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Original Article

Upregulation of cathepsin S in psoriatic keratinocytes

Alexander Schönefuß[1]*, Wiebke Wendt[2]*, Benjamin Schattling[1], Roxane Schulten[1], Klaus Hoffmann[3], Markus Stuecker[3], Christian Tigges[3], Hermann Lübbert[1,2] and Christine Stichel[2]

[1]Department of Animal Physiology, Ruhr-University of Bochum, Bochum, Germany;
[2]Biofrontera Bioscience GmbH, Leverkusen, Germany;
[3]Department of Dermatology and Allergology, Ruhr-University Bochum, Bochum, Germany
Correspondence: Christine C. Stichel, Animal Physiology, Biology, ND5/132, Ruhr-University Bochum, D-44780 Bochum, Germany,
Tel.: +49-234-32-25829, Fax: +49-234-32-14189, e-mail: c.stichel-gunkel@biofrontera.com
**These authors contributed equally to this work.*

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Abstract: Cathepsin S (CATS) is a cysteine protease, well known for its role in MHC class II-mediated antigen presentation and extracellular matrix degradation. Disturbance of the expression or metabolism of this protease is a concomitant feature of several diseases. Given this importance we studied the localization and regulation of CATS expression in normal and pathological human/mouse skin. In normal human skin CATS-immunostaining is mainly present in the dermis and is localized in macrophages, Langerhans, T- and endothelial cells, but absent in keratinocytes. In all analyzed pathological skin biopsies, i.e. atopic dermatitis, actinic keratosis and psoriasis, CATS staining is strongly increased in the dermis. But only in psoriasis, CATS-immunostaining is also detectable in keratinocytes. We show that cocultivation with T-cells as well as treatment with cytokines can trigger expression and secretion of CATS, which is involved in MHC II processing in keratinocytes. Our data provide first evidence that CATS expression (i) is selectively induced in psoriatic keratinocytes, (ii) is triggered by T-cells and (iii) might be involved in keratinocytic MHC class II expression, the processing of the MHC class II-associated invariant chain and remodeling of the extracellular matrix. This paper expands our knowledge on the important role of keratinocytes in dermatological disease.

Key words: cysteine protease – keratinocyte – psoriasis – T-cell

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Introduction

Controlled proteolysis is vital (fundamental) to the development, differentiation, regeneration and homeostasis of the skin. Investigations over the last decade have shown that one group of lysosomal proteases, the cathepsins (CAT), plays a major role in these proteolytic processes both intra- and extracellular (1).

Cathepsins comprise one of the largest and best characterized family of lysosomal enzymes composed of cysteine, aspartic and serine peptidases. The cysteine proteases represent the major group of cathepsins. At the gene level there are 11 human members (CATB, C, F, H. K, L, O, S, V, W and X) known (2,3). As a number of other proteases, CAT are synthesized as inactive preproenzymes, which require proteolytic removal of the N-terminal propeptide for their activity. This activation can be autocatalytic or facilitated by other proteases (4). Aside from their housekeeping lysosomal function in the metabolic turnover of proteins cathepsins possess highly specific and directed proteolytic activities. In the skin they are involved in extracellular matrix remodelling, keratinocyte differentiation, hair follicle cycle, apoptosis and MHCII-mediated antigen presentation (5,6). In the latter process CATS has been implicated in the last step of Ii degradation in B-cells and dendritic cells thereby generating the class II-associated invariant chain peptides (CLIP) (7). Therefore, CATS is most important for CD4-positive T-cell function in lymphatic tissues. In other cell types, e.g. macrophages, or tissues, e.g. thymus, other cathepsins like CATB, CATL, CATF and CATV assume the role of CATS (4,8–10).

In line with these physiological functions CATS is preferentially localized in phagocytic and antigen-presenting cells

Abbreviations: AD, atopic dermatosis; AK, actinic keratosis; AP, antigen-presenting cell; CATS, cathepsin S; CK, cytokeratin; CLIP, class II invariant chain-associated peptide; DRG, dorsal root ganglion; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; HLA-DR, human leukocyte antigen DR alpha chain; Ii, MHCII-associated invariant chain; Ii-p10, p10 fragment of Ii; Ii-p35, p35 isoform of Ii; INFg, interferon-γ; MF, mature form of CATS; MHCII, major histocompatibility complex class II; PF, proform of CATS; PS, psoriasis; Tryp, tryptase.

in various tissues including the skin. Nevertheless, CATS also appears to be expressed in a number of non-immune cells, like neurons, endothelial cells and vascular smooth-muscle cells (11–15). With respect to the skin CATS was only detected in cell cultures, the human keratinocyte cell line HaCaT and freshly isolated human keratinocytes (16). Moreover, these authors also showed that the expression of CATS in cultured keratinocytes is regulated by cytokines implying a major importance in the pathogenesis of dermatoses which are accompanied by inflammation. Surprisingly nothing is known regarding CATS expression and function in normal and pathological skin.

With these strong implications for a role of CATS skin physiology we decided to study the expression of CATS in normal skin and three skin diseases; psoriasis, atopic dermatitis and actinic keratosis. In addition we analyzed the regulation and role of this protease in epi-/dermal processes using a cell culture approach.

Materials and methods

Clinical material

Formalin-fixed, paraffin-embedded and fresh psoriasis (PS) and actinic keratosis (AK) biopsy material (abdomen, arm, leg, scalp, face) were obtained from the Clinic for Dermatology and Allergology, Ruhr-University Bochum (Germany). Eight psoriasis (psoriasis vulgaris; ICD-10: L40.0, duration of disease 2 months–4 years) and seven actinic keratoses (ICD-10: L57.0) were selected. All biopsies were taken with the informed patient's consent and approval of the local medical ethical committee. The diagnoses were made by experienced dermatopathologists (MS, CT).

Samples of normal human skin ($n = 4$) were obtained from operative tissue (breast, abdomen, forehead, scalp) with informed consent and approval of the local medical ethical committee. No cases had a history of inflammatory skin disease or any kind of skin cancer. Skin probes were either fresh frozen and prepared for Western blot analysis or fixed in 4% paraformaldehyde, embedded in paraffin and cut into 5 μm thick sections.

Animal treatment

NC/Nga mice (weighing 25 g) were purchased from Charles River Lab. (Sulzbach, Germany). They were housed in a 12:12 h light:dark cycle with free access to food and water. Ambient temperature was maintained at 24°C.

Atopic dermatitis (AD) was induced according to a previously described method (17). Mice ($n = 5$) were first sensitized by application of 20 μl of 5% oxazolone (4-ethoxymethylene-2-phenyl-2-oxazolin-5-one, Sigma-Aldrich, Munich, Germany) dissolved in ethanol on both flanks. Starting from day-7 after sensitization mice were treated topically with 60 μl of 0.1% oxazolone once every other day for an additional 2 weeks. Control animals ($n = 5$) were treated with ethanol only.

Two days after treatment animals were perfused transcardially with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB). Skins were immediately removed and postfixed overnight at 4°C. Tissue blocks were embedded in paraffin and serially cut into 3 μm thick sections.

Cell cultures

In this study we used a keratinocyte (HaCaT, passages 79–86) and a T-lymphoma cell line. The HaCaT line of spontaneously immortalized human keratinocytes (18), kindly provided by Prof. Fusenig (German Cancer Research Center, Heidelberg, Germany), was cultured in Dulbecco's modified Eagle medium (DMEM) with 10% fetal calf serum (FCS). The human T-cell leukaemia line HuT-78 (LGC Promochem, Wesel, Germany) was grown in RPMI-1640 (Invitrogen, Karlsruhe, Germany) supplemented with 10% FCS (PAA Laboratories GmbH, Cölbe, Germany). Both cell lines were subcultivated two to three times per week.

Stimulation/coculture of cells

HaCaT cells, cultivated up to 90% confluency, were stimulated with 5 ng/ml recombinant, human interferon-γ (INFg; Invitrogen, Karlsruhe, Germany) or 5 ng/ml recombinant, human tumor necrosis factor-α (TNFa; Invitrogen) in serum-free DMEM for 24 h at 37°C. For the inhibitor studies, cells were pretreated with 40 μM of the CATS inhibitor Z-Val-Val-Nle-diazomethylketone (Bachem, Weil am Rhein, Germany) in serum-free DMEM for 30 min. Subsequently, IFNg was added as described above.

For the cocultivation studies, HaCaT cells grown in six-well plates were washed twice with PBS. Subsequently, 3×10^6 HuT cells were suspended in 3 ml RPMI containing 10% FCS and placed within an insert well (Becton Dickinson Labware, Franklin Lakes, NJ, USA; 0.4 μm pore size). The cell culture insert was then placed on top of the HaCaT cells to avoid physical contact of the different cell lines (indirect coculture). Additional 3 ml RPMI containing 10% FCS were added to the HaCaT monolayer. HuT and HaCaT cells were harvested separately for Western Blot analysis 48 h later.

Reverse transcription (RT)-PCR

Total RNA from HaCaT cells was prepared. Cells were homogenized with 1 ml TRIzol (Invitrogen) per 100 mg of tissue and incubated for 5 min at RT. After adding 0.2 ml chloroform per ml TRIzol the phases were separated by centrifugation. The colourless supernatant was removed and total RNA was precipitated by adding iso-propanol 1:1 followed by a second centrifugation step. The RNA pellet was washed in 70% ethanol, air dried and dissolved in water.

Total RNA (2 μg) from HaCaT cells was used for the reverse transcription reaction. The reverse transcription

reaction was done by using the RevertAid First Strand cDNA Synthesis Kit (Fermentas, St Leon-Rot, Germany) according to the manufacturer's recommendations.

The RT-PCR reaction was performed using a Robocycler 96 (Stratagene, La Lolla, USA). Each sample contained 0.5 μl of cDNA, 2 mM $MgCl_2$, 2 mM dNTP's, 1 U Taq-polymerase (all Fermentas) and 10 pmol of CATS primers (nucleotides 574-593 and 775-794; Acc. No.: AJ002386). This solution was buffered by 10-fold PCR buffer with $(NH_4)_2SO_4$ (Fermentas). PCR amplification was initiated by incubation at 95°C and followed by 37 cycles: 45 s at 95°C, 45 s at 65°C, 45 s at 72°C. PCR products were analysed by agarose gel electrophoresis.

Western blot analysis

Sample preparation

Cell culture supernatants of HaCaT cells, grown in serum-free medium, were diluted 1:1 with 2 × Laemmli sample buffer. The adherent cells were detached from the culture dish in Dulbecco's phosphate buffer saline without Ca^{2+}/Mg^{2+}(D-PBSl Invitrogen) with a plastic cell scraper (TPP, Trasadingen, Switzerland). The obtained HaCaT cell suspensions and HuT cells grown in suspension were centrifuged for 5 min in a precooled centrifuge at 900 *g*. Cell pellets were resuspended in D-PBS diluted 1:1 with 2 × Laemmli sample buffer and boiled for 10 min. Protein determination was performed by the method of Neuhoff et al. (19).

Skin samples were homogenized 20 times in cold hypotonic buffer containing 10 mM HEPES/KOH pH 7.9, 10 mM KCL, 2 mM $MgCl_2$, 0.1 mM DTT, 0.1% (v/v) Nonidet P40 and 1 fold complete protease inhibitor cocktail (Roche, Grenzach-Wyhlen, Germany). After homogenization suspensions were incubated on ice for 15 min, subsequently vortexed for 2–3 s and centrifuged for 2 min at 12.000 *g* in a precooled centrifuge. The supernatant of these samples contained all the soluble parts of the cells. Supernatants were collected and mixed 1:1 with Laemmli buffer.

Immunoblotting

Proteins were electrophoretically separated on a 10 or 12% polyacrylamide gel containing SDS and transferred onto a PVDF-membrane (Carl Roth, Karlsruhe, Germany). After blocking in 1.5% milk powder and 1% bovine serum albumine (BSA) in TBS–Tween (0.1% Tween, 20 mM TBS), incubation with the primary antibodies (see Table 1) was conducted in blocking buffer overnight at 4°C. Proteins were detected using HRP-coupled secondary antibodies and the ECL-Plus system (Amersham Bioscience, München, Germany). Protein load was controlled by detection with mouse anti-chicken α-tubulin. To control protein loading, the tubulin amounts in each sample were determined using a mouse anti-chicken α-tubulin antibody.

To confirm the specificity of the anti-CATS antibody Western Blot analyses with different recombinant cathepsins (R&D Systems, Wiesbaden, Germany) were performed (Fig. S2i).

Histology, immunohisto- and -cytochemistry

The histopathological characterization of the skin samples (see Supporting Information), the regional and cellular

Table 1. Antibodies used in this study for Western blot analyses and immunohisto-/cytochemistry

Antigen	Antibody				
	Abbreviation	Source	Type	Dilution	Application
Alpha-tubulin	aTub	Sigma	Mouse, monoclonal	1:400.000	WB
Cathepsin S	CATS	R&D systems	Goat, polyclonal	1:200	WB
CD74 (PIN.1)	Ii	Santa Cruz	Mouse, monoclonal	1:200	WB
HLA-DRα (DA6.147)	MHCII	Santa Cruz	Mouse, monoclonal	1:200	WB
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	Santa Cruz	Goat, polyclonal	1:200	WB
Cathepsin S	CATS	Santa Cruz	Goat, polyclonal	1:200	IHC, ICC
Cytokeratin 1	CK1	Vector	Mouse, monoclonal	1:300	IHC
Cytokeratin 6	CK6	Abcam	Mouse, monoclonal	1:100	IHC
Cytokeratin 14	CK14	Vector	Mouse, monoclonal	1:300	IHC
Filaggrin	Fila	Vector	Mouse, monoclonal	1:1.000	IHC
HLA-DRα (DA6.147)	MHCII	Santa Cruz	Mouse, monoclonal	1:500	IHC
Interleukin 4	IL4	Santa Cruz	Goat, polyclonal	1:400	IHC
Leucocyte surface antigen p150,95	CD11c	Pierce	Hamster, monoclonal	1:200	IHC
Macrophage antigen CD68	CD68	Serotec	Mouse, monoclonal	1:100	IHC
T-cell receptor complex	CD3	Serotec	Rat, monoclonal	1:100	IHC
Thymocyte antigen CD1a	CD1a	Abcam	Mouse, monoclonal	1:100	IHC
Tryptase	Tryp	Abcam	Mouse, monoclonal	1:5.000	IHC
von Willebrand factor	vWF	Sigma	Rabbit, polyclonal	1:4.000	IHC

expression analyses of CATS and the phenotypic characterization of CATS expressing cells were performed by histochemical (0.01% toluidine blue for mast cells) or immunohisto-/-cytochemical methods using a panel of antibodies as described in Table 1.

Single immunohistochemical stainings of skin tissues were performed on deparaffinized sections (3–5 μm thick) after rapid melanin bleach according to the method described by (20). After antigen retrieval (5 min cooking in 0.01 M citrate buffer, pH 6.0) and incubation with 3% normal serum, sections were treated with the first antibody (see Table 1), followed by incubation with corresponding CY3-labeled (1:500; Dianova, Hamburg, Germany) or biotinylated secondary antibodies (Vector Laboratories Inc., Burlingame, CA, USA), and the ABC reagent (Axxora, Grünberg, Germany) or fluorescein isothiocyanate-labeled Avidin (1:400; Axxora). Peroxidase reaction was carried out with 3,3′-diaminobenzidine as the chromogene and was intensified with silver–gold (21). Specificity of the stainings was confirmed by omission of the primary antibodies and by preabsorption with a fivefold (by weight) excess of specific blocking peptides for 2 h at RT (Fig. S2g,h).

Data analysis

Peroxidase-labelled immunhistochemistry sections were visualized at the microscopic level (Axioskop2; Zeiss, Oberkochen, Germany) under brightfield illumination and Normarski optics, while fluorescent structures were analyzed by epifluorescence (Axioskop2). Images were captured with an imaging system (JVC, KY-F75U camera) connected to a computer equipped with an imaging program (Diskus 4.50; Hilgers, Königswinter, Germany).

Western Blot experiments were quantified with regard to CATS protein expression after normalization of each sample against the corresponding tubulin or GAPDH band using the image analysis software TINA 2.09 (Raytest, Straubenhardt, Germany). Data were analysed by means of one-way analysis of variance (ANOVA) or Kruskal–Wallis one-way analysis of variance on ranks (*H*-test) to determine statistical significance. All pairwise multiple comparison procedures (Tukey test or Student–Newman–Keuls method) were used for *post hoc* comparisons when significance (at the $P < 0.05$ level) was indicated. Comparisons between two groups were made using Student's *t*-test or Mann–Whitney *U*-test if tests on normality and/or equal variances failed. All statistical analyses were conducted with SIGMASTAT 3.1 software (Systat Software GmbH, Erkrath, Germany).

Results

CATS expression in normal skin

The expression of CATS in normal skin was examined using immunohistochemistry and Western Blot analyses.

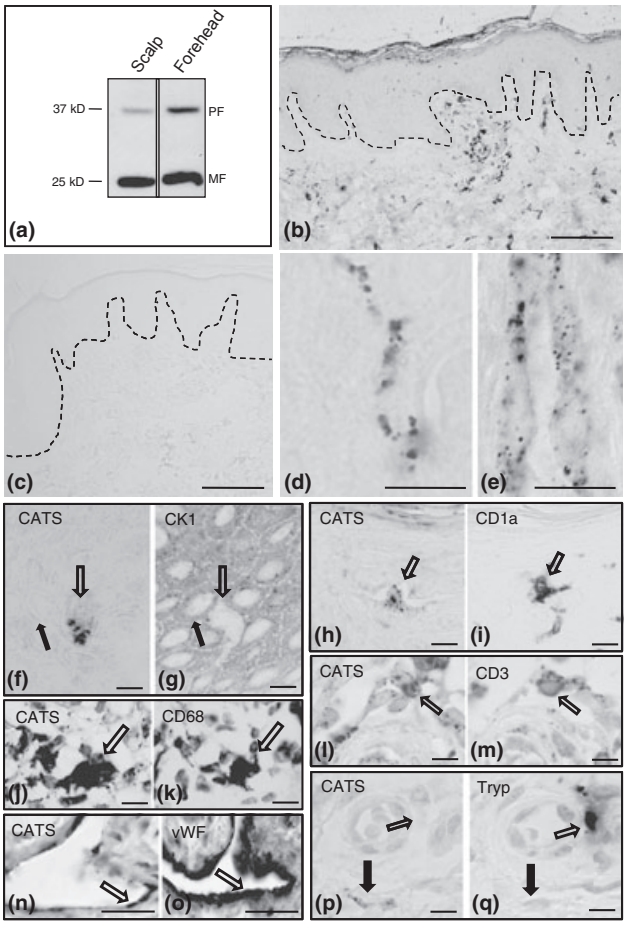

Figure 1. CATS expression in normal skin. CATS expression in nonpathological human skin. (a) Representative CATS Western blot analyses of skin samples from two different sites of human head (scalp and forehead). Signals of the proform (PF) and mature form (MF) could be detected in both samples. (b) Overview of the distribution of CATS-expressing structures in the skin. Immunohistochemistry reveals a large number of CATS-positive cells in the dermis and single cells in the epidermis. (c) Control sections were free of immunostaining. (d, e) High-power micrographs of CATS-immunopositive cells in the epidermis (d) and dermis (e). Note the granular immunodeposits in the cytoplasm of the cells. (f–q) Pairs of serial thin (3 μm) sections stained for CATS (f, h, j, l, n, p) and the cell type-specific markers CK1 (g), CD1a (i), CD68 (k), CD3 (m), von Willebrand factor (o) and tryptase (q) show expression of CATS in Langerhans cells (h, i), macrophages (j, k), T-cells (l, m) and endothelial cells (n, o), but not in keratinocytes (f, g) and mast cells (p, q). Arrows mark the same cell in neighboring sections. Dashed lines indicate the course of the basal membrane between epidermis and dermis. Scale bars: b, c (100 μm); d, e, n–q (10 μm); f–m (20 μm).

The latter revealed signals of the proform and the mature form in all skin samples analyzed (Fig. 1a), whereas the preproform could not be detected. Light immunohistochemistry indicated that CATS protein is present within cells distributed in the epidermis and dermis (Fig. 1b). While most of the dermal cells localized around blood vessels, the number of immunopositive cells in the epidermis

was small and were found in the stratum spinosum. The regional and cellular staining pattern of CATS was independent of body location.

Immunopositive cells were of small size and the reaction product appeared as granules within the cytoplasm of these cells (Fig. 1d,e). To determine the phenotype of the CATS(+)-cells we performed single stainings of adjacent serial 3–5 μm thick sections with skin cell type-specific markers. Immunostainings with the keratinocyte marker cytokeratin 1 (CK1) and the Langerhans cell marker (CD1a) revealed that all CATS(+)-cells in the epidermis of normal skin are Langerhans cells and hence that keratinocytes are free of CATS (Fig. 1f–i). In the dermis CATS(+)-cells expressed the macrophage marker CD68 (Fig. 1j,k), the T-cell marker CD3 (Fig. 1l,m) or the endothelial cell marker vWF (Fig. 1n,m), but did not colocalize with the mast cell marker tryptase (Tryp) (Fig. 1p,q).

CATS upregulation in psoriatic keratinocytes

To assess whether CATS is regulated in dermatologic diseases we examined several types of pathological skin. We analyzed skin samples from patients with psoriasis and actinic keratosis and from a mouse model for atopic dermatitis. Besides clinical histological diagnosis the skin biopsy specimens were immunohistochemically phenotyped using antibodies against cytokeratins and inflammatory cells (see Figs S1 and S2a–f). All biopsies showed the typical histopathological appearance of the respective disease (22–25).

In all analyzed pathological skin samples we observed a quantitative upregulation of CATS(+)-cells in the dermis (Fig. 2a–c). This dermal upregulation was small in AK (Fig. 2b), but substantial in PS and AD (Fig. 2a,c). To our surprise the dermal changes of CATS in PS were accompanied by a strong upregulation in the epidermis. Psoriatic keratinocytes in the epidermis exhibited CATS(+)-granules in the cytoplasm (Fig. 2d,e), while CATS was undetectable in keratinocytes of normal or of actinic keratosis and atopic dermatitis skin biopsies (Fig. 2). The upregulation of CATS in the epidermis was accompanied by an upregulation of MHCII in keratinocytes (Fig. 2i,j) and an infiltration of CD3(+) T-cells (Fig. 2k–m and Fig. S1b)

CATS expression in HaCaT cells

In order to set up an *in vitro* culture system for studies of CATS regulation and function in keratinocytes we used the HaCaT cell line and evaluated the basal expression level of CATS in the unstimulated cells first. RT-PCR and Western Blot analyses showed CATS transcript (Fig. 3a) and rather weak protein (Fig. 3b) expression in HaCaT cells. In accordance to normal skin, signals of the pro- and mature form were detected (Fig. 3b), but no signal in the supernatant was found.

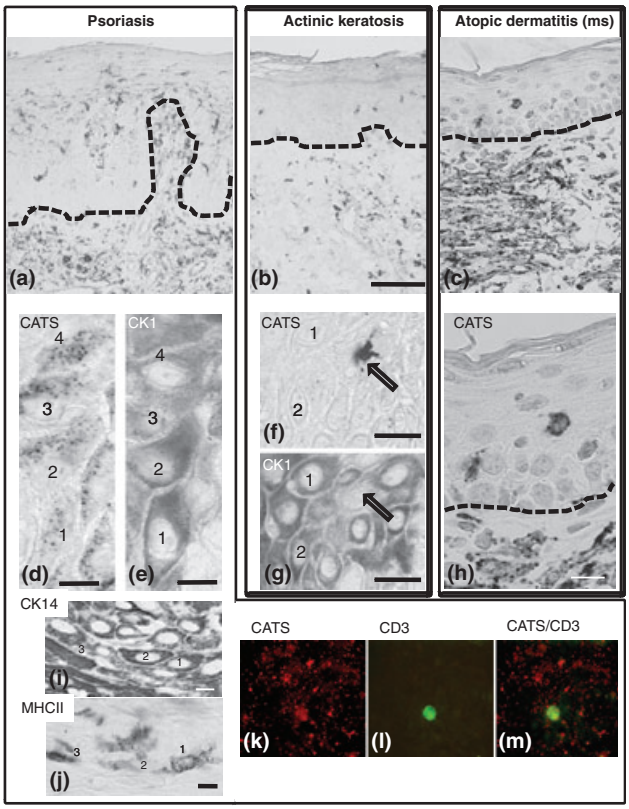

Figure 2. CATS expression in skin diseases. (a–c) Survey micrographs illustrate the CATS expression in skin samples of psoriasis (a), actinic keratosis (b) and mouse atopic dermatitis (c). In psoriatic skin the number of CATS-immunopositive cells is markedly increased. (d–g) Neighboring 3 μm thick sections immunostained for CATS (d, f) and the keratinocyte marker CK1 (e, g) show the expression of CATS in psoriatic keratinocytes but not in those of actinic keratosis. Arrows and numbers mark the same cell in neighboring sections. (h) High power micrograph illustrates single, non-keratinocytic CATS-immunopositive cells in the epidermis of atopic dermatitis. Dashed lines indicate the course of the basal membrane between epidermis and dermis. (i, j) Neighboring sections of psoriasis sample immunostained for the keratinocyte marker CK14 (i) and MHCII (j) show the expression of MHCII in psoriatic keratinocytes. Numbers mark the same cell in neighboring sections. (k–m) Double immunfluorescence staining for CATS and CD3 shows infiltration of T-cells in psoriatic epidermis. Scale bars: a, b (50 μm); c (20 μm); d–m (10 μm).

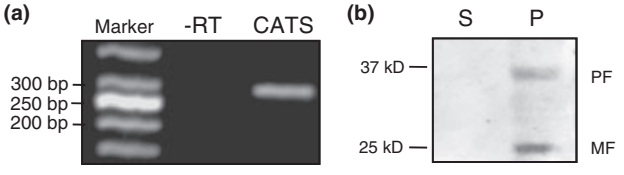

Figure 3. HaCaT cells express CATS. (a) CATS transcript was detected in HaCaT cells using RT-PCR. In order to control that no genomic sequences were amplified, a control without reverse transcriptase (-RT) was performed. (b) The presence of the CATS protein (PF, proform; MF, mature form) was verified by Western Blot analysis. The same blot was stripped and re-probed for GAPDH expression to normalize protein loading and transfer. S, culture supernatant; P, cell pellet.

Coculture with T-cells or treatment with cytokines induces a strong upregulation of CATS expression in HaCaT keratinocytes

Next we asked the questions if the T-cells which markedly infiltrate the epidermis of psoriatic skin lesions (see Fig. 2 and Fig. S1b; for review see 22) might be able to induce CATS expression in keratinocytes.

We therefore investigated the influence of the T-cell line HuT on the CATS expression level in HaCaT keratinocytes using a coculture model. We cultured the HuT cells in insert wells above the HaCaT cells. Under these conditions HuT cells induced an upregulation of CATS expression in HaCaT cells (Fig. 4a). The expression of the CATS forms was increased 1.2–2.5 fold (Fig. 4b). These results suggest that T-cells induce CATS expression by soluble factors secreted into the medium.

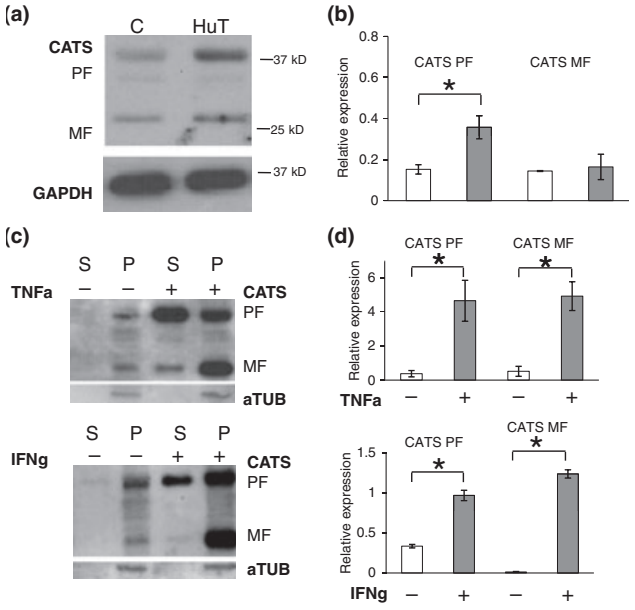

Figure 4. Effect of T-cell coculture or treatment with the cytokines TNFa or IFNg on CATS expression in HaCaT cells. (a, b) Western blot analysis of CATS expression (a) and the respective quantification of the band intensities (b) for whole cell lysates HaCaT cells cultured in the presence of the T-cells HuT. Indirect (no cell–cell contact) coculturing of these cells induced an upregulation of the pro- and mature form of CATS in HaCaT cells. Expression levels were normalized relative to the corresponding GAPDH band. Bars represent the mean ± SEM of three independent experiments. Statistics: student's *t*-test. (c) Similarly, treatment with TNFa or IFNg induced a substantial increase of both forms of CATS. Moreover, after stimulation with either cytokine CATS appeared in the supernatant of the cells. (d) Quantification of CATS proform and mature form band intensities in whole cell lysates of untreated versus treated HaCaT cells. Expression levels were normalized relative to the corresponding a-tubulin (aTUB) band. Bars represent the mean ± SEM of four independent experiments. Statistics: *U*-test: $*P < 0.05$; $**P < 0.01$. (c) control (no HuT cells); S, culture supernatant; P, cell pellet.

To further explore the T-cell derived factors that stimulate CATS expression we studied the effect of the two major cytokines TNFa and IFNg, which are secreted by T-cells in large quantities in psoriatic lesions (for reviews see 22,26). Both cytokines markedly increased the expression of CATS in HaCaT-cells. Western Blot analyses revealed an upregulation of the pro- and the mature form after treatment (Fig. 4c,d). Interestingly, we also noted the appearance of CATS in the supernatant of treated cells. While Western Blot analyses failed to detect CATS in the supernatant of untreated HaCaT cells, we noted the proform and mature form of CATS in the supernatant of both, TNFa- and IFNg-treated keratinocytes (Fig. 4c).

CATS activity regulates the level of MHCII expression and Ii processing in keratinocytes

CATS plays a crucial central role in antigen representation by MHCII, controlling both the expression and the differentiation of this complex (8,27). For a long time it has been assumed that the MHCII complex is predominantly expressed in professional antigen-presenting cells (APCs) like dendritic cells, macrophages and B cells and only recently it has been shown to be constitutively expressed in keratinocytes (28). Moreover the upregulation of the MHCII complex by keratinocytes in several dermatoses including psoriasis is well documented (29–31). Therefore we studied the functional role of CATS in MHCII complex processing in keratinocytes. We stimulated HaCaT cells with IFNg in the presence or absence of a specific CATS-inhibitor and analyzed the MHCII expression (represented by the human leukocyte antigen DR alpha chain; HLA-DRA) and the degradation of the invariant chain Ii.

In the absence of the inhibitor IFNg treatment resulted in a strong induction of MHC class II and Ii protein expression in HaCaT cells (Fig. 5a,b; control). To determine whether cathepsin inhibition results in modulation of this IFNg-induced protein upregulation we preincubated the cells with the specific CATS-inhibitor Z-Val-Val-Nle-diazomethylketone. Inhibitor preincubation induced a strong decrease of the IFNg-induced MHCII upregulation (Fig. 5a). Next, we focused on the processing of the invariant chain and analyzed whether similar to professional antigen-presenting cells CATS participates in the cleavage of the invariant chain (Ii) in keratinocytes. For this purpose, accumulation of Ii degradation fragments was investigated by Western Blot analysis using an antibody directed against the cytoplasmic tail of Ii. Treatment with IFNg induced an upregulation of the unprocessed p33/35 isoforms of Ii, but no Ii degradation intermediates (Fig. 5b). However, additional preincubation with the specific CATS inhibitor Z-Val-Val-Nle-diazomethylketone resulted in an accumulation of the degradation product Ii-p10. This increase was accompanied by a significant decrease in the

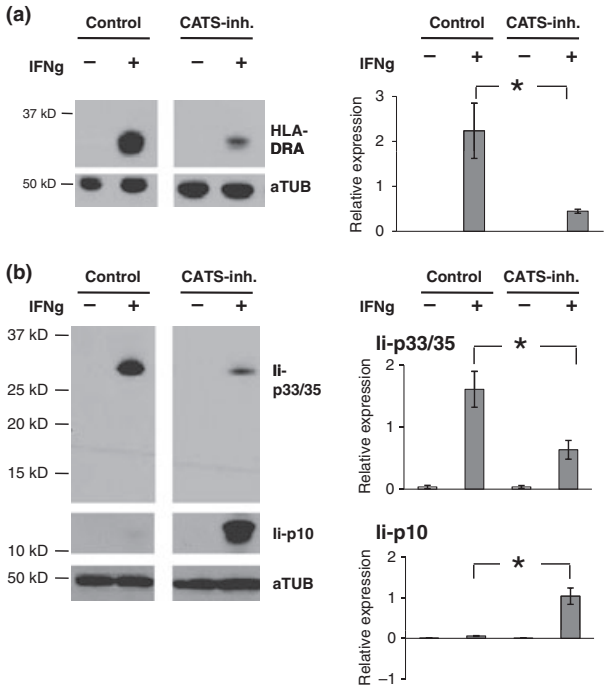

Figure 5. CATS is involved in MHCII expression and Ii processing in HaCaT keratinocytes. IFNg-stimulated or unstimulated HaCaT cells were treated with the specific CATS-inhibitor Z-Val-Val-Nle-diazomethylketone (CATS-inh.). (a) The inhibitor significantly diminished the IFNg-induced upregulation of the MHC class II molecule HLA-DR (*$P < 0.05$). (b) Moreover, inhibitor preincubation induced a downregulation of the invariant chain Ii-p33/35 with concomitant accumulation of the Ii processing intermediate Ii-p10 indicating that inhibition of CATS blocks the conversion of Ii-p10 into CLIP. Expression levels were normalized relative to the corresponding a-tubulin (aTUB) band. Bars represent the mean ± SEM of three independent experiments. Statistics: ANOVA.*$P < 0.05$.

Ii-p33/35 form (Fig. 5b). Thus, inhibition of CATS activity in HaCaT cells attenuates IFNg-induced upregulation of MHCII molecules and blocks conversion of Ii-p10 into class II invariant chain-associated peptide (CLIP) resulting in an accumulation of the Ii-p10 intermediate degradation product of Ii.

Discussion

Human skin contains a number of proteases, which fulfill a broad spectrum of biological processes (for reviews see 6,32). Dysregulation of these proteases may result in the development of severe skin pathologies or even tissue loss (for reviews see 33–35). For example, human psoriatic tissues lack normal expression of the cysteine protease caspase 14 and induction of this protease has been shown to reduce psoriasiform lesions in the flaky skin mouse model (36). Cysteine proteases, including the caspases and cathepsins, comprise one of the largest and best characterized families of peptidases, but little is known about their role in skin pathology.

In this manuscript we concentrate on CATS, a cathepsin which plays a major role in immune system function (37). We provide first evidence that (i) CATS expression is induced in psoriatic keratinocytes, but not in actinic keratosis and atopic dermatitis keratinocytes, (ii) T-cells and the cytokines IFNg and TNFa trigger the keratinocytic CATS expression and (iii) CATS is involved in MHC class II expression and invariant chain (Ii) degradation in keratinocytes.

Until present most analyses of cathepsin expression have been performed in dermal cell culture and only two studies show expression of cathepsins B, C, D, H, L in human and rat epidermis (38,39). Our study revealed that unlike the latter cathepsins, CATS is not expressed in normal keratinocytes. However, we detected CATS in phagocytic/antigen-presenting cells distributing predominantly in the dermis and only rarely in the epidermis. This cellular expression pattern fits to the well-known pattern of CATS expression in various other organs, like the brain, spleen and lung (12,40,41).

This distribution pattern changed substantially in the pathological skin. In all analyzed skin diseases the number of CATS-positive antigen-presenting cells increased in the dermis. In addition, CATS expression could be demonstrated in psoriatic keratinocytes. Similar to our data of CATS, previous reports showed an increased expression level and a redistribution of the cathepsins L, B and D in the psoriatic epidermis (38,42,43). Moreover, besides cathepsins, keratinocytes produce a number of other proteases, which serve important roles during epidermal differentiation and the generation of a permeability barrier (6). Expression analyses of these enzymes revealed that some matrix metalloproteases and serine proteases (44–47) add to the list of upregulated proteases in psoriatic keratinocytes. Thus, we can summarize that similar to other tissues (11–13,48,49), inflammatory changes in the skin are accompanied by a strong induction of CATS.

The PS-specific expression of CATS in keratinocytes raises the questions about the induction mechanism as well as the role of this enzyme in the pathophysiology of PS. Taking into account that PS in contrast to AD and AK is characterized by an infiltration of T-cells and Langerhans cells into the epidermis (see also Figs S1 and S2) and that these cell types are known to interact closely with keratinocytes during PS pathogenesis (22,50–54) we analysed whether T-cells are able to induce CATS expression. Our results clearly support this assumption. In this study we showed that T-cells are able to stimulate CATS expression in keratinocytes via the secretion of cytokines, like TNFa and IFNg. These results are supported by the finding that IFNg stimulates CATS activity in HaCaT cells (16). The IFNg-induced upregulation of CATS has been demonstrated to take place at the transcriptional level. Interestingly the promoter structure suggests that the CATS gene can be

specifically regulated, e.g. via binding of transcriptional regulators to an IFN-stimulated response element (55,56). The T-cell line applied in this study, HuT-78, constitutively expresses TNFa and IFNg (unpublished observations (57)).

However, the role of CATS in PS is still unclear. The best documented function for CATS is its role in antigen processing and presentation, which is also reflected by the prominent CATS expression in phagocytic and antigen-presenting cells. It has been shown that the proteolytic actions performed by CATS within early endosomes of most of the professional APCs comprise degradation of MHC class II-associated invariant chain (1,8,27). Given this function, the upregulation of MHC class II-immunoreactivity in the psoriatic epidermis reported in this study and the ability of keratinocytes to synthesize MHC class II (16,30,58,59) we addressed the question whether CATS fulfills similar tasks in keratinocytes. Using a specific CATS inhibitor we showed that CATS has a distinct role in Ii processing as well as in MHC class II expression in keratinocytes. A previous study showed a simultaneous upregulation of CATS and MHC class II molecules in IFNg-stimulated keratinocytes but did not disclose a direct connection between the upregulation of these molecules (16). Our study provides first evidence that CATS accounts for regular peptide loading and MHC class II expression in keratinocytes thereby controlling the MHC class II antigen presentation machinery. A specific role for CATS during antigen presentation in human keratinocytes is also supported by other studies. Contrary to the situation in mouse, processing of Ii chain in human epithelial cells is mostly dependent on CATS as demonstrated for human thymic epithelial cells (60). In contrast to other cathepsins, only CATS is selectively upregulated in human keratinocytes upon IFNg stimulation (16).

Furthermore, we found evidence for a substantial release of CATS after stimulation with TNFa or IFNg. The unusual activity of CATS at neutral pH raises the possibility that it degrades proteins in the extracellular space. In fact, studies have shown a crucial function of CATS in the degradation of the extracellular matrix (61–63). By remodeling the extracellular matrix CATS might be implicated in processes like the transepidermal migration of keratinocytes (64,65), the maintenance of the permeability barrier (66,67) and even the regulation of T-cell functions (68,69).

During the past years keratinocytes emerged as active players in several inflammatory skin diseases. Upon stimulation they secrete proinflammatory mediators and express various adhesion molecules (70,71). Our data expands these well-known functions and give evidence that during PS pathogenesis keratinocytes change phenotype. They gain antigen-presenting capacities and secrete the protease CATS. Regulating the expression of CATS by either anticytokine therapy (72) or the recently developed CATS-inhibitors (73–75) could represent important strategies for the therapy of psoriasis. IFNg and TNFa work both upstream to CATS, but TNAa inhibitors cause severe side effects in some patients (76,77). As CATS inhibitors will act more downstream in the pathological cascade, will not directly target T-cells and will be specific for CD4-positive T-cells, side effects commonly associated with immunosuppression should be reduced.

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References

1 Brix K, Dunkhorst A, Mayer K, Jordans S. Cysteine cathepsins: cellular roadmap to different functions. Biochimie 2008: **90**: 194–207.
2 Turk B, Turk D, Turk V. Lysosomal cyteine proteases: more than scavengers. Biochem Biophys Acta 2000: **1477**: 98–111.
3 Deussing J, Kouadio M, Rehman S, Werber I, Schwinde A, Peters C. Identification and characterization of a dense cluster of placenta-specific cysteine peptidase genes and related genes on mouse chromosome 13. Genomics 2002: **79**: 225–240.
4 Vasiljeva O, Reinheckel T, Peters C, Turk D, Turk V, Turk B. Emerging roles of cysteine cathepsins in disease and their potential as drug targets. Curr Pharm Des 2007: **13**: 385–401.
5 Dickinson D P. Cysteine peptidases of mammals: their biological roles and potential effects in the oral cavity and other tissues in health and disease. Crit Rev Oral Biol Med 2002: **13**: 238–275.
6 Zeeuwen P L. Epidermal differentiation: the role of proteases and their inhibitors. Eur J Cell Biol 2004: **83**: 761–773.
7 Villadangos J A, Bryant R A, Deussing J *et al.* Proteases involved in MHC class II antigen presentation. Immunol Rev 1999: **172**: 109–120.
8 Honey K, Rudensky A Y. Lysosomal cysteine proteases regulate antigen presentation. Nat Rev Immunol 2003: **3**: 472–482.
9 Shi G P, Bryant R A, Riese R *et al.* Role for cathepsin F in invariant chain processing and major histocompatibility complex class II peptide loading by macrophages. J Exp Med 2000: **191**: 1177–1186.
10 Tolosa E, Li W, Yasuda Y *et al.* Cathepsin V is involved in the degradation of invariant chain in human thymus and is overexpressed in myasthenia gravis. J Clin Invest 2003: **112**: 517–526.
11 Lemere C A, Munger J S, Shi G P *et al.* The lysosomal cysteine protease, cathepsin S, is increased in Alzheimer's disease and Down syndrome brain. An immunocytochemical study. Am J Pathol 1995: **146**: 848–860.
12 Wendt W, Lübbert H, Stichel C C. Upregulation of cathepsin S in the aging and pathological nervous system of mice. Brain Res 2008: **1232**: 7–20.
13 Leichsenring A, Bäcker I, Wendt W *et al.* Differential expression of cathepsin S and X in the spinal cord of a rat neuropathic pain model. BMC Neurosci 2008: **12**: 80.
14 Sukhova G K, Shi G P, Simon D I, Chapman H A, Libby P. Expression of the elastolytic cathepsins S and K in human atheroma and regulation of their production in smooth muscle cells. J Clin Invest 1998: **102**: 576–583.
15 Cheng X W, Kuzuya M, Nakamura K *et al.* Localization of cysteine protease, cathepsin S, to the surface of vascular smooth muscle cells by association with integrin alphanubeta3. Am J Pathol 2006: **168**: 685–694.
16 Schwarz G, Boehncke W H, Braun M *et al.* Cathepsin S activity is detectable in human keratinocytes and is selectively upregulated upon stimulation with interferon-gamma. J Invest Dermatol 2002: **119**: 44–49.
17 Man M Q, Hatano Y, Lee S H *et al.* Characterization of a hapten-induced, murine model with multiple features of atopic dermatitis: structural, immunologic, and biochemical changes following single versus multiple oxazolone challenges. J Invest Dermatol 2008: **128**: 79–86.
18 Boukamp P, Petrussevska R T, Breitkreutz D, Hornung J, Markham A, Fusenig N E. Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. J Cell Biol 1988: **106**: 761–771.
19 Neuhoff V P K, Zimmer H G, Mesecke S. A simple and vertsatile, sensitive and volume-independent method for quantitative protein determination with independence of other external influences. Hoppe-Seyler's Z Physiol Chem 1979: **360**: 1657–1670.
20 Frangioni G, Borgioli G. One hour bleach for melanin. Stain Technol 1988: **63**: 325–326.
21 Stichel C C, Singer W, Zilles K. Ultrastructure of PkC(II/III)-immunopositive structures in rat primary visual cortex. Exp Brain Res 1990: **82**: 575–584.
22 Lowes M A, Bowcock A M, Krueger J G. Pathogenesis and therapy of psoriasis. Nature 2007: **445**: 866–873.

23 Meagher L J, Wines N Y, Cooper A J. Atopic dermatitis: review of immunopathogenesis and advances in immunosuppressive therapy. Australas J Dermatol 2002: **43**: 247–254.
24 Fu W, Cockerell C J. The actinic (solar) keratosis: a 21st-century perspective. Arch Dermatol 2003: **139**: 66–70.
25 Roewert-Huber J, Stockfleth E, Kerl H. Pathology and pathobiology of actinic (solar) keratosis - an update. Br J Dermatol 2007: **157** (Suppl 2): 18–20.
26 Ghoreschi K, Weigert C, Rocken M. Immunopathogenesis and role of T cells in psoriasis. Clin Dermatol 2007: **25**: 574–580.
27 Hsing L C, Rudensky A Y. The lysosomal cysteine proteases in MHC class II antigen presentation. Immunol Rev 2005: **207**: 229–241.
28 Danielyan L, Tolstonog G, Traub P *et al.* Colocalization of glial fibrillary acidic protein, metallothionein, and MHC II in human, rat, NOD/SCID, and nude mouse skin keratinocytes and fibroblasts. J Invest Dermatol 2007: **127**: 555–563.
29 Lampert I A. Expression of HLA-DR (Ia like) antigen on epidermal keratinocytes in human dermatoses. Clin Exp Immunol 1984: **57**: 93–100.
30 Gottlieb A B, Lifshitz B, Fu S M, Staiano-Coico L, Wang C Y, Carter D M. Expression of HLA-DR molecules by keratinocytes, and presence of Langerhans cells in the dermal infiltrate of active psoriatic plaques. J Exp Med 1986: **164**: 1013–1028.
31 Day M J. Expression of major histocompatibility complex class II molecules by dermal inflammatory cells, epidermal Langerhans cells and keratinocytes in canine dermatological disease. J Comp Pathol 1996: **115**: 317–326.
32 Fraki J E, Schechter N M, Lazarus G S. Human skin proteinases as inflammatory mediators. Br J Dermatol 1983: **109** (Suppl 25): 72–76.
33 Berdowska I. Cysteine proteases as disease markers. Clin Chim Acta 2004: **342**: 41–69.
34 Magert H J, Drogemuller K, Raghunath M. Serine proteinase inhibitors in the skin: role in homeostasis and disease. Curr Protein Pept Sci 2005: **6**: 241–254.
35 Herouy Y. Matrix metalloproteinases in skin pathology (Review). Int J Mol Med 2001: **7**: 3–12.
36 Hsu S, Dickinson D, Borke J *et al.* Green tea polyphenol induces caspase 14 in epidermal keratinocytes via MAPK pathways and reduces psoriasiform lesions in the flaky skin mouse model. Exp Dermatol 2007: **16**: 678–684.
37 Gupta S, Singh R K, Dastidar S, Ray A. Cysteine cathepsin S as an immunomodulatory target: present and future trends. Expert Opin Ther Targets 2008: **12**: 291–299.
38 Kawada A, Hara K, Kominami E, Hiruma M, Noguchi H, Ishibashi A. Processing of cathepsins L, B and D in psoriatic epidermis. Arch Dermatol Res 1997: **289**: 87–93.
39 Sato K, Waguri S, Ohsawa Y *et al.* Immunocytochemical localization of lysosomal cysteine and aspartic proteinases, and ubiquitin in rat epidermis. Arch Histol Cytol 1997: **60**: 275–287.
40 Petanceska S, Canoll P, Devi L A. Expression of rat cathepsin S in phagocytic cells. J Biol Chem 1996: **271**: 4403–4409.
41 Petanceska S, Burke S, Watson S J, Devi L. Differential distribution of messenger RNAs for cathepsins B, L and S in adult rat brain: an in situ hybridization study. Neurosci 1994: **59**: 729–738.
42 Haider A S, Peters S B, Kaporis H *et al.* Genomic analysis defines a cancer-specific gene expression signature for human squamous cell carcinoma and distinguishes malignant hyperproliferation from benign hyperplasia. J Invest Dermatol 2006: **126**: 869–881.
43 Bylaite M, Moussali H, Marciukaitiene I, Ruzicka T, Walz M. Expression of cathepsin L and its inhibitor hurpin in inflammatory and neoplastic skin diseases. Exp Dermatol 2006: **15**: 110–118.
44 Jensen P J, Baird J, Belin D *et al.* Tissue plasminogen activator in psoriasis. J Invest Dermatol 1990: **95**: 13S–14S.
45 Fleischmajer R, Kuroda K, Hazan R *et al.* Basement membrane alterations in psoriasis are accompanied by epidermal overexpression of MMP-2 and its inhibitor TIMP-2. J Invest Dermatol 2000: **115**: 771–777.
46 Suomela S, Kariniemi A L, Impola U *et al.* Matrix metalloproteinase-19 is expressed by keratinocytes in psoriasis. Acta Derm Venereol 2003: **83**: 108–114.
47 van Lingen R G, van de Kerkhof P C, Seyger M M *et al.* CD26/dipeptidyl-peptidase IV in psoriatic skin: upregulation and topographical changes. Br J Dermatol 2008: **158**: 1264–1272.
48 Taleb S, Lacasa D, Bastard J P *et al.* Cathepsin S, a novel biomarker of adiposity: relevance to atherogenesis. FASEB J 2005: **19**: 1540–1542.
49 Zheng T, Kang M J, Crothers K *et al.* Role of cathepsin S-dependent epithelial cell apoptosis in IFN-gamma-induced alveolar remodeling and pulmonary emphysema. J Immunol 2005: **174**: 8106–8115.
50 Arnold R, Seifert M, Asadullah K, Volk H D. Crosstalk between keratinocytes and T lymphocytes via Fas/Fas ligand interaction: modulation by cytokines. J Immunol 1999: **162**: 7140–7147.
51 Gudjonsson J E, Johnston A, Sigmundsdottir H, Valdimarsson H. Immunopathogenic mechanisms in psoriasis. Clin Exp Immunol 2004: **135**: 1–8.
52 Komine M, Karakawa M, Takekoshi T *et al.* Early inflammatory changes in the ''perilesional skin'' of psoriatic plaques: is there interaction between dendritic cells and keratinocytes? J Invest Dermatol 2007: **127**: 1915–1922.
53 Baadsgaard O, Fisher G J, Voorhees J J, Cooper K D. Interactions of epidermal cells and T cells in inflammatory skin diseases. J Am Acad Dermatol 1990: **23**: 1312–1316.
54 Bata-Csorgo Z, Hammerberg C, Voorhees J J, Cooper K D. Intralesional T-lymphocyte activation as a mediator of psoriatic epidermal hyperplasia. J Invest Dermatol 1995: **105**: 89S–94S.
55 Shi G P, Webb A C, Foster K E *et al.* Human cathepsin S: chromosomal localization, gene structure, and tissue distribution. J Biol Chem 1994: **269**: 11530–11536.
56 Storm van's G K, Layne M D, Ye Q *et al.* IFN regulatory factor-1 regulates IFN-gamma-dependent cathepsin S expression. J Immunol 2002: **168**: 4488–4494.
57 Stoof T J, Mitra R S, Sarma V, Dixit V M, Nickoloff B J. Keratinocyte activation following T-lymphocyte binding. J Invest Dermatol 1992: **98**: 92–95.
58 Albanesi C, De P O, Girolomoni G. Resident skin cells in psoriasis: a special look at the pathogenetic functions of keratinocytes. Clin Dermatol 2007: **25**: 581–588.
59 Onuma S. Immunohistochemical studies of infiltrating cells in early and chronic lesions of psoriasis. J Dermatol 1994: **21**: 223–232.
60 Bania J, Gatti E, Lelouard H *et al.* Human cathepsin S, but not cathepsin L, degrades efficiently MHC class II-associated invariant chain in nonprofessional APCs. Proc Natl Acad Sci U S A 2003: **100**: 6664–6669.
61 Yasuda Y, Kaleta J, Bromme D. The role of cathepsins in osteoporosis and arthritis: rationale for the design of new therapeutics. Adv Drug Deliv Rev 2005: **57**: 973–993.
62 Abdul-Hussien H, Soekhoe R G, Weber E *et al.* Collagen degradation in the abdominal aneurysm: a conspiracy of matrix metalloproteinase and cysteine collagenases. Am J Pathol 2007: **170**: 809–817.
63 Shi G P, Sukhova G K, Kuzuya M *et al.* Deficiency of the cysteine protease cathepsin S impairs microvessel growth. Circ Res 2003: **92**: 493–500.
64 O'Toole E A. Extracellular matrix and keratinocyte migration. Clin Exp Dermatol 2001: **26**: 525–530.
65 Li W, Fan J, Chen M, Woodley D T. Mechanisms of human skin cell motility. Histol Histopathol 2004: **19**: 1311–1324.
66 Doering T, Proia R L, Sandhoff K. Accumulation of protein-bound epidermal glucosylceramides in beta-glucocerebrosidase deficient type 2 Gaucher mice. FEBS Lett 1999: **447**: 167–170.
67 Menon G K, Feingold K R, Elias P M. Lamellar body secretory response to barrier disruption. J Invest Dermatol 1992: **98**: 279–289.
68 Gorski A, Kupiec-Weglinski J W. Extracellular matrix proteins, regulators of T-cell functions in healthy and diseased individuals. Clin Diagn Lab Immunol 1995: **2**: 646–651.
69 Matysiak W, Stepien-Sopniewska B, Mrowiec T. Extracellular matrix proteins as regulators of the immune response. Pol Arch Med Wewn 1995: **94**: 173–177.
70 Steinhoff M, Brzoska T, Luger T A. Keratinocytes in epidermal immune responses. Curr Opin Allergy Clin Immunol 2001: **1**: 469–476.
71 Albanesi C, Scarponi C, Giustizieri M L, Girolomoni G. Keratinocytes in inflammatory skin diseases. Curr Drug Targets Inflamm Allergy 2005: **4**: 329–334.
72 Skurkovich S V, Skurkovich B, Kelly J A. Anticytokine therapy – new approach to the treatment of autoimmune and cytokine-disturbance diseases. Med Hypotheses 2002: **59**: 770–780.
73 Muqit M M, bou-Sleiman P M, Saurin A T *et al.* Altered cleavage and localization of PINK1 to aggresomes in the presence of proteasomal stress. J Neurochem 2006: **98**: 156–169.
74 Markt P, McGoohan C, Walker B *et al.* Discovery of novel cathepsin S inhibitors by pharmacophore-based virtual high-throughput screening. J Chem Inf Model 2008: **48**: 1693–1705.
75 Gauthier J Y, Black W C, Courchesne I *et al.* The identification of potent, selective, and bioavailable cathepsin S inhibitors. Bioorg Med Chem Lett 2007: **17**: 4929–4933.
76 Bongartz T, Sutton A J, Sweeting M J, Buchan I, Matteson E L, Montori V. Anti-TNF antibody therapy in rheumatoid arthritis and the risk of serious infections and malignancies: systematic review and meta-analysis of rare harmful effects in randomized controlled trials. JAMA 2006: **295**: 2275–2285.
77 Solomon D H. The comparative safety and effectiveness of TNF-alpha antagonists [corrected]. J Manag Care Pharm 2007: **13**: S7–S18.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Characterization of normal, psoriatic and actinic keratosis skin.

Figure S2. Characterization of atopic dermatitis (ms) and specificity controls.

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