CHARACTERIZATION OF MOLECULES SHOWING ALTERED EXPRESSION PROFILE IN PSORIASIS

Ph. D. Thesis

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I. Gellért L, Fuzik J, **Göblös A**, Sárközi K, Marosi M, Kis Z, Farkas T, Szatmári I, Fülöp F, Vécsei L, Toldi J. Neuroprotection with a new kynurenic acid analog in the four-vessel occlusion model of ischemia. European Journal of Pharmacology 667(1-3): 182-7, 2011

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II. Szabó EZ, Manczinger M, **Göblös A**, Kemény L, Lakatos L. Switching on RNA silencing suppressor activity by restoring Argonaute binding to a viral protein. Journal of Virology 86(15):8324-7, 2012

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LIST OF ABBREVIATIONS

AEC: amino-9-ethylcarbazole

AIM2: absent in melanoma 2

AP: alkaline phosphatase

ASC: adaptor protein

BSA: bovine serum albumin

CARD: caspase activation and recruitment domain

CARD18: caspase recruitment domain family member 18, Iceberg

COP: CARD only protein

CCL20: C-C motif chemokine-20

CXCL8: interleukin-8

CXCL10: C-X-C motif chemokine-10

DAMP: damage-associated molecular pattern

DAPI: 4,6-diamidino-2-phenylindole

DC: dendritic cell

DIG: digoxigenin

DNase: deoxyribonuclease

ELISA: enzyme-linked immunosorbent assay

GM-CSF: granulocyte macrophage colony-stimulating factor

HaCaT: human, adult, low calcium, high temperature, human adult skin immortal keratinocyte cell line

hnRNPs: heterogeneous nuclear ribonucleoproteins

Hfq: bacterial RNA binding protein – host factor Q

HPV-Ker: human papillomavirus E6 oncogene immortalized keratinocyte cell line

IFN-γ: interferon- γ

IHC: immunohistochemistry

IL-1: interleukin-1

IL-3: interleukin-3

IL-6: interleukin-6

IL-18: interleukin-18

LNA: locked nucleic acid

lncRNA: long non-coding RNA

LPS: lipopolysaccharide

ncRNA: non-coding RNA

NHEK: normal human epidermal keratinocytes

NLR: Nod-like receptor

NKT: natural killer cell

NPM (B23): nucleophosmin

Nod: nucleotide-binding oligomerization domain

PAMP: pathogene-associated molecular pattern

PBS: phosphate-buffered saline

PCR: polymerase chain reaction

poly(dA:dT): polydeoxyadenylic acid–polydeoxythymidylic acid double-stranded homopolymer

PRINS: psoriasis susceptibility-related RNA gene induced by stress

PRR: pathogen recognition receptor

PYD: pyrin domain

PYHIN: Pyrin and HIN domain family Gene Family

SSC: saline–sodium citrate buffer

TBS: Tris-buffered saline

Tc: cytotoxic T cells

Th: helper T cells

TNF-α: tumor necrosis factor-α

TS: tape stripping

UV: ultra violet

VLA-1: very late antigen 1

1. INTRODUCTION

1.1. Psoriasis

Psoriasis is a common, lifelong skin disease, affecting approximately 2% of the population [1] and predominantly presented in the Caucasian population. The disease is less common in Asians (about 0.1%) and is rarely seen in Africans [2]. The prevalence of psoriasis is approximately the same in males and females [3]. Psoriasis has been defined as a chronic inflammatory disease with important immune-mediated features that involves a multifactorial cellular and molecular network. Psoriasis is generally characterized by exacerbation and remission and usually persists through life [4]. Psoriasis compromises innate and adaptive immune system components and has a well-defined network of chemokines and messengers. The cause of psoriasis is complex, with evidence that the interplay of the multigenic susceptibility and environmental as well as life-style factors lead to the development of the symptoms.

Psoriasis can be provoked by many triggers such as mild trauma (for example scratching), sunburn, chemical irritants, infections and factors impairing skin barrier function [5]. Systemic drugs like β-blockers, lithium and non-steroidal anti-inflammatory agents can also exacerbate the disease [6].

The clinical manifestation of psoriasis has many faces; the disease is featured by variable morphology, distribution and severity. The most common form of the disease is chronic plaque psoriasis (*psoriasis vulgaris*), which manifests as red plaques with silvery-white scales (Fig. 1. A, B). The well-demarcated regions of inflamed skin (involved skin) are clearly distinguished from the normal appearing skin (non-involved skin). *Psoriasis vulgaris* affects approximately 85 to 90% of all patients with the disease [1,7]. Psoriasis can affect any skin site, however typical locations include the extensor surfaces of forearms and shins, peri-umbilical, peri-anal, and retro-auricular regions and scalp. Most of the affected individuals have a mild form psoriasis defined as a percentage of affected body surface area (BSA) even or less than 10%. Up to one third of patients have moderate-to-severe form of psoriasis affecting more than 10% of the body surface [8].

The plaques are the result of increased keratinocyte proliferation, where up to an eight-fold increase in epidermal cell turnover has been demonstrated [9] leading to a thickening of the epidermis (acanthosis) with elongated rete ridges (Fig. 1. C). The differentiation of keratinocytes is extremely altered in psoriasis [4]. The scales are the result

of a hyperproliferative epidermis with premature maturation of keratinocytes and incomplete cornification with retention of nuclei in the stratum corneum (parakeratosis). The redness of the lesions is due to increased dermal angiogenesis leading to the formation of complex underlying vascular system that reaches the skin surface through a markedly thickened epithelium. The increased vascularity allows for a greater influx of inflammatory cells into the skin, further driving the inflammation.

Besides the cutaneous symptoms, nail involvement occurs in about 50% of patients with psoriasis [10] and ranges from pits and yellowish discoloration to a sever nail deformity. Approximately 30 % of patients with psoriasis also have joint involvement [11].

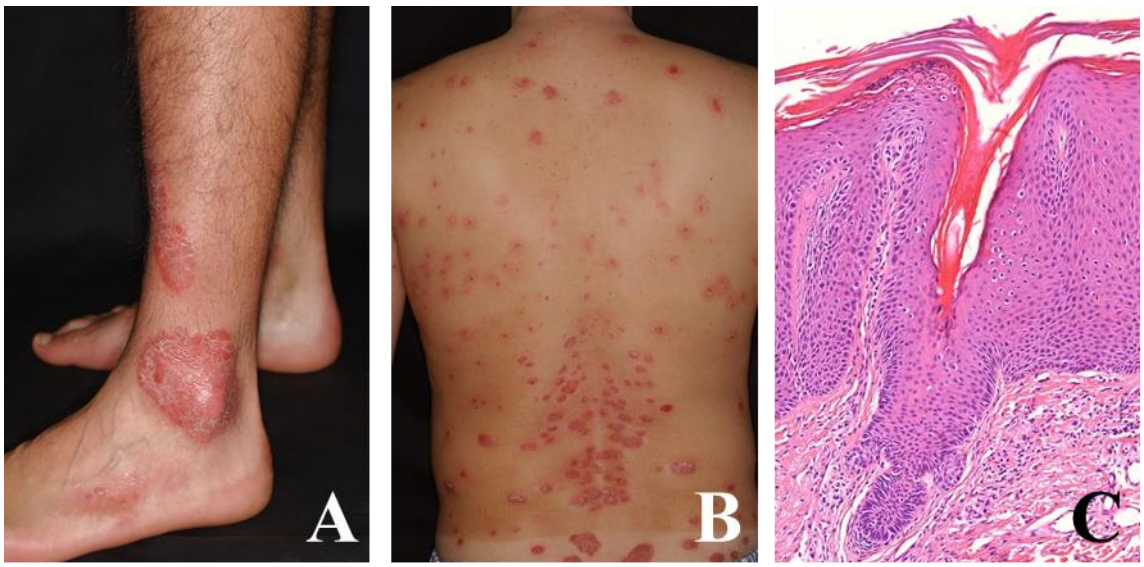

Figure 1. Clinical and histological features of psoriasis. (A, B) Clinical presentation of psoriasis showing demarcated red plaques with silver scales. (C) Haematoxylin and eosin staining of psoriatic lesional skin showing marked epidermal thickening (Clinical and histological photographs are derived from the photo archive of the Department of Dermatology and Allergology, University of Szeged)

Psoriasis is associated with a high degree of morbidity; patients are embarrassed about the appearance of their skin, and there are also side effects of medications. Moreover, patients with psoriasis have reduced levels of employment and income as well as a decreased quality of life [12]. In addition to skin plaques, psoriasis can present with various co-morbidities including arthritis, atherosclerosis, inflammatory bowel disease, obesity, type 2 diabetes mellitus and depression [1,13–15].

The current existing therapies only relieve symptoms but cannot cure the disease. The therapeutic costs are high, moreover some treatments carry substantial side effects.

Therefore the better understanding of the immunopathogenesis of psoriasis is essential for the development of improved therapies.

1.1.1. Pathophysiology of psoriasis

The pathophysiology of psoriasis is complex and dynamic. Various combinations of selective abnormalities of the immune system and the epithelial cells give rise to the psoriatic phenotype [16]. The recent psoriasis research era proposes that combination of environmental, genetic and modifying factors as well as immunologically mediated inflammation confers susceptibility to the disease [17,18]. Genome-wide scans for associated genes have identified several immune-related genes [19,20], and some susceptibility factors related to basic functional abnormalities of keratinocytes [16]; providing a mechanistic link between genetics, immunity, and epithelial cells.

The epidermal hyperplasia along with abnormal maturation of keratinocytes leads to the development of the characteristic thick scaly plaques. Psoriasis shows characteristic histopathological changes in almost every cutaneous cell types. The complex interplay of cutaneous cells (dendritic cell, T-cell, antigen-presenting cells, neutrophilic granulocytes, keratinocytes, vascular endothelial cells, and the cutaneous nervous system) is mediated by many cytokines (e.g., interleukin-1, interleukin-6, IFN-γ, TNF-α, etc.) and chemokines (e.g., interleukin-8 [CXCL8], CXCL10, CCL20, etc.) that orchestrate the pathologic changes in the skin.

Environmental factors such as physical injury or bacterial infection breaks the immunogenic tolerance and starts a set of events that triggers psoriasis in genetically predisposed individuals [7,21]. In the initiation phase, stressed keratinocytes release self-DNA that forms complex with cathelicidin (LL37), which in turn activates innate immune sentinels to produce pro-inflammatory cytokines that mediate the activation of dermal dendritic cells (dermal DCs). Activated dermal DCs migrate to the draining lymph nodes and induce the differentiation of naïve T-cells such as type 17 helper T cells (Th17) or type 17 cytotoxic T cells (Tc17) and type 1 helper T cells (Th1) or type 1 cytotoxic T cells (Tc1). The activated T-cells migrate *via* lymphatic and blood vessels into the dermis, attracted by keratinocyte derived chemokines leading to the formation of plaques.

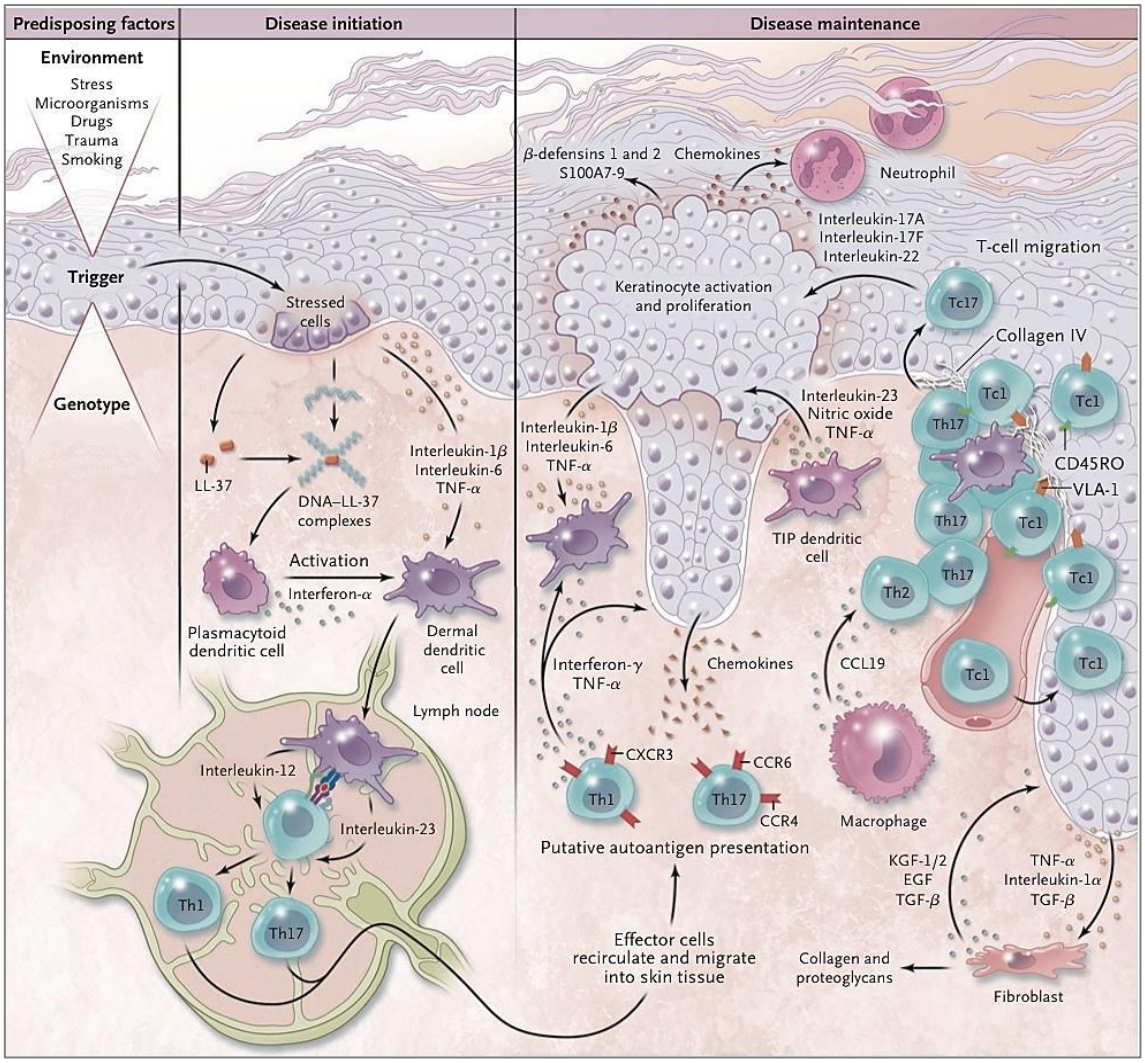

Figure 2. Formation of psoriatic lesions from initiation to maintenance of the disease

(modified figure based on the publication Nestle et al, 2009) [7]

One of the key processes during disease maintenance is the production of immune mediators by T-cells that stimulate keratinocyte proliferation and further cytokine and chemokine production of keratinocytes, fibroblasts, NKT cells and DCs. The migration of T-cells from the dermis into the epidermis is controlled through the interaction of very late antigen 1 (VLA-1) on T-cells and collagen IV at the basement membrane on blood vessels and contribute to the disease progression. Feedback loops involving keratinocytes, fibroblasts and endothelial cells contribute to tissue reorganization, endothelial cell activation and proliferation, and deposition of the extracellular matrix (Fig. 2.). Thus, a rich interface between effectors of the innate and adaptive immune system shapes the psoriatic inflammatory process.

1.1.2. The significance of altered molecular patterns in non-involved skin

Great majority of the researchers have mainly focused on the examination of the psoriatic involved skin, however, the healthy looking (non-involved) psoriatic skin also carries molecular alterations that could contribute to the pathogenesis of psoriasis. The altered skin tissue homeostasis of the normal looking skin of psoriatic patients has key importance in the initiation of the disease. The normal looking skin contains abundant stores of T lymphocytes [22] as well as resident population of DCs [23], suggesting that the skin might be a potential site for the direct triggering of immune responses [21]. Keratinocytes of the non-involved psoriatic epidermis are inherently oversensitive to proliferative signals, and this elevated level of sensitivity plays a crucial role in the development of psoriatic lesions [24,25]. The aim of our workgroup is to identify and characterize abnormal molecular patterns in non-lesional psoriatic keratinocytes contributing to the initiation of the disease phenotype and factors that make these keratinocytes prone to respond with hyperproliferation to cytokines produced by skin infiltrating lymphocytes. For that we previously performed a differential display experiment to compare the gene expression pattern of non-lesional psoriatic epidermis and control healthy epidermis, and then some years later we examined the gene expression differences in non-lesional and normal healthy epidermis upon cytokine induction, using cDNA microarray method. These two experiments identified several molecular patterns in psoriatic non-lesional epidermis that are likely to contribute to inherent abnormal reactivity of keratinocytes to various stimuli. Among others, the differential display experiment identified a novel non-coding RNA gene, PRINS (psoriasis susceptibility-related RNA gene induced by stress) that is significantly overexpressed in psoriatic non-lesional epidermis compared either to healthy or psoriatic lesional epidermis [26]. The cDNA microarray revealed that the expression of CARD18 (caspase recruitment domain family member 18, Iceberg) mRNA is also altered in psoriatic non-lesional epidermis upon T lymphokine induction compared to normal epidermis [27]. In the following sections of the dissertation we establish a detailed overview of the above mentioned molecules, their molecular characteristics as well as their possible contribution to psoriasis.

1.2. Non-coding RNAs

Over the last decade, advances in genome-wide analysis of the eukaryotic transcriptome have revealed that up to 90% of the human genome are transcribed, however, GENCODE-annotated exons of protein-coding genes only cover 2.94% the genome, while the remaining are transcribed as noncoding RNAs (ncRNAs) [28]. Noncoding transcripts are further divided into housekeeping ncRNAs and regulatory ncRNAs. Housekeeping ncRNAs, which are usually considered constitutive, include ribosomal, transfer, small nuclear and small nucleolar RNAs. Regulatory ncRNAs are generally divided into two subclasses based on their length. Those less than 200 nucleotides are usually referred to as short/small ncRNAs, and those longer than 200 bases are known as long noncoding RNAs (lncRNAs) [29]. lncRNAs have broad spectrum of function: they can serve as signals for cellular information, decoys of RNA and protein molecules - thereby inhibiting their functions -, guides for recruitment of transcriptional and epigenetic factors, scaffolds for macromolecular assemblies with varied functions [30]. The specific functions of lncRNAs are determined by many factors, such as their tissue-specific expression, and the physiological status of the cells [31].

1.2.1. PRINS, a newly identified long non-coding RNA

PRINS (Psoriasis susceptibility-related RNA gene INnduced by Stress), identified by our research group is a 3681-nucleotide-long ncRNA molecule, transcribed by RNA polymerase II. BLAST searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi) revealed that the PRINS gene is specific for anthropoid primates, its rodent orthologue could not be identified. Our studies demonstrated a higher PRINS expression in the uninvolved psoriatic epidermis than in either the psoriatic involved or healthy epidermis [26]. These results suggest that the overexpression of PRINS in the uninvolved psoriatic epidermis may play a role in psoriasis susceptibility rather than in the precipitation of psoriatic lesions, and are in agreement with the previous observations that the keratinocytes of the uninvolved epidermis differ from healthy keratinocytes in their responses to external stimuli [24,32,33]. Real time reverse transcription-PCR analysis showed that stress signals such as ultraviolet-B irradiation, viral infection (herpes simplex virus), and translational inhibition increased the RNA expression level of PRINS. Gene-specific silencing of PRINS by RNA interference revealed that down-regulation of PRINS impairs cell

viability after serum starvation but not under normal serum conditions [26], suggesting that PRINS functions as a noncoding regulatory RNA and plays a protective role in cells exposed to stress. Moreover, silencing of PRINS gene resulted in altered HeLa cell morphology and gene expression alterations, among others in the case of G1P3, an interferon-inducible anti-apoptotic gene [34]. It has been reported that long mRNA-like ncRNAs form complexes with proteins [35–37], and act as regulators of various cellular functions [38]. Therefore we have performed an *in vitro* binding assay which revealed that PRINS interacts physically with the molecular chaperone protein nucleophosmin (NPM, B23) in HaCaT and normal human epidermal keratinocyte (NHEK) lysates and we were first to describe the upregulation of this protein in psoriasis [39].

1.2.2. Nucleophosmin, the direct interacting partner of PRINS non-coding RNA

NPM, a multifunctional nucleolar phosphoprotein [40], has a potential role as a positive regulator in cell proliferation [41,42], moreover it is implicated in mRNA processing [43,44] and also in the acute response of mammalian cells to environmental stress, when it stimulates DNA repair and reduces apoptosis [45]. A number of studies have shown that UV irradiation results in a rapid nucleoplasmic translocation of the otherwise predominantly nucleolar protein NPM in fibroblasts [45,46], and cancer cells [47,48]. Multiple functions of NPM conferred by the shuttling of protein between the nucleus and cytoplasm (Figure 3.) [49].

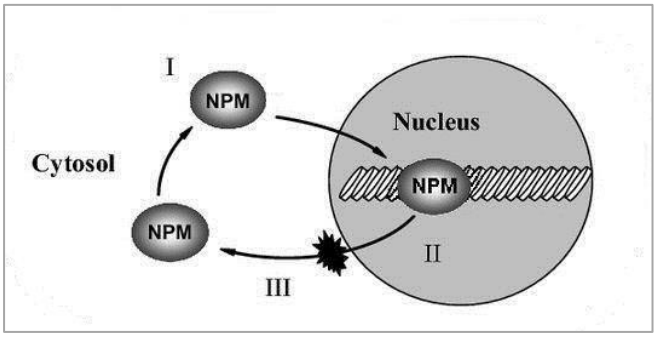

Figure 3. Multiple functions of NPM during shuttling between the nucleus and cytoplasm

I. Post-transcriptional regulation of specific mRNAs **II.** Nuclear functions as a histone chaperone and as a transcription regulator **III.** Roles in cell cycle regulation and centrosomal duplication during cell proliferation (Modified figure based on the publication of Perbal et al, 2008)

It has been demonstrated that NPM forms oligomers involving highly conserved short loops, the ionic interactions between the monomeric subunits leading to the formation of a thermo-stable, chemically resistant pentamers; it has also been shown that NPM pentamers can form decamers *in vitro* by packing two pentamers on top of each other in a sandwich-like structure [50]. Similar protein oligomer-RNA complexes have been documented previously such as the hexameric or heptameric complexes of Sm proteins that function as RNA chaperons and fulfill a number of central tasks in various types of RNA processing [51], and the bacterial Hfq protein that forms hexamers and also functions as an RNA chaperone complex [52]. The heterogeneous nuclear ribonucleoproteins (hnRNPs) play an active part in post-transcriptional gene regulation, e.g. RNA splicing and regulation of the stability and translation of target mRNAs. Most hnRNPs are primarily localized in the nucleoplasm, and can shuttle between the nucleus and the cytoplasm [53].

1.3. Inflammation, inflammasome signaling

Innate immune responses have the ability to both combat infectious microbes and drive pathological inflammation. Inflammation is a non-specific physiological response triggered by noxious stimuli and conditions, such as infection, injury, tissue stress or malfunction [54]. Acute inflammation occurs within a few minutes following the injury of tissue, initiated by tissue-associated cells. Although acute inflammation is normally self-limiting and beneficial to host defense and healing, excessive or continuous inflammatory responses are associate with impaired regulation of inflammatory signaling and subsequently may results in chronic inflammatory or autoimmune disease [55,56].

The innate immune system is composed of germline-encoded pattern recognition receptors (PRR) that collectively serve as sensors for monitoring the extracellular and intracellular compartments for signs of infection or tissue injury [57]. PRRs recognize damage-associated molecular patterns (DAMP) and pathogen-associated molecular patterns (PAMP) [58]. A key component of cytosolic surveillance is the inflammasome, a large multimolecular complex that controls the activation of the proteolytic enzyme caspase-1 [59]. Caspase-1 in turn regulates maturation of the pro-inflammatory cytokines interleukin-1β (IL-1β), IL-18, or the rapid inflammatory form of cell death, called pyroptosis [60].

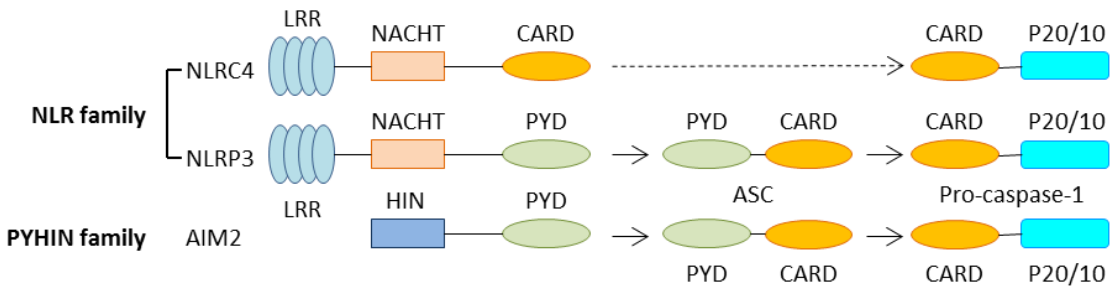

Figure 4. Inflammasome components and domain structure

Inflammasomes come in at least two distinct parts: the NLR family members contain nucleotide binding and oligomerization domains (NACHTs), leucine-rich-repeats and pyrin or card domains (PYDs or CARDs), and the PYHIN family members contain PYD and HIN domains. Most NLRs and PYHINs lack CARD domain and require ASC, an adaptor molecule that contains PYD and CARD domains. The PYD of ASC interacts with the PYD of NLRs or PYHINs, whereas the CARD of ASC recruited to pro-caspase-1 CARD domain (Figure 4.). The recruitment of pro-caspase-1 into a multiprotein inflammasome complex induces auto proteolytic cleavage of pro-caspase-1. Caspase-1 is an aspartate-specific cysteine protease that cleaves its substrates, the preform of IL-1β and IL-18 [57,61]. IL-1β is produced mainly by monocytes, but also by macrophages, dendritic cells and a variety of other cells in the body [62]. A minute amount of IL-1β *in vivo* can evoke fever, hypotension, release of adrenocorticotrophic hormone and production of cytokines which in turn induce various inflammatory and immune responses [63]. Increased IL-1β production has been reported in patients with various infections, inflammation, trauma, ischemic diseases, tumors, intravascular coagulation, autoimmune disorders, UV radiation, graft-versus-host disease, transplant rejection, and in healthy subjects after strenuous exercise [64].

1.3.1. AIM2 inflammasome mediated processes in psoriasis

The importance of innate immune dysregulation in psoriasis has long been recognized [65–67]. The activated state of the innate immune system is represented by the activity of NKT cells, dendritic cells, neutrophils and keratinocytes, and an increased expression of cytokines [68]. Pro-inflammatory cytokines, such as IL-1β and IL-18, have been shown to play an important role in the pathogenesis of psoriasis, i.e., initiating and mediating the infiltration of immune cells and stimulating keratinocytes to proliferate [17,69,70] . Elevated

IL-1β and IL-18 expressions have been observed in inflamed skin; moreover, the increased IL-1β is produced mostly by keratinocytes [71,72]. Certain cytokines, inflammasomes and inflammatory caspases, such caspase-1, are also described as potential inducers and regulators of skin inflammation in contact hypersensitivity and in psoriasis [68,73].

Cytosolic DNA is a potent inducer of the innate immune response and has been proposed to be involved in the pathogenesis of psoriasis [7,74,75]. Keratinocytes express and secrete four different deoxyribonucleases (DNases), which remove extracellular DNA by degradation. In psoriatic skin, three of these DNases do not exhibit DNase activity and one seems to have reduced DNA-affinity. The optimum pH of these DNA binding proteins likely changes during keratinization [76], resulting in aberrant DNase activity and the presence of excess DNA fragments in the cytosol.

The DNA-sensor-containing absence in melanoma 2 (AIM2) inflammasome is triggered by both self-derived (Figure 5.) and pathogen-released (*Francisella tularensis*, *Listeria monocytogenes*, vaccinia virus) cytosolic double-stranded DNA [77,78]. Recently, abundant cytosolic DNA and increased AIM2 expressions were detected in keratinocytes in psoriatic lesions but not in healthy skin [75], and suggested to contribute to the abnormal IL-1β secretion in psoriasis.

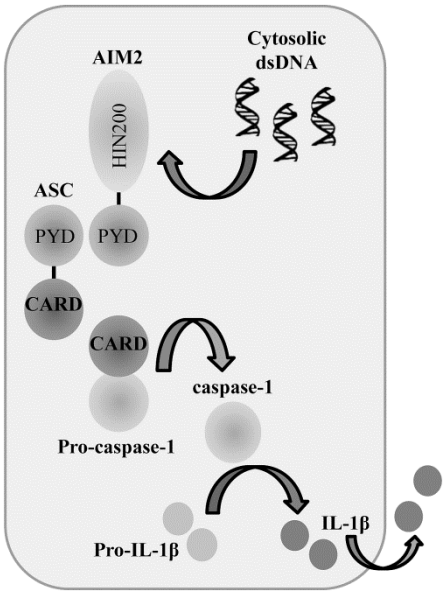

Figure 5. The signaling of the DNA-sensor-containing AIM2 inflammasome

1.3.2. CARD18 (Iceberg), a novel negative caspase recruitment domain

Our recent large-scale gene expression study has revealed that the caspase recruitment domain family member 18 (CARD18, Iceberg) transcript is differentially expressed in psoriatic non-involved epidermis compared to healthy epidermis [27]. CARD18, a member of CARD-only protein (COP) family, consists of only one CARD domain which exhibits sequence similarity (53%) [79] to the CARD prodomain of caspase-1. COP family members probably arose through gene duplication and are restricted to the higher primates [80]. CARD18 serves as a decoy protein which can modify the activity of inflammasomes by inhibiting the generation of active IL-1β and IL-18 through direct interaction with pro-caspase-1 [81,82]. CARD18 is detected mainly in placenta and in many human cell lines and its expression is upregulated by proinflammatory stimuli (LPS and TNF-α) in THP-1 monocytes [79]. Moreover, CARD18 inhibited the LPS- or INF-ɣ induced secretion of IL-1β in monocytes [82]. These data suggest that CARD18 may be a part of a negative feedback loop in several professional and non-professional cell types.

2. AIMS

2.1. To investigate the physical and functional characteristics of PRINS non-coding RNA and its interacting partner, nucleophosmin (NPM)

- To examine the histological and cellular distribution of PRINS non-coding RNA in different tissue types

- To study the intracellular localization of NPM under normal conditions and after UV-B irradiation in human keratinocytes

- To analyze the effect of PRINS gene silencing on NPM UV-B induced shuttling in human keratinocytes

2.2. To investigate the characteristics and functions of the CARD18 molecule in normal human keratinocytes and in the skin

- To study the basal expression of CARD18 and its expression changes upon T-cell lymphokine treatment in healthy and psoriatic non-involved epidermis

- To analyze the distribution of CARD18 expression under normal conditions and after mild injury (tape stripping) in healthy and psoriatic non-involved as well as involved epidermis

- To study the cellular expression of CARD18 in spontaneously differentiating normal human epidermal keratinocytes

- To analyze the gene expression of molecules involved in inflammasome signaling in keratinocytes exposed to cytosolic DNA treatment

- To explore whether the silencing of CARD18 has an effect on keratinocyte innate immune functions

3. MATERIALS AND METHODS

3.1. Patients

Shave biopsy samples for organotypic skin cultures were taken from the non-involved buttock area of five young male psoriasis patients and five age- and gender-matched healthy controls. Subsequently, an organotypic skin culture system was established as has been described previously [83].

For immunohistochemical staining, patients with moderate-to-severe psoriasis vulgaris (n=2) and healthy (n=2) volunteers were enrolled in the study (CARD18 staining). Psoriatic patients had not undergone treatment for 4 weeks before sampling.

In another set of experiments, the skin of psoriatic patients (n=2) and healthy volunteers (n=2) were subjected to tape stripping (TS). Punch biopsies were taken from the area once before and twice after (24 and 48 hours) the treatment (CARD18 staining).

Written informed consent was obtained from all donors involved in the study. The study was approved by the Human Investigation Review Board of the University of Szeged, complying with the ethical standards of research and in accordance with the Helsinki Declaration.

3.2. Cell cultures

Normal human epidermal keratinocytes (NHEKs) were separated from skin specimens obtained from the Plastic Surgery Unit of our department. The epidermis and the dermis were separated by overnight incubation in Dispase (Roche Diagnostics, Manheim, Germany), and keratinocytes were obtained after maceration in 0.25% trypsin. Cells were grown in 75 cm^2 cell culture flasks and were maintained in keratinocyte serum-free medium (Gibco Keratinocyte SFM Kit; Life Technologies, Copenhagen, Denmark), supplemented with 1% antibiotic/antimycotic solution (PAA Laboratories GmBH, Pasching, Austria) and 1% L-glutamine (PAA Laboratories) at 37 °C in a humidified atmosphere with 5% CO_2. The calcium concentration of the medium was <0.1 mM. The medium was changed every 2 days. Third passage keratinocytes were used for experiments.

A previously described *in vitro* keratinocyte differentiating model was used [84]. For the induction of AIM2 inflammasome, cells were subjected to 5 ng/ml tumor necrosis factor

(TNF) α (R&D Systems, Minneapolis, MN, USA) and 5 ng/ml interferon (IFN) ɣ (R&D Systems) pretreatment and subsequently transfected with 1 μg/ml polydeoxyadenylic acid–polydeoxythymidylic acid double-stranded homopolymer (poly(dA:dT, Sigma Aldrich, Saint Louis, MO, USA) transfection using X-tremeGENE 9 transfection reagent (Roche).

Treatment of keratinocytes with psoriasis-related cytokines (here referred to as T-cell lymphokines) was carried out as described previously [27]. Cells were stimulated with the mixture of 0.3 ng/ml IL-3 (R&D Systems), 1 ng/ml granulocyte macrophage colony-stimulating factor (GM-CSF, R&D Systems) and 1 ng/ml IFN-ɣ to the medium. The supernatants were collected and cells were harvested at the indicated time points after treatments.

The HPV-Ker cell line was immortalized by the HPV E6 oncogene as described previously [85]. HPV-Ker cells were grown in 75 cm^2 cell culture flasks and maintained in keratinocyte serum-free medium (Gibco® Keratinocyte SFM Kit; Life Technologies, Copenhagen, Denmark) supplemented with 1% antibiotic/antimycotic solution (PAA, Pasching, Austria) and 1% L-glutamine (PAA, Pasching, Austria) at 37 °C in a humidified atmosphere containing 5% CO_2. The medium was changed every two days.

Cells were subjected to UV-B irradiation (312 nm, 40 mJ/cm^2) in phosphate-buffered saline (PBS; 153 mM NaCl, 7.67 mM Na_2HPO_4, 2.67 mM NaH_2PO_4) at room temperature (RT). Immediately after irradiation, the PBS was aspirated off, and culture medium was added to the culture dishes. Cells were harvested at the indicated times after UV-B exposure.

3.3. *In Situ* Hybridization

Custom LNA mRNA detection probes for PRINS with the sequence 5'-3': /5DigN/AAGCTTCTGTCCTCATTAGTCTTC/3Dig_N/ and scrambled control sequence 5'-3': /5DigN/AAGCCTCCGTTCTTATTAGTCTTC/3Dig_N/ were ordered from Exiqon (Exiqon A/S, Vedbaek, Denmark).

For *in situ* hybridization experiments, patients with moderate-to-severe psoriasis vulgaris (n=6) and healthy (n=10) volunteers were enrolled in the study.

For the detection of PRINS expression in different human tissue types tissue chips were used (n=2). Formaldehyde-fixed blocks measuring 4 mm in diameter of normal breast,

cerebellum, cerebrum, gallbladder, kidney, large bowel, small bowel, lung, lymph node, skin, stomach, testicle and uterus samples were placed into a tissue microarray (TMA) block. The fixation of samples was standardized (4% buffered formaldehyde for 24 hours at RT, in a volume of 1:10). After paraffin embedding, the TMA was cut into 4-μm-thick sections, which were rehydrated with increasing concentrations of alcohol.

HPV-Ker cells were used for the intracellular detection of PRINS expression. HPV-Ker cells were trypsinized and harvested by centrifugation, and resuspended in PBS. 10^5 cells were centrifuged onto a slide by using a cytocentrifuge (Cytopro™, Wescor, Logan, UT, USA) and dried overnight at RT. The slides were fixed in 2% paraformaldehyde for 20 min at RT.

After 2 × 5-min PBS washes, slides – both with the tissue chips samples and HPV-Ker cells - were treated for 15 min and for 5 min, respectively, with 20 μg/mL proteinase K in PBS at 37 °C. Digoxigenin-labeled LNA probes suspended in hybridization buffer (50% formamide) were denatured at 95 °C for 2 min. Hybridization was performed at 60 °C overnight. After hybridization, samples were washed in 5 × SSC (saline–sodium citrate buffer) for 15 min, and then 2 × 30 min in 0.2 × SSC at 60 °C. After a 10-min PBS wash, samples were equilibrated in 10% goat serum-containing blocking buffer (0.5% blocking reagent (Roche, Budapest, Hungary) in PBS containing 0.1% Tween 20) for 1 hour at RT and then incubated with anti-DIG alkaline phosphatase (AP) conjugate (Roche Diagnostics, Manheim, Germany) diluted 1:500 with blocking solution for 1 h. After antibody incubation, samples were washed in PBS containing 0.1% Tween-20 for 2 × 10 min and in PBS for 2 × 10 min. Coloring reactions were performed overnight with BM purple AP substrate (Roche Diagnostics, Manheim, Germany). Slides were mounted with Glycergel (Dako Denmark A/S, Glostrup, Denmark). Pictures were taken with the use of a Zeiss AxioImager fluorescent light microscope (Carl Zeiss MicroImaging, Thornwood, NY, USA) fitted with a PixeLINK CCD camera (PixeLINK, Ottawa, Ontario, Canada).

3.4. Real-time RT-PCR

Total RNA was isolated from cells using TRIzol® Reagent (Invitrogen Corp., Carlsbad, CA, USA), following the manufacturer's instructions. cDNA was synthesized from 1 μg total RNA with the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). Real-time RT-PCR experiments were carried out with the Universal Probe Library system (F. Hoffmann-La Roche AG, Basel, Switzerland) or with a Cy5-labeled probe for

PRINS expression studes (CCTTCATCTCACACACCTACGCAG). Primers for the real-time RT-PCR experiments are listed in Table 1.

	Forward	Reverse	Probe
PRINS	GAGGCCAGCAGTTTCTACAG	AGGGACAACCACATCAAAGC	Cy5-labeled
CARD18	AAGACCCCTCAACTTGCCTCA	CTCCCTTGGAAGAAGCTCTG	UPL 50
AIM2	AGTCCTCTGCTAGTTAAGCTCTCTG	TCTGACAACTTTGGGATCAGC	UPL 87
caspase-1	CCTTAATATGCAAGACTCTCAAGGA	TAGCTGGGTTGTCCTGCACT	UPL 17

Table 1. Primers used to real-time RT-PCR experiments

PCR assays were performed with the C1000 Touch Thermal Cycler (Bio-Rad Laboratories). The expression of each gene was normalized to the 18S ribosomal RNA gene. Relative mRNA levels were calculated by the ΔΔCt method. Data from different treatments were compared using one-tailed t test. Differences were considered significant when $P \leq 0.05$.

3.5. Immunohistochemistry

Paraffin-embedded specimens from healthy, involved and non-involved psoriatic skin were obtained for CARD18 immunohistochemistry (IHC). Samples were fixed in 4% buffered formaldehyde for 24 h. The tissue block was subjected to paraffin embedding and 4-μm-thick sections were cut and placed on silanized slides, dewaxed in xylene for 3 x 5 min and rehydrated in decreasing concentrations of ethanol. Tissue retrieval was performed in citrate buffer (10 mM, pH 6.0). For nonspecific antigen blocking, 1% horse serum was used for 30 min. Sections were incubated with anti-CARD18 goat polyclonal IgG antibody (Santa Cruz Biotechnology, Dallas, TX, USA; 1:300) at 4°C overnight. The anti-CARD18 goat polyclonal antibody was not included in the staining procedure when sections were stained for the matching negative controls. After three washing steps with phosphate-buffered saline (PBS), biotinylated anti-goat IgG (1:200, Vector Laboratories Inc., Burlingame, CA, USA) was applied for 30 min at room temperature. After one PBS wash, sections were incubated in Extravidin Peroxidase (1:400, Sigma Aldrich) for 30 min at room temperature. Visualization was performed by adding AEC reagent (0.05% 3-amino-9-ethylcarbazole dissolved in N,N-dimethylformamide, 0.01% H_2O_2, and 0.05 M acetate buffer). Sections were counterstained with hematoxylin for 10 seconds and

subsequently analyzed with a Zeiss Axio Imager fluorescent light microscope (Carl Zeiss MicroImaging, Thornwood, NY, USA) fitted with a Carl Zeiss AxioCam MRc camera.

3.6. Immunofluorescence stainings

NHEK cells and HPV-Ker were trypsinized, harvested by centrifugation and resuspended in PBS. 10^5 cells were centrifuged onto a slide using a cytocentrifuge (Cytopro™, Wescor, Logan, UT, USA) and dried overnight at RT. The slides were fixed in 2% paraformaldehyde for 20 min at room temperature.

For the detection of NPM expression, nonspecific antigens were blocked for 30 min at RT in 1% goat serum containing 0.5% BSA-TBS. Slides were incubated overnight at 4 °C with anti-NPM monoclonal antibody (Sigma Aldrich, St. Louis, MO, USA; 1:500) in 0.5% BSA-TBS. Anti-mouse Alexa Fluor 546 goat anti-mouse IgG secondary antibody (Sigma Aldrich, St. Louis, MO, USA; 1:500) was applied for 3 hours at RT.

For CARD18 detection, nonspecific antigens were blocked for 30 min at room temperature in 1% donkey serum containing 0.5% BSA-TBS. Slides were incubated overnight at 4^oC with anti-CARD18 goat polyclonal IgG antibody (Santa Cruz Biotechnology; 1:250) in 0.5% BSA-TBS. Anti-goat Alexa Fluor 546 donkey secondary antibody (Sigma Aldrich) was applied for 1 hour at room temperature.

Cell nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI, Sigma Aldrich, 1:100) and mounted with Fluoromount-G (Southern Biotech, Birmingham, AL, USA). Pictures were taken with the aid of a Zeiss Axio Imager fluorescent light microscope (Carl Zeiss MicroImaging) fitted with a Carl Zeiss AxioCam MRc 5 camera.

For the semiquantitative analysis of the intracellular localization of NPM (nucleolar or nuclear), at least 25 fields of view were counted per each group by two independent examiners. Cells were sorted and counted by observing the localization of NPM immunostaining in the nucleolus or in the nucleoplasm.

3.7. Gene specific silencing

Gene-specific silencing of the non-coding RNA PRINS was performed with a vector-based method described previously [26]. The most effective PRINS silencing was achieved in supplement-free HPV-Ker cell cultures at ~70% confluency, and the transient transfection with the *in vitro* pSilencer™ 2.1-U6 hygro vector (Ambion Inc., Austin, TX,

USA) was therefore carried out with these cells. The siRNA sequence targeting PRINS gene silencing was as follows: AK696, TTTCTGGAATGATGTCCAA. The scrambled sequence (SC1313) was used as control: AACTTTATCTCGGATCTAT. Cells were transiently transfected with the plasmids following an X-tremeGENE 9 DNA transfection protocol, as described by the manufacturer (Roche Diagnostics, Mannheim, Germany). Transfection efficiency was on average 85%, as checked with a GFP reporter construct (Lonza, Basel, Switzerland). The effectiveness of the silencing was measured by real time RT-PCR (Supp. Fig.1).

Gene-specific silencing of CARD18 was performed with an siRNA transfection method. NHEK cells were transiently transfected at approximately 70% confluency using the X-tremeGENE siRNA Transfection Reagent (Roche), according to the manufacturer's instructions. For gene-specific silencing of CARD18, an siRNA duplex was applied: ICEBERG siRNA (h), sc-105550, and control siRNA-A, sc-37007 (Santa Cruz Biotechnoloby). The most effective CARD18 silencing was achieved in serum-free culture medium without additive and supplements. The effectiveness of the silencing was measured by real-time RT-PCR (Supp. Fig.2).

3.8. ELISA

Cell supernatants were collected and the debris was removed by centrifugation (5000 rpm, 4 min, 4°C). The amount of IL-1β was determined by ELISA (IL-1β ELISA Duo Set, R&D Systems), according to the manufacturer's instructions.

4. RESULTS

4.1. PRINS ncRNA shows variable histological and cellular expression level

The previous real-time RT-PCR studies of our workgroup have demonstrated differences in PRINS expression in various human tissue samples [26]. For a detailed analysis of cellular and histological distribution of PRINS ncRNA we have applied ISH. To investigate the PRINS expression pattern in different tissue types, we applied paraffin-embedded tissue chips containing thirteen sections from different healthy human organs on one slide. The samples were exposed to the same conditions during ISH which led to good comparability of different tissue types. The intracellular distribution of PRINS was also examined in cultured NHEKs.

The ISH experiments demonstrated variable levels of expression in different human tissue samples: high levels of PRINS expression were detected in the gut, lung, lymph node, uterus, testicle and skin (Figure 6A–H), whereas no staining was observed in the cerebrum and cerebellum (data not shown). The breast, kidney, stomach, and gallbladder tissue specimens displayed only moderate PRINS expressions (data not shown). These findings are in agreement with our previous results revealing variable PRINS gene expression levels in different healthy human organs.

We paid a special attention to the skin samples and their careful analysis revealed that the dermal and epidermal expression of PRINS significantly differed. Within the epidermis, a strong staining intensity was similar in the various layers, however, stratum granulosum and stratum lucidum exhibited somewhat more intensive PRINS ISH staining (Figure 6G,H). This staining is regarded specific, since the scrambled control probe did not result in the same staining intensity in these layers. There was only a moderate positivity in the dermis.

In vitro cultured keratinocytes (Figure 6I) exhibited strong nucleolar (indicated by small arrows) and perinuclear PRINS positivity and moderate homogeneous cytoplasmic staining. The cellular expression profile of PRINS is in agreement with the staining pattern of keratinocytes seen in the epidermal layers of skin samples.

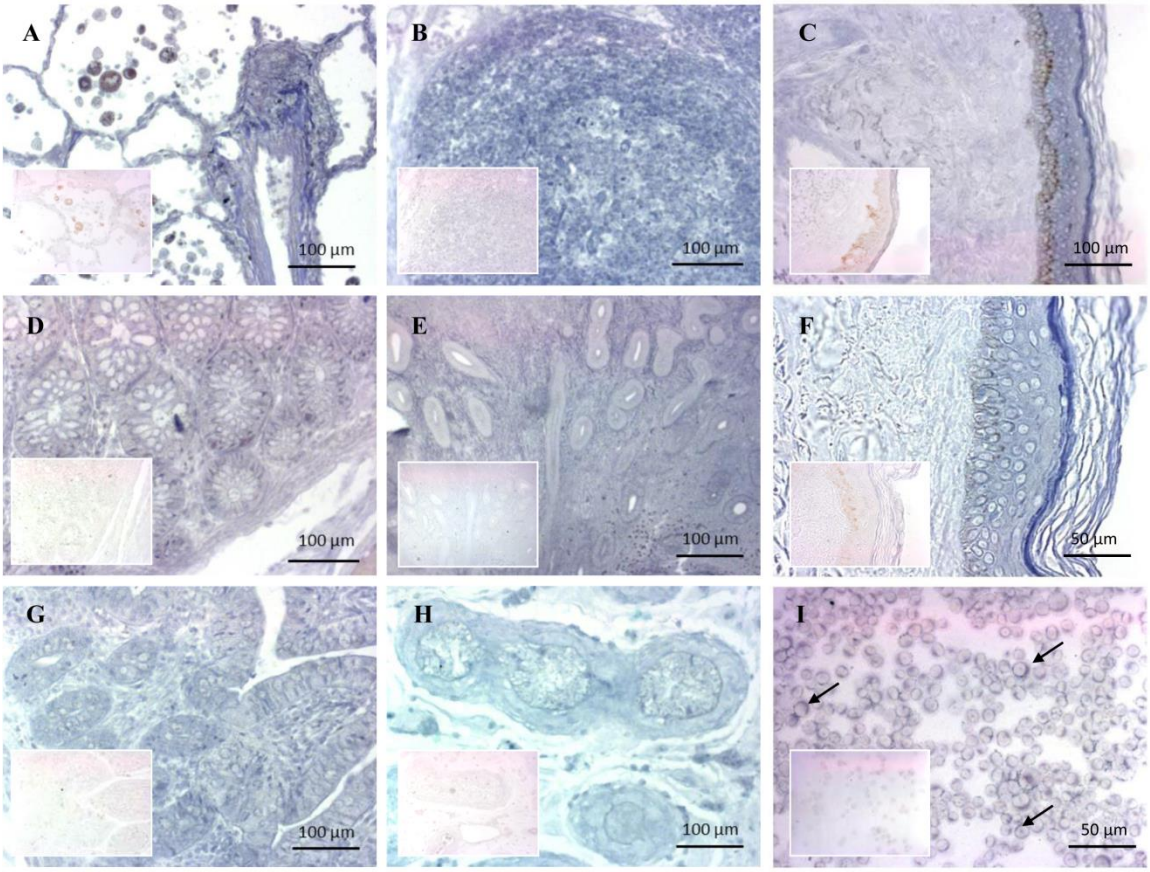

Figure 6. Detection of PRINS expression in various human tissue samples by ISH. The sections were incubated with an LNA RNA detection probe for PRINS and for the control staining (see insets at the same magnification) we used a scrambled control sequence. Relatively strong PRINS positivity was seen in the (A) lungs; (B) the large bowel; (C) the small bowel; (D) the lymph nodes; (E) the uterus; (F) the testicles; (G,H) the skin; and (I) cultured keratinocytes.

4.2. PRINS expression differs in healthy and psoriatic skin

With a quantitative RT-PCR approach our workgroup earlier showed that PRINS was expressed more strongly in the psoriatic uninvolved epidermis than in either the normal or the involved epidermis[26]. To visualize their differences, ISH experiments were performed to compare the expressions of PRINS in normal healthy (n = 10), psoriatic uninvolved (n = 6) and psoriatic involved (n = 6) skin samples.

The ISH results partially confirmed the real time RT-PCR findings, indicating a moderately elevated level of PRINS expression in the uninvolved (Figure 7B) and involved (Figure 7C) epidermis relative to the healthy epidermis (Figure 7A). However, the differences in expression between the involved and uninvolved epidermis were not as

pronounced as with the real time RT-PCR approach. The explanation for this may lie in the differential sensitivity of the two methods.

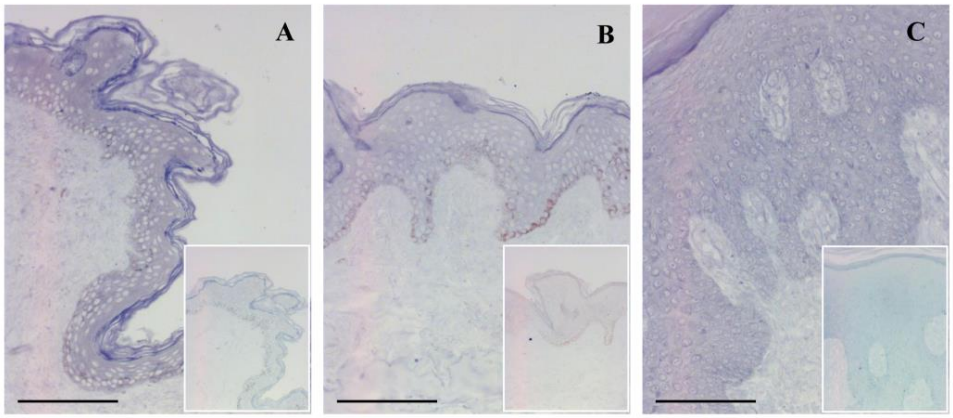

Figure 7. Detection of PRINS expression in psoriasis. ISH for PRINS expression was performed on healthy normal (n = 10), psoriatic uninvolved (n = 6) and psoriatic involved (n = 6) skin specimens. Sections were incubated with the PRINS-specific LNA probe or with the control scrambled (see insets at the same magnification) LNA probe. Representative staining for each tissue types: (A) normal healthy skin; (B) psoriatic uninvolved skin; and (C) psoriatic involved skin. Bar = 100 μm.

4.3. Intracellular localization of NPM protein in keratinocytes upon UV exposure

By performing three independent binding experiments, our colleague Krisztina Szegedi identified nucleophosmin as a putative PRINS-binding protein [39]. NPM is a ubiquitously expressed nuclear phosphoprotein which shuttles continuously between the nucleus and the cytoplasm[86]. Within the nucleus, NPM is predominantly localized in the nucleoli, with its highest levels in the granular component that contains the more mature pre-ribosomal particles[40], but a significant fraction of NPM can also be detected in the nucleoplasm. NPM is a multifunctional protein with multiple locations in the cell. During the cell cycle, NMP is dynamically localized[87] and it is also redistributed from the nucleolus in response to cytotoxic drugs and genotoxic stress. In fibroblasts and cancer cells, UV irradiation results in a rapid nucleoplasmic translocation of the nucleolar NPM[46,88]. To investigate whether we could observe a similar intracellular localization of NPM in keratinocytes after UV-irradiation, we applied an immunocytochemistry method. In these experiments, we used HPV-Ker cells, a keratinocyte cell line newly established by our workgroup [85]. The UV responses of NHEKs and HPV-Ker cells were compared.

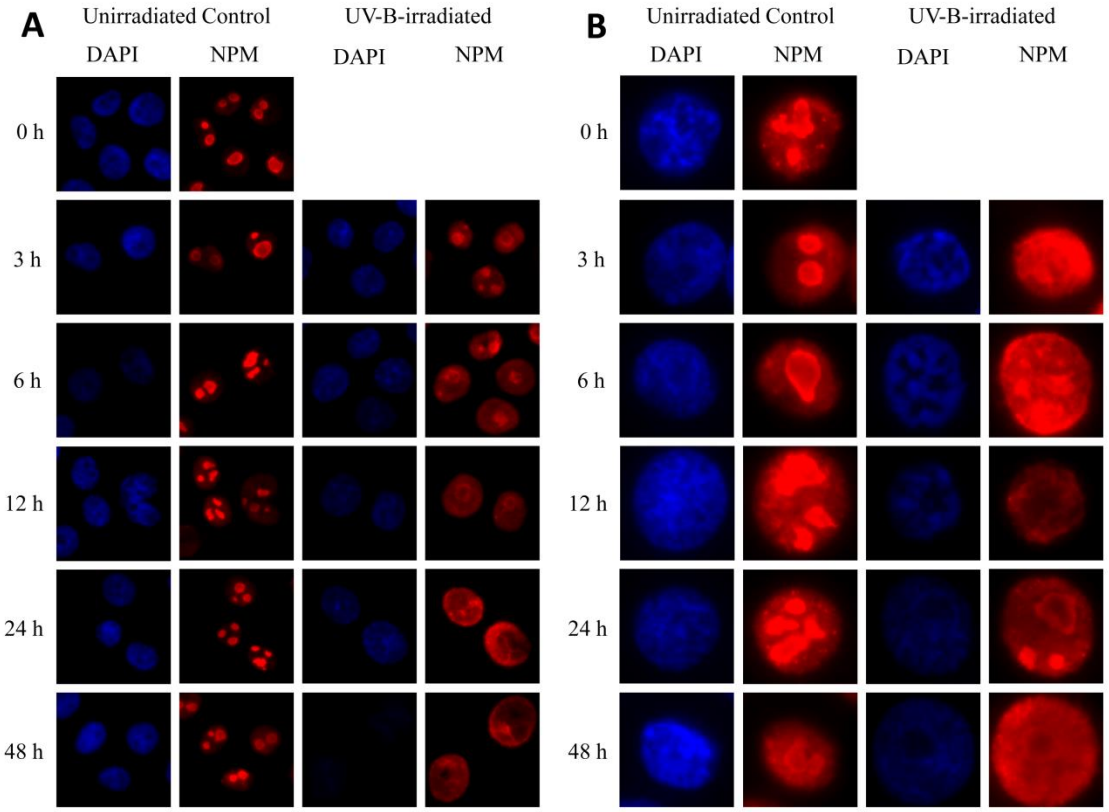

Figure 8. UV-B radiation induces the intracellular trafficking of nucleoplasmic NPM in keratinocytes. Unirradiated and UV-B-irradiated (312 nm, 40 mJ/cm2) cells were followed for the indicated periods of time, fixed and immunostained for NPM. DNA was stained with DAPI. (A) HPV-Ker cells (B) NHEK cells. Magnification: 20X

UV-B exposure caused a relocalization of NPM from the nucleolus to the nucleoplasm in HPV-Ker cells (Figure 8A). The UV-B-induced subcellular shuttling could be detected quite early after UV-B irradiation: slight translocational changes were observed 3 h after treatment. The immunofluorescence intensity of NPM staining in the nucleoplasm was the most abundant at 12 and 24 h. Forty-eight hours following UV-B treatment, the protein had mostly returned to the nucleolus. The time course studies showed that the stimulation of NPM shuttling by UV-B irradiation was a rapid and transient process. The intracellular localization pattern of NPM after UV-B exposure was similar in both the cultured NHEKs (Figure 8B) and the HPV-Ker cells, which suggests that HPV-Ker cells could be appropriate models for further studies on intracellular NPM trafficking.

4.4. PRINS modifies the UV-B-induced intracellular shuttling of NPM

To determine whether PRINS had any effect on the intracellular trafficking of NPM, we silenced the expression of PRINS in growth factor-deprived HPV-Ker cells with a vector-based method[26] and studied the NPM shuttling under normal conditions and after UV-B exposure. NPM shuttling was compared in HPV-Ker cells transfected with a specific PRINS-silencing construct (AK696) and in HPV-Ker cells transfected with a control construct (SC1313).

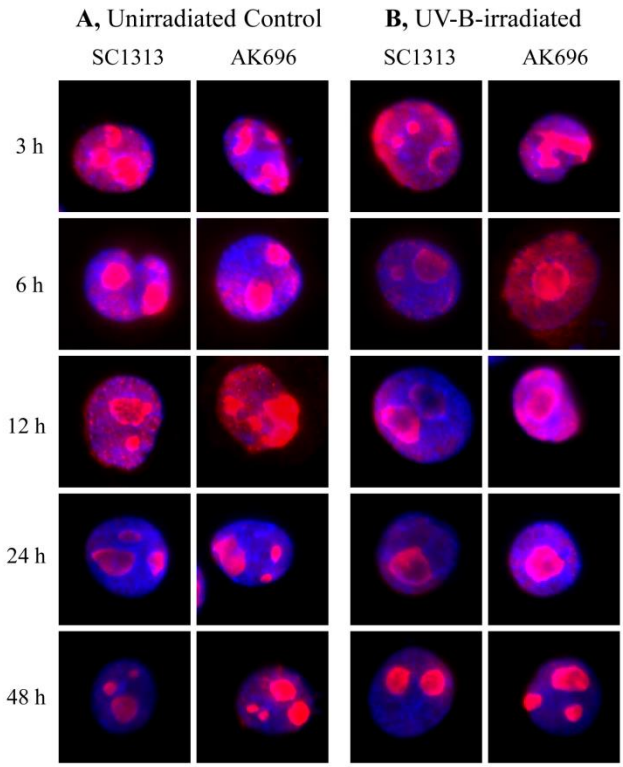

Figure 9. Silencing of PRINS expression modifies the UV-B-induced trafficking of NPM. HPV-Ker cells were transfected with a PRINS gene-specific silencing vector (AK696). Control cells were transfected with a vector containing a scrambled sequence (SC1313). One day after the transfection, the unirradiated (A) and the UV-B-irradiated (B) HPV-Ker cells were followed for the indicated periods of time, fixed and immunostained for NPM. DNA was stained with DAPI. Magnification: 20X

In untreated samples, NPM was localized mostly within the nucleolus in both the SC1313- and the AK696-transfected cells (Figure 9A). The relocalization of NPM from the nucleolus to the nucleoplasm in the control SC1313-transfected cells at the indicated

times after UV-B exposure was similar to our preliminary observations in HPV-Ker cells. As compared with the control cells, cells transfected with the PRINS silencing vector (AK696) (Figure 9B) showed a moderate retention of NPM in the nucleolus following UV-B treatment.

To validate the immunocytochemical observations, a semiquantitative analysis was performed (Figure 10). The nuclear transition of NPM was inhibited in AK696-transfected cells after UV-B irradiation. Interestingly, we observed that the transfection itself slightly modified the shuttling of NPM even in the UV-B negative group.

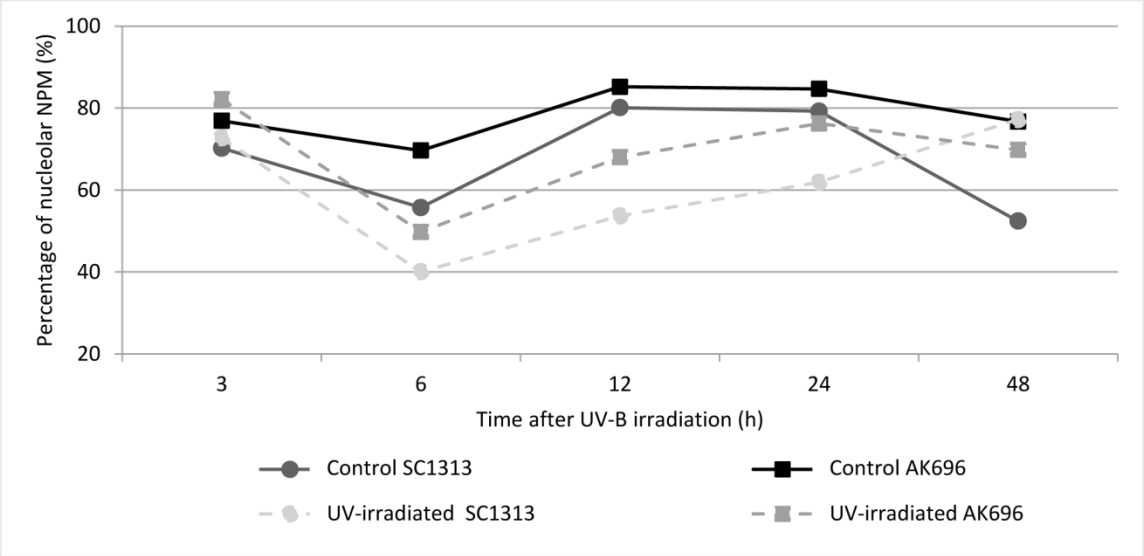

Figure 10. A semiquantitative analysis of the cells was performed following the immunostaining.
In every group, 25 fields of view were counted. Mean values are plotted on the chart.

4.5. CARD18 gene expression differs in healthy and psoriatic non-involved epidermis upon T-cell lymphokine treatment

We and others have presented evidence that psoriatic non-involved epidermal keratinocytes carry molecular and cellular alterations that contribute to the occurrence of psoriatic symptoms. Along with this work we have recently reported the results of a cDNA microarray experiments in which we detected that 61 annotated genes were differentially expressed in psoriatic non-involved and healthy epidermis upon T-cell lymphokine treatment [27]. In this experiment, organotypic skin cultures were treated with a

cytokine mixture (GM-CSF, IL-3, IFN-ɣ) that has been shown to mimic the effect of psoriatic T-cell lymphokines [89]. CARD18 was identified as differentially expressed gene in this experiment. CARD18 encodes a protein that belongs to the COP family, inhibiting inflammasome activation directly by interacting with pro-caspase-1 and serves as a negative regulator of IL-1β maturation [60].

To validate the cDNA microarray results, real-time RT-PCR analysis was carried out to measure the abundance of CARD18 mRNA (Figure 10). For that we used the original RNA samples derived from organotypic skin cultures (healthy epidermis, n=5 and psoriatic non-involved epidermis, n=5).

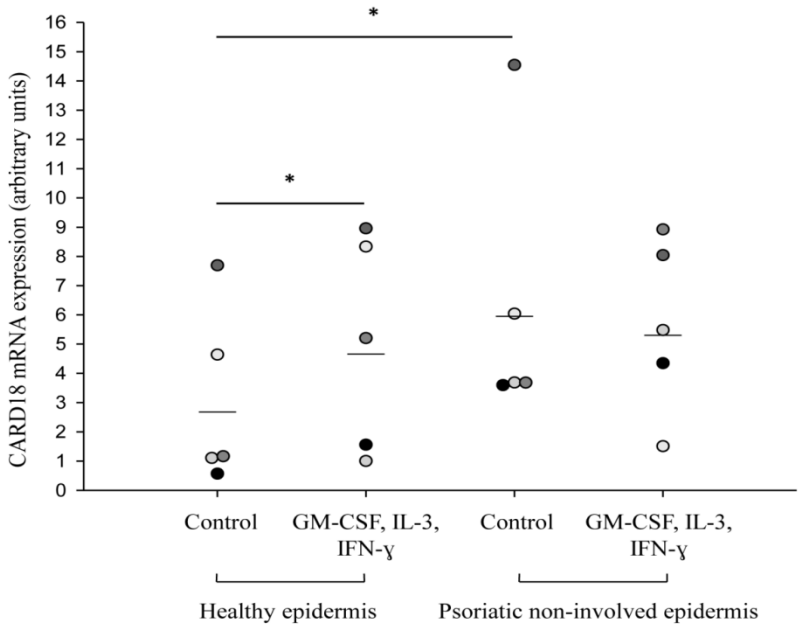

Figure 11. CARD18 gene expression in healthy and non-involved psoriatic epidermis. Total RNA was isolated from the epidermal compartment of organotypic skin cultures (n=5). CARD18 gene expression changes in response to T-cell lymphokine (GM-CSF, IL-3, IFN-ɣ) treatment in healthy epidermis and in non-involved psoriatic epidermis. Horizontal lines represent mean values.

Our results confirmed the cDNA microarray results: we observed a 2-fold elevation of CARD18 mRNA level in psoriatic non-involved epidermis compared to healthy epidermis. Although the basal expression levels were relatively high in the psoriatic non-involved epidermis, it was not further induced in response to T-cell lymphokines. This was in contrast to the findings for healthy skin, where lower basal expression levels were induced with a 1.65-fold elevation in response to the same treatment (Figure 11).

4.6. CARD18 is abundantly expressed in differentiating keratinocytes

To examine the characteristics of CARD18 in NHEKs, we examined mRNA and protein changes in spontaneously differentiating NHEKs. In spontaneously differentiating third-passage NHEKs, the differentiation process was detected by increaseing keratin 10 as a differentiation marker, and by decreasing alpha5 integrin expression as a proliferation-related marker (data not shown). The 0-day samples were taken from subconfluent cultures. To determine the mRNA expression of CARD18, real-time RT-PCR was carried out using gene-specific primers. The *in vitro* gene-expression study revealed low-level CARD18 mRNA expression in the proliferative state of cells, and this expression continuously increased during differentiation up to 43.2-fold elevation in the 10-day samples (Fig. 12A).

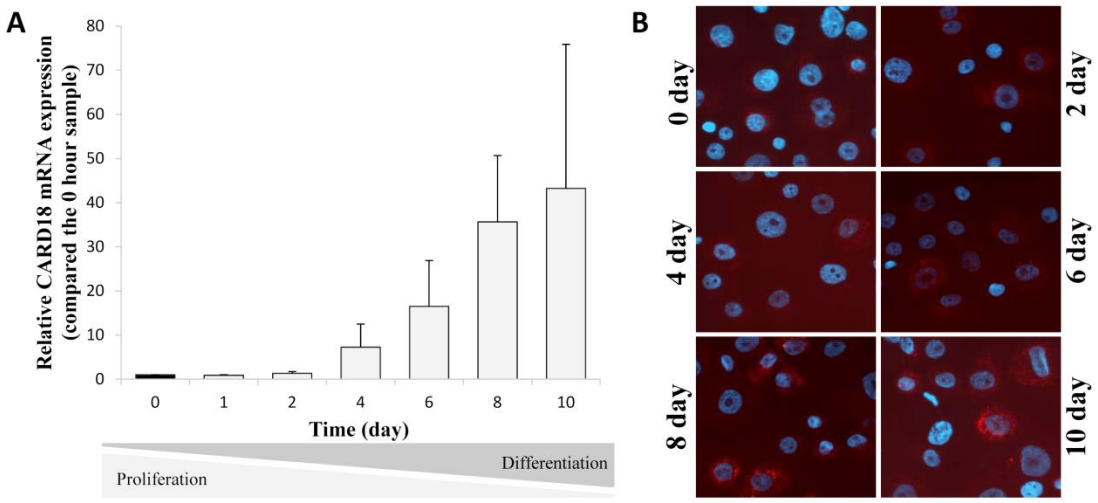

Figure 12. Expression of CARD18 mRNA and protein during keratinocyte proliferation and differentiation. Cultured NHEKs (n=3) were grown till subconfluency before samples were taken (0 day). (A) Changes in CARD18 mRNA expression were analyzed by real-time RT-PCR at the indicated time points. Relative expression is shown compared to the 0-day sample. (B) CARD18 intracellular protein localization was detected in NHEKs (n=3, one representative picture is shown) at the indicated time points. Samples were fixed and immunostained for CARD18 (red). DAPI was used for nuclear counterstaining. Magnification: 20X

To examine the expression of CARD18 protein, immunofluorescence staining was applied to keratinocytes, revealing that moderate positive CARD18 protein expression in

samples taken on days 0–4 gradually increased in the 6–10-day samples (Fig. 12B). Thus, CARD18 protein expression followed a similar pattern observed for the mRNA expression during the 10-day course. The immunocytochemistry staining allowed the intracellular distribution to be determined and revealed that CARD18 was located in the cytoplasm of NHEKs, as it was observed in the epidermal keratinocytes of the *ex vivo* samples (see later; Fig.13-14).

4.7. CARD18 expression differs in healthy and diseased skin

Using an organotypic skin model, we previously determined that CARD18 mRNA abundance was higher in psoriatic non-involved epidermis compared to healthy epidermis. To compare CARD18 protein levels in healthy and diseased skin, immunohistochemical (IHC) staining was performed on paraffin-embedded samples from healthy (Fig. 13A), psoriatic non-involved (Fig. 13B) and psoriatic involved skin samples (Fig. 13C) in an independent experiment.

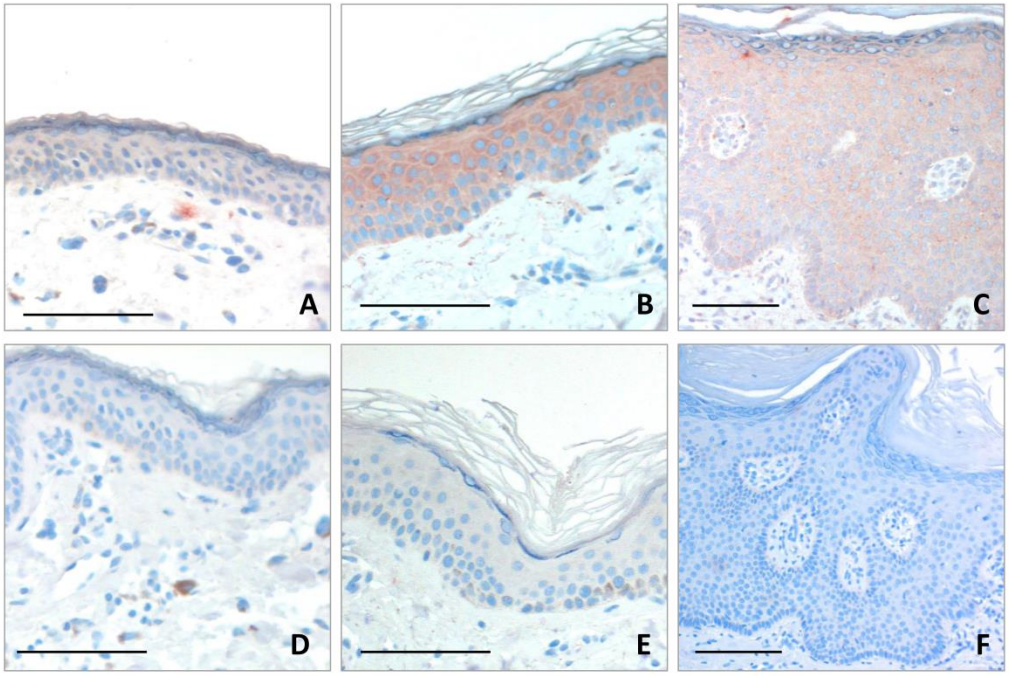

Figure 13. Immunohistochemical staining of CARD18 in healthy and psoriatic skin. CARD18 protein levels were analyzed in healthy volunteers (n=10) and patient with psoriasis (n=6). One representative picture is shown of healthy (A), psoriatic non-involved (B), and psoriatic involved (C) skin. Negative controls were performed for healthy (D), psoriatic non-involved (E) and psoriatic involved skin (F). Bar = 100 μm

In all examined sections, CARD18 appeared exclusively in the epidermis, specifically in the cytoplasm of epidermal keratinocytes. IHC revealed differential CARD18 expression in different skin samples: elevated levels were observed in the psoriatic non-involved and psoriatic-involved epidermis compared to healthy epidermis (Fig 13B and 13C vs 13A). These results confirmed our real-time RT-PCR findings. No differences were detected in the different keratinocyte layers of the epidermis; however, the level of CARD18 staining was somewhat higher in the suprabasal layers of psoriatic non-involved and involved epidermis. Figure 13D-F provide the data from negative controls for figures 13A-C.

4.8. CARD18 expression is differentially induced in healthy, psoriatic non-involved and psoriatic involved epidermis after tape stripping (TS)

Mechanical stress causing disruption of the skin barrier leads to molecular responses, including a rapid increase in DNA synthesis and early over-expression of pro-inflammatory cytokines [90,91]. It has long been known that baseline proliferative activity of keratinocytes of non-involved skin of psoriatic patients does not differ from keratinocytes in skin of healthy individuals; however, the proliferative response to TS or other trauma is significantly higher in psoriatic non-involved skin compared to healthy skin [92–94]. Our aim was to study weather CARD18 has a differential expression in these skin types upon TS. Therefore CARD18 protein expression was observed in mechanically stimulated skin from healthy, involved and non-involved skin of psoriatic donors. Punch biopsies were taken before the procedure and two times after TS (24 and 48 hours). Compared to untreated sections (Fig. 14A, D, G), CARD18 protein expression level was induced 24 hours after TS in healthy (Fig. 14B), psoriatic non-involved (Fig. 14E) and psoriatic involved (Fig. 14H) samples, as well. However, the elevation of CARD18 expression in treated and untreated psoriatic non-involved samples was not as pronounced as in healthy or psoriatic involved skin. Forty-eight hours after the treatment, CARD18 expression remained elevated only in the psoriatic non-involved samples (Fig. 14F). Results from negative-control staining of the TS experiment proved the specificity of stainings (Supp. Fig. 3).

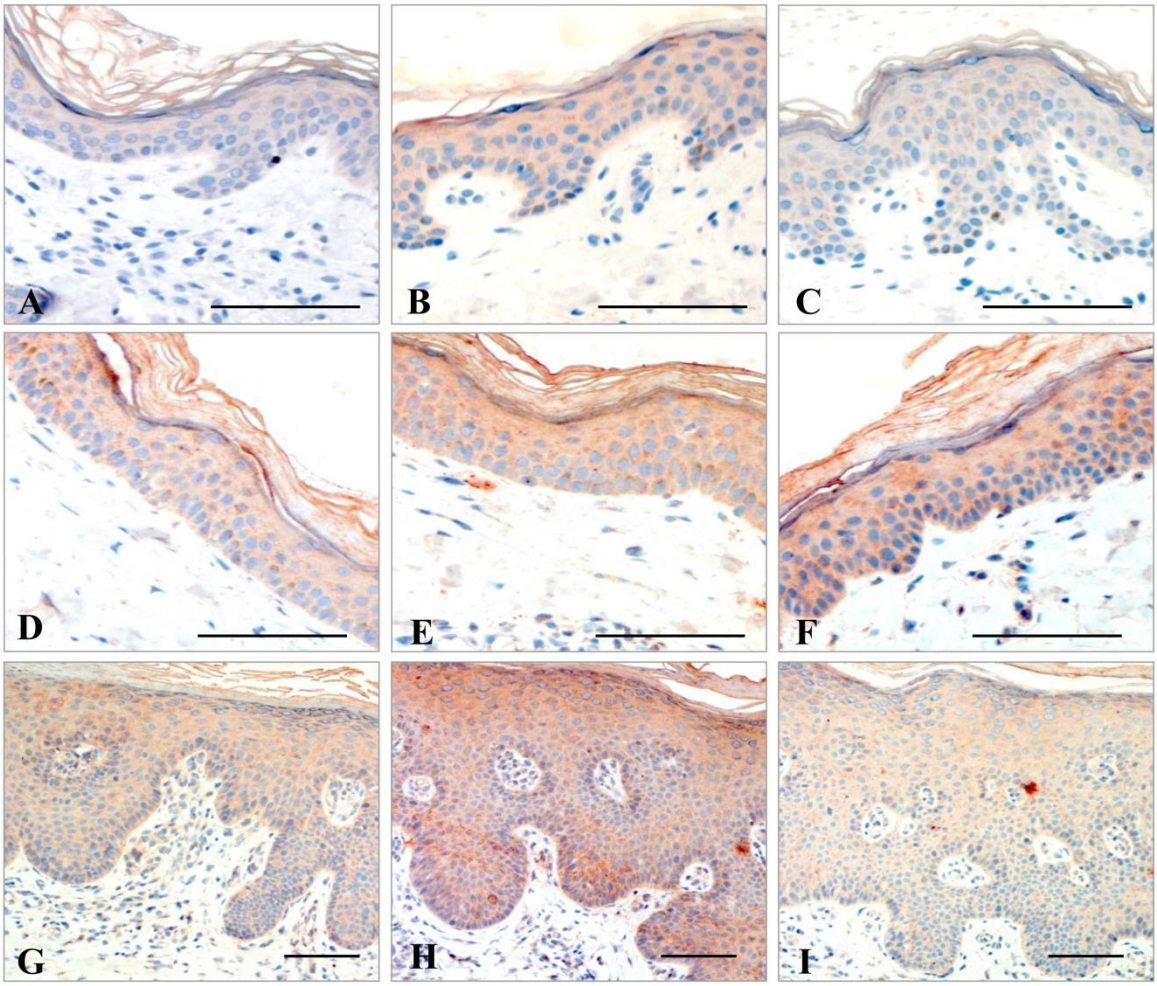

Figure 14. CARD18 expression upon acute barrier disruption. Tape stripping (TS) method was carried out on the skin of healthy (n=2, one representative picture is shown) and psoriatic volunteers (n=3, one representative picture is shown). Punch biopsies were taken at the indicated time points. Skin samples were subjected to immunohistochemical staining of CARD18: healthy untreated (A), healthy TS at 24 h (B), healthy TS at 48 h (C), psoriatic non-involved untreated (D), psoriatic non-involved TS at 24 h (E), psoriatic non-involved TS at 48 h (F), psoriatic involved untreated (G), psoriatic involved TS at 24 h (H), psoriatic involved TS at 48 h (I). Bar = 100 μm

4.9. T-cell lymphokine treatment does not induce altered inflammasome signaling in keratinocytes

To elucidate the cellular processes in which CARD18 participates and to gain further insight into its role in the pathomechanism of psoriasis, we investigated CARD18 expression in response to a T-cell lymphokine mixture containing GM-CSF, IL-3 and IFN-ɣ, a cytokine mix that has already been characterized in psoriasis [89]. Keratinocytes,

important immune-competent cells, are able to sense danger signals and mediate immune responses through the activation of pro-inflammatory signaling pathways [17], resulting in the expression of inflammasome components and secretion of pro-inflammatory cytokines [95]. Twelve hours after cultured NHEKs were incubated with the T-cell lymphokine mixture, a significant increase in CARD18 expression was observed compared to the untreated cells (Figure 15), and these results were in accordance to those obtained with healthy epidermis in organotypic skin culture [27].

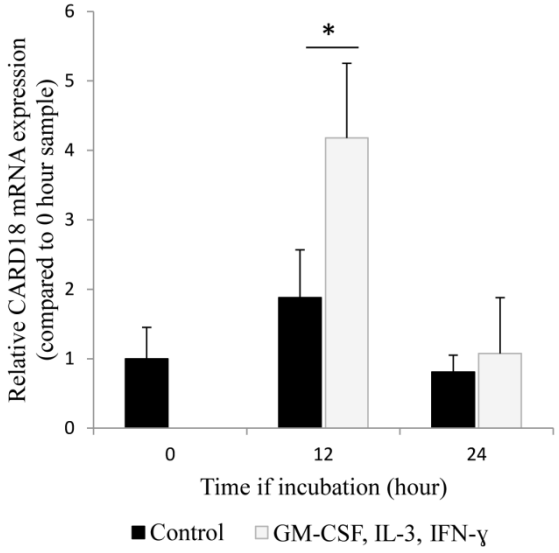

Figure 15. CARD18 mRNA expression upon T-cell lymphokine treatment in NHEKs. NHEK cultures (n=3) were incubated with GM-CSF, IL-3, and IFN-ɣ. Samples were taken at the indicated time points. CARD18 mRNA expression was analyzed by real-time RT-PCR at the indicated times. Expression is relative to the 0 hour sample. * P>0.05

The same T-lymphokine treatment was previously shown to up-regulate IL-1β gene expression [27]. It has been reported that the inflammatory IL-1β cytokine has an important role in skin inflammation and that posttranscriptional activation of IL-1β is mediated by inflammasome activation and caspase-1 proteolytic cleavage. In addition, the relationship between the AIM2 inflammasome and cutaneous inflammation in psoriatic skin has been established [75].

Therefore we investigated AIM2 and caspase-1 gene expression together with IL-1β secretion in NHEKs during T-cell lymphokine treatment. The treatment had no effect either on AIM2 (Supp.Fig. 4A) and caspase-1 (Supp.Fig. 4B) mRNA expression and or on IL-1β secretion (data not shown).

4.10. Modifying effect of CARD18 silencing on molecules involved in cytosolic DNA-triggered responses of NHEKs

Since the T-cell lymphokine treatment had no effect on inflammasome signaling molecules, we turned our attention to other psoriasis-associated aspects of cellular responses. Cytosolic DNA has been recently identified as a trigger for AIM2 inflammasome activation. It has been also demonstrated that it is abundant in psoriatic-involved epidermis [75,96]. To mimic the effect of cytosolic DNA, a synthetic dsDNA analogue poly(dA:dT) was transfected into cultured keratinocytes.

A significant increase in IL-1β release - up to 50 pg/ml (Fig. 16A) - as well as 3–5 fold increase in caspase-1 mRNA expression (Fig. 16C) and a 200-fold increase in AIM2 mRNA expression (Fig. 16D) were observed, although changes in CARD18 mRNA were not detected (Fig. 16B).

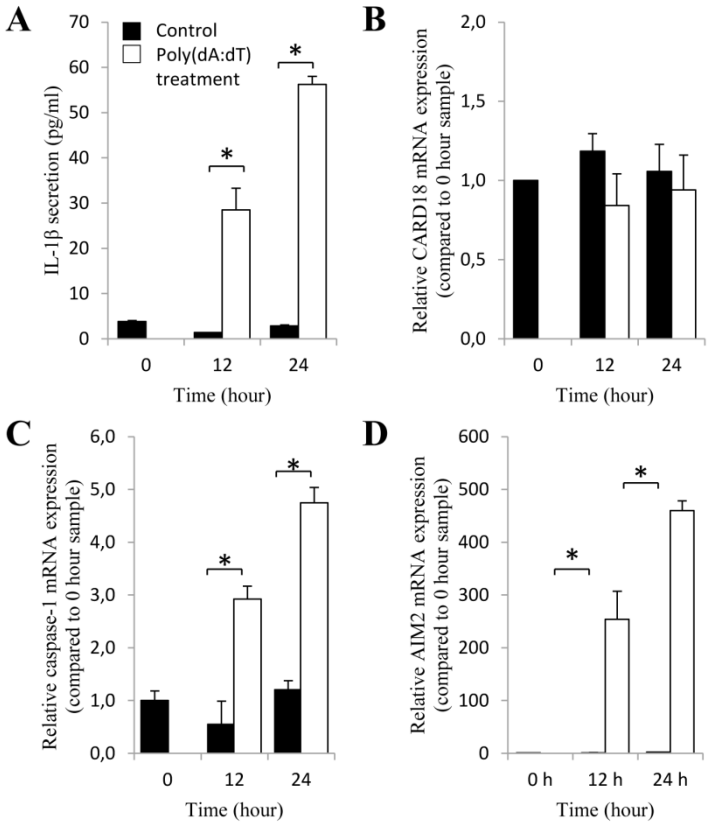

Figure 16. The effect of synthetic DNA analogue (poly(dA:dT)) transfection in NHEKs. NHEK cultures (n=3) were transfected with poly(dA:dT) before taking samples at the indicated times. IL-1β secretion (A) was measured by ELISA. CARD18 (B), caspase-1 (C) and AIM2 (D) mRNA expressions were analyzed by real-time RT-PCR. Expression is relative to the 0 hour sample. * P>0.05

To provoke enhanced inflammatory reactions, a combined IFN-γ and TNF-α pre-treatment was performed before poly(dA:dT) transfection. IFN-γ is known to induce AIM2 gene expression [97], whereas TNF-α mediates pro-IL-1β transcription [98], and both cytokines are consistently elevated in psoriatic skin. The co-treatment resulted in increased IL-1β secretion - up to 100 pg/ml (Fig. 17A) - and increased the expression of caspase-1 and AIM2 mRNAs by 13-fold (Fig. 17C) and 250-fold (Fig. 17D), respectively. CARD18 mRNA level was also increased by six fold (Fig. 17B).

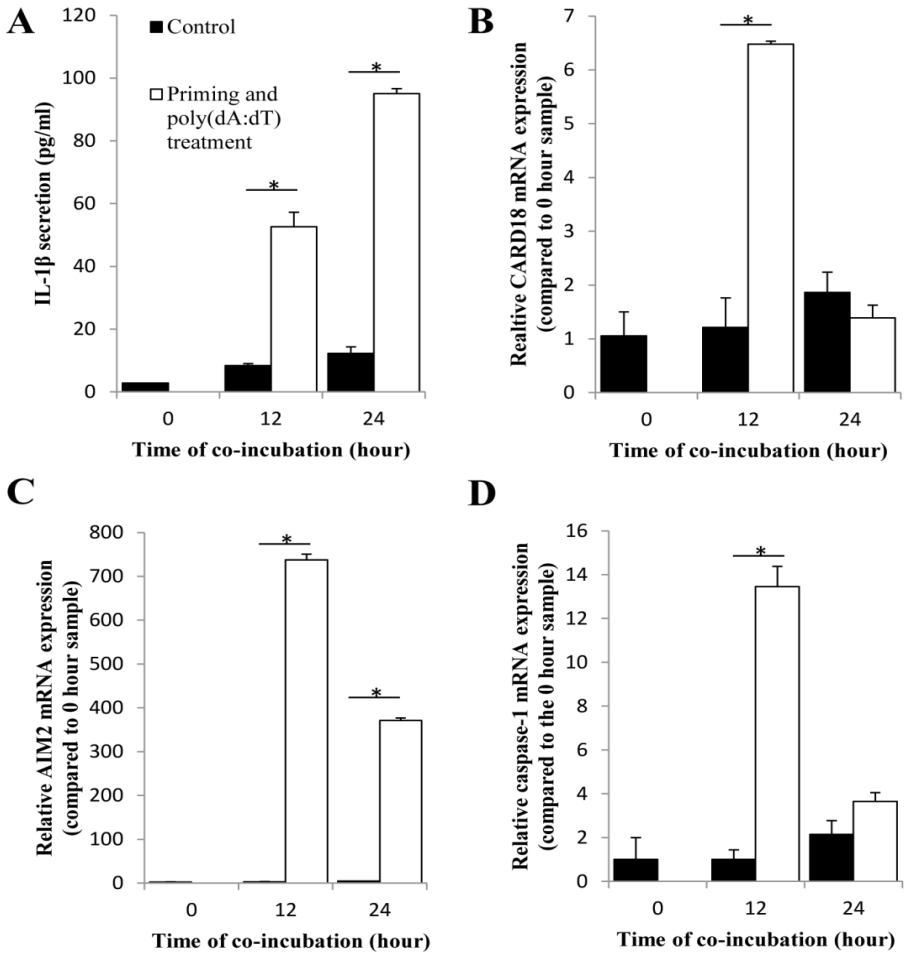

Figure 17. The response of cytokine-primed keratinocytes to synthetic DNA analogue (poly(dA:dT)) transfection. NHEK cultures (n=3) were incubated with IFN-ɣ and TNF-α for 24 hours, followed by poly(dA:dT) transfection. Samples were taken at the indicated times. IL-1β secretion (A) was measured by ELISA method. CARD18 (B), caspase-1 (C) and AIM2 (D) mRNA expression was determined by real-time RT-PCR. Relative expressions are shown compared to the 0 hour sample. * P>0.05

These results encouraged us to further characterize the function of CARD18 in keratinocytes in which inflammatory processes had been induced. CARD18 expression was silenced by siRNA 24 hours before IFN-γ and TNF-α treatment and subsequent poly(dA:dT) transfection. The applied siRNA treatment effectively down-regulated CARD18 expression to 50% of the levels observed in the control cells transfected with the scrambled DNA sequence (Fig. 18B).

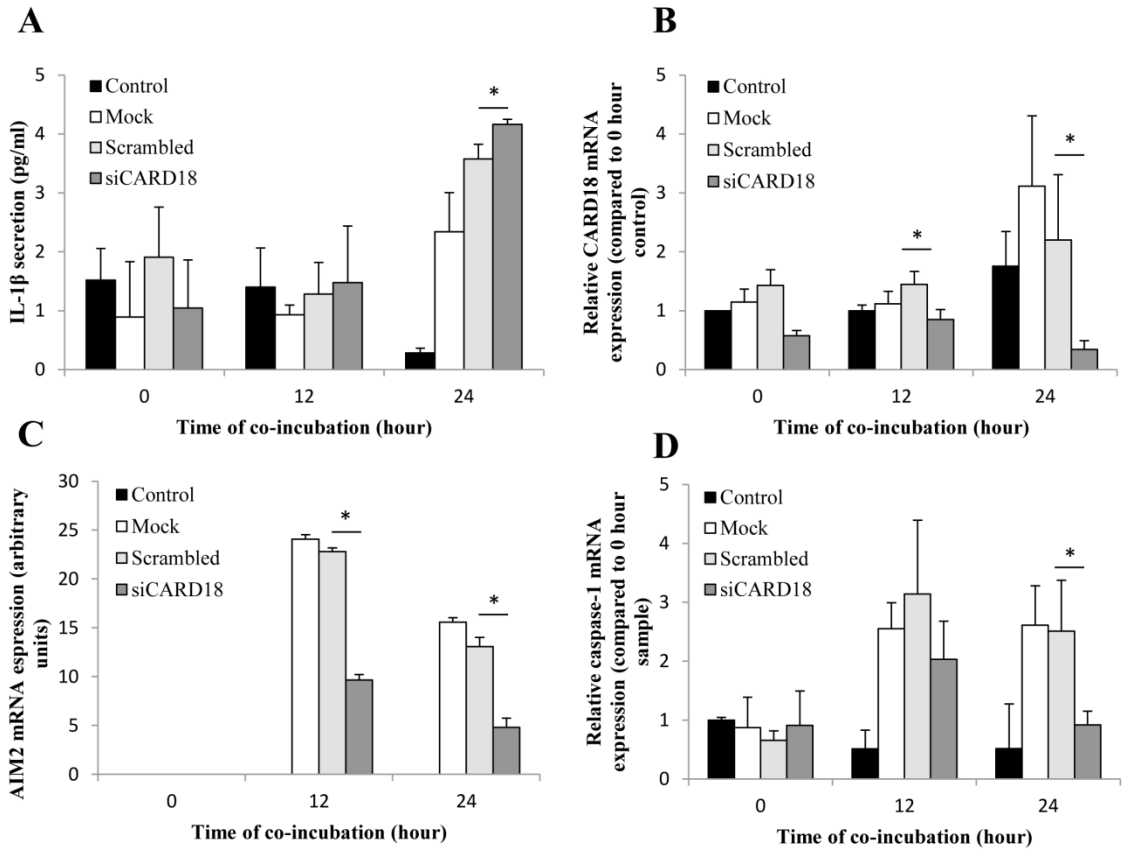

Figure 18. The effect of CARD18 gene-specific silencing in cytokine-primed poly(dA:dT) transfected NHEKs. CARD18 expression was silenced in NHEK cultures (n=3). CARD18 siRNA, scrambled control and mock-transfected cells were subsequently treated with a mixture of IFN-ɣ and TNF-α (12 hours) and transfected with poly(dA:dT). Samples were taken at the indicated times. IL-1β secretion (A) was measured by ELISA. CARD18 (B), caspase-1 (C) and AIM2 (D) mRNA expression was analyzed by real-time RT-PCR. Expression is relative to the 0 hour sample, except for AIM2, where arbitrary units were used. * P>0.05

CARD18 silencing resulted in a significant decrease in AIM2 gene expression 12 and 24 hours after treatment (Fig. 18C) and significantly reduced caspase-1 mRNA

expression (Fig. 18D) 24 hours after the treatment. For further confirmation, we determined whether the silencing of CARD18 had any effect on IL-1β secretion: our results indicated that downregulation of CARD18 expression resulted in an elevated IL-1β production in keratinocytes (Fig. 18A). These results indicate that CARD18 might indeed contribute to the fine-tuning of inflammatory processes in keratinocytes and suggest that — similar to the role in professional immune cells [81] — functions as a negative regulator of inflammasome activation in keratinocytes.

5. DISCUSSION

Psoriasis is an inflammatory skin disease affecting approximately 2% of the population. Symptoms of the disease may vary from mild to life-threatening severe forms, but any forms of the disease may have tremendous effects on life quality. Psoriasis is a multifactorial disorder: genetic susceptibility, environmental and life-style factors all contribute to the development of the disease which is characterized by inflammation and the thickening of the epidermis due to keratinocyte hyperproliferation. The two main cell types playing key roles in disease pathogenesis are keratinocytes and the so-called professional immune cells.

Accumulating evidence suggest that altered skin tissue homeostasis, especially keratinocyte-specific alterations of the normal looking skin of psoriatic patients are key in the initiation of the disease phenotype. The „immune era" of psoriasis research unquestionably brought a breakthrough in the new, targeted therapies of the disease, but to identify novel targets for intervention and possibly for prevention, we must understand the role of aberrant keratinocyte functions in the course of the disease. The newly emerging era of drug development in psoriasis targets abnormal intracellular processes and favors the application of small molecules.

In the last decade we have performed several experiments to discover abnormal molecular patterns of psoriatic non-lesional keratinocytes. These large-scale gene expression studies identified the two molecules that are in the focus of this PhD thesis. According to our results, both PRINS non-coding RNA and CARD18 molecule showed altered expression profile in psoriatic non-involved epidermis compared to healthy epidermis, so we aimed to investigate the function of these two molecules in keratinocytes and in psoriasis.

Previously, our research group investigated the tissue specificity of PRINS expression by real-time RT PCR. Those results revealed that the PRINS non-coding RNA is expressed in various human tissues types and the level of expression showed marked differences.

In the present study we have analyzed the histological distribution of PRINS on thirteen different tissue types using *in situ* hybridization method. The ISH experiments

demonstrated variable staining pattern and intensity in human tissue samples: high levels of PRINS were detected in the gut, lungs, lymph nodes, uterus, testicles and skin, however no staining was observed in the cerebrum and cerebellum. These results are in agreement with the previous real-time RT-PCR data. In human skin we saw a strong PRINS staining intensity in the epidermis. In contrast to that, there was a very low - sometimes undetectable – PRINS expression in the dermis. Taken together, the *in situ* hybridization experiments demonstrated that PRINS is expressed in various tissue types but the level of expression shows great variety; moreover, the staining specificity suggests that PRINS may have an important function in the human epidermis.

After the comparison of the different tissue types, we have studied the differential distribution of PRINS in healthy and diseased skin samples. The ISH results indicated a slightly elevated PRINS expression in psoriatic involved and non-involved epidermis compared to the healthy epidermis. The different distribution and expression of PRINS in the epidermal keratinocytes could be correlated with the abnormal keratinocyte functions seen in psoriasis and with the skin-relevant stress factors inducing the epidermal stress response.

We have previously demonstrated that the PRINS ncRNA interacts physically with the chaperone molecule nucleophosmin (NPM) [39]. The crucial regulatory roles of NPM in cell proliferation and cellular stress response have already been described [99,100]. It has been also established that the nucleolar–nucleoplasmic shuttling of the multifunctional phosphoprotein NPM is affected by ultraviolet (UV) light in fibroblasts and cancer cells. Since there was no data about NPM shuttling characteristics in keratinocytes, we have investigated the NPM localization in HPV-Ker and NHEK cells upon UV-B exposure. Our study of the intracellular trafficking of NPM revealed similar NPM localization pattern in HPV-Ker and NHEK cells that was seen in fibroblasts and cancer cells; particularly, UV-irradiation caused a relocalization of NPM from the nucleolus to the nucleoplasm.

During our functional experiments we have silenced the gene expression of PRINS. The gene-specific silencing of the PRINS expression in UV-B-irradiated keratinocytes resulted in the retention of NPM in the nucleolus, suggesting that PRINS is physically and functionally linked to NPM.

The data we have reported here suggest that PRINS might be part of a regulatory complex formed with NPM. The resulting complex may play a role in the regulation of proliferation and differentiation and in the stress response of cells. We hypothesize that

the abnormal functioning of this complex contributes to the pathogenesis of both malignant and benign hyperproliferative diseases, such as psoriasis.

CARD18 has been identified as a negative regulator of inflammasome activation through direct interaction with the CARD domain of pro-caspase-1 and abrogation of IL-1β and Il-18 production in macrophages [79]. We have identified CARD18 as a highly expressed transcript in psoriatic non-involved epidermis and demonstrated its abnormal regulation in psoriatic non-involved epidermis [27].

IL-1β is an important mediator in the induction and maintenance of psoriasis [101,102] and represents a potential therapeutic target; therefore several studies have targeted possible inhibitors of IL-1β [103–105]. IL-1β and IL-18 are generated by a number of different inflammasomes in response to various environmental influences and this process is likely further modulated by additional genetic factors [106]. The importance of these multi-protein platforms has been reported in several inflammatory diseases, including psoriasis [107,108]. Specific inflammasome inhibitors rectify the balance between beneficial and deleterious IL-1β and IL-18 production. To understand the pathologic functions of CARD18 in skin inflammation, we studied CARD18 under normal and inflammatory conditions both *in vitro* and *in vivo*.

In organotypic skin cultures, basal CARD18 mRNA expression level was higher in psoriatic non-involved epidermis than in healthy epidermis. In addition, samples of different origin exhibited different responses to lymphokine induction. Healthy epidermis exhibited lower CARD18 mRNA basal expression, which was induced by the cytokine treatment. In contrast, the higher basal expression of CARD18 mRNA in psoriatic non-involved samples was not further increased following T-cell lymphokine treatment.

In human skin, CARD18 protein and mRNA exhibited similar accumulation patterns: histological staining showed higher CARD18 expression in non-involved and involved epidermis than in healthy epidermis. Mild injury (i.e., TS) induced CARD18 protein expression in all examined skin samples; however, the level of induction was different: the modest elevation was detected in psoriatic non-involved epidermis compared to healthy and psoriatic involved epidermis. The higher basal level and the lack of response of CARD18 gene and protein expression in psoriatic non-involved epidermis might indicate a “ceiling effect” or impaired regulation of inflammatory signaling, similar to the

phenomenon that has been described for IL-1β production in keratinocytes and in salivary glands [27,109].

Although keratinocytes are able to express inflammasome components and secrete IL-1β, we observed very low — sometimes undetectable — levels of AIM2, caspase-1 and CARD18 mRNA and IL-1β secretion in untreated keratinocytes. Compared to the untreated cells, however, treatment with a combination of inflammation-related stress factors significantly increased both the expression of inflammasome components and IL-1β secretion. The applied triggering factors, such as IFN-γ and TNF-α priming followed by synthetic dsDNA analogue poly(dA:dT) transfection are also known to contribute to the pathogenesis of psoriasis [7,75].

Furthermore, to gain some information about the function of CARD18 in keratinocytes under inflammatory conditions, we silenced CARD18 expression. CARD18 silencing resulted in a modest but significant elevation of IL-1β secretion in poly(dA:dT)-treated cells, suggesting that, similar to professional immune cells, CARD18 indeed has a negative regulatory role in keratinocyte innate immune functions. Interestingly, reduced inflammasome activation was detected as decreased AIM2 and caspase-1 gene expression in the CARD18-silenced cells. We hypothesize that IL-1β secretion is a highly regulated process both in professional and in non-professional immune cells, such as keratinocytes. Thus, the silencing of CARD18 might up- and down-regulate multiple process simultaneously, resulting in only a mild change in IL-1β secretion. The reduced inflammasome activation data suggest the present of a negative feedback loop: elevated active caspase-1 down-regulates caspase-1 and AIM2 gene expression through a yet unknown control mechanism. Juruj et al have recently published results supporting this hypothesis: the authors demonstrated that caspase-1 negatively regulated the formation or stability of the AIM2 inflammasome complex, and that this regulation was specific to the AIM2 inflammasome pathway in macrophages [110].

Nair and coworkers demonstrated that caspase-1, AIM2 and IL-1β mRNAs are present at low levels in healthy and non-involved skin, and that the expression of these molecules increases significantly in psoriatic involved skin [111]. Moreover, caspase-1 protein expression is increased in involved psoriatic skin compared to non-involved psoriatic skin, whereas procaspase-1 expression is unchanged. Under normal conditions, caspase-1 activity has not been detected in the skin [70]. During inflammation, AIM2 inflammasome [112] and caspase-1 become activated both in professional immune cells and in keratinocytes and induce the abnormal inflammatory responses observed in inflammatory

skin diseases. The regulation of caspase-1 is undoubtedly an important checkpoint in IL-1β production and, thus, in the pathogenesis of chronic inflammatory skin diseases, such as psoriasis. By performing a set of *in vitro* experiments in this study, we have demonstrated that CARD18, an endogenous decoy protein proposed to interfere with caspase-1 activation, is part of the inflammasome regulatory processes that has been previously described [70,75]. Moreover, our primary expression experiments clearly showed that CARD18 exhibits aberrant expression and inductivity in psoriatic epidermis. Although knowledge of CARD18 and other COP family members is limited, these small proteins could be promising targets for treating inflammatory diseases. Our findings regarding misregulated CARD18 expression in psoriasis suggest that this molecule may contribute to disease pathogenesis and could be a potential target for therapy.

6. SUMMARY

In our work, we have investigated the characteristics and function of two molecules, PRINS and CARD18, which may contribute to the stress responses of epithelial cell and the initiation of psoriasis. Both molecules have been identified previously by our research group as differentially expressed transcripts in healthy and psoriatic epidermis.

We have earlier described a novel non-coding RNA, PRINS that was overexpressed in non-lesional psoriatic epidermis and its expression was induced by various stress factors such as serum starvation, contact inhibition, UV-B irradiation, and microbial agents in HaCaT cells. In this work we analyzed PRINS expression in thirteen different tissue types. *In situ* hybridization experiments demonstrated variable staining patterns in human tissue samples, moreover, we observed tissue specific expression of PRINS in human skin: in the epidermis strong PRINS staining was seen in contrast to the dermis, where a very slight PRINS expression could be detected. Next, we compared the healthy and psoriatic skin: a low PRINS expression elevation was observed in psoriatic involved and non-involved epidermis compared to the healthy epidermis. The previous examination of our workgroup identified nucleophosmin (NPM) as a PRINS-interacting partner. We investigated the subcellular localization changes of NPM protein upon UV-irradiation. We demonstrated an induction of NPM shuttling from the nucleolus to the nucleoplasm in keratinocytes. To explore whether PRINS ncRNA has any functional role in UV-mediated cellular stress responses, we silenced the PRINS gene expression in HPV-Ker cells and monitored NPM intracellular localization. Silencing of the PRINS non-coding RNA expression in the UV-B irradiated keratinocytes resulted in the retention of NPM in the nucleolus, suggesting that PRINS is physically and functionally linked to NPM, thus plays a role in the NPM-mediated cellular stress response.

CARD18 is known as a negative regulatory molecule that inhibits inflammatory events by terminating inflammasome activation due to a direct interaction with pro-caspase-1. During the investigation of molecular mechanisms in keratinocytes that contribute to the pathogenesis of psoriasis, we found that CARD18 expression differs in healthy and psoriatic skin; moreover, CARD18 demonstrated altered response under inflammatory conditions in healthy and psoriatic skin. In healthy skin, low basal CARD18 expression was detected, which showed significant elevation in response to inflammatory stimuli (lymphokine treatment or mechanical injury). In contrast with this, higher basal expression was observed in psoriatic non-involved skin, but no further CARD18

induction could be detected. The investigation of cellular inflammatory processes revealed that psoriasis-associated danger signals triggered the expression of inflammasome components (AIM2, caspase-1) and CARD18 as well as IL-1β production of keratinocytes. Furthermore, gene-specific silencing of CARD18 in cells treated with cytosolic DNA (poly(dA:dT)) resulted in increased IL-1β secretion, suggesting a negative regulatory role for CARD18 in keratinocyte inflammatory signaling. The differential regulation of CARD18 in healthy and psoriatic uninvolved epidermis may contribute to psoriasis susceptibility. Furthermore, our *in vitro* results indicate that CARD18 may contribute to the fine tuning of keratinocyte innate immune processes.

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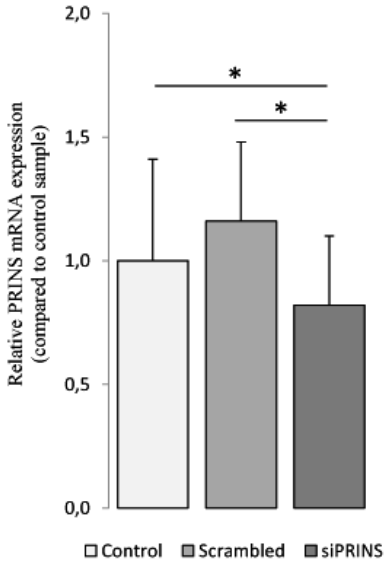
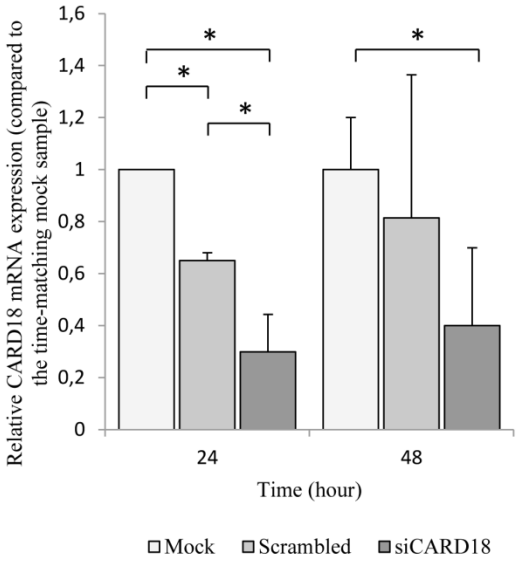
APPENDIX

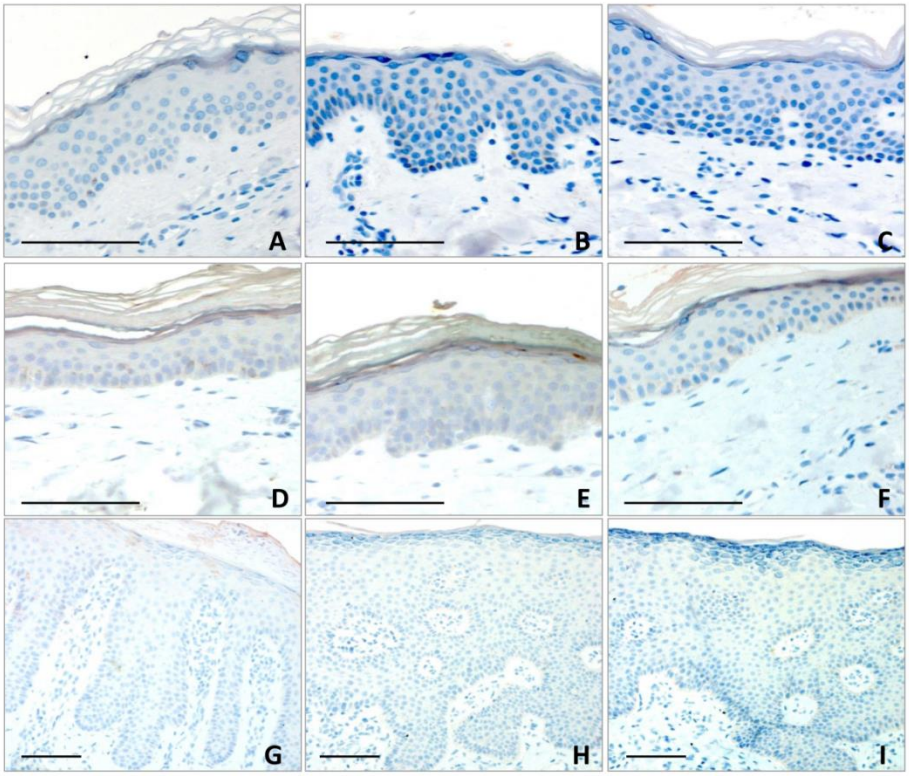
Supplementary data



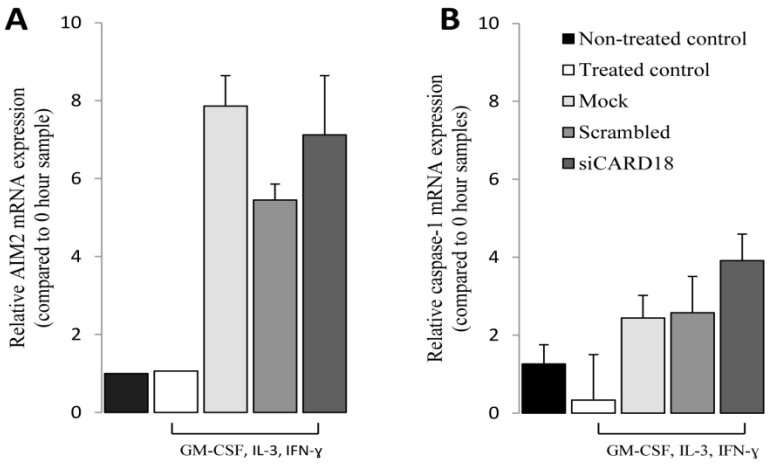
Supplementary Fig. 1. The efficacy of PRINS gene-specific silencing.
PRINS expression was determined by real-time RT-PCR in HPV-Ker cells incubated with transfection reagent (control), transfected with scrambled sequence, or transfected with PRINS siRNA duplex. Expression is relative to mock-transfected cells. * P>0.05



Supplementary Fig. 2. The efficacy of CARD18 gene-specific silencing.
CARD18 expression was determined by real-time RT-PCR in NHEK cells incubated with transfection reagent (mock), transfected with scrambled sequence, or transfected with CARD18 siRNA duplex. Expression is relative to mock-transfected cells. * P>0.05



Supplementary Fig. 3. Negative-control staining of CARD18 upon acute barrier disruption. Skin samples: healthy untreated (A), healthy TS at 24 h (B), healthy TS at 48 h (C), psoriatic non-involved untreated (D), psoriatic non-involved TS at 24 h (E), psoriatic non-involved TS at 48 h (F), psoriatic involved untreated (G), psoriatic involved TS at 24 h (H), psoriatic involved TS at 48 h (I). Bar = 100 μm



Supplementary Fig. 4. The effect of CARD18 gene-specific silencing in NHEKs treated with T-cell lymphokine. Following CARD18 silencing, NHEK cultures (n=3) were incubated with GM-CSF, IL-3, and IFN-ɣ for 12 hours. AIM2 (A) and caspase-1(B) mRNA expression was analyzed by real-time RT-PCR at the indicated time. Expression is relative to the 0 hour sample. * P>0.05

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Article

Expression and Functional Studies on the Noncoding RNA, PRINS

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Abstract: PRINS, a noncoding RNA identified earlier by our research group, contributes to psoriasis susceptibility and cellular stress response. We have now studied the cellular and histological distribution of PRINS by using *in situ* hybridization and demonstrated variable expressions in different human tissues and a consistent staining pattern in epidermal keratinocytes and *in vitro* cultured keratinocytes. To identify the cellular function(s) of PRINS, we searched for a direct interacting partner(s) of this stress-induced molecule. In HaCaT and NHEK cell lysates, the protein proved to be nucleophosmin (NPM) protein as a potential physical interactor with PRINS. Immunohistochemical experiments revealed an elevated expression of NPM in the dividing cells of the basal layers of psoriatic involved skin samples as compared with healthy and psoriatic uninvolved samples. Others have previously shown that NPM is a ubiquitously expressed

nucleolar phosphoprotein which shuttles to the nucleoplasm after UV-B irradiation in fibroblasts and cancer cells. We detected a similar translocation of NPM in UV-B-irradiated cultured keratinocytes. The gene-specific silencing of PRINS resulted in the retention of NPM in the nucleolus of UV-B-irradiated keratinocytes; suggesting that PRINS may play a role in the NPM-mediated cellular stress response in the skin.

Keywords: noncoding RNA; nucleophosmin; keratinocyte proliferation; psoriasis; UV-B irradiation

1. Introduction

Psoriasis is a multifactorial, hyperproliferative, chronic inflammatory skin disease that affects 1%–3% of the adult Caucasian population (OMIM 177,900, www.ncbi.nlm.nih.gov), with a substantial negative impact on the patient's quality of life [1]. Immunoregulatory abnormalities [2] as well as environmental and genetic factors [3,4] contribute jointly to the development of psoriasis. Hyperproliferation of the keratinocytes in the psoriatic plaques is triggered by infiltrating T-lymphocytes at the dermal–epidermal junction [5]. The keratinocytes of the uninvolved psoriatic epidermis are inherently oversensitive to proliferative signals, and this elevated level of sensitivity plays a crucial role in the development of psoriatic lesions [6,7]. Thus, resident skin cells and infiltrating immune cells cooperate in the formation of psoriatic lesions, but the exact molecular mechanisms that regulate the interactions between these cells are still far from understood. The identification of genes and proteins with altered expressions, either in the uninvolved or in the involved psoriatic skin, may therefore facilitate an understanding of the pathogenesis of this disease. While investigations of the molecular mechanisms behind psoriasis have identified numerous disease-associated genes and proteins [8–12], it is still unclear how the altered expressions of some genes contribute to the pathogenesis of psoriasis, how the differentially expressed molecules exert their action and what other molecules they may interact with.

We demonstrated earlier that PRINS, (psoriasis susceptibility-related RNA gene induced by stress), a noncoding RNA (ncRNA) first identified by our research group, is expressed more strongly in the uninvolved psoriatic epidermis than in the psoriatic involved or healthy epidermis, suggesting that the overexpression of PRINS in the uninvolved psoriatic epidermis may play a role in psoriasis susceptibility. Our *in vitro* experiments also revealed that PRINS functions as a regulatory RNA, playing a protective role in cells exposed to stress [13].

Since their first detection [14] numerous mRNA-like ncRNA transcripts have been found in different cell types. Besides alternative splicing and promoter-driven regulation, ncRNA molecules comprise a new level of regulation of protein-coding genes [15]. As yet few papers have provided partial or full descriptions of the structural complexes formed by ncRNAs and their protein partners [16–18], and their participation and roles in different cellular processes are still poorly understood. PRINS an RNA polymerase II transcribed RNA, is a 3681 nucleotide-long molecule. We originally described it in HaCaT keratinocytes where it participates in cellular stress response. BLAST searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi) revealed that the PRINS gene is specific for

anthropoid primates, its rodent ortholog could not be identified [13]. Our studies demonstrated a higher PRINS expression in the uninvolved psoriatic epidermis than in either the psoriatic involved or healthy epidermis [13]. These results suggest that the overexpression of PRINS in the uninvolved psoriatic epidermis may play a role in psoriasis susceptibility rather than in the precipitation of psoriatic lesions, and are in agreement with the previous observations that the keratinocytes of the uninvolved epidermis differ from healthy keratinocytes in their responses to external stimuli [6,19,20]. We additionally showed that PRINS may possess regulatory functions: the expression of the antiapoptotic G1P3 gene was found to be under the control of PRINS in various cell types [21].

We now report on the cellular and histological distribution of PRINS, determined by using *in situ* hybridization (ISH). Moreover, we present the results of an *in vitro* binding assay which suggests that PRINS interacts physically with the molecular chaperone protein nucleophosmin (NPM, B23) in HaCaT and normal human epidermal keratinocyte (NHEK) lysates and describe the first upregulation of this protein in psoriasis.

NPM is a nucleolar phosphoprotein [22] with a potential role as a positive regulator in cell proliferation [23,24]. A number of studies have shown that UV irradiation results in a rapid nucleoplasmic translocation of the otherwise predominantly nucleolar protein NPM in fibroblasts [25,26] and cancer cells [27,28].

Our experiments indicate a similar pattern of intracellular localization of NPM after UV-B exposure in cultured keratinocytes, and reveal that the gene-specific silencing of PRINS modifies the UV-induced intracellular shuttling of NPM suggesting that NPM is a physical and functional interactor of PRINS.

2. Results and Discussion

2.1. The Histological and Cellular Distribution of PRINS

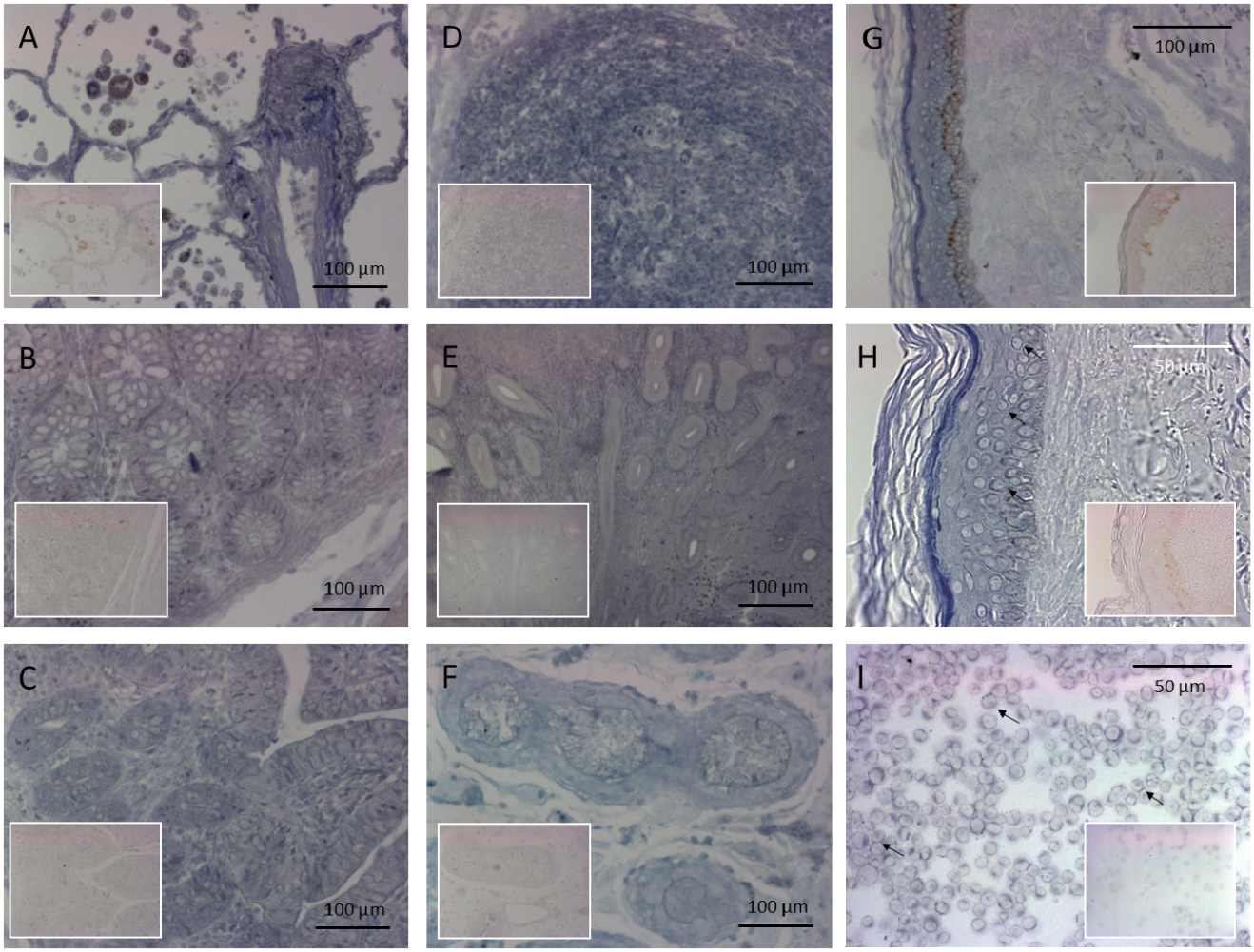
By using real-time RT-PCR, we earlier demonstrated differences in PRINS expression in various human tissue samples [13]. For a more detailed analysis of PRINS expression we have now applied ISH. To investigate the pattern of PRINS expression pattern in different tissue types, we applied paraffin-embedded tissue chips containing thirteen sections from different healthy human organs on one slide. The samples were exposed to the same conditions during ISH, and this led to good comparability. The intracellular distribution of PRINS was also examined in cultured NHEKs.

The ISH experiments demonstrated variable levels of expression in different human tissue samples: high levels of PRINS expression were detected in the gut, lungs, lymph nodes, uterus, testicles and skin (Figure 1A–H), whereas no staining was observed in the cerebrum and cerebellum (data not shown). The breast, kidney, stomach, and gallbladder tissue specimens displayed only moderate PRINS expressions (data not shown). These findings are in agreement with our previous results revealing variable PRINS gene expression levels in different healthy human organs. ISH demonstrated a relatively high PRINS positivity in the healthy epidermis.

The dermal and epidermal expression of PRINS significantly differed while a strong staining intensity in the epidermis was similar in the various layers. The stratum granulosum and stratum lucidum exhibited somewhat darker PRINS ISH staining (Figure 1G,H), which seemed to be specific,

since the scrambled control probe did not result in the same staining intensity in these layers. There was only a moderate positivity in the dermis. In *in vitro* cultured keratinocytes (Figure 1I) exhibited strong nucleolar (indicated by small arrows) and perinuclear PRINS positivity and moderate homogeneous cytoplasmic staining. The cellular expression profile of PRINS is in agreement with the epidermal staining pattern.

Figure 1. Detection of psoriasis susceptibility-related RNA gene induced by stress (PRINS) expression in various human tissue samples by ISH. The sections were incubated with an LNA RNA detection probe for PRINS and for the control staining (see insets at the same magnification) we used a scrambled control sequence. Relatively strong PRINS positivity was seen in the (**A**) lungs; (**B**) the large bowel; (**C**) the small bowel; (**D**) the lymph nodes; (**E**) the uterus; (**F**) the testicles; (**G**,**H**) the skin; and (**I**) cultured keratinocytes.



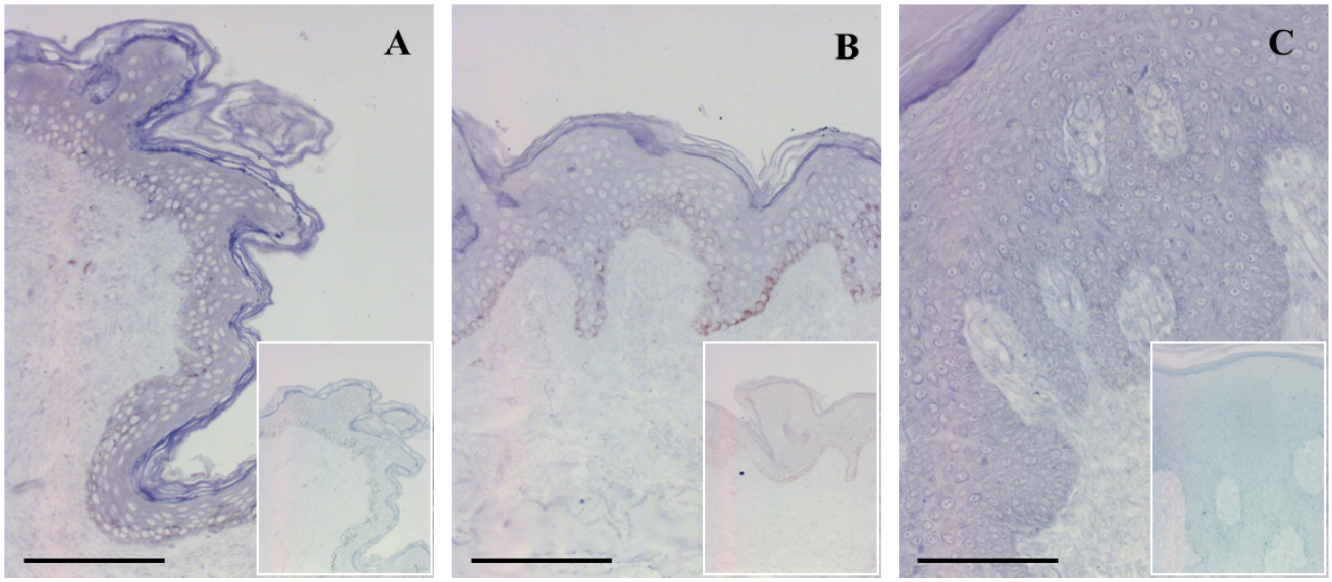
2.2. PRINS Expression in Healthy and Psoriatic Skin

ISH experiments were performed to compare the expressions of PRINS in normal healthy (n = 10), psoriatic uninvolved (n = 6) and psoriatic involved (n = 6) skin samples. With a quantitative RT-PCR approach we earlier showed that PRINS was expressed more strongly in the psoriatic uninvolved epidermis than in either the normal or the involved epidermis [13].

The ISH results partially confirmed the Q-RT-PCR findings, indicating a moderately elevated level of PRINS expression in the uninvolved (Figure 2B) and involved (Figure 2C) epidermis relative to the healthy epidermis (Figure 2A). However, the differences in expression between the involved and uninvolved epidermis were not as pronounced as with the Q-RT-PCR approach. The explanation for this may lie in the different sensitivities of the two methods.

The differential distribution of PRINS (a multiple stress-related ncRNA) in the epidermal keratinocytes could be correlated with the abnormal keratinocyte functions seen in psoriasis and with the skin-relevant stress factors inducing the epidermal stress response.

Figure 2. Detection of PRINS expression in psoriasis. ISH for PRINS expression was performed on healthy normal (n = 10), psoriatic uninvolved (n = 6) and psoriatic involved (n = 6) skin specimens. Sections were incubated with the PRINS-specific LNA probe or with the control scrambled (see insets at the same magnification) LNA probe. Representative stainings for each tissue types: (**A**) normal healthy skin; (**B**) psoriatic uninvolved skin; and (**C**) psoriatic involved skin. Bar = 100 μm.



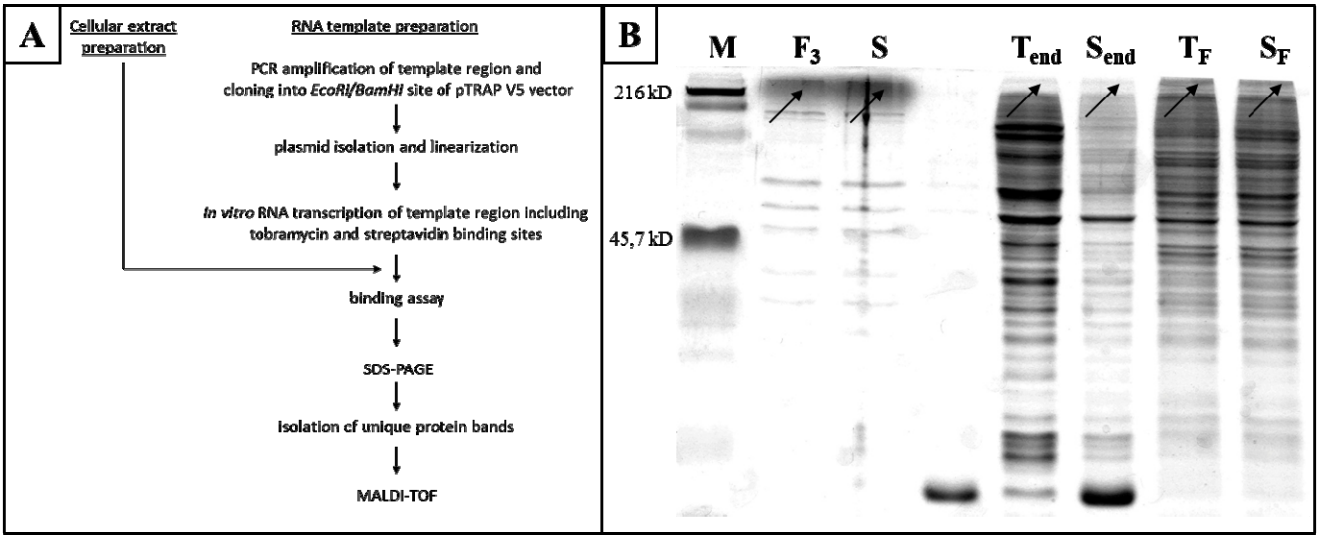
2.3. In Vitro *Identification of NPM as a PRINS-Interacting Protein*

Our previous data suggested roles for PRINS in psoriasis susceptibility and the cellular stress response. It is also accepted that long mRNA-like ncRNAs form complexes with proteins [16–18], and act as regulators of various cellular functions [29].

To investigate the molecular mechanisms involved in the stress-related functions of PRINS, we first searched for its intracellular counterparts. In an *in vitro* experiment (Figure 3A) we used a 39mer RNA transcript of PRINS; this region harbors the 19mer sequence of the gene successfully used for the gene-specific silencing of PRINS [13]. For the identification of PRINS-interacting proteins, *in vitro* transcribed RNA (PRINS AK676 sequence) was added to a tobramycin affinity matrix and the cell lysates were also added to the column. After several washing steps, the RNP particles were eluted from the tobramycin matrix and transferred to a streptavidin affinity matrix. Control elutes were taken from both the tobramycin and streptavidin columns, where the cellular extracts were added to the affinity

matrix without the bound *in vitro* transcribed PRINS sequence. The elutes obtained with the RNP-complex purification kit were run on SDS-PAGE (Figure 3B). Bands (one with a molecular weight of >216 kD and another of ~50 kD) that appeared in the PRINS- binding fraction (S, F_3) but not for the control samples (T_{end}, S_{end}) were cut out from the gel and further analyzed with matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer. The *in vitro* binding assay was performed and consecutive experiments with three independent cell lysates (two from HaCaT keratinocytes and one from NHEKs).

Figure 3. Identification of the direct interacting partner of PRINS. The *in vitro* binding assay and the consecutive experiments with the three independent cell lysates (two from HaCaT keratinocytes and one from cultured NHEK) were performed as depicted in the Figure. A 39mer RNA transcript of PRINS was used. The elutes obtained with the RNP-complex purification kit were run on SDS-PAGE. Bands (arrows) that appeared in the PRINS binding fraction but not for the control samples, were cut out from the gel and further analyzed with MALDI-TOF. (**A**) Flow chart of the *in vitro* experiments. (**B**) Following the binding assay in HaCaT cells, the proteins in the eluates were separated on 7.5% SDS-PAGE and stained with Coomassie Brilliant Blue. NPM from a ~250-kD band was identified by MALDI-TOF (shown by black arrows). S, elute from the 1st affinity matrix (tobramycin) bound with the *in vitro* transcribed PRINS and cellular extract. F_3, elute from the 2nd affinity matrix (streptavidin) incubated with elute "S". T_F, flow-through from the tobramycin affinity matrix incubated with the cellular extract. S_F, flow-through from the streptavidin affinity matrix incubated with the cellular extract. T_{end}, elute from the tobramycin affinity matrix incubated with the cellular extract. S_{end}, elute from the streptavidin affinity matrix incubated with the cellular extract



NPM was identified as a putative PRINS-binding protein in all three independent binding experiments. NPM, a multifunctional phosphoprotein, is implicated in mRNA processing [30,31] and also in the acute response of mammalian cells to environmental stress, when it stimulates DNA repair and reduces apoptosis [26]. It has been demonstrated that NPM forms oligomers involving highly conserved short loops, the ionic interactions between the monomeric subunits leading to the formation

of a thermo-stable, chemically resistant pentamer; it has also been shown that NPM pentamers can form decamers *in vitro* by packing two pentamers on top of each other in a sandwich-like structure [32]. This explains why we identified NPM from a 250 kD band by MALDI-TOF and suggests a putative structure for the oligomer that is capable of housing long oligonucleotides such as long ncRNAs. Similar protein oligomer-RNA complexes have been documented previously such as the hexameric or heptameric complexes of Sm proteins that function as RNA chaperones and fulfill a number of central tasks in various types of RNA processing [33], and the bacterial Hfq protein that forms hexamers and also functions as an RNA chaperone complex [34]. The heterogeneous nuclear ribonucleoproteins (hnRNPs) play an active part in post-transcriptional gene regulation, e.g., RNA splicing and regulation of the stability and translation of target mRNAs. Most hnRNPs are primarily localized in the nucleoplasm, and can shuttle between the nucleus and the cytoplasm [35].

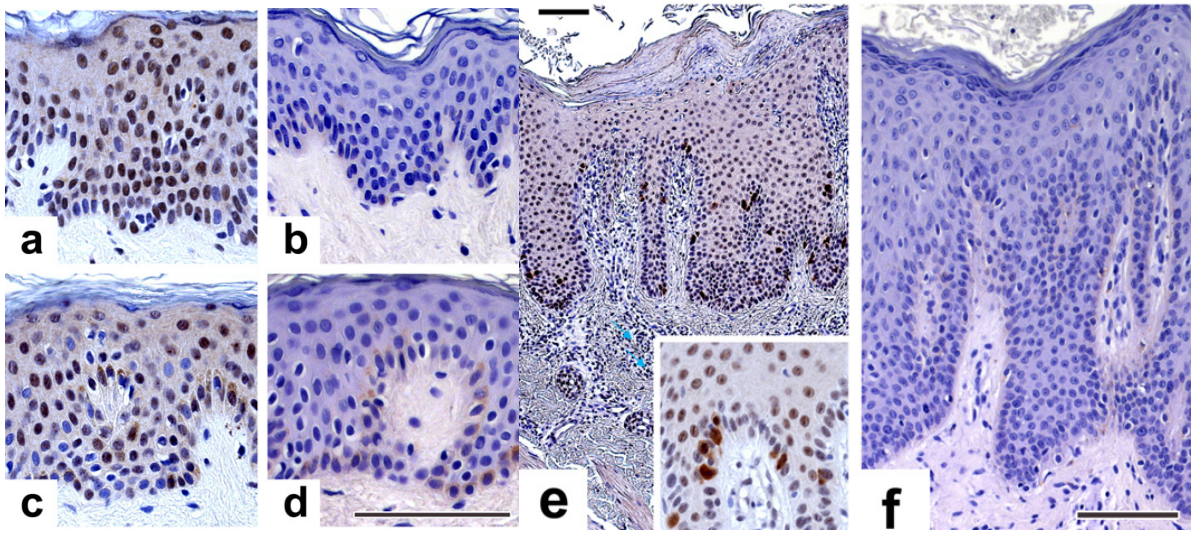
2.4. NPM Is Overexpressed in Psoriatic Involved Epidermis

The psoriasis-associated expression of PRINS has been clearly established by Sonkoly *et al.* [13]. We asked the question of whether NPM gave a characteristic pattern of staining that might correlate with the known overexpression of PRINS in the psoriatic uninvolved epidermis. NPM immunopositivity was apparent in all the studied skin samples. In both the healthy (Figure 4a) and the psoriatic uninvolved (Figure 4c) skin, the keratinocytes presented a nuclear staining throughout all of the epidermal layers. Although there were some subgranular nuclei that exhibited a high density of brown color in the healthy epidermis, a higher rate of positivity was noted among the spinous suprabasal keratinocytes than among the granular and subgranular keratinocytes in the uninvolved epidermis (Figure 4c). This finding is in agreement with previously reported ISH results [36] that indicated the highest accumulation of NPM transcript in the nuclei of epithelial cells. In the dermis, infiltrating immune cells and blood displayed showed pronounced nuclear NPM staining (small arrows). The cellular distribution of PRINS and that of NPM in the healthy epidermis showed similarities, supporting our results concerning their physical interaction.

In the psoriatic involved skin, the different layers of the epidermis exhibited different NPM protein expression patterns. In the extended spinous layer, only moderate NPM staining was detected, while in the basal layer and in the immediate suprabasal layer NPM was expressed at high levels. The most interesting phenomenon was seen in the basal layer of the psoriatic involved epidermis (Figure 4e), where certain nests of dividing keratinocytes presented marked cytoplasmic immunopositivity besides nuclear staining. This staining pattern finally resulted in a very interesting “speckled” overall image in the psoriatic involved epidermis. This finding is in accord with the results of Amin and co-workers who recently reported that NPM is localized at the chromosome periphery during mitosis [37], when the nuclear membrane is demolished. We hypothesize that the interesting “speckled” staining pattern of the basal keratinocytes in the involved psoriatic epidermis reflects this phenomenon.

In a previous study of the expression of NPM, exclusive nuclear staining was seen in squamous cell carcinoma, whereas in basal cell carcinoma samples a certain level of cytoplasmic NMP immunopositivity was also apparent [38]. These observations suggest that NPM may play a role in normal keratinocyte proliferation and its overexpression is a key motive in certain hyperproliferative and malignant skin diseases.

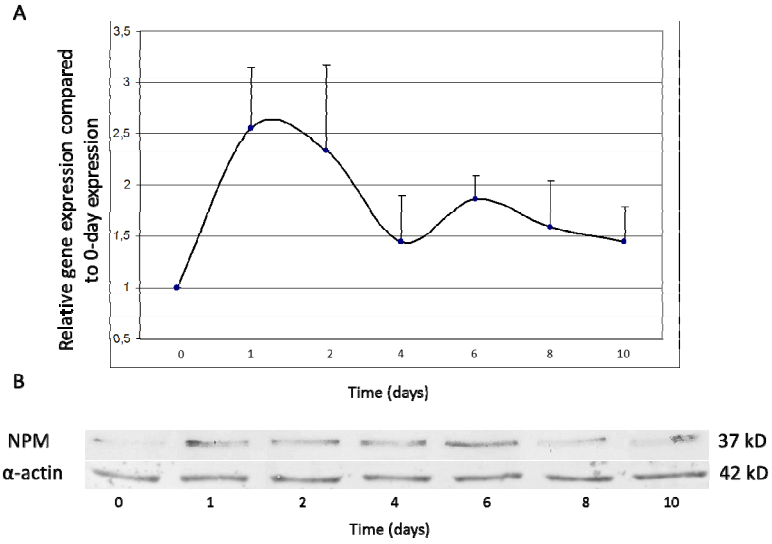
Figure 4. Immunohistochemical analysis of nucleophosmin (NPM) protein expressions in healthy and psoriatic skin samples. Paraffin-embedded tissues of healthy, non-lesional and lesional psoriatic skin samples were used for NPM-specific immunohistochemistry. After nonspecific antigen blocking, sections were incubated with anti-NPM mouse monoclonal antibody at a dilution of 1:500. The incubation with secondary antibody was followed by the development of a DAB color reaction, the intensity of which reaction was controlled under a light microscope. Sections were counterstained with hematoxylin. In the healthy and psoriatic uninvolved skin, the keratinocytes presented nuclear staining throughout all of the epidermal layers. In the healthy epidermis, some subgranular nuclei showed a high density of NPM, while in the uninvolved epidermis, a higher rate of spinous suprabasal nuclear staining was observed. In the psoriatic involved skin, the various layers of the epidermis exhibited a different NPM protein expression pattern. The keratinocytes presented pronounced cytoplasmic immunopositivity besides nuclear staining. Bar = 100 μm; (**a**) healthy epidermis; (**b**) isotype control for healthy epidermis; (**c**) psoriatic non-lesional; (**d**) isotype control for psoriatic non-lesional; (**e**) psoriatic lesional epidermis; (**f**) isotype control for lesional psoriatic epidermis.



2.5. NPM Expression Decreases as Keratinocytes Differentiate

Since the epidermal keratinocytes were found to express NPM at high levels, we investigated whether this expression was dependent on the proliferation/differentiation state of the keratinocytes. In a set of *in vitro* experiments on spontaneously differentiating 3rd passage NHEKs, the process of differentiation was followed via the increase in a differentiation marker (keratin 10) and the decrease in a proliferation-related marker (alpha5 integrin) (Figure S1). The 0-h samples were taken from subconfluent cultures. Real-time RT-PCR experiments (n = 3) (Figure 5A) and Western blot experiments (n = 3) (Figure 5B) revealed that the NPM mRNA and protein expressions were highest on days 1 and 2 and then gradually declined during the 10-day follow-up.

Figure 5. Expression of NPM protein during keratinocyte proliferation/differentiation *in vitro*. Cultured normal human epidermal keratinocyte (NHEK) cultures (n = 3) were grown until subconfluency, when the 0-h sample was taken. (**A**) Changes in the expression of NPM mRNA were analyzed by real-time RT-PCR at the indicated times. Relative expression is shown compared to the 0-h sample. (**B**) At the indicated times, cells were harvested and lysed, then subjected to denaturing SDS-PAGE analysis and electroblotted to nitrocellulose. NPM protein was detected with a mouse monoclonal anti-NPM antibody and visualized with the NBT/BCIP system.



The transcriptional level upregulation of NPM occurred in proliferating cells and, although a slight decline in NPM level was observed, the protein remained at a high level in the less proliferating, more differentiated keratinocytes when the alpha5 integrin was already decreasing [39], suggesting that the NPM protein is relatively stable once expressed in the cells. The fact that the mRNA level decreased rapidly (day 4), while the protein level in the keratinocytes declined in a prolonged, more moderate manner (day 8), indicates that posttranscriptional, translational regulation and posttranslational modifications such as phosphorylation during the maturation and processing of the protein might play important roles in the NPM protein expression in keratinocytes. NPM is known to be phosphorylated by protein kinase CK2 in the interphase [40] and by $p34^{cdc2}$ kinase during mitosis [41,42]. It has also been shown that the level of NPM phosphorylation correlates with the cellular proliferative activity and is enhanced at mitosis [43–46]. However, a precise determination of exactly which phosphorylation pathway(s) play(s) a role in NPM stabilization in the keratinocytes requires further experiments.

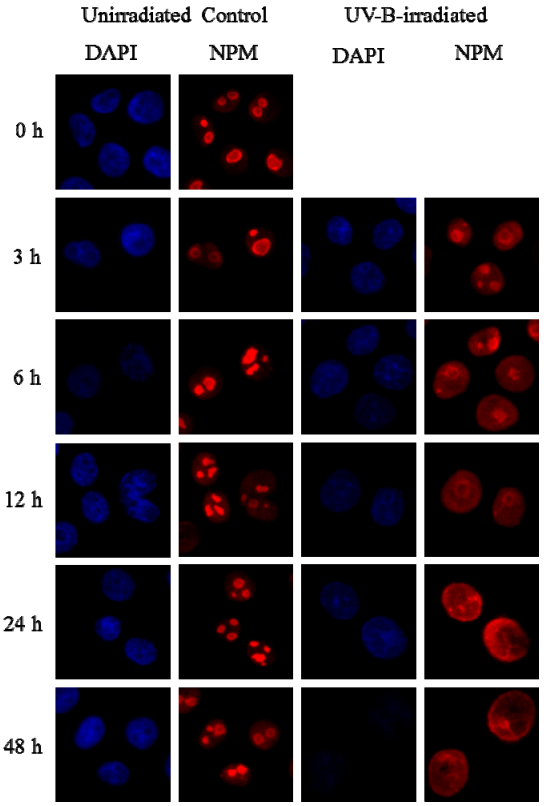
2.6. Intracellular Localization of NPM Protein in Keratinocytes

NPM is a ubiquitously expressed nuclear phosphoprotein which shuttles continuously between the nucleus and the cytoplasm [47]. Within the nucleus, NPM is predominantly localized in the nucleoli, with its highest levels in the granular component that contains the more mature preribosomal particles [22], but a significant fraction of NPM can also be detected in the nucleoplasm. NPM is a multifunctional protein with multiple locations in the cell. During the cell cycle, NMP is dynamically

localized [37] and it is also redistributed from the nucleolus in response to cytotoxic drugs and genotoxic stress. In fibroblasts and cancer cells, UV irradiation results in a rapid nucleoplasmic translocation of the nucleolar NPM [25,27]. To investigate whether we could observe a similar intracellular localization of NPM in keratinocytes after UV-irradiation, we applied an immunocytochemistry method. In these experiments, we used a newly established keratinocyte cell line, HPV-Ker [48]. The UV responses of NHEKs and HPV-Ker cells were compared. The viability and the expression of several UV-induced genes were identical in the two cell types. Moreover, the stress-induced p53 gene expression in the HPV-Ker cells presented the same UV-B inducibility as that in the NHEKs. The functional similarities between the NHEKs and the HPV-Ker cells suggested that HPV-Ker cells are good model cells for the study of UV-induced keratinocyte functions.

UV-B exposure caused a relocalization of NPM from the nucleolus to the nucleoplasm in HPV-Ker cells (Figure 6). The UV-B-induced subcellular shuttling could be detected quite early after UV-B irradiation: 3 h after treatment, slight translocational changes were observed. The immunofluorescence in the nucleoplasm was most increased at 12 and 24 h. Forty-eight hours following UV-B treatment, the protein had mostly returned to the nucleolus. The time course studies showed that the stimulation of NPM shuttling by UV-B irradiation was a rapid and transient process. The intracellular localization pattern of NPM after UV-B exposure was similar in both the cultured NHEKs (Figure S2) and the HPV-Ker cells, which suggests that HPV-Ker cells could be appropriate models for further studies of intracellular NPM trafficking.

Figure 6. UV-B radiation induces the intracellular trafficking of nucleoplasmic NPM in HPV-Ker cells. Unirradiated and UV-B-irradiated (312 nm, 40 mJ/cm^2) HPV-Ker cells were followed for the indicated periods of time, fixed and immunostained for NPM. DNA was stained with 4,6-diamidino-2-phenylindole (DAPI).



2.7. PRINS Modifies the UV-B Irradiation-Induced Intracellular Shuttling of NPM

To determine whether PRINS had any effect on NPM intracellular trafficking, we silenced the expression of PRINS in growth factor-deprived HPV-Ker cells with a vector-based method [13] and studied the NPM shuttling under normal conditions and after UV-B exposure. NPM shuttling was compared in HPV-Ker cells transfected with a specific PRINS-silencing construct (AK696) and in HPV-Ker cells transfected with a control construct (SC1313). Real-time RT-PCR revealed successful gene-specific PRINS silencing (Figure S3A), which resulted in a moderate induction of NPM expression (Figure S3B).

In untreated samples, the NPM was localized mostly within the nucleolus in both the SC1313 and the AK696-transfected cells (Figure 7A). The relocalization of NPM from the nucleolus to the nucleoplasm in the control SC1313-transfected cells at the indicated times after UV-B exposure was similar to our preliminary observations in HPV-Ker cells. As compared with the control cells, the AK696-transfected cells (Figure 7B) showed a moderate retention of NPM in the nucleolus following UV-B treatment. To validate the immunocytochemical observations, a semiquantitative analysis was performed (Figure 7C). The nuclear transition of NPM was inhibited in AK696-transfected cells after UV-B irradiation. Interestingly, we observed that the transfection itself slightly modified the shuttling of NPM even in the UV-B negative group.

Figure 7. Silencing of the PRINS expression modifies the UV-B-induced trafficking of NPM. HPV-Ker cells were transfected with a PRINS gene-specific silencing vector (AK696). Control cells were transfected with a vector containing a scrambled sequence (SC1313). One day after the transfection, the unirradiated (**A**) and the UV-B-irradiated (**B**) HPV-Ker cells were followed for the indicated periods of time, fixed and immunostained for NPM (red). DNA was stained with DAPI (blue). Following the immunostaining, we performed a semiquantitative analysis of the cells. In every group, 25 fields of view were counted. Mean values are plotted on the chart (**C**).

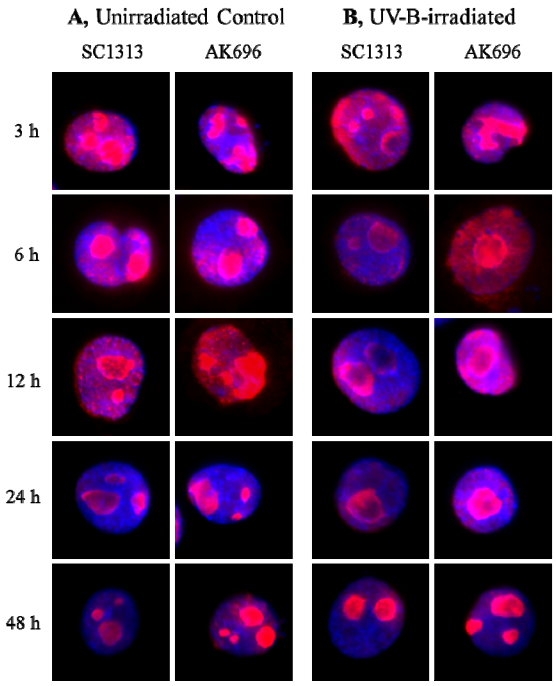
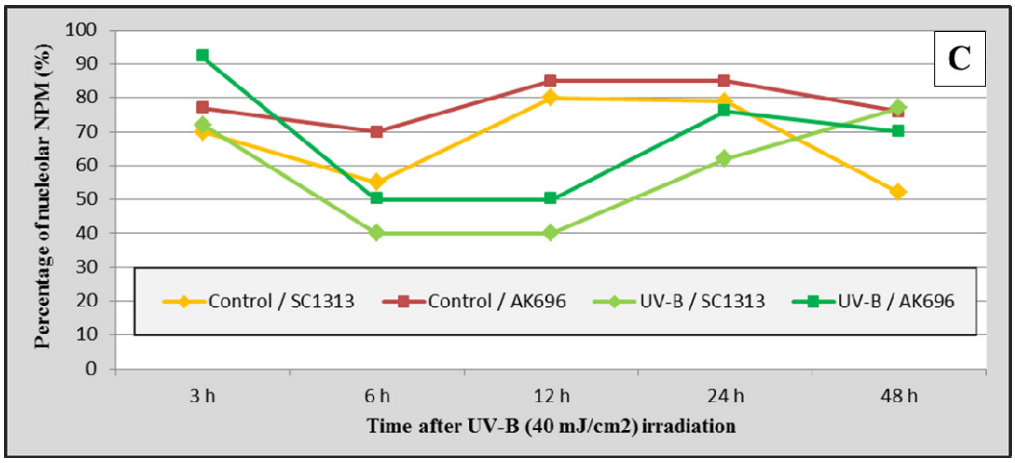


Figure 7. *Cont.*



Besides the above-described NPM immunocytochemical detection, the shuttling of the protein was also studied by using an NPM-GFP chimeric construct. The results of the two experimental approaches were identical: both suggested that PRINS contributes to the UV-B-induced intracellular trafficking of NPM (Figure S4).

Our data indicate that PRINS does not act alone, but forms a complex with the NPM protein and contributes to its stress-related intracellular trafficking.

2.8. In Silico *Prediction and Gene-Specific Silencing Experiments Suggest that PRINS Might Be Part of the Transcription Regulatory CTCF/NPM Complex and Acts as a Negative Regulator of Cell Cycle Progression*

It has recently been demonstrated that NPM is a major interacting protein of the zinc finger protein CCCTC binding factor (CTCF), which is known to be a critical mediator of multiple epigenetic processes [49]. The gene-specific silencing of NPM causes severe mitotic defects and delayed mitotic progression, suggesting a crucial regulatory role in cell proliferation [37].

Interestingly, when we performed an *in silico* prediction, transcriptional regulators with 11 highly conserved zinc finger domains were among the potential PRINS-binding proteins and the NPM-interacting CTCF binding factor belongs in this zinc finger protein family (Table S1).

CyclinD1, a known target gene of the NPM/CTCF factor regulatory complex, is upregulated in psoriasis [50]. In our preliminary experiments, the gene-specific silencing of PRINS resulted in a substantial induction of cyclin D1 expression (data not shown). Moreover, we also showed that the silencing of PRINS resulted in a slight elevation of NPM expression (Figure S3B). This finding is in good agreement with the differentiation-related expression of PRINS: it is expressed most strongly in serum-starved, contact-inhibited HaCaT keratinocytes and its expression decreases as the cells start to proliferate after release from cell quiescence; moreover, PRINS is strongly expressed in the uninvolved psoriatic epidermis and its expression is substantially downregulated in the hyperproliferative involved epidermis [13]. These data indicate that PRINS might be part of the transcription regulatory CTCF/NPM complex, and as part of this molecular complex it plays a negative regulatory role in the cell cycle progression. The involvement of long ncRNAs in molecular regulatory complexes has already been documented in the literature [16].

3. Experimental Section

3.1. Cell Cultures

HaCaT keratinocytes, the immortalized human keratinocyte cell line kindly provided by Dr. Fusenig, N.E. (Heidelberg, Germany), was cultured to subconfluency in T75 tissue culture flasks (Corning Incorporated, Corning NY USA) and maintained in keratinocyte serum-free medium (Gibco® Keratinocyte SFM Kit; Life Technologies, Copenhagen, Denmark) supplemented with 1% antibiotic/antimycotic solution (PAA, Pasching, Austria) and 1% L-glutamine (PAA, Pasching, Austria) at 37 °C in a humidified atmosphere containing 5% CO_2. The medium was changed every two days. Cells were synchronized as described previously [39].

NHEKs were obtained from normal human epidermis and cultured as described earlier [51]. Briefly, NHEKs were separated from a skin specimen obtained from the Plastic Surgery Unit of our Dermatology Department. The epidermis and the dermis were separated by overnight incubation in Dispase (Roche Diagnostics, Manheim, Germany), and the keratinocytes were obtained after a maceration in 0.25% trypsin. Cells were maintained in keratinocyte serum-free medium (Gibco® Keratinocyte SFM Kit; Life Technologies, Copenhagen, Denmark), which contains EGF and BPE as supplements. The calcium concentration of the medium is <0.1 mM. For the experiments 3rd passage keratinocytes were used. In our *in vitro* differentiating model, fresh medium was added to subconfluent 3rd passage keratinocytes and samples were taken from the culture at the indicated time points. The rapid upregulation of alpha5 integrin on day 1 indicated intensive keratinocyte proliferation and, as the culture became confluent on day 2, the expression of alpha5 integrin gradually decreased (Figure S1).

The HPV-Ker cell line was immortalized by the HPV E6 oncogene as described previously [48]. HPV-Ker cells were grown in 75 cm^2 cell culture flasks and maintained in keratinocyte serum-free medium (Gibco® Keratinocyte SFM Kit; Life Technologies, Copenhagen, Denmark) supplemented with 1% antibiotic/antimycotic solution (PAA, Pasching, Austria) and 1% L-glutamine (PAA, Pasching, Austria) at 37 °C in a humidified atmosphere containing 5% CO_2. The medium was changed every two days.

Cells were subjected to UV-B irradiation (312 nm, 40 mJ/cm^2) in phosphate-buffered saline (PBS; 153 mM NaCl, 7.67 mM Na_2HPO_4, 2.67 mM NaH_2PO_4) at room temperature (RT). Immediately after irradiation, the PBS was aspirated off, and culture medium was added to the culture dishes. Cells were harvested at the indicated times after UV-B exposure.

3.2. In Situ *Hybridization*

Custom LNA mRNA detection probes for PRINS with the sequence 5'-3': /5DigN/AAGCTTCTGTCCTCATTAGTCTTC/3Dig_N/ and scrambled control sequence 5'-3': /5DigN/AAGCCTCCGTTCTTATTAGTCTTC/3Dig_N/ were ordered from Exiqon (Exiqon A/S, Vedbaek, Denmark).

Tissue chips: Formaldehyde-fixed blocks measuring 4 mm in diameter of normal breast, cerebellum, cerebrum, gallbladder, kidney, large bowel, small bowel, lung, lymph node, skin, stomach, testicle and uterus samples were placed into a tissue microarray (TMA) block. The fixation of samples was standardized (4% buffered formaldehyde for 24 h at RT, in a volume of 1:10). After paraffin

embedding, the TMA was cut into 4-μm-thick sections, which were rehydrated with increasing concentrations of alcohol.

HPV-Ker cells were trypsinized and harvested by centrifugation, and resuspended in PBS. One hundred thousand cells were centrifuged onto a slide by using a cytocentrifuge (Cytopro™, Wescor, Logan, UT, USA) and dried overnight at RT. The slides were fixed in 2% paraformaldehyde for 20 min at RT.

After 2 × 5-min PBS washes, tissue chip slides and slides with HPV-Ker cells were treated for 15 min and for 5 min, respectively, with 20 μg/mL proteinase K in PBS at 37 °C. Digoxigenin-labeled LNA probes suspended in hybridization buffer (50% formamide) were denatured at 95 °C for 2 min. Hybridization was performed at 60 °C overnight. After hybridization, samples were washed in 5 × SSC (saline–sodium citrate buffer) for 15 min, and then 2 × 30 min in 0.2 × SSC at 60 °C. After a 10-min PBS wash, samples were equilibrated in 10% goat serum-containing blocking buffer (0.5% blocking reagent (Roche, Budapest, Hungary) in PBS containing 0.1% Tween 20) for 1 h at RT and then incubated with anti-DIG alkaline phosphatase (AP) conjugate (Roche Diagnostics, Manheim, Germany) diluted 1:500 with blocking solution for 1 h. After antibody incubation, samples were washed in PBS containing 0.1% Tween-20 for 2 × 10 min and in PBS for 2 × 10 min. Coloring reactions were performed overnight with BM purple AP substrate (Roche Diagnostics, Manheim, Germany). Slides were mounted with Glycergel (Dako Denmark A/S, Glostrup, Denmark). Pictures were taken with the use of a Zeiss AxioImager fluorescent light microscope (Carl Zeiss MicroImaging, Thornwood, NY, USA) fitted with a PixeLINK CCD camera (PixeLINK, Ottawa, ON, Canada).

3.3. In Vitro *Binding Assay*

These experiments were conducted in accordance with the instruction manual of the Dual TRAP™ RNP Purification KIT (Cytostore Inc., Calgary, AB, Canada). The flow chart of the procedure is shown in Figure 2B. RNA template preparation for the binding assay was carried out as follows: genomic DNA was isolated from healthy human donor blood with a Perfect gDNA Blood Mini kit (Eppendorf, Mannenheim, Germany) in accordance with the manufacturer's protocol. PCR amplification of the template region of the PRINS gene was performed with the following protocol: a denaturing step at 94 °C for 5 min, 30 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s and one cycle of terminal extension at 72 °C for 2 min. The sequences of the EcoRI-AK676 5' FWD (5' GGA ATT CTC CGT CTT AAA GGA AAA AAA TTT CTG 3') and Bam-AK715 3' REV (5' CGG GAT CCT AGT CCC TCT CTC TGA TTT ATT G 3') primers used for this PCR reaction The PRINS PCR amplicon was cloned into the EcoRI and BamHI site of the pPTRAP V5 vector (Cytostore Inc., Calgary, AB, Canada). Transcription was performed on the the pTRAP V5 vector linearized with the XhoI restriction enzyme (Sigma Aldrich, St. Louis, MO, USA). The efficiency of transcription was tested on 10% PAGE. We used the tagged RNA directly (with the reaction mixture) without further purification in the RNA/protein co-incubation and purification procedure.

The *in vitro* transcribed RNA (PRINS AK676 sequence) was added to the tobramycin affinity matrix, and co-incubated with cellular extracts (HaCaT cell extract $n = 2$; NHEK extract $n = 1$). The washing steps and the elution of the RNP particles were carried out as described in the users' manual.

The eluate (S) was transferred to the streptavidin affinity matrix. A second round of washings and elution were carried out (elute F_3). Control elutes were obtained from both the tobramycin (T_{end}) and the streptavidin (S_{end}) columns, which did not bind the *in vitro* transcribed PRINS sequence: the cellular extracts flowed through them. In general, we carefully followed the instructions in the manual of the RNP purification kit,; however, some modifications were made as follows: during the purification of the PRINS RNA/protein complex, when the pre-binding of RNA transcripts to the 1st affinity matrix was performed, the *in vitro* transcribed RNA was incubated with RNAse inhibitor in Vial A for 60 min. Co-incubation of the cell extract with the *in vitro* transcribed RNA on the column was carried out overnight and this was followed by a two-step (2 × 30) elution.

Proteins in the elutes were separated on 7.5% SDS-PAGE and stained with Coomassie Brilliant Blue. As marker we, used Kaleidoscope prestained standards (Bio-Rad Laboratories, Cressier, Switzerland) containing seven individually color-coded proteins. The bands of interest were cut out and identified by MALDI-TOF mass spectrometry.

3.4. Real-Time RT-PCR

Total RNA was isolated from cells through the use of TRIzol TM Reagent (Invitrogene Corp., Carlsbad, CA, USA), following the manufacturer's instructions. cDNA was synthesized from 1 µg total RNA with the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). Real-time RT-PCR experiments were carried out with the Universal Probe Library system (F. Hoffmann-La Roche AG, Basel, Switzerland). The PRINS primers were FWD: GAGGCCAGCAGTTTCTACAG and REV: AGGGACAACCACATCAAAGC and the Cy5-labeled CCTTCATCTCACACACCTACGCAG probe; NPM primers were FWD: GGAGGAGGATGTGAAACTCTTAAG and REV: CTCTTCATCATCATCGTCATCATC and the FAM-labeled probe was ATATCTGGAAAGCGGTCTGCCC; each probe was purchased from Integrated DNA Technologies Inc., Coralville, IA, USA. PCR assays were performed with an iQ5 Real-Time PCR Detection Machine (Bio-Rad). The relative mRNA levels were calculated by the ΔΔCt method [52]. The expression of each gene was normalized to the 18S ribosomal RNA gene.

3.5. Western Blot Analysis

During Western blot analysis, equal amounts of proteins were separated on 10% SDS-PAGE gel, transferred to Pure Nitrocellulose Membrane (Bio-Rad Laboratories) and immunoblotted with anti-NPM (Sigma Aldrich, St. Louis, MO, USA) antibody diluted 1:500. Anti-mouse IgG alkaline phosphate conjugate (Sigma Aldrich, St. Louis, MO, USA) was used as a secondary antibody, and signals were visualized with Sigma Fast TM BCIP/NBT (Sigma Aldrich, St. Louis, MO, USA). A-Actin was used as a loading control; α-actin-specific antibody was purchased from Sigma.

3.6. Immunohistochemistry

Paraffin-embedded tissues of healthy, involved and uninvolved psoriatic skin samples measuring 5 mm × 10 mm × 4 mm were obtained for NPM immunohistochemistry. The fixative was 4% buffered formaldehyde for 24 h. The tissue block was subjected to paraffin embedding and 4-µm-thick sections

were placed on silanized slides, dewaxed in xylene for 3 × 5 min and rehydrated in decreasing concentrations of ethanol and methanol for 2 × 3 min and 2 × 2 min, respectively. Tissue endogenous peroxidase was blocked in a mixture of 90 mL of methanol and 3 mL of H_2O_2 for 5 min. For nonspecific antigen blocking, a mixture of 1% BSA and 0.1% Azide was used for 10 min. After nonspecific antigen blocking, sections were incubated with anti-NPM mouse monoclonal IgG antibody (Sigma Aldrich, St. Louis, MO, USA). The antibody was used in a dilution of 1:25 in LabVision Solution® (LabVision Corporation, Fremont, CA, USA) overnight.

For more sensitive reactions, HRP polymer (Envision® system; Dako, Denmark) was applied for 30 min. The intensity of the DAB reaction (RealEnvision® system; Dako, Denmark) was controlled under a light microscope. Sections were counterstained with hematoxylin for 1 min.

3.7. Gene-Specific Silencing

Gene-specific silencing of PRINS was performed with a vector-based method described previously [13]. The most effective PRINS silencing was achieved in supplement-free HPV-Ker cell cultures at ~70% confluency, and the transient transfection with the *in vivo* pSilencer™ 2.1-U6 hygro vector (Ambion Inc., Austin, TX, USA) was therefore carried out with these cells. The siRNA sequence targeting PRINS gene silencing was as follows: AK696, TTTCTGGAATGATGTCCAA. The scrambled sequence (SC1313) was used as control: AACTTTATCTCGGATCTAT. Cells were transiently transfected with plasmids following an X-tremeGENE 9 DNA transfection protocol, as described by the manufacturer (Roche Diagnostics, Mannheim, Germany). The transfection efficiency was on average 85%, as checked with a GFP reporter construct (Lonza, Basel, Switzerland). The effectiveness of the silencing was measured by RT-PCR (Figure S4).

3.8. Immunocytochemistry

Control and UV-treated HPV-Ker cells and NHEK cells were trypsinized and harvested by centrifugation, and resuspended in PBS. One hundred thousand cells were centrifuged onto a slide using a cytocentrifuge (Cytopro™, Wescor, Logan, UT, USA) and dried overnight at RT. The slides were fixed in 2% paraformaldehyde for 20 min at RT, and then washed in TBS. Nonspecific antigens were blocked for 30 min at RT in 1% goat serum containing 0.5% BSA-TBS. Slides were incubated overnight at 4 °C with anti-NPM monoclonal antibody (Sigma Aldrich, St. Louis, MO, USA; 1:500) in 0.5% BSA-TBS. Anti-mouse Alexa Fluor 546 goat anti-mouse IgG secondary antibody solutions (Sigma Aldrich, St. Louis, MO, USA; 1:500) were applied for 3 h at RT. Cell nuclei were counterstained with DAPI (Sigma Aldrich, St. Louis, MO, USA, 1:100) and mounted with Fluoromont-G (Southern Biotech, Birmingham, AL, USA). Pictures were taken with the aid of a Zeiss AxioImager fluorescent light microscope (Carl Zeiss MicroImaging, Thornwood, NY, USA) fitted with a PixeLINK CCD camera (PixeLINK, Ottawa, ON, Canada).

For the semiquantitative analysis of the intracellular localization of NPM (nucleolar or nuclear), at least 25 fields of view were counted per each group by two independent examiners. Cells were sorted and counted by observing the localization of NPM immunostaining in the nucleolus or in the nucleoplasm.

3.9. In Silico *Prediction of PRINS-Binding Proteins*

In silico prediction of the putative PRINS-binding proteins was carried out with the MatBase program (www.genomatix.de).

4. Conclusions

We have demonstrated here that the ncRNA PRINS interacts physically and functionally with the chaperone molecule NPM. The crucial regulatory roles of NPM in cell proliferation and cellular stress response have already been demonstrated [53−55] and studied in detail. In agreement with previous findings, we revealed that NPM is associated with proliferation of the keratinocytes. The data we have reported here suggest that PRINS might be part of a regulatory complex formed with NPM. The resulting complex may play a role in the regulation of proliferation and differentiation and in the stress response of cells. We hypothesize that the abnormal functioning of this complex contributes to the pathogenesis of both malignant and benign hyperproliferative diseases, such as psoriasis.

Acknowledgments

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Conflict of Interest

The authors declare no conflict of interest.

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Keratinocytes express functional CARD18, a negative regulator of inflammasome activation, and its altered expression in psoriasis may contribute to disease pathogenesis

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ABSTRACT

Caspase recruitment domain family member 18 (CARD18, Iceberg) is known as a negative regulatory molecule that inhibits inflammatory events by terminating inflammasome activation due to a direct interaction with pro-caspase-1.

During the investigation of molecular mechanisms in keratinocytes that contribute to the pathogenesis of psoriasis, we found that CARD18 expression differs in healthy and psoriatic skin; moreover, CARD18 demonstrated altered response under inflammatory conditions in healthy and psoriatic skin. In healthy skin, low basal CARD18 expression was detected, which showed significant elevation in response to inflammatory stimuli (lymphokine treatment or mechanical injury). In contrast, higher basal expression was observed in psoriatic non-involved skin, but no further induction could be detected.

We demonstrated that keratinocytes express CARD18 both at mRNA and protein levels and the expression increased in parallel with differentiation. The investigation of cellular inflammatory processes revealed that psoriasis-associated danger signals triggered the expression of inflammasome components (AIM2, Caspase-1) and CARD18 as well as IL-1β production of keratinocytes. Furthermore, gene-specific silencing of CARD18 in cells treated with cytosolic DNA (poly(dA:dT)) resulted in increased IL-1β secretion, suggesting a negative regulatory role for CARD18 in keratinocyte inflammatory signaling.

The differential regulation of CARD18 in healthy and psoriatic uninvolved epidermis may contribute to the susceptibility of psoriasis. Furthermore, our *in vitro* results indicate that CARD18 may contribute to the fine tuning of keratinocyte innate immune processes.

1. Introduction

The innate immune system constitutes the first line of defense that detects pathogen- and damage-associated molecular patterns. Inflammation is a protective physiological response; however, impaired activation and/or down-regulation of inflammatory signaling may result in inflammatory diseases, some of which involve multiple organs. Inflammasomes, located in the cytosol, are part of the innate immune system. These multi-molecular complexes are responsible for the recognition of various cytoplasmic danger signals and provoke inflammatory responses by recruiting and activating pro-caspase-1 through autocatalytic cleavage (Schroder and Tschopp, 2010). The activation of caspase-1 ultimately leads to the processing and, thus, secretion of pro-inflammatory cytokines, most importantly interleukin (IL) 1β and IL-18, and also induces

Abbreviations: AIM2, absence in melanoma 2; CARD18, caspase recruitment domain family member 18; COP, CARD-only protein; DAPI, 4,6-diamidino-2-phenylindole; DNase, deoxyribonucleases; GM-CSF, granulocyte macrophage colony-stimulating factor; IL, interleukin; IFN-γ, interferon-γ; TNF, tumor necrosis factor; IHC, immunohistochemistry; NHEK, normal human epidermal keratinocytes; PBS, phosphate-buffered saline; poly(dA:dT), polydeoxyadenylic acid–polydeoxythymidylic acid double-stranded homopolymer; TS, tape stripping.

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pyroptotic cell death (Bauernfeind et al., 2011). IL-1β affects nearly all cell types and is fundamental for innate as well as adaptive immunity (Dinarello, 1996). Increased IL-1β abundance plays an important role in a number of chronic and acute inflammatory diseases (Li et al., 2008), such as psoriasis.

Psoriasis is a life-long skin disease with chronic inflammatory and immune-mediated characteristics, affecting approximately 2% of the population (Christophers, 2001). The disease usually manifests as raised, erythematous oval plaques on the skin (Nestle et al., 2009a). In addition to decreasing the quality of life, psoriasis is also associated with a high degree of co-morbidities, including arthritis, atherosclerosis, inflammatory bowel disease, obesity, type 2 diabetes and depression. The clinical phenotype of these co-morbidities illustrates the importance of the dysfunctional immune system (Rivas Bejarano and Valdecantos, 2013).

The importance of innate immune dysregulation in psoriasis has long been recognized (Bos et al., 2005; Nickoloff, 1999; Nickoloff et al., 2000). The activated state of the innate immune system is represented by the activity of natural killer cells, dendritic cells, neutrophils and keratinocytes, and an increased expression of cytokines (Iversen and Johansen, 2008). Pro-inflammatory cytokines, such as IL-1β and IL-18, have been shown to play an important role in the pathogenesis of psoriasis, *i.e.*, initiating and mediating the infiltration of immune cells and stimulating keratinocytes to proliferate (Johansen et al., 2007; Mee et al., 2007; Nestle et al., 2009b). Elevated IL-1β and IL-18 expression has been observed in inflamed skin; moreover, the increased IL-1β is produced mostly by keratinocytes (Koizumi et al., 2001; Renne et al., 2010). Certain cytokines, inflammasomes and inflammatory caspases, such as caspase-1, have been described as potential inducers and regulators of skin inflammation in contact hypersensitivity and in psoriasis (Iversen and Johansen, 2008; Salskov-Iversen et al., 2011).

Cytosolic DNA is a potent inducer of the innate immune response and has been proposed to be involved in the pathogenesis of psoriasis (Dombrowski et al., 2011; Lande et al., 2007; Nestle et al., 2009a). The DNA-sensor-containing absence in melanoma 2 (AIM2) inflammasome is triggered by both self-derived and pathogen-released (*Francisella tularensis*, *Listeria monocytogenes*, vaccinia virus) cytosolic double-stranded DNA (Fernandes-Alnemri et al., 2009; Rathinam and Fitzgerald, 2010). Recently, abundant cytosolic DNA and increased AIM2 expression were found to be present in keratinocytes in psoriatic lesions but not in healthy skin (Dombrowski et al., 2011), and have been suggested to contribute to the abnormal IL-1β secretion in psoriasis. Keratinocytes express four different deoxyribonucleases (DNases), which remove extracellular DNA by degradation. In psoriatic skin, three of these DNases do not exhibit DNase activity and one seems to have reduced DNA-affinity. The optimum pH of these DNA-binding proteins likely changes during keratinization (Reimer et al., 1978), resulting in aberrant DNase activity and the presence of excess DNA fragments in the cytosol.

Our recent large-scale gene expression study has revealed that the caspase recruitment domain family member 18 (CARD18, Iceberg) transcript is differentially expressed in psoriatic non-involved epidermis compared to healthy epidermis (Szabó et al., 2014). CARD18, a member of CARD-only protein (COP) family, has only one CARD domain which exhibits sequence similarity to the CARD prodomain of caspase-1. COP family members probably arose through gene duplication and are restricted to the higher primates (Kersse et al., 2007). CARD18 serves as a decoy protein which can modify the activity of inflammasome by inhibiting the generation of active IL-1β and IL-18 through direct interaction with pro-caspase-1 (Druilhe et al., 2001; Humke et al., 2000). Further investigation of the molecular biology and functions of CARD18 should shed light on yet unknown basic innate immune mechanisms and could lead to the identification of possible therapeutic target(s). Therefore, we investigated CARD18 expression in cultured keratinocytes exposed to various psoriasis-related stress factors and sentinels in psoriasis. We also addressed whether CARD18 contributes to AIM2 inflammasome-mediated keratinocyte functions, and if this small molecule could modify inflammatory processes in keratinocytes.

2. Materials and methods

2.1. Patients

Shave biopsy samples for organotypic skin cultures were taken from the non-involved buttock area of five young male psoriasis patients and five age- and gender-matched healthy controls. Subsequently, organotypic skin cultures were established, as has been described previously (Szabó et al., 2014).

For immunohistochemical staining, patients with moderate-to-severe psoriasis vulgaris (n = 2) and healthy (n = 2) volunteers were enrolled in the study.

In another set of experiments, the skin of psoriatic patients (n = 2) and healthy volunteers (n = 2) were subjected to tape stripping (TS). Punch biopsies were taken from the area once before and twice after (24 and 48 h) the treatment.

Written informed consent was obtained from all donors involved in the study. Psoriatic patients had not undergone treatment for 4 weeks before sampling. The study was approved by the Human Investigation Review Board of the University of Szeged, complying with the ethical standards of research and in accordance with the Helsinki Declaration.

2.2. Cell cultures

Normal human epidermal keratinocytes (NHEKs) were separated from skin specimens obtained from the Plastic Surgery Unit of our department. The epidermis and the dermis were separated by overnight incubation in Dispase (Roche Diagnostics, Manheim, Germany), and keratinocytes were obtained after maceration in 0.25% trypsin. Cells were grown in 75 cm^2 cell culture flasks and were maintained in keratinocyte serum-free medium (Gibco Keratinocyte SFM Kit; Life Technologies, Copenhagen, Denmark), supplemented with 1% antibiotic/antimycotic solution (PAA Laboratories GmBH, Pasching, Austria) and 1% L-glutamine (PAA Laboratories) at 37 °C in a humidified atmosphere with 5% CO_2. The calcium concentration of the medium was <0.1 mM. The medium was changed every 2 days. Third passage keratinocytes were used for experiments.

A previously described, an *in vitro* keratinocyte-differentiating model was used (Pivarcsi et al., 2001). For the induction of AIM2 inflammasome, cells were subjected to 5 ng/ml tumor necrosis factor (TNF) α (R&D Systems, Minneapolis, MN, USA) and 5 ng/ml interferon (IFN) γ (R&D Systems) pretreatment and 1 μg/ml polydeoxyadenylic acid–polydeoxythymidylic acid double-stranded homopolymer (poly(dA:dT)), (Sigma Aldrich, Saint Louis, MO, USA) transfection. The supernatants were collected and cells were harvested at the indicated time points after treatments.

2.3. Real-time RT-PCR

Total RNA was isolated from cells using TRIzol® Reagent (Invitrogen Corp., Carlsbad, CA, USA), following the manufacturer's instructions. cDNA was synthesized from 1 μg total RNA with the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). Real-time RT-PCR experiments were carried out with the Universal Probe Library system (F. Hoffmann-La Roche AG, Basel, Switzerland). The following primers were used: CARD18

forward AAGACCCCTCAACTTGCCTCA and reverse CTCCCTTGGAAGAAGCTCTG, (UPL probe number 50); AIM2 forward AGTCCTCTGCTAGTTAAGCTCTCTG and reverse TCTGACAACTTTGGGATCAGC (UPL probe number 87); and caspase-1 forward CCTTAATATGCAAGACTCTCAAGGA and reverse TAGCTGGGTTGTCCTGCACT (UPL probe number 17). PCR assays were performed with the C1000 Touch Thermal Cycler (Bio-Rad Laboratories). The expression of each gene was normalized to the 18S ribosomal RNA gene. Relative mRNA levels were calculated by the $\Delta\Delta$Ct method. Data from different treatments were compared using one-tailed *t*-test. Differences were considered significant when $P \leq 0.05$.

2.4. Immunohistochemistry

Paraffin-embedded specimens from healthy, involved and non-involved psoriatic skin were obtained for CARD18 immunohistochemistry (IHC). Samples were fixed in 4% buffered formaldehyde for 24 h. The tissue block was subjected to paraffin embedding and 4-μm-thick sections were placed on silanized slides, dewaxed in xylene for 3×5 min and rehydrated in decreasing concentrations of ethanol. Tissue retrieval was performed in citrate buffer (10 mM, pH 6.0). For nonspecific antigen blocking, 1% horse serum containing 0.5% BSA-TBS was used for 30 min. Sections were incubated with anti-CARD18 goat polyclonal IgG antibody (Santa Cruz Biotechnology, Dallas, TX, USA; 1:300) at 4 °C overnight. The anti-CARD18 goat polyclonal antibody was not included in the staining procedure when sections were stained for the matching negative controls. After three washing steps with phosphate-buffered saline (PBS), biotinylated anti-goat IgG (1:200, Vector Laboratories Inc., Burlingame, CA, USA) was applied for 30 min at room temperature. After one PBS wash, sections were incubated in ExtrAvidin peroxidase (1:400, Sigma Aldrich) for 30 min at room temperature. Visualization was performed by adding AEC reagent (0.05% 3-amino-9-ethylcarbazole dissolved in N,N-dimethylformamide, 0.01% H_2O_2, and 0.05 M acetate buffer). Sections were counterstained with hematoxylin for 10 s and subsequently analyzed with a Zeiss Axio Imager fluorescent light microscope (Carl Zeiss MicroImaging, Thornwood, NY, USA) fitted with a Carl Zeiss AxioCamMRc camera.

2.5. Immunofluorescence staining

NHEK cells were trypsinized, harvested by centrifugation and resuspended in PBS before 10^5 cells were centrifuged onto a slide using a cytocentrifuge (Cytopro™, Wescor, Logan, UT, USA) and dried overnight at room temperature. The slides were fixed in 2% paraformaldehyde for 20 min at room temperature. Nonspecific antigens were blocked for 30 min at room temperature in 1% donkey serum containing 0.5% BSA-TBS. Slides were incubated overnight at 4 °C with anti-CARD18 goat polyclonal IgG antibody (Santa Cruz Biotechnology; 1:250) in 0.5% BSA-TBS. Anti-goat Alexa Fluor 546 donkey secondary antibody solutions (Sigma Aldrich) were applied for 1 h at room temperature. Cell nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI, Sigma Aldrich, 1:100) and mounted with Fluoromount-G (Southern Biotech, Birmingham, AL, USA). Pictures were taken with the aid of a Zeiss Axio Imager fluorescent light microscope (Carl Zeiss MicroImaging) fitted with a Carl Zeiss AxioCamMRc 5 camera.

2.6. ELISA

Cell supernatants were centrifuged (5000 rpm, 4 min, 4 °C) to pellet cell debris and the amount of IL-1β was determined by ELISA (Human IL-1β ELISA Duo Set, R&D Systems), according to the manufacturer's instructions.

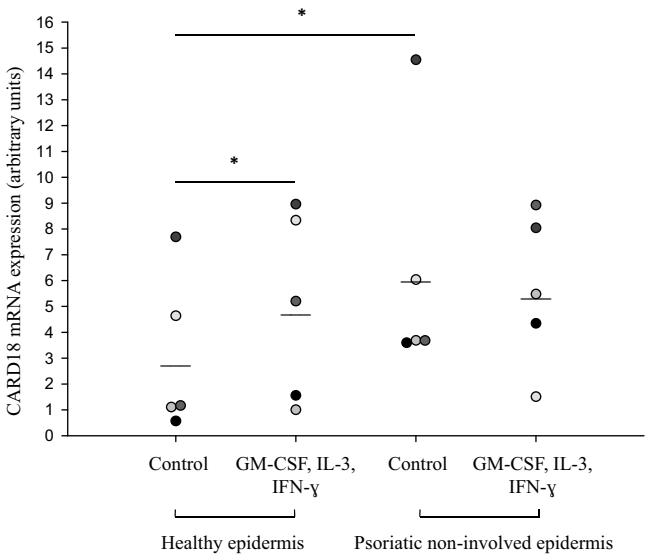

Fig. 1. CARD18 gene expression in healthy and non-involved psoriatic epidermis. Total RNA was isolated from the epidermal compartment of organotypic skin cultures (n = 5). CARD18 gene expression patterns in response to T-cell lymphokine (GM-CSF, IL-3, IFN-γ) treatment in healthy epidermis and in non-involved psoriatic epidermis. Horizontal lines represent mean values.* $P > 0.05$.

2.7. Gene specific silencing

Gene-specific silencing was performed with an siRNA transfection method. NHEK cells were transiently transfected at approximately 70% confluency using the X-tremeGENE siRNA Transfection Reagent (Roche), according to the manufacturer's instructions. For gene-specific silencing of CARD18, siRNA duplex was applied: ICEBERG siRNA (h), sc-105550, and control siRNA-A, sc-37007 (Santa Cruz Biotechnology). The most effective CARD18 silencing was achieved in serum-free culture medium without additive and supplements. The effectiveness of the silencing was measured by real-time RT-PCR (Supplementary Fig. 1).

3. Results

3.1. CARD18 gene expression differs in healthy and psoriatic non-involved epidermis upon T-cell lymphokine treatment

There is evidence that psoriatic non-involved epidermal keratinocytes carry molecular and cellular alterations that contribute to the occurrence of psoriatic symptoms. We have recently reported the results of a cDNA microarray experiments in which 61 annotated genes were differentially expressed in psoriatic non-involved and healthy epidermis upon T-cell lymphokine treatment (Szabó et al., 2014). In this experiment, organotypic skin cultures were treated with a cytokine mixture (GM-CSF, IL-3, IFN-γ) that has been shown to mimic the effect of psoriatic T-cell lymphokines (Bata-Csorgo et al., 1995). CARD18, identified as a differentially expressed gene in our experiment, encodes a protein that belongs to the COP family. CARD18 is a negative regulator of IL-1β maturation, inhibiting inflammasome activation directly by interacting with pro-caspase-1 (Bauernfeind et al., 2011). To validate the cDNA microarray results, real-time RT-PCR analysis was carried out to measure the abundance of CARD18 mRNA (Fig. 1) using original RNA samples derived from organotypic skin cultures (healthy epidermis, n = 5, and psoriatic non-involved epidermis, n = 5). Our results confirmed the cDNA microarray results: we observed a 2-fold elevation of CARD18 mRNA level in psoriatic non-involved epidermis compared to healthy epidermis. Although the basal expression levels were relatively high in the psoriatic non-involved

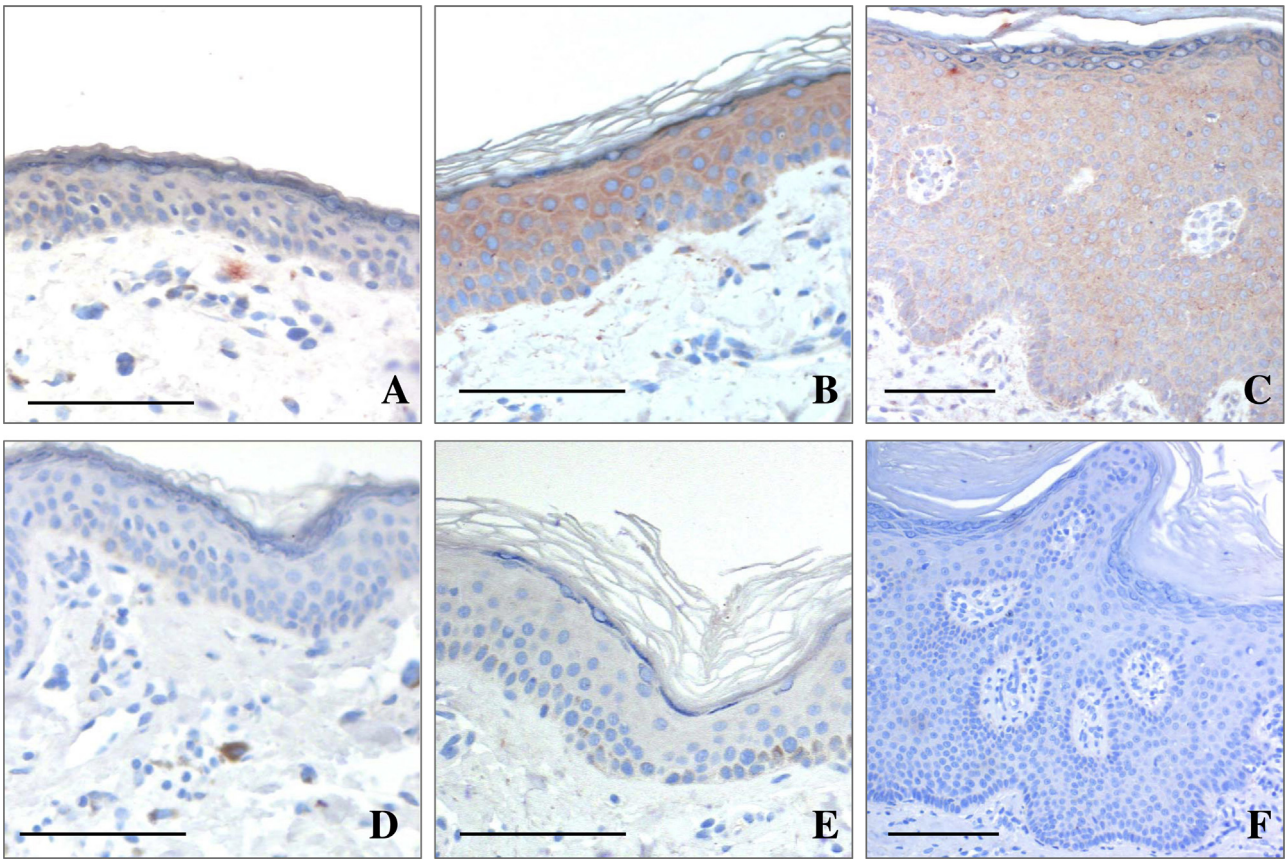

Fig. 2. Immunohistochemical staining of CARD18 in healthy and psoriatic skin. CARD18 protein levels were analyzed in healthy (A), psoriatic non-involved (B), and psoriatic involved (C) skin. Negative controls were performed for healthy (D), psoriatic non-involved (E) and psoriatic involved skin (F). Bar = 100 μm.

epidermis, it was not further induced in response to T-cell lymphokines. This was in contrast to the findings for healthy skin, where lower basal expression levels were induced with an average 1.65-fold elevation in response to the same treatment.

3.2. CARD18 expression differs in healthy and diseased skin

Using an organotypic skin model, we determined that CARD18 mRNA abundance was higher in psoriatic non-involved epidermis compared to healthy epidermis. To compare CARD18 protein levels in healthy and diseased skin, immunohistochemical (IHC) staining was performed on paraffin-embedded samples from healthy (Fig. 2A), psoriatic non-involved (Fig. 2B) and psoriatic involved skin samples (Fig. 2C) in an independent experiment. In all examined sections, CARD18 appeared exclusively in the epidermis, specifically in the cytoplasm of the epidermal keratinocytes. IHC revealed differential CARD18 expression in different skin samples: elevated levels were observed in the psoriatic-involved and non-involved epidermis compared to the healthy epidermis (Fig. 2B and 2C vs 2A). These results confirmed our real-time RT-PCR findings. No differences were detected in the different keratinocyte layers of the epidermis; however, the level of CARD18 staining was somewhat higher in the suprabasal layers of psoriatic non-involved and involved epidermis. Fig. 2D–F provides the data from negative controls for Fig. 2A–C.

3.3. CARD18 expression is differentially induced in healthy, psoriatic non-involved and psoriatic involved epidermis after tape stripping (TS)

Mechanical stress causing disruption of the skin barrier leads to molecular responses, including a rapid increase in DNA synthesis and early over-expression of pro-inflammatory cytokines (Marionnet et al., 2003; Wood et al., 1997). It has long been known that baseline proliferative activity of keratinocytes of non-involved skin of psoriatic patients does not differ from keratinocytes in skin of healthy individuals; however, the proliferative response to TS or other trauma is significantly higher in psoriatic non-involved skin compared to healthy skin (Hatta et al., 1997; van de Kerkhof et al., 1983; Wiley and Weinstein, 1979). CARD18 protein expression was observed in mechanically stimulated skin from healthy and non-involved skin of psoriatic donors. Punch biopsies were taken before the procedure and two times after TS (24 and 48 h). Compared to untreated sections (Fig. 3A, D, G), CARD18 protein expression level was induced 24 h after TS in healthy (Fig. 3B), psoriatic non-involved (Fig. 3E) and psoriatic involved (Fig. 3H) samples, as well. However, the elevation of CARD18 expression in treated and untreated psoriatic non-involved samples was not as robust as in healthy or psoriatic involved skin. Forty-eight hours after the treatment, CARD18 expression remained elevated only in the psoriatic non-involved samples (Fig. 3F). Negative-control staining of the TS experiment is provided in Supplementary data (Supplementary Fig. 2).

3.4. CARD18 is abundantly expressed in differentiating keratinocytes

To examine the characteristics of CARD18 in NHEKs, which are non-professional immune cells, we examined mRNA and protein changes in spontaneously differentiating NHEKs. In spontaneously differentiating third-passage NHEKs, the differentiation process was detected as an increase in a differentiation marker, keratin 10, and the decrease in a proliferation-related marker, alpha5 integrin (data not shown). The 0-day samples were taken from

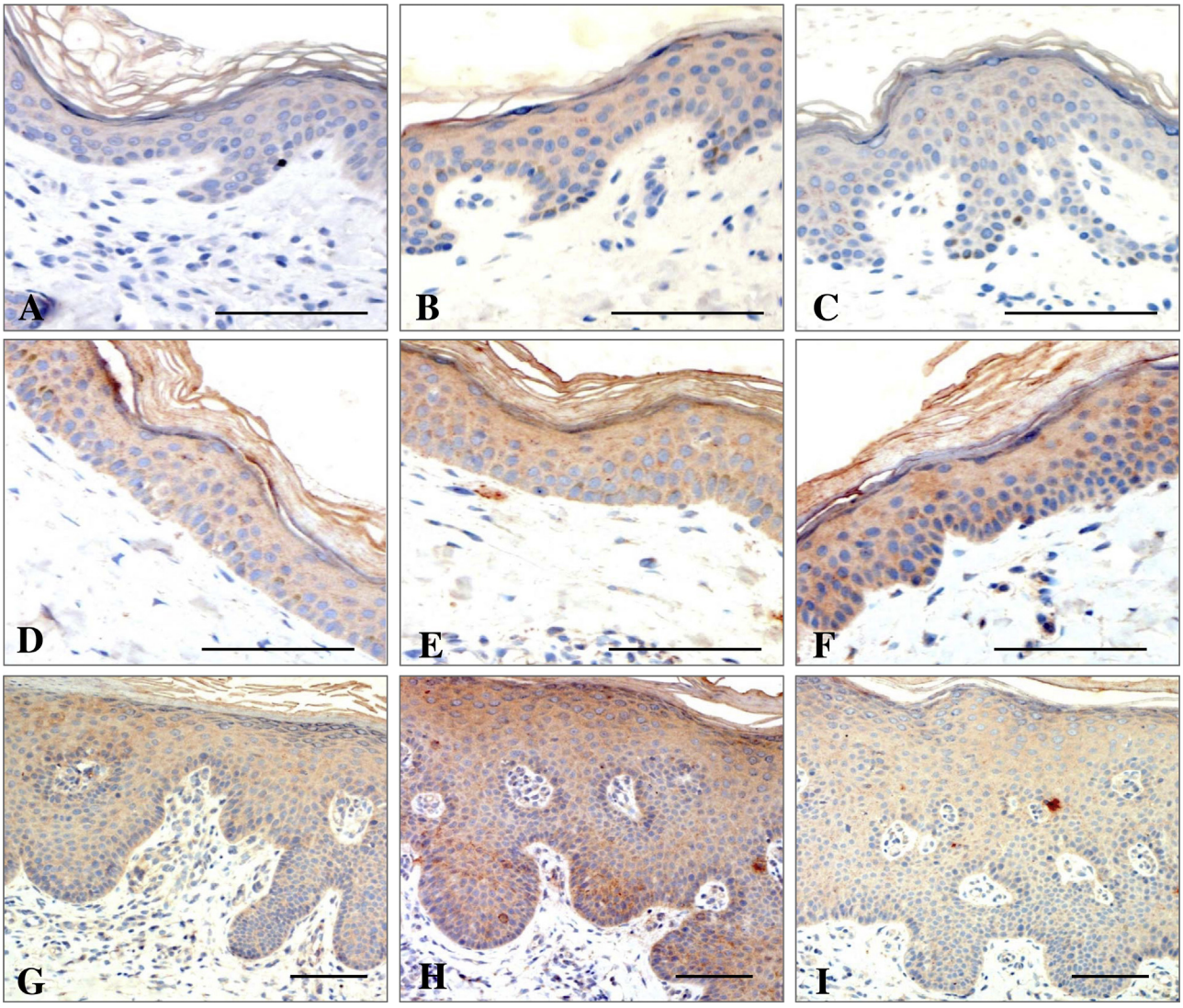

Fig. 3. CARD18 expression upon acute barrier disruption. Tape stripping (TS) method was carried out on the skin of healthy (n = 2, one representative picture is shown) and psoriatic volunteers (n = 2, one representative picture is shown). Punch biopsies were taken at the indicated time points. Skin samples were subjected to immunohistochemical staining of CARD18: healthy untreated (A), healthy TS at 24 h (B), healthy TS at 48 h (C), psoriatic non-involved untreated (D), psoriatic non-involved TS at 24 h (E), psoriatic non-involved TS at 48 h (F), psoriatic involved untreated (G), psoriatic involved TS at 24 h (H), psoriatic involved TS at 48 h (I). Bar = 100 μm.

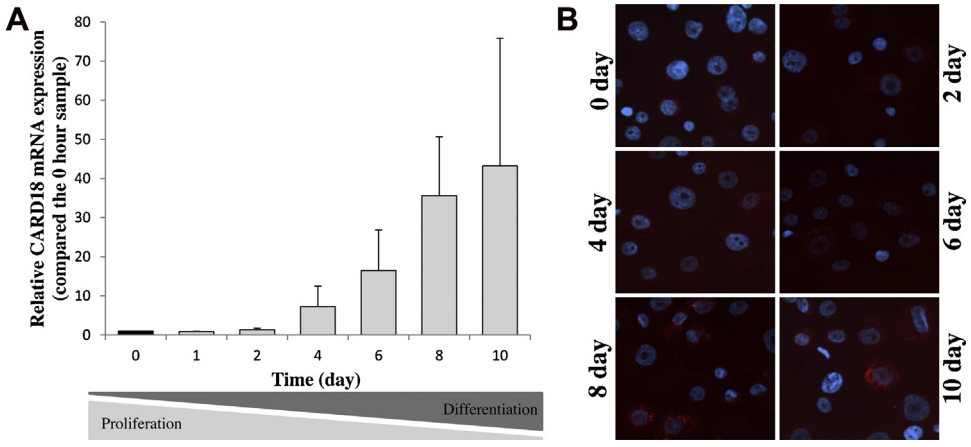

Fig. 4. Expression of CARD18 mRNA and protein during keratinocyte proliferation and differentiation. Cultured NHEKs (n = 3) were grown to subconfluency before samples were taken (0 day). (A) Changes in CARD18 mRNA expression were analyzed by real-time RT-PCR at the indicated time points. Relative expression is shown compared to the 0-day sample. (B) CARD18 intracellular protein localization was detected in NHEKs (n = 3, one representative picture is shown) at the indicated time points. Samples were fixed and immunostained for CARD18 (red). DAPI was used for nuclear counterstaining. Magnification: 20×.

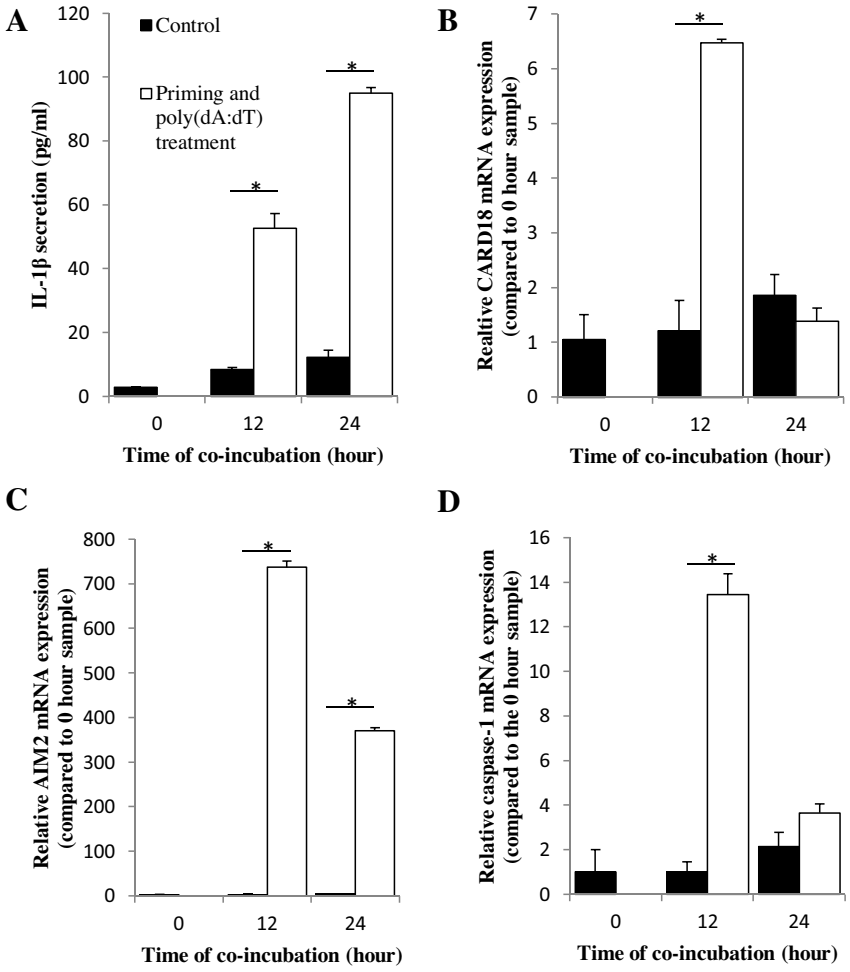

Fig. 5. The response of cytokine-primed keratinocytes to synthetic DNA analogue (poly(dA:dT)) transfection. NHEK cultures (n = 3) were incubated with IFN-γ and TNF-α for 24 h, followed by poly(dA:dT) transfection. Samples were taken at the indicated time points. IL-1β secretion (A) was measured by ELISA method. CARD18 (B), caspase-1 (C) and AIM2 (D) mRNA expression was determined by real-time RT-PCR. Relative expressions are shown compared to the 0 h sample. * $P > 0.05$.

subconfluent cultures. To determine the mRNA expression of CARD18, real-time RT-PCR was carried out using gene-specific primers. The *in vitro* gene-expression study revealed low-level CARD18 mRNA expression in the proliferative state of cells, and this expression continuously increased during differentiation: up to 43.2-fold elevation in the 10-day samples (Fig. 4A).

To examine the expression of CARD18 protein, immunofluorescence staining was applied to keratinocytes, revealing that moderate positive CARD18 protein expression in samples taken on days 0–4 gradually increased in the 6–10-day samples (Fig. 4B). Thus, CARD18 protein expression followed a similar pattern observed for the mRNA expression during the 10-day course. The immunocytochemistry staining allowed the intracellular distribution to be determined and revealed that CARD18 was located in the cytoplasm of NHEKs, as it was observed in the epidermal keratinocytes of the *ex vivo* samples.

3.5. CARD18 modifies the expression of molecules involved in cytosolic DNA-triggered responses of NHEKs

To elucidate the cellular processes in which CARD18 participates and to gain further insight into its role in the pathomechanism of psoriasis, we investigated CARD18 expression in response to certain psoriasis-associated signals. Keratinocytes, as important immune-competent cells, are able to sense danger signals and mediate immune response through the activation of pro-inflammatory signaling pathways (Nestle et al., 2009b), resulting in the expression of inflammasome components and secretion of pro-inflammatory cytokines (Feldmeyer et al., 2007).

Cytosolic DNA has been identified recently as a trigger for AIM2 inflammasome activation and is abundant in psoriatic-involved epidermis (Chiliveru et al., 2014; Dombrowski et al., 2011). To mimic the effect of cytosolic DNA, a synthetic dsDNA analogue poly(dA:dT) was transfected into cultured keratinocytes. A significant increase in IL-1β release – up to 50 pg/ml (Supplementary Fig. 3A) – as well as 3–5 fold increase in caspase-1 expression (Supplementary Fig. 3C) and a 200-fold increase in AIM2 expression (Supplementary Fig. 3D) were observed, although changes in CARD18 mRNA were not detected (Supplementary Fig. 3B).

To provoke enhanced inflammation, IFN-γ and TNF-α pretreatment was performed before poly(dA:dT) transfection. IFN-γ is known to induce AIM2 gene expression (Lee et al., 2012), whereas TNF-α mediates pro-IL-1β transcription (Mills and Dunne, 2009), and both cytokines are consistently elevated in psoriatic skin. The co-treatment with IFN-γ, TNF-α and poly(dA:dT) resulted in increased IL-1β secretion – up to 100 pg/ml (Fig. 5A) – and increased the expression of caspase-1 and AIM2 mRNAs by 13-fold (Fig. 5C) and 250-fold (Fig. 5D), respectively. CARD18 mRNA level was also increased by six fold (Fig. 5B).

These results encouraged us to further characterize the function of CARD18 in keratinocytes in which inflammatory processes had been induced. CARD18 expression was silenced by siRNA

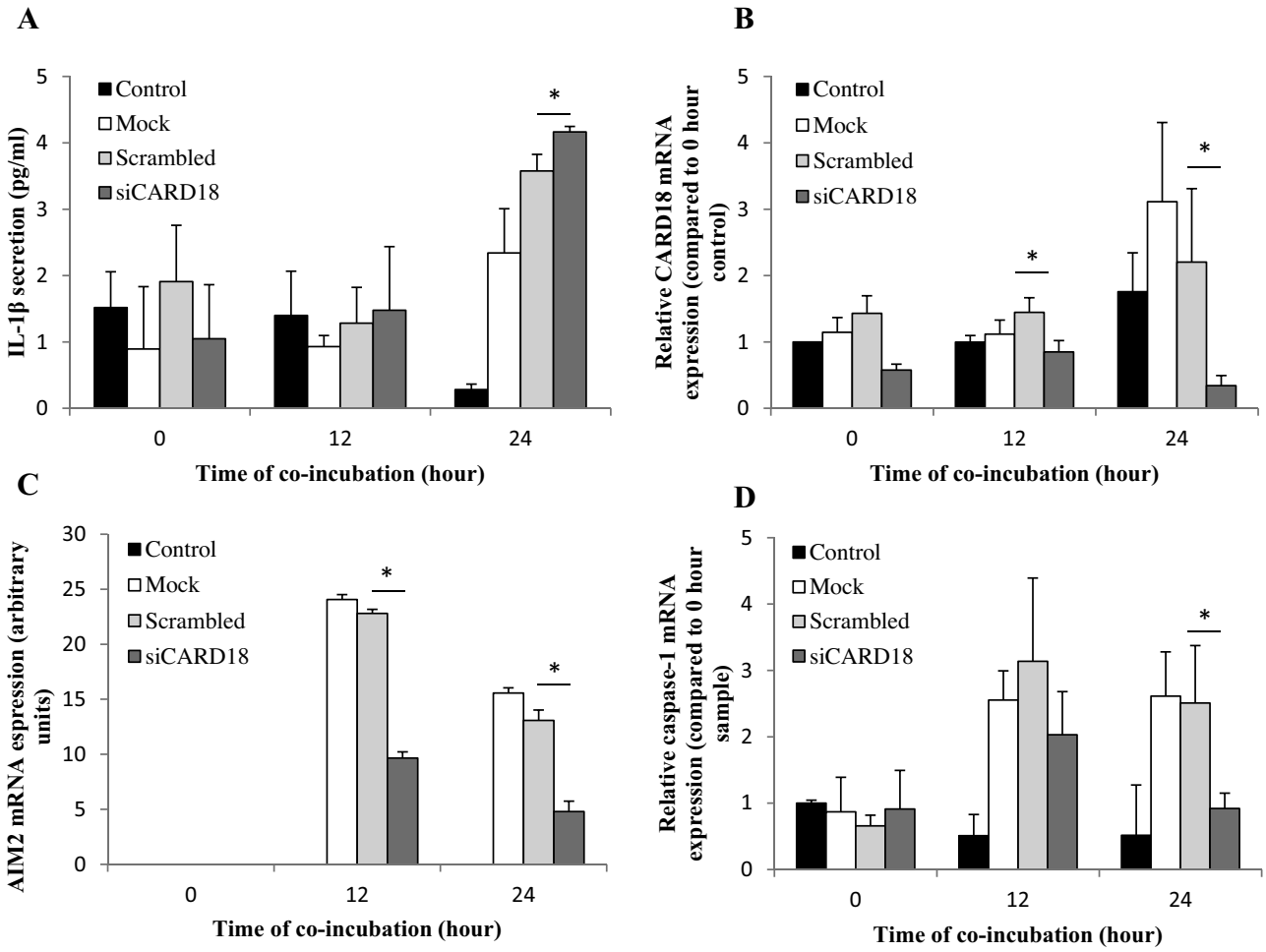

Fig. 6. The effect of CARD18 gene-specific silencing in cytokine-primed poly(dA:dT) transfected NHEKs. CARD18 expression was silenced in NHEK cultures (n = 3). CARD18 siRNA, scrambled control and mock-transfected cells were subsequently treated with a mixture of IFN-γ and TNF-α (12 h) and transfected with poly(dA:dT). Samples were taken at the indicated times. IL-1β secretion (A) was measured by ELISA. CARD18 (B), caspase-1 (C) and AIM2 (D) mRNA expression was analyzed by real-time RT-PCR. Expression is relative to the 0 h sample, except for AIM2, where arbitrary units were used. * $P > 0.05$.

24 h before IFN-γ and TNF-α treatment and the subsequent poly(dA:dT) transfection. The applied siRNA effectively downregulated CARD18 expression to 50% of the levels observed for the scrambled control cells (Fig. 6B).

The CARD18 silencing resulted in a significant decrease in AIM2 gene expression 12 and 24 h after treatment (Fig. 6C) and significantly reduced caspase-1 mRNA expression (Fig. 6D) 24 h after the treatment. For further confirmation, we determined whether the silencing of CARD18 had any effect on IL-1β secretion: our results indicated that downregulation of CARD18 expression affected an elevation of IL-1β production in keratinocytes (Fig. 6A). These results indicate that CARD18 might indeed contribute to the fine-tuning of inflammatory processes in keratinocytes and suggest that – similar to the role in professional immune cells (Humke et al., 2000) – functions as a negative regulator of inflammasome activation in keratinocytes.

4. Discussion

IL-1β is an important mediator in the in the induction and maintenance of psoriasis (Bernard et al., 2012; Feldmeyer et al., 2010) and represents a potential therapeutic target; therefore, several studies have targeted possible inhibitors of IL-1β (Mansouri et al., 2015; Tamilselvi et al., 2013; Yun et al., 2015). IL-1β and IL-18 are generated by a number of different inflammasomes in response to various environmental influences and this process is likely further modulated by additional genetic factors (Masters, 2013). The importance of these multi-protein platforms has been reported in several inflammatory diseases, including psoriasis (Dombrowski et al., 2011; Mason et al., 2011). Specific inflammasome inhibitors rectify the balance between beneficial and deleterious IL-1β and IL-18 production. CARD18 has been identified as a negative regulator of inflammasome activation through direct interaction with the CARD domain of pro-caspase-1 and abrogation of IL-1β and Il-18 production in macrophages (Humke et al., 2000). We have identified CARD18 as a highly expressed transcript in psoriatic non-involved epidermis and demonstrated its abnormal regulation in psoriatic non-involved epidermis (Szabó et al., 2014). To understand the pathologic functions of CARD18 in skin inflammation, we studied CARD18 under normal and inflammatory conditions both *in vitro* and *in vivo*.

In organotypic skin cultures, CARD18 mRNA basal expression level was higher in psoriatic non-involved epidermis than healthy epidermis. In addition, samples of different origin exhibited different responses to lymphokine induction. The higher basal expression of CARD18 mRNA in psoriatic non-involved samples was not further increased following T-cell lymphokine treatment. In contrast, healthy epidermis exhibited lower CARD18 mRNA basal expression, which was induced by the cytokine treatment.

In human skin, CARD18 protein and mRNA exhibited similar accumulation patterns: histological staining showed higher CARD18 expression in non-involved and involved epidermis than in healthy epidermis. Mild injury (*i.e.*, TS) induced CARD18 protein expression in all examined skin samples. However, the level of induction was different: modest elevation was detected in psoriatic non-involved epidermis compared to healthy and psoriatic involved epidermis. The higher basal level and the lack of response of CARD18 gene and protein expression in psoriatic non-involved epidermis might indicate a "ceiling effect" or impaired regulation of inflammatory signaling, similar to the phenomenon that has been described for IL-1β production in keratinocytes and in salivary glands (Mastrolonardo et al., 2007; Szabó et al., 2014).

Although keratinocytes are able to express inflammasome components and secrete IL-1β, we observed very low – sometimes undetectable – levels of AIM2, caspase-1 and CARD18 mRNA and IL-1β secretion in uninduced keratinocytes. Compared to the untreated cells, however, treatment with a combination of inflammation-related stress factors significantly increased both the expression of inflammasome components and IL-1β secretion. The applied triggering factors, such as IFN-γ and TNF-α priming followed by poly(dA:dT) transfection are also known to contribute to the pathogenesis of psoriasis (Dombrowski et al., 2011; Nestle et al., 2009a).

To gain some information about the function of CARD18 in keratinocytes under inflammatory conditions, we silenced CARD18 expression. CARD18 silencing resulted in a modest but significant elevation of IL-1β secretion in poly(dA:dT)-treated cells, suggesting that, similar to professional immune cells, CARD18 indeed has a negative regulatory role in keratinocyte innate immune functions. Interestingly, reduced inflammasome activation was detected as decreased AIM2 and caspase-1 gene expression in the CARD18 silenced cells. We hypothesize that IL-1β secretion is a highly regulated process both in professional and in non-professional immune cells, such as keratinocytes. Thus, the silencing of CARD18 might up- and down-regulate multiple process simultaneously, resulting in only a mild change in IL-1β secretion. The reduced inflammasome activation data suggest the present of a negative feedback loop: elevated active caspase-1 down-regulates caspase-1 and AIM2 gene expression through a yet unknown control mechanism. Juruj et al. have recently published results supporting this hypothesis: the authors demonstrated that caspase-1 negatively regulated the formation or stability of the AIM2 inflammasome complex, and that this regulation was specific to the AIM2 inflammasome pathway in macrophages (Juruj et al., 2013).

Nair and coworkers demonstrated that caspase-1, AIM2 and IL-1β mRNAs are present at low levels in healthy and non-involved skin, and that the expression of these molecules increases significantly in psoriatic involved skin (Nair et al., 2009). Moreover, caspase-1 protein expression is increased in involved psoriatic skin compared to non-involved psoriatic skin, whereas procaspase-1 expression is unchanged. Under normal conditions, caspase-1 activity has not been detected in the skin (Johansen et al., 2007). During inflammation, AIM2 inflammasome (De Koning et al., 2012) and caspase-1 become activated both in professional immune cells and keratinocytes and induce the abnormal inflammatory responses observed in inflammatory skin diseases. The regulation of caspase-1 is undoubtedly an important checkpoint in IL-1β production and, thus, in the pathogenesis of chronic inflammatory skin diseases, such as psoriasis. By performing a set of *in vitro* experiments in this study, we have demonstrated that CARD18, an endogenous decoy protein proposed to interfere with caspase-1 activation, is part of the inflammasome regulatory processes that has been previously described (Dombrowski et al., 2011; Johansen et al., 2007). Moreover, our primary expression experiments clearly showed that CARD18 exhibits aberrant expression and inductivity in psoriatic epidermis. Although knowledge of CARD18 and other COP family members is limited, these small proteins could be promising targets for treating inflammatory diseases. Our findings regarding misregulated CARD18 expression in psoriasis suggest that this molecule may contribute to disease pathogenesis and could be a potential target for therapy.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molimm.2016.03.009.

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