, contact eczema; LP, lichen planus; Pso, psoriasis vulgaris.
[1]Count: differential expression (based on a log2 score) of the specific gene in disease versus healthy control (Skin Patho PIQOR microarray, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany).

well as in CE. HLA-related genes, including HLA-DPa1, HLA-DRA, and HLA-DFB1, were typically found in LP and CE, whereas type I/III IFN-associated genes, including CXCL9, ISG15, and Mx1/IFI78, were specifically found in LP, Pso, and CE but not in AD and HC. These results are in accordance with earlier studies concerning gene markers in these other inflammatory diseases and support the informative value of our approach (Pedersen *et al.*, 2007; Wenzel *et al.*, 2008; Kamsteeg *et al.*, 2010).

IL-17/TNFα-associated genes are specifically expressed in psoriasis skin samples

In psoriasis, the unsupervised cluster analysis revealed a large set of mainly IL-17/TNFα-associated inflammatory genes specifically expressed in this disease (detailed data are provided in Supplementary 2 online). Interestingly, this cluster included a number of genes regulated only by a combined effect of TNFα and IL-17, including the inflammatory response genes S100A15 (Koebnerisin), S100A12, the platelet-derived growth factor (PLAT), and the cytokines IL-19 and IL-36Ra. Further, genes in this cluster were LCN2 (Lipocalin 2), which is regulated by IL-17 alone, and the cytokine IL-36γ, which may be induced by IL-17 or TNFα alone, as well as by combined treatment with TNFα and IL-17 (Chiricozzi *et al.*, 2011).

SAM analyses identify potential discriminators between psoriasis and other inflammatory skin disorders

In the next step, we performed a set of Significance Analysis of Microarrays (SAM) (Tusher *et al.*, 2001) comparing the gene expression results in psoriasis with that of the other inflammatory diseases (AD, LP, and CE) in order to identify potential markers for psoriasis (Figure 1b and Supplementary Table 2 online). These analyses revealed the upregulation of several specific genes in the psoriasis samples, most of them in concordance with the genes seen in the psoriasis-specific cluster achieved by the unsupervised cluster analysis (Figure 1a), including IL-36γ, IL-36Ra, S100A15, LCN2, ATP12A, GJB6, TGM3, and CD24.

Volcano plot identifies IL-36γ/IL-1F9 as a specific psoriasis marker

To focus on single genes that might serve as specific psoriasis biomarkers, we used a volcano plot to visualize the most significantly and differentially expressed genes in psoriasis versus the other inflammatory skin diseases (Figure 1c) (Chen *et al.*, 2007). This approach revealed IL-36γ/IL-1F9 as the clear top outlier, and therefore we selected this gene as the most promising candidate for our subsequent analysis. Interestingly, the expression of IL-36γ was only moderately associated with the expression of other potential psoriasis markers, including S100-A7, -A8, -A9, -A15, DEFB4, and IL-19 ($\rho = 0.43$–0.64, $P = 0.018$–0.001, see Supplementary 2 online).

Immunohistology confirms the strong expression of IL-36γ in psoriasis

To confirm the expression of IL-36γ on the protein level, we performed immunohistological analyses in the corresponding skin samples. These analyses clearly reflected the picture of IL-36γ expression observed in the gene expression analyses (Figure 2a). Psoriasis samples showed a strong expression of IL-36γ in the upper epidermal layer, mostly in four and more cell layers, whereas this staining was only weakly found in the control diseases (AD, LP, and CE) and absent in HCs (Figure 2b). As depicted in Figure 2c, the gene expression score in these samples was closely associated with the histological score (Spearman's $\rho = 0.71$, $P < 0.01$). In addition, we analyzed the lesional IL-36γ expression in pustular psoriasis, which was similar to the Pso findings (Supplementary 3 online).

Low IL-36γ protein expression in other erythrosquamous skin disorders

In the next step, we included additional erythrosquamous skin diseases with potential differential diagnoses to psoriasis (pityriasis lichenoides, subacute cutaneous lupus erythematosus, tinea, and mycosis fungoides, $n = 5$, respectively) in the immunohistological analyses. In all these entities, only weak

Table 2. Potential discriminators of psoriasis versus other inflammatory skin disorders

	Pso versus AD				Pso versus LP				Pso versus CE				Pso versus HC			
	Up Pso		Up AD		Up Pso		Up LP		Up Pso		Up CE		Up Pso		Up HC	
	Gene	Rate[1]	Gene	Rate	Gene	Rate	Gene	Rate	Gene	Rate	Gene	Rate	Gene	Rate	Gene	Rate
1	IL-36γ	10.2	CCL18	7.3	S100A15	11.2	HLA-DPB1	7.1	IL-36γ	9.2	CD52	7.1	S100A8	28.3	IL-37	9.7
2	DEFB4	9.7	CRIP1	7.0	SPRR2	10.0	CD52	6.8	S100A15	8.0	CRIP1	6.2	S100A9	25.2	SERPINA12	9.2
3	S100A15	8.4	CCL27	7.0	DEFB4	9.7	CCL18	6.7	GJB6	7.4	CCL27	6.0	S100A7	23.0	CST6	9.0
4	LCN2	8.0	CCL17	5.3	LCN2	9.4	MMP9	6.1	DEFB4	7.2	CCL18	5.9	SPRR2	18.3	CCND1	8.9
5	GJB6	7.8	ALCAM	4.9	IL-36γ	9.3	HLA-DPA1	6.1	FABE	7.1	CCL17	5.7	FABE	17.1	—	—
6	CRABP2	6.8	LGALS1	4.8	S100A9	8.4	RARRES3	6.0	IL-36Ra	6.4	KAP10	5.4	LCE3D	17.0	—	—
7	IL-36Ra	6.7	CCL13	4.7	ATP12A	8.0	CXCL9	5.9	SPRR2	6.1	TNFC	5.3	KRT16	14.9	—	—
8	FABE	6.7	MS4A6A	4.6	TGM3	7.5	TNFC	5.8	ARG	6.0	TIMP1	5.2	KRT6	14.7	—	—
9	SPRR2	6.5	PRRX1	4.5	MGC70195	7.4	KAP10	5.8	CD24	6.0	CORO1A	5.0	AKR1B10	14.3	—	—
10	ATP12A	6.5	POSTN	4.4	CD24	7.3	CRIP1	5.7	HBP17	5.8	ALCAM	5.0	IFI27	13.8	—	—

Abbreviations: AD, atopic dermatitis; CE, contact eczema; HC, healthy controls; LP, lichen planus; Pso, psoriasis vulgaris; Up, upregulation.
[1]Rate: the observed relative expression rate as calculated by SAM analyses.

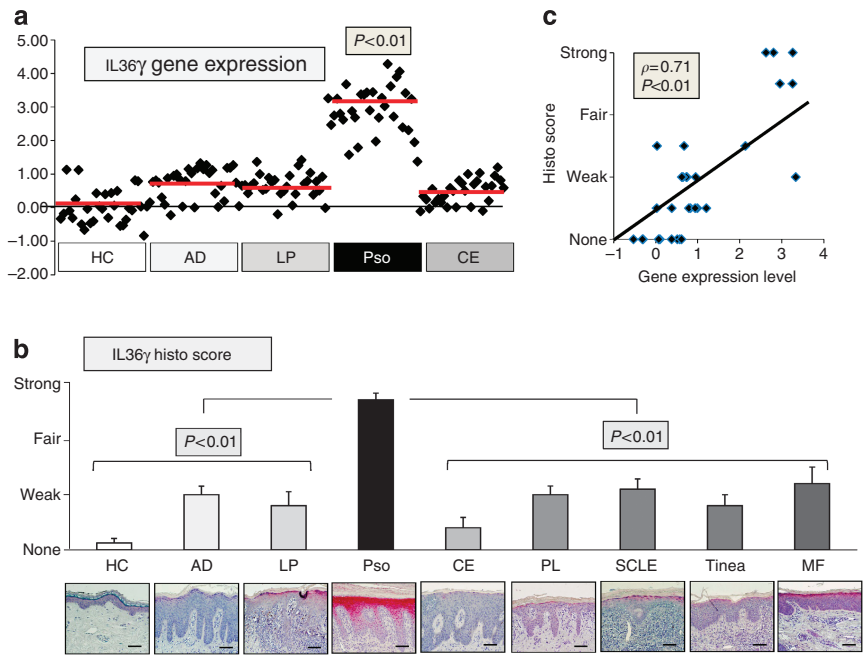
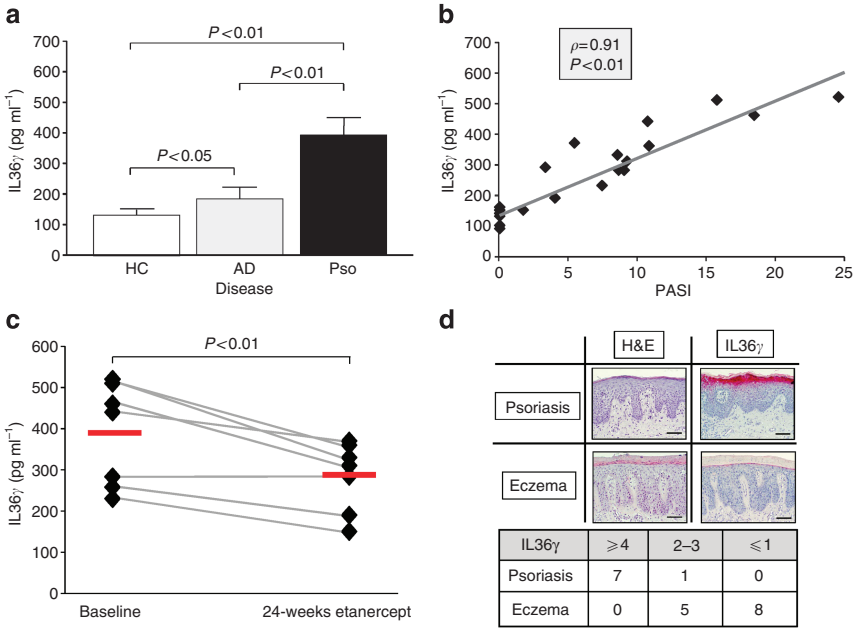


Figure 2. IL-36γ gene and protein expression in psoriasis and other skin diseases. (**a**) Individual gene expression score of IL-36γ in psoriasis skin lesions and controls ($n=30$ for each subset). Comparison of IL-36γ gene expression in psoriasis versus HC, AD, LP, and CE: each comparison significant with $P<0.01$ (Mann–Whitney *U*-test). (**b**) Protein expression of IL-36γ in different erythrosquamous skin diseases (mean expression ± SEM; $P<0.01$, Mann–Whitney *U*-test) and corresponding representative immunohistological micrographs (original magnification ×200, scale bar = 0.1 mm). $n=10$ for HC, AD, LP, Pso, and CE, respectively, and $n=5$ for PL, SCLE, Tinea, and MF, respectively. (**c**) Correlation (Spearman's ρ) between gene expression and histological score. Included are 40 of the original samples, with $n=10$ for HC, AD, LP, and Pso, respectively. HC, healthy controls; AD, atopic dermatitis; LP, lichen planus; Pso, psoriasis vulgaris; CE, contact eczema; PL, pityriasis lichenoides; SCLE, subacute cutaneous lupus erythematosus; MF, mycosis fungoides.

expression of IL-36γ was observed (Supplementary Figure 2b online).

IL-36γ serum levels in peripheral blood are enhanced and correlate with disease activity

IL-36γ ELISA analyses of peripheral blood serum samples (taken from psoriasis patients in different stages of disease, as well as from healthy donors and patients who suffered from AD) were performed to investigate a potential systemic relevance of this cytokine. As depicted in Figure 3a, IL-36γ levels in untreated psoriasis patients were significantly elevated when compared with AD and HC ($P<0.01$). Importantly, the IL-36γ serum levels in all psoriasis patients (under treatment and untreated) correlated closely to the disease



IL36γ	⩾4	2–3	⩽1
Psoriasis	7	1	0
Eczema	0	5	8

Figure 3. IL-36γ peripheral blood serum levels and predictive value of IL-36γ immunohistochemistry. (**a**) Mean IL-36γ blood serum levels ± SEM in untreated patients with psoriasis (Pso, $n=7$), atopic dermatitis (AD, $n=5$), and healthy controls (HCs, $n=7$). (**b**) Correlation (Spearman's ρ) between PASI (psoriasis area and severity index) score and IL-36γ serum levels in treated and untreated individuals ($Pso_{untreated}$ $n=7$, $Pso_{treated}$ $n=7$, and HCs, $n=7$). (**c**) Individual and mean IL-36γ serum levels in untreated (baseline) and etanercept-treated (after 24 weeks of treatment) psoriasis patients. Wilcoxon signed-rank test. (**d**) Representative micrographs of psoriasis and eczema skin biopsies (hematoxylin and eosin staining, IL-36γ immunohistochemistry, original magnification × 200, scale bar = 0.1 mm); table of predictive value of IL-36γ immunohistochemistry (divided into three categories depending on the amount of cell layers stained: ⩾4/2–3/⩽1 cell layers; $n=21$). Pso, psoriasis vulgaris.

activity as determined by psoriasis area and severity index (depicted in Figure 3b, Spearman's $\rho=0.91$, $P<0.01$).

Significant decrease in IL-36γ serum levels under anti-TNFα treatment

To investigate the impact of an anti-inflammatory drug on the IL-36γ serum levels, serum samples of seven psoriasis patients before (''baseline'') and after 24-week (''under treatment'') treatment with the anti-TNFα drug etanercept were analyzed. Here the anti-TNF-treatment led to a significant decline of the IL-36γ serum levels (see Figure 3c). Detailed data are provided in Supplementary 4 online.

Predictive diagnostic value of IL-36γ staining in skin biopsies

Finally, we investigated the predictive diagnostic value of immunohistological IL-36γ staining as a diagnostic marker in unclear cases. We identified 21 samples in our database with a clinical follow up of at least 2 years, which had been difficult to classify in the primary histological biopsy (taken between 2009 and 2011) but were diagnosed either ''psoriasis'' or ''eczema'' in the meantime. These biopsies were stained for IL-36γ expression, blinded, and evaluated by two experienced dermatopathologists (AD and JW). A strong expression of IL-36γ (⩾4 cell layers) was specifically found in psoriasis ($n=7$), whereas low expression (⩽1 cell layer) was only found in eczema ($n=8$). Six cases showed a marginal IL-36γ expression (2–3 cell layers) and could not be reliably classified based on their IL-36γ staining.

Beyond the threshold values (⩾4 and ⩽1, respectively), the staining was entirely consistent with the clinical diagnosis—i.e., in 15 out of 21 samples, we were able to assign a specific diagnosis based solely on IL-36γ staining. Twenty-nine percent of the cases remained unclassified (Figure 3d). Using the given critical value (⩾4), IL-36γ had a highly specific positive predictive diagnostic value for psoriasis.

DISCUSSION

In the present study, gene expression analyses in patients with different inflammatory skin diseases revealed a cluster of genes significantly upregulated in psoriasis, which are particularly regulated by IL-17 and TNFα (Chiricozzi *et al.*, 2011). Among these, IL-36γ was found to be the most specifically regulated gene in psoriasis when compared with other inflammatory skin diseases. This finding was confirmed by immunohistochemistry (IHC) on the protein level. Importantly, IL-36γ blood serum levels were closely associated with the psoriasis disease activity (as measured by psoriasis area and severity index) and decreased under treatment with anti-TNFα drugs, and IL-36γ IHC was useful in tissue diagnosis of psoriasis from eczema.

Biological function of IL-36γ

IL-36γ belongs to the newly identified IL-36 cytokine family, formerly known as IL-1F cytokines, which is related to the IL-1 family. The IL-36 family comprises the agonistic cytokines IL-36α (=IL-1F6, also called IL-1ε), IL-36β (=IL-1F8), and IL-36γ (=IL-1F9), as well as the antagonistic cytokine IL-36Ra (=IL-1F5, also called IL-36RN and IL-1δ) (Dinarello *et al.*,

2010). Recent evidence indicates that the IL-36 cytokines activate similar intracellular signals as IL-1 and are involved in the regulation of innate, as well as adaptive immune responses (Vigne *et al.*, 2011). The agonistic IL-36R ligands stimulate pro-inflammatory pathways by binding to IL-1 receptor-like 2 (IL-1RL2) and IL-1RAcP resulting in the activation of mitogen-activated protein kinase and NF-kB signal transduction. They signal through the mitogen-activated protein kinase, c-Jun N-terminal kinase, and extracellular signal–regulated kinase1/2 pathways and enhance the secretion of pro-inflammatory factors including IL-6 and IL-8 (Towne *et al.*, 2004; Boraschi and Tagliabue, 2013).

The IL-36s are expressed in a restricted manner, primarily by keratinocytes in the skin but also by other epithelial tissue including bronchial epithelia, suggesting that these proteins are involved in first-line defenses against microorganisms (Gresnigt and van de Veerdonk, 2013). Interestingly, IL-36 cytokines also have synergistic effects on the induction of antimicrobial peptides by IL-17A or TNFα, indicating that Th17 cytokines and IL-36 can reinforce similar responses in keratinocytes (Carrier *et al.*, 2011).

IL-36γ expression is upregulated by toll-like receptor ligands including polyinosinic-polycytidylic acid (poly(I:C)) and flagellin, supporting the notion that IL-36γ might act as an alarmin and is expressed in response to activation of the innate immune system (Lian *et al.*, 2012). IL-36γ induces the production of several pro-inflammatory cytokines, including IL-12, IL-6, TNFα, and IL-23, and contributes to skin inflammation by acting on keratinocytes, dendritic cells, and, indirectly, on T lymphocytes (Vigne *et al.*, 2011; Foster *et al.*, 2014). Vice versa, IL-17 and TNFα, which both are typically expressed by immune cells, are able to enhance the expression of IL-36γ by keratinocytes (Chiricozzi *et al.*, 2011; Johnston *et al.*, 2013). Therefore, IL-36γ appears to have a central amplifying position in pro-inflammatory pathways at the interface between innate and adaptive immunity (Vigne *et al.*, 2011; Lowes *et al.*, 2013).

Role of IL-36γ in inflammatory skin diseases

The first evidence for a potential role of the IL-36 family in inflammatory skin diseases emerged in 2007 when Blumberg *et al.* (2007) generated transgenic mice expressing IL-36α/IL-1F6 in basal keratinocytes. These mice developed a psoriasis-like skin phenotype characterized by acanthosis, hyperkeratosis, and the presence of a mixed inflammatory cell infiltrate. In addition, the authors found an increased expression of IL-36α in human psoriatic skin. These findings were supported by two subsequent studies: Johnston *et al.* (2011) found an increased expression of all IL-36 cytokines (α, β, and γ) and of IL-36Ra in human psoriasis skin lesions ($n=20$), as well as in two psoriasis mouse models (KC-Tie2 mice and imiquimod-treated C57BL/6 mice) by PCR and IHC. He *et al.* (2013) showed an enhanced expression of IL-36γ in human psoriasis skin lesions.

These results are confirmed by our own findings, revealing IL-36γ as a highly expressed marker within psoriasis skin lesions. Unlike other markers described earlier in psoriasis, including, for example, the S100 proteins A7, A8, and A9 (Semprini *et al.*, 1999; Liu *et al.*, 2007; Glaser *et al.*, 2009; Kerkhoff *et al.*, 2012), IL-36γ was highly specific for psoriatic inflammation but only weakly expressed in other inflammatory skin diseases (AD, CE, and LP). These data are in accordance with the results by Kamsteeg *et al.* (2010), who found a high expression of IL-36γ in psoriasis but not in eczematous diseases. In addition to these findings, our IHC results demonstrate the potential impact of this marker in the discrimination of psoriasis against other most common erythrosquamous skin diseases, including also pityriasis lichenoides, subacute cutaneous lupus erythematosus, tinea, and mycosis fungoides.

Recent studies implicate a central role for the IL-36s in the pro-inflammatory network in psoriasis. Overexpression of IL-36 in mouse skin leads to a disease quite similar to human plaque psoriasis, and inhibition of IL-36 in human psoriatic skin ameliorates the inflammation (Towne and Sims, 2012). This psoriasis-like dermatitis in mice is driven by IL-36-mediated DC keratinocyte cross talk (Tortola *et al.*, 2012). Human keratinocytes derived from patients with psoriasis have been shown to express significantly higher levels of IL-36γ in response to IL-17 compared with those isolated from healthy donors (Muhr *et al.*, 2011). Vice versa, aberrant function of the IL-36Ra (=IL-36RN) results in an unregulated secretion of pro-inflammatory cytokines and the clinical picture of a severe psoriasis with pustular lesions (Marrakchi *et al.*, 2011; Onoufriadis *et al.*, 2011). These findings make the IL-36 system a promising drug target in psoriasis. This is supported by the recent development of an anti-IL-36-receptor antibody (WO2013074569) for clinical use in psoriasis (Wolf and Ferris, 2014).

IL-36γ as a potential biomarker in psoriasis

Our data demonstrate that IL-36γ is not only a specific psoriasis marker for skin lesions but that IL-36γ peripheral blood serum levels are closely correlated with the disease activity and clearly decrease under treatment with the anti-TNFα drug etanercept. These latter findings are supported by Johnston *et al.* (2011) who detected a decrease in lesional expression of IL-36γ mRNA in the skin under treatment with this drug. Furthermore, we demonstrated that IL-36γ might be a helpful diagnostic marker in the at times difficult histological differential diagnosis between eczematous psoriasis and psoriasiform eczema. Here strong expression of IL-36γ ($\geqslant 4$ cell layers) in IHC was found to be a highly specific diagnostic marker for psoriasis.

Conclusion

Our results demonstrate that psoriasis skin lesions are characterized by the expression of a specific gene cluster, including several IL-17/TNFα-associated cytokines. Among these, IL-36γ is the most outstanding marker. IL-36γ expression in psoriasis skin lesions is significantly enhanced when compared with other erythrosquamous skin diseases. IL-36γ peripheral blood serum levels are closely associated with disease activity (psoriasis area and severity index) and decline under anti-TNFα treatment. Finally, IL-36γ IHC has a highly positive predictive value to discriminate psoriasis from eczema skin lesions in unclear cases.

These features highlight IL-36γ as a valuable future biomarker in psoriasis patients, both for diagnostic purposes and for monitoring of disease activity during the clinical course. Furthermore, IL-36γ might also provide a future drug target, because of its potential amplifier role in TNFα and IL-17 pathways in psoriatic skin inflammation.

MATERIALS AND METHODS

Skin samples

Skin samples of 150 donors (30 patients with Pso, AD, CE, LP, and HC, respectively) were analyzed. Only biopsies taken from untreated patients with typical skin lesions were included. Diagnosis was confirmed by standard histological techniques in every case. The study was approved by the local ethic committee in Bonn (No. 12201) and fulfils the Declaration of Helsinki Principles. All patients signed a consent form. Skin samples were divided into two parts immediately after excision. One part was flash frozen in liquid nitrogen and later processed for mRNA isolation. The second part was fixed in 5% formalin solution over night and was subjected to conventional histological investigation and IHC. The details of this study have been described before (Wenzel *et al.*, 2008). In addition, skin samples of other erythrosquamous skin diseases (pityriasis lichenoides, $n=5$), subacute cutaneous lupus erythematosus ($n=5$), Tinea ($n=5$), mycosis fungoides ($n=5$), and previously unclassified psoriasiform dermatitis ($n=21$) were included from the authors archives.

RNA isolation

Isolation of total RNA from skin excision biopsies was performed using TriReagent (Sigma, St Louis, MO) and the NucleoSpin 96 RNAKit (Macherey and Nagel, Dueren, Germany). RNA was quantified by photometrical measurement, and the integrity was assessed using a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA).

Gene expression analyses

The Skin Patho PIQOR (Parallel Identification and Quantification of RNAs) microarray (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) was used for gene expression analyses. Here Cy5-labeled RNA from disease samples was hybridized against a Cy3-labeled common skin reference pool, as described before (Wenzel *et al.*, 2008). Hybridization, scanning, and data analysis were performed in accordance with the MIAME (Minimum information about a microarray experiment) standards. All raw data are available at GEO (Gene Expression Omnibus, http://www.ncbi.nlm.nih.gov/geo/), accession number GSE63741.

Histology and immunohistology

Sections were prepared from formalin-fixed, paraffin-embedded skin biopsies. Standard hematoxylin and eosin, as well as periodic acid Schiff staining were performed for diagnostic purposes. The protein expression of IL-36γ was analyzed by IHC using the monoclonal mouse IgG1 anti-human-IL-36γ antibody ab156783 (Abcam Inc., Cambridge, MA) without pretreatment with a dilution of 1:500. The lesional IL-36γ expression was scored semiquantitatively (0 = no expression; 1 = weak expression; 2 = fair expression and 3 = strong expression) by two experienced dermatopathologists (JW and AD) (Wenzel *et al.*, 2005). Visualization was performed using the REAL staining kit (DAKO, Hamburg, Germany) with Fast Red as chromogen.

IL-36γ blood serum concentrations

Blood samples of patients with psoriasis in stage of untreated active disease (''baseline''), as well as after 24 weeks of systemic treatment with etanercept (''24-week treatment'') (at the dose of 50 mg twice a week for 12 weeks followed by 50 mg once a week) were collected in our outpatient clinic (Pso untreated: $n=7$, Pso treated: $n=7$). In addition, the psoriasis area and severity index score was determined for each patient to measure the disease activity at the different time points. Furthermore, serum samples from untreated AD patients ($n=5$) and healthy donors (HCs, $n=7$) were taken for control purposes. Serum samples were obtained from peripheral blood, centrifuged at 1,500g at 4 °C for 15 minutes, and subsequently stored at −20 °C until analysis. The serum concentrations of IL-36γ were measured in duplicates by ELISA using a commercial kit (SEL621Mu, Cloud-Clone, Houston, Tx) according to the manufacturer's protocol.

Statistical analysis

Gene expression analyses. Primary statistical analyses were performed by using the TMEV software, version 4.9 (Dana-Farber Cancer Institute, Boston, MA). Unsupervised hierarchical cluster analysis with Pearson's correlation coefficient was used to investigate the average linkage of the genes within the different subsets (Eisen *et al.*, 1998). To identify potential marker genes in psoriasis, Significance Analysis for Microarrays was used to identify genes specifically expressed in the different subsets (expression > 2-fold enhanced, $P<0.01$) (Tusher *et al.*, 2001). This method included two-sided unpaired *t*-tests for each of the 1,539 unique genes represented on the microarray chip using the R programing environment and the Bioconductor platform. Raw *P*-values were corrected for multiple testing by the Bonferroni method (Tusher *et al.*, 2001). Subsequently, a volcano plot was created as a widely used approach to visualize the significance of differentially expressed genes in microarray analysis (Chen *et al.*, 2007). Corrected *P*-values are represented on the *y*-axis of the volcano plot after a negative log10 transformation as a common standard. Gene expression values were log2 transformed to ensure normal distribution of the data as a prerequisite for the subsequent statistical methods according to standard procedures in microarray analysis.

Statistics for IHC and ELISA analysis. Using SPSS 22, the non-parametrical Mann–Whitney *U*-test was performed to compare the mean staining intensity of IL-36γ in the different skin diseases, as well as to compare mean serum levels as determined by ELISA. The Wilcoxon signed-rank test was used to compare IL-36γ serum levels before and after Etanercept treatment. Correlation analyses were performed by calculating Spearman's rho. *P*-level <0.05 was considered to be significant (*) and *P*-level <0.01 as highly significant (**).

CONFLICT OF INTEREST

The authors state no conflict of interest. AB is an employee of Miltenyi Biotec, Germany.

ACKNOWLEDGMENTS

We thank Nadine van Holt for her most valuable support in preparation of the manuscript, Kai Hofmann and Stefan Tomiuk for their excellent bioinformatics

analysis of microarray data, Sabrina Schmitz for outstanding technical assistance in gene expression profiling, and Silvia Rüberg for her great assistance with the GEO submission. This work was supported by a grant of the Deutsche Forschungsgemeinschaft (DFG) to JW (WE-4428), by the René Touraine Foundation (for AD), and by the PTJ reference number 0306v12 as part of the Technology and Innovation Program (TIP) North-Rhine Westphalia (gene expression analyses). MH is a member of the DFG excellence cluster ImmunoSensation.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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