Investigations on cytosolic nucleic acid fragment induced innate immune functions of keratinocytes

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PhD Thesis

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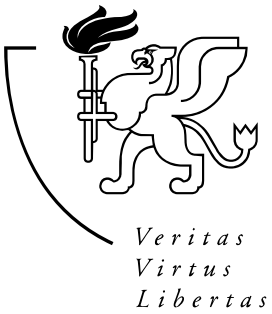

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List of publications

Scientific papers included in this thesis

I. **J. Danis**, L. Janovák, B. Gubán, A. Göblös, K. Szabó, L. Kemény, Z. Bata-Csörgő, M. Széll: Differential Inflammatory-Response Kinetics of Human Keratinocytes upon Cytosolic RNA- and DNA-Fragment Induction, *Int J Mol Sci* 19(3):774 (2018)
IF:3.226*

II. **J. Danis**, A. Göblös, Z. Bata-Csörgő, L. Kemény, M. Széll: PRINS Non-Coding RNA Regulates Nucleic Acid-Induced Innate Immune Responses of Human Keratinocytes, *Front Immunol* 8:1053 (2017)
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III. M. Széll, **J. Danis**, Z. Bata-Csörgő, L. Kemény: PRINS, a primate-specific long non-coding RNA, plays a role in the keratinocyte stress response and psoriasis pathogenesis, *Pflug Arch Eur J Phys* 468(6), p. 935–943 (2016) *[Invited Review]*
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Publications not directly related to the thesis

IV. **J. Danis**, M. Széll: Functional relevance of pyknons in tumor formation, *Non-coding RNA Investig* 2:3 (2018) *[Invited Editorial]*

V. **J. Danis**, M. Széll: VELUCT, a long non-coding RNA with an important cellular function despite low abundance, *J Thorac Dis* 9(10):3638-3640 (2017) *[Invited Editorial]*

VI. A. Göblös, **J. Danis**, K. Vas, Z. Bata-Csörgő, L. Kemény, M. Széll: Keratinocytes express functional CARD18, a negative regulator of inflammasome activation, and its altered expression in psoriasis may contribute to disease pathogenesis, *Mol Immunol* 73, p. 10–18. (2016)
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VII. **Danis J.**, Forczek E., Bari F.: A telemedicina alkalmazása a bőrgyógyászatban: a teledermatológia [Telemedicine in dermatological practice: teledermatology] *Orv Hetil* 157:(10) pp. 363-369. (2016)
IF:0.349
(Independent citation: 0 Self citation: 1 Cumulative: 1)

VIII. **J. Danis**, T. Turányi: Sensitivity Analysis of Bacterial Chemotaxis Models, Procedia Computer Science 7 p.233-234. (2011)
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Tartalomjegyzék

1. Introduction

1.1 The skin

As the interacting surface between the body and the environment, the skin provides a physical and biochemical barrier and a sensory-receptive area, ensures adequate hydration and is responsible for the synthesis of vitamins and hormones, moreover actively defends the body against a variety of environmental, chemical and physical stimuli by acting as an active member of the innate immune system (1). The fine structure of the skin shows considerable regional variations in epidermal and dermal thickness, distribution of epidermal appendages and melanocyte content. Anatomically the skin can be divided into three layers, the epidermis and the dermis divided by the basal membrane, an extracellular matrix that is rich in type IV collagen and laminin and the subcutaneous tissue or hypodermis (2). The subcutaneous tissue is responsible for attaching the skin to the underlying bone and muscle and supplies the above layers with blood vessels and nerves. The subcutaneous tissue consists of loose connective tissue with fibroblasts, macrophages and adipocytes (3).

The dermis consists of connective tissue and harbors mechanoreceptors that provide the sense of touch and heat through nociceptors and thermoreceptors. It also contains the hair follicles, sweat glands, sebaceous glands, apocrine glands, lymphatic vessels and blood vessels (1).

The epidermis is composed mainly by keratinocytes, which undergo a specific differentiation program that determines a partition of epidermis into five layers (Fig. 1.). The innermost layer, called “stratum basale” or “stratum germinativum” is made up by keratinocytes that are still adherent to the basement membrane by hemidesmosomes and undergo constant proliferation forming the second layer, named “stratum spinosum”. Keratinization is initiated in this layer by the production of lamellar bodies through the Golgi apparatus. The third layer is the “stratum granulosum”, where the keratinocytes lose the nucleus and release the lamellar bodies to create a lipid barrier. The fourth layer, “stratum lucidum” is only present in palms and soles. The uppermost layer of the epidermis and the skin, “stratum corneum” is formed by keratin-filled dead keratinocytes (corneocytes) that had been covered by keratin and serve as the physical and biochemical barrier against external factors. The epidermis host not only

keratinocytes, but immune cells, such as Langerhans-cells and lymphocytes and cells of the peripheral nervous system (Merkel cells) (1,2).

The fine structure of the skin shows remarkable alterations in chronic skin diseases. In psoriasis the increased mitotic rate and premature maturation of keratinocytes leads to epidermal thickening (acanthosis) and the incomplete cornification of the cells leads to the retention of nuclei in the stratum corneum (parakeratosis) and the reduction or absence of the stratum granulosum (Fig. 1.). The redness of the lesion is due to the increased angiogenesis in the lesions, which allows for a greater influx of inflammatory cells into the dermis (4).

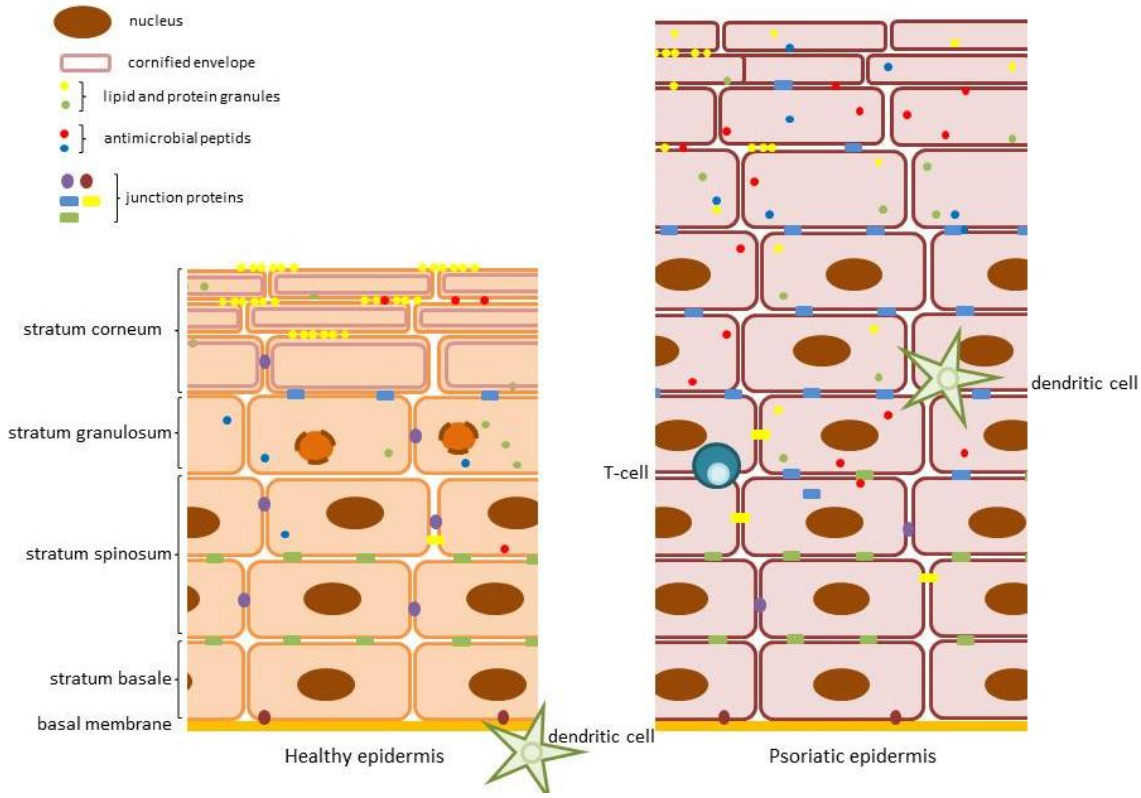

Figure 1. The structure of the epidermis in healthy and psoriatic conditions. In the healthy epidermis the proliferative keratinocytes of the stratum basale constantly proliferate and the daughter cells are moving up and form the second layer, named stratum spinosum. In the stratum granulosum keratinocytes lose their nucleus. The uppermost layer stratum corneum is formed by dead keratinocytes filled with keratin-filaments forming the cornified envelope. In psoriasis immune cells infiltrate the epidermis, while the increased mitotic rate of keratinocytes leads to epidermal thickening and the disturbed maturation leads to the retention of the nuclei in the stratum corenum.

1.1.1 Immune functions of keratinocytes

The epidermis not only forms a physical and chemical barrier, but also an immune barrier against the invading pathogens. Besides skin resident professional immune cells in the epidermis e.g. Langerhans-cells, keratinocytes are also immunocompetent cells. They express a wide range of pattern recognition receptors (PRRs) and are responsive to various pathogen and danger associated molecular patterns (PAMPs and DAMPs) (6–9). These receptors make keratinocytes the first immune sentinels against pathogens, through their quick and efficient sensing and response. Keratinocytes express different Toll-like receptors (TLR), RIG-like (RLRs) receptors, and NOD-like (NLR) receptors recognizing a wide range of microbial compounds (7–11). Activation of the receptors triggers the induction of nuclear factor kappa B (NF-κB) and interferon regulatory factor, which induce the expression of inflammatory genes, mainly tumor necrosis factor α (TNF-α) and type I interferons (IFN). NLRs and PYHIN proteins form multiprotein complexes, called inflammasomes, which are responsible for cleaving the unprocessed pro-interleukin (IL) -1β and pro-IL-18 forms into mature, biologically active cytokines (12). Unlike professional immune cell types, keratinocytes express the precursors of these cytokines constantly, which in contact with the inflammasome activators instantly get activated and secreted, enabling the neighboring cells to respond by increased inflammatory cytokine production (13–15). The overactivation of these innate immune responses and the dysregulated interplay of the innate and adaptive immune responses can lead to chronic inflammatory diseases, such as psoriasis (4).

1.1.2 Nucleic acid fragment induced immune responses in keratinocytes

RNA and DNA fragments are derived mainly from the genetic material of various bacterial and viral pathogens and as PAMPs activate the immune reactions of keratinocytes. The range of cell entry and replication mechanisms led to a wide range of sensors for nucleic acids, which are mainly endosomal and cytosolic receptors. Since the microbial genomes are built from the same nucleotides as the host cell’s, small differences in their size and chemical modifications, as well as intracellular localization are used to distinguish between self and foreign (16). Recent evidence indicate that in response to infection mitochondrial DNA can be released into the cytosol as DAMPs, contributing to the innate immune response (17). Moreover, the incomplete degradation of the genomic material of the dying cells can lead to

the accumulation of nucleic acid fragments in the intracellular space. These self-derived fragments are also recognized as DAMPs, leading to a prolonged inflammation in chronic inflammatory diseases (14,18).

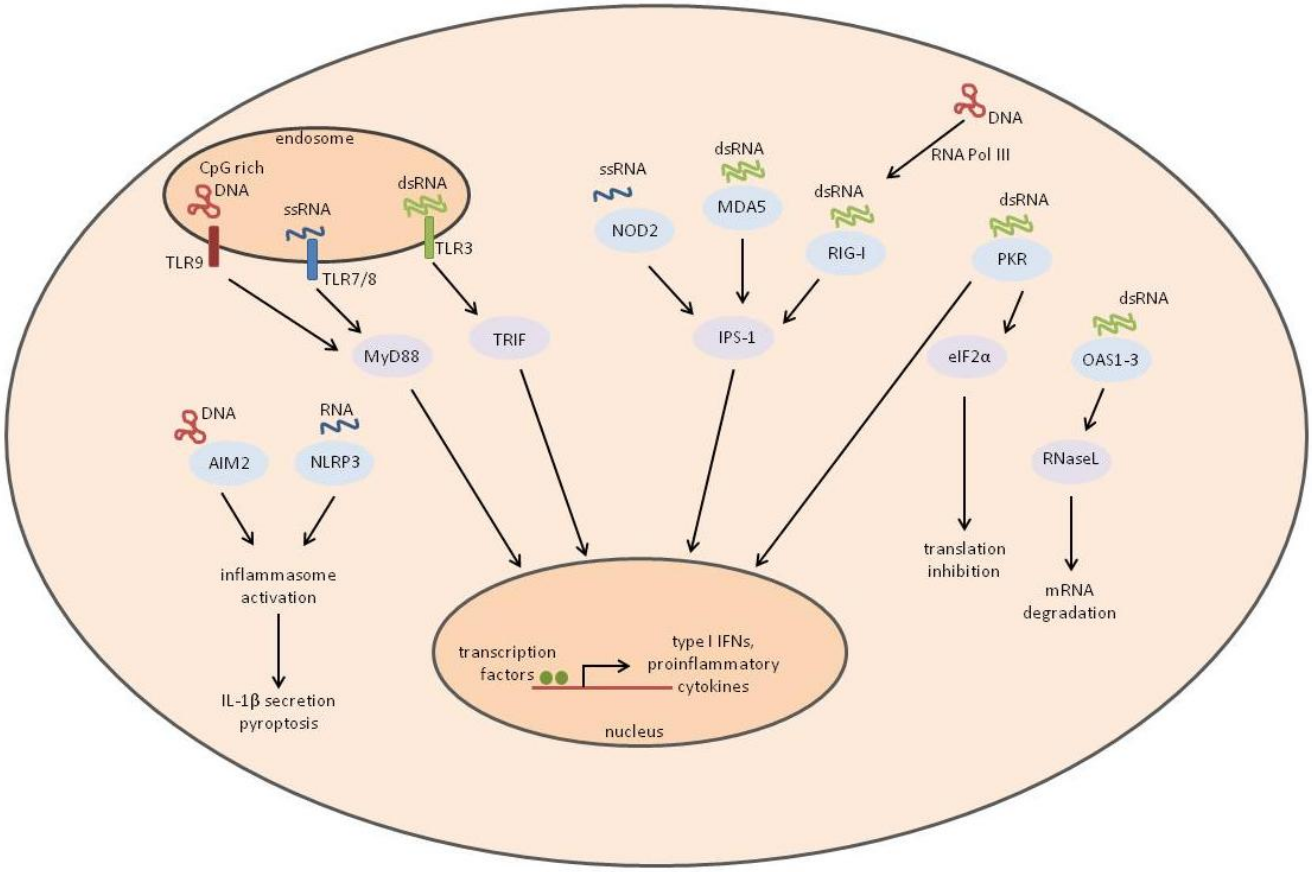

Figure 2. Cellular RNA and DNA sensors. Receptors in the endosome (TLR9, TLR7/8, TLR3) and cytosolic receptors (light blue) are activated by DNA and RNA fragments and through the activation of adaptor molecules (light purple) leads to the transcription of proinflammatory cytokines and type I interferons (IFNs). dsRNA – double-stranded RNA, dsDNA – double-stranded DNA, ssRNA – single-stranded RNA, TLR – Toll-like receptor, NOD2 – nucleotide oligomerization domain, RIG-I – retinoic acid inducible gene I, MDA5 – melanoma differentiation associated gene-5, PKR – protein kinase RNA activated, OAS1-3 – 2′-5′-oligoadenylate synthetase, AIM2 – absent in melanoma 2, NLRP3 – NOD-like receptor 3, MyD88 – myeloid differentiation primary response 88, TRIF – TIR (Toll/interleukin-1 receptor) domain-containing adaptor protein inducing interferon β, IPS-1 – interferon-beta promoter stimulator 1, eIF2α – eukaryotic translation initiation factor 2α, RNaseL – ribonuclease L, RNA Pol III – RNA polymerase III. Figure is based on Olejniczak et al, 2012 (19).

Nucleic acid induced reactions have been mainly studied by using synthetic RNA analogue poly(I:C) and DNA analogue poly(dA:dT), which both induce type I interferon (IFN-α/β) and inflammatory cytokine expression in keratinocytes (11,20–23). Poly(I:C) is recognized primarily by toll-like receptor 3 (TLR3) (24), although, TLR3-independent sensing of poly(I:C) has also been observed with involvement of retinoic acid induced gene I (RIG-I) and melanoma differentiation-associated gene 5 (25). Poly(dA:dT) recognition partially overlaps with poly(I:C) recognition, since RIG-I serves as a receptor after poly(dA:dT) has been transcribed by RNA polymerase III into double-stranded (ds) RNA molecules (26). Cyclic GMP-AMP synthase, a newly described cytosolic DNA receptor implicated in antiviral responses, binds dsDNA sequences independently and activates interferon regulatory factor 3 in cooperation with interferon-γ-inducible protein 16 (27). Poly(dA:dT) is the inducer of the absent in melanoma 2 (AIM2) inflammasome (14,28), while poly(I:C) was described as an activator of the promiscuous signal activated NOD-like receptor pyrin domain containing 3 (NLRP3) inflammasome (29), leading to a subsequent IL-1β release in keratinocytes.

The basal expression of most inflammatory cytokines in keratinocytes is low and is regulated in response to stimuli at the transcriptional level. The activated PRRs induce signal transduction pathways of the NF-κB, mitogen activated protein kinases (MAPK) and signal transducers of activator of transcription (STAT) signal transduction pathways, which have been reported to participate in nucleotide-induced inflammatory cytokine expression in several cell types (20,23,26,29,30). Poly(I:C) was found to induce NF-κB, p38, MAPK and STAT-1 signaling in keratinocytes, whereas, in melanocytes, poly(dA:dT) induces NF-κB, p38 and c-Jun N-terminal kinase (JNK) signaling, which differentially regulates cytokine expression (20,23).

1.1.3 Altered keratinocyte functions in psoriasis

Psoriasis is a chronic inflammatory skin disease caused by the deregulated interplay of professional immune cells and keratinocytes. The disease affects approximately 2-3 % of the Caucasian population. The etiology of the disease is complex, with the evidence that the interplay of environmental and life-style factors in individuals carrying multigenic susceptibility loci leads to the development of the symptoms. Psoriatic symptoms characterized by scaly, red, well-demarcated plaques on the skin, which can be triggered by

mild trauma, such us scratching or sunburn, chemical irritants and infections, but some systemic drugs can also initiate these symptoms (4).

One of the endogenous triggering factors thought to initiate the disease are self-derived cytosolic nucleotide fragments (11,13,14,18). Receptors for RNA and/or DNA fragments (10,11,31), moreover their activators, self-derived RNA and DNA fragments and RNA:DNA duplexes are highly abundant in the lesional epidermis (14,32). During normal cornification, keratinocytes express deoxyribonucleases (DNases) (33); however, it was recently shown that reduced keratinocyte DNase activity in psoriasis results in suppressed DNA degradation and, as a consequence, parakeratosis (34) and the presence of excess DNA fragments in the cytosol. Similarly, disturbed ribonuclease activities were described in psoriatic skin (35,36), which might result in excess RNA fragments. In the initiation phase, an antimicrobial peptide cathelicidine (LL-37) was shown to form a complex with the excess nucleotide fragments in the epidermis, and activate the pro-inflammatory responses of plasmacitoid dendritic cells (pDCs) and keratinocytes. The activated pDCs migrate to the lymph nodes and activate the naïve helper T cells (T_h cells), such as $T_{h1,}$ T_{h17}, which migrate to the dermis guided by keratinocyte derived cytokines and chemokines. The immune reactions initially induced by nucleotide fragments in keratinocytes are supported by the T_{h1} and T_{h17} derived cytokines and stimulate the hyperproliferation of keratinocytes.

The keratinocytes of the non-involved psoriatic epidermis already carry inherited changes in their molecular patterns compared to keratinocytes of the healthy epidermis, and are oversensitive to proliferative signals, which contribute to the development of the disease (37). The aim of our workgroup is to characterize these abnormal molecular patterns in the psoriatic non-lesional epidermis. To this end we previously performed a differential display (38,39) and a cDNA microarray (40) experiment comparing the gene expression of psoriatic non-involved and healthy epidermis. These experiments revealed deregulated extracellular matrix expression (38), identified a yet unknown long non-coding RNA, named PRINS (39), revealed splicing disturbances (41) and showed altered expression of inflammatory mediators IL-1β and IL-23 (40) and the inflammasome regulator caspase recruitment domain family member (CARD) 18 (28).

1.2 Non-coding RNAs

With the completion of the human genome project, it has become obvious that protein-coding genes comprise only 2% of the genome, although the majority of the genome is transcribed into RNA. RNA molecules that lack protein-coding potential are collectively referred to as non-coding (nc) RNAs. In addition to the well-known housekeeping rRNAs, tRNAs and small nuclear RNAs (snRNAs), the most intensively studied subgroup of ncRNAs are the microRNAs (miRNAs), which are well characterized by their size and uniform function. Non-coding RNAs larger than 200 nucleotides are referred to as long ncRNAs (lncRNAs). They are markedly heterogeneous in size and cellular function, thus their classification is rather complicated (42,43). The varying and still evolving taxonomy of lncRNA reflects the novelty of the field.

Currently lncRNA biology faces two challenges: the validation of lncRNAs identified by large-scale gene expression studies and the confirmation of their functionality in health and in disease. There is still a debate whether lncRNAs are functional or just transcriptional noise rapidly removed by cellular quality control mechanisms (44). Although various criteria, including expression levels, splicing variants and sequence conservation, have been proposed to differentiate between functional and non-functional lncRNAs, a consensus has not yet been achieved. The number of lncRNA genes (>70 000) in the human genome outnumbers protein-coding genes (<20 000) (45), but studies of their functional roles and detailed mechanisms account for less than 0.1% of all predicted lncRNAs (43). Thus an enormous effort is still needed to determine their specific functions, which is made more difficult by the low-level expression seen for most lncRNAs (45) and high inter-individual differences in lncRNA expression (46).

In the last decade, genome-scale transcription studies have uncovered non-coding RNAs as previously unrecognized players in the dysregulation of inflammatory functions (47–50) and disease pathogenesis (51–55).

1.2.1 PRINS long non-coding RNA

Our research group was the first to identify an lncRNA potentially contributing to psoriasis susceptibility: PRINS, the *psoriasis susceptibility related non-coding RNA induced by stress*. PRINS was identified by a differential display experiment as highly expressed in psoriatic

non-lesional epidermis compared to healthy epidermis (39). The 3’ end of the PRINS transcript is identical to the AK022045 RNA sequence. Real-time RT-PCR data confirmed that the highest PRINS expression can be found in psoriatic non-lesional epidermis, while there is a gradual decrease in its expression in psoriatic lesional epidermis and healthy epidermis, which suggested its possible contribution to psoriasis susceptibility (39). The expression of PRINS was found to be modified by a diverse set of cellular stressors, including starvation, ultraviolet B (UVB) irradiation, translation inhibition (56) and hypoxia (57), and silencing of PRINS in HaCaT cells during stress exposure decreased cellular viability (39). These results suggested its contribution to cellular stress responses.

Using various bioinformatical tools, we have analyzed genomic features of the PRINS gene (Fig. 3. A), which is located on the short arm of human chromosome 10 (map position 10p12.31) and is composed of two exons and an intron of approximately 7 kb in length (58).

The entire PRINS lncRNA resides in an intron of the recently annotated KIAA1217 gene, also known as SKT, which is involved in early stages of embryogenesis (59). Proximal to KIAA1217 is the OUT deubiquitinase 1 gene, whereas distal to KIAA1217 is the Rho GTPase activating protein 21. Interestingly, the miR603 miRNA is located in a KIAA1217 intron, 3' of the PRINS coding region. A transcription start site was identified 6 kb proximal to the putative 5' end of the PRINS gene using the ENCODE database (60). This region is marked by a high density of binding sites for several transcription factors, including GATA2, Fos, HDAC2 and STAT-3, and histone modification sites associated with active transcription, such as mono- and tri-methylation of lysine 4 of histone H3 (H3K4me1/3) and acetylation of lysine 9 and 27 (H3K9Ac, H3K27Ac), suggesting that a strongly regulated active promoter might be associated with the lncRNA. The region adjacent to the 3' end of the PRINS lncRNA gene also contains histone modification sights, which, due to the close 3' proximity to the PRINS lncRNA gene, might be an enhancer element. Approximately two thirds of functioning human lncRNAs contain at least one element derived from a transposable element, which are seldom found in protein-coding genes (61). This is also true for the PRINS lncRNA, which contains three *Alu* elements comprising approximately one third of its sequence (Fig. 3. B). It is well established that transposable elements have been very important in the evolution of lncRNAs (62). PRINS lncRNA gene is most probably a primate-specific sequence and transposition was the major mechanism of its origin. Orthologues could

be found only in the genomes of primates with variations in the extent of similarity (Fig. 3. B). PRINS and the orthologues with the highest similarity among primate species reside on the short arm of chromosome 10.

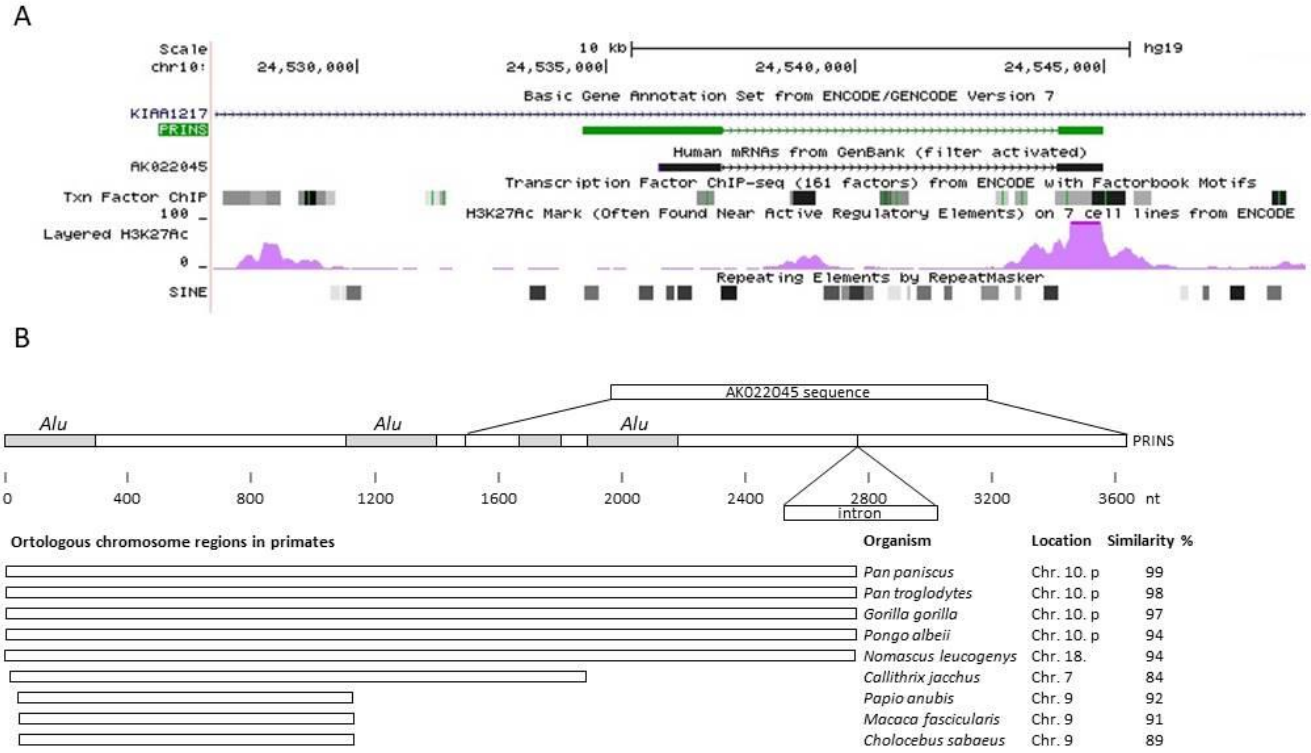

Figure 3. Major characteristics of the PRINS lncRNA identified by USCS Genome Browser (A) and NCBI BLAST (B). (A) PRINS gene is located in an intron of the KIAA1217 gene on Chromosome 10. The 3' end of the PRINS gene sequence is identical with the AK022045 RNA sequence. High density of transcription factor binding sites (Txn Factor ChIP) and acetylation of Histon H3 lysine 27 (H3K27Ac) in NHEKs was identified approximately 6 kb upstream from the PRINS gene and at the 3' end of the gene, suggesting an active transcription. Short interspersed nuclear elements (SINE) are found on the sequence of PRINS, which were identified by BLAST **s**imilarity search as *Alu* elements within the PRINS lncRNA sequence (B). The PRINS gene is localized on the p12.1 arm of human Chr. 10, which is highly conserved in human and four other primate species. Although partial sequence similarity was found, it is largely due to the conservation of *Alu* elements in other primate species, where the PRINS sequence was distributed on other chromosomes.

To identify interacting partners of PRINS, a cDNA microarray (63) and an *in vitro* protein binding assay (64) were previously carried out. The cDNA microarray identified the interferon-α inducible protein 6 (IFI6, also known as G1P3), which was downregulated in HeLa cells by PRINS silencing (63). The *in vitro* protein binding assay identified nucleophosmin (NPM), as a protein binding to PRINS. Functional studies showed, that silencing of PRINS resulted in alterations of UVB induced shuttling of NPM, showing the physical and functional interaction of PRINS and NPM (64). The subcellular localization of PRINS and the NMP protein also facilitates these functions. PRINS lncRNA is mainly localized in the nucleolus of normal human cultured keratinocytes; although, moderate perinuclear and cytoplasmic expression was also detected by *in situ* hybridization (64). This is in good agreement with reports that non-coding RNAs localize mainly to the nucleus (65). The PRINS sequence might also determine its cellular localization, as it includes the AGCCC pentamer with the sequence restrictions at positions –8 (T or A) and –3 (G or C) of a motif which was reported to be crucial for nuclear localization of lncRNAs (66). Our previous *in situ* hybridization results are in good agreement with this sequence-based analysis (64).

Together, the results from our previous experiments indicate that the evolutionarily young, primate- specific PRINS is one of the lncRNAs differentially expressed in psoriasis (51), and it plays a role in keratinocyte stress response. Its elevated expression in psoriatic non-lesional epidermis might contribute to the altered stress response of psoriatic keratinocytes and, thus, to disease pathogenesis.

Recent publications on PRINS non-coding RNA supported its involvement in inflammatory cellular processes. Microbial stimuli decreased PRINS expression in macrophages (67) and normal human epidermal keratinocytes (NHEKs) (56). Moreover PRINS was recently shown to potentially interact with chemokine (C-C motif) ligand 5 (CCL-5, also known as RANTES) in kidney epithelial cells (57). PRINS was also shown to be involved in the resistance against TNF-α induced apoptosis of colorectal cancer cells (68). These results suggest the possible contribution of PRINS to inflammatory processes.

2. Aims

Our aims were to characterize nucleotide fragments induced immune responses in human keratinocytes by studying multiple regulatory layers of these processes. Therefore, we aimed to

- characterize the nucleotide fragment induced cytokine expression profile of different human keratinocyte cell types
- analyze the nucleotide fragment induced signal transduction pathways, and how they contribute to the nucleotide fragment induced cytokine expression in keratinocytes
- determine how dsDNA fragments affect the gene expression of PRINS long non-coding RNA in human keratinocytes
- determine how PRINS long non-coding RNA can affect the dsDNA induced cytokine expression in human keratinocytes

3. Materials and Methods

3.1 Cell cultures

Normal human epidermal keratinocytes (NHEKs), the HaCaT cell line (69) and the HPV-KER cell line (70) were used for the experiments. NHEKs were separated from skin specimens obtained from the Plastic Surgery Unit of our Department. Written informed consent was obtained from all investigated individuals. The study was approved by the Human Investigation Review Board of the University of Szeged and complied with the ethical standards of research and in accordance with the Helsinki Declaration. The epidermis was separated from the dermis with overnight incubation in dispase (Roche Diagnostics, Manheim, Germany), and keratinocytes were obtained after maceration in 0.25% trypsin. All cell types were grown in 75 cm^2 cell culture flasks. NHEKs and HPV-KER cells were maintained in keratinocyte serum-free medium containing epidermal growth factor and bovine pituitary factor (Gibco Keratinocyte SFM Kit; Life Technologies, Copenhagen, Denmark) and supplemented with 1% antibiotic/antimycotic solution (PAA Laboratories GmBH, Pasching, Austria) and 1% L-glutamine (PAA Laboratories). HaCaT cells were grown in DMEM with 4.5 g/l glucose supplemented with 1% antibiotic/antimycotic solution, 1% L-glutamine and 10% fetal bovine serum. Cells were maintained at 37°C in a humidified atmosphere with 5% CO_2. The medium was changed every 2 days.

3.2 Stimulation of the cells

HPV-KER cells, HaCaT cells or third passage NHEKs were seeded into 6-well plates at a density of 200 000 cells/ml. Cells were transfected with 0.666 μg/ml polydeoxyadenylic acid-polydeoxythymidylic acid double-stranded homopolymer [poly(dA:dT)] (InvivoGene, San Diego, CA, USA) or with 0.666 μg/ml polyinosinic–polycytidylic acid [poly(I:C)] (Sigma Aldrich, Saint Louis, MO, USA) using the X-tremeGene 9 transfection reagent (Roche Diagnostics). Cells were harvested at indicated time points. In some cases, before transfection with poly(dA:dT), cells were primed in supplement-free medium by addition of 5ng/ml tumor necrosis factor-α (TNF-α) and 5ng/ml interferon-γ (IFN-γ) for 24 hours, as indicated in Figure legends.

For inhibition studies, cells were incubated 1 hour prior to poly(dA:dT)/poly(I:C) transfection with inhibitors for NF-κB (Bay11-7085, 10 μM; MedChem Express, Monmouth Junction, NJ, USA), STAT-1 (Fludarabine, 10 μM; Sigma Aldrich), STAT-3 (Stattic, 5 μM; Sigma Aldrich), MEK1/2 (PD98059, 20 μM; Sigma Aldrich), JNK (SP600125, 10μM; Tocris Bioscience, Bristol, UK) and p38 (SB203580, 10 μM; Tocris Bioscience).

3.3 RNA isolation and RT-PCR

Total RNA was isolated from cells using TRIzol® Reagent (Invitrogen Corp., Carlsbad, CA, USA), following the manufacturer's instructions. Turbo DNA-free Kit (Ambion, Life Technologies) was used for the removal of contaminating DNA. cDNA was synthesized from 1 μg total RNA using 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 (Roche Diagnostics) using a C1000 Touch Thermal Cycler (Bio-Rad Laboratories), using primers listed in Table 1. The expression of each gene was normalized to the expression of the 18S rRNA gene. Relative mRNA levels were calculated by the ΔΔCt method.

Table 1. Primers and probes used for real-time RT-PCR experiments

Manufacturer	mRNA	Strand	Primer sequences	Probe no.
Roche Diagnostics - Universal Probe Library	18S rRNS	Forward	CGC TCC ACC AAC TAA GAA CG	77
		Reverse	CTC AAC ACG GGA AAC CTC AC	
	TNF-α	Forward	CAG CCT CTT CTC CTT CCT GAT	29
		Reverse	GCC AGA GGG CTG ATT AGA GA	
	IL-1α	Forward	GGT TGA GTT TAA GCC AAT CCA	6
		Reverse	TGC TGA CCT AGG CTT GAT GA	
	IL-1β	Forward	AAA GCT TGG TGA TGT CTG GTC	10
		Reverse	AAA GGA CAT GGA GAA CAC CAC T	
	IL-6	Forward	CAG GAG CCC AGC TAT GAA CT	45
		Reverse	GAA GGC AGC AGG CAA CAC	
	CCL5	Forward	TGC CCA CAT CAA GGA GTA TTT	59
		Reverse	CTT TCG GGT GAC AAA GAC G	
Thermo Fischer Scientific	IL-8	TaqMan Gene Expression Assays		Hs00174103_m1
	PRINS	TaqMan Gene Expression Assays		Hs03671803_s1
	TNF-α	TaqMan Gene Expression Assays		Hs00174128_m1

3.4 Detection of NF-κB induction

Luciferase assays were performed to determine the NF-κB activity in response to poly(I:C) and poly(dA:dT) treatment. The HPV-KER cell line was transfected with the NF-κB reporter construct vector pNF-κB-luc Cis-Reporter Plasmid (Stratagene, La Jolla, CA, USA) and the pGL4.75 [hRluc/CMV] plasmid (Promega, Madison, WI, USA) with the use of the X-tremeGene9 transfection reagent. The treated cells were washed twice with PBS, lysed with passive lysis buffer (Biotium, Hayward, CA, USA) and the luciferase activities in the lysates were measured using the Firefly & Renilla Dual Luciferase Assay Kit (Biotium) and Thermo Luminoskan Ascent (Thermo Scientific, Rockford, IL, USA), according to the manufacturer's instructions. All samples were measured three times and the luciferase activity derived from the NF-κB-luc plasmid was normalized to the activity of the Renilla luciferase activity from pGL4.75 [hRluc/CMV] plasmid.

3.5 Western blot analysis

HPV-KER cells were harvested at indicated time points after poly(dA:dT) or poly(I:C) transfection and lysed in lysis buffer supplemented with 0.5% SDS and 1% Halt™ Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific). Equal amounts of protein in ProTrack Loading Buffer (Lonza, Basel, Switzerland) were separated on a 7.5% TGX Fast Cast Gel and transferred to nitrocellulose membrane (0.45 μm; Bio-Rad Laboratories). After blocking the membrane in 5% non-fat milk in Tris-Buffered Saline containing 0.2% Tween-20, primary antibodies were incubated overnight at 4°C with constant agitation. HRP-conjugated secondary antibodies were incubated for 60 minutes at room temperature (Table 2.). Signal was visualized with SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Scientific) on a C-Digit Blot Scanner (LI-COR Corp. Lincoln, NE, USA).

Table 2. List of primary and secondary antibodies used for western blot experiments.

Primary antibody	Manufacturer	Dilution	Secondary antibody	Manufacturer	Dilution
anti-actin	Sigma Aldrich	1:2000	anti-rabbit HRP	Southern Biotech	1:2000
anti-phospho-IκBα	Santa Cruz Biotechnology	1:1000	anti-mouse HRP	Southern Biotech	1:2000
anti-phospho-ERK1/2	BioLegend	1:1000	anti-mouse HRP	Southern Biotech	1:2000
anti-phospho-STAT-1 (S727)	Cell Signaling Technology	1:1000	anti-rabbit HRP	Southern Biotech	1:2000
anti-phospho-STAT-3 (S727)	Cell Signaling Technology	1:1000	anti-rabbit HRP	Southern Biotech	1:2000
anti-phospho-JNK (T183/Y185)	Bio-Techne	1:500	anti-rabbit HRP	Southern Biotech	1:2000
anti-phospho-p38 alpha (T180/Y182)	Bio-Techne	1:1000	anti-mouse HRP	Southern Biotech	1:2000

3.6 Gene-specific overexpression

For overexpressing PRINS, the AK022045 cDNA sequence (Biological Resource Center (NBRC) National Institute of Technology and Evaluation, Chiba, Japan) was cloned into a pcDNA3.1(+) vector. The empty pcDNA3.1(+) vector served as a control.

The ΔPRINS construct was created by GeneArt gene synthesis (Thermo Scientific) by replacing the AK022045 region (position 538–622) with the following scrambled sequence, and cloned into a pcDNA3.1(+) vector: 5’-GTGCGTGGCGGAGACGTGGTGGTAGAC CGAATTGAGGAGGATCCGAAGGTTAGACGTAGGCGATCGCCGCTTCGGACGCGG TCGC-3’.

Transient transfection of NHEK cells was carried out at approximately 70% confluency in parallel to cytokine priming, using the X-tremeGENE HP DNA transfection protocol, as described by the manufacturer (Roche Diagnostics). The transfection efficiency was 85% on average, as determined by the transfection of a GFP reporter construct (Lonza, Basel, Switzerland) and analysis of GFP expression by flow cytometry. The effectiveness of overexpression was investigated with real-time RT-PCR (Fig. 4.).

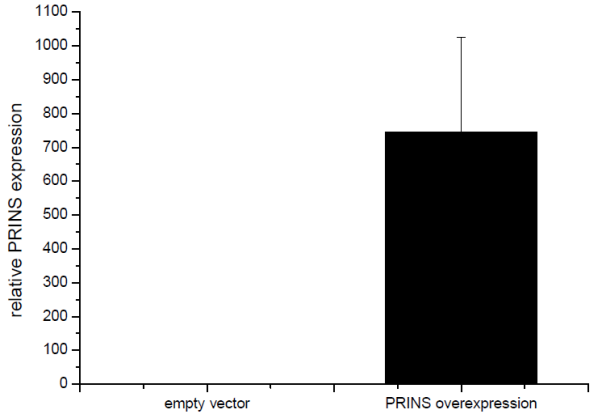

Figure 4. The evaulation of the effectiveness of PRINS overexpression by real-time RT-PCR. NHEKs were transfected with a pcDNA3.1(+) vector containing the PRINS cDNA, while the empty pcDNA3.1(+) vector was used as control. The PRINS expression of the empty vector transfected samples displays the basal expression of PRINS in keratinocytes with Δc_T~33±3, while after overexpression of PRINS Δc_T~24±3.

3.7 *In silico* prediction of interacting sites

Sequence complementarity between PRINS (AK022045) and the mRNA of IL-1α (M28983.1), IL-1β (NM_000576.2), IL-6 (NM_000600.4), IL-8 (NM_000584.3), TNF-α (NM_000594.3) and CCL-5 (NM_002985.2) was analyzed using two algorithms: RIsearch (71), which uses a simplified nearest-neighbor energy model, and INTARNA (72–74), which calculates the free-energy values of the interaction based on predicted global and local structures of mRNAs. The regions predicted by both programs were considered as putative interaction sites.

3.8 *In vitro* transcription and *in vitro* binding assay

PRINS and ΔPRINS RNA sequences were produced by *in vitro* transcription from pcDNA3.1(+) containing the AK022045 or ΔPRINS cDNA sequence, using Transcript Aid T7 In Vitro Transcription Kit (Thermo Scientific). Products were purified by the GeneJET RNA Purification Kit (Thermo Scientific). Quality and sequence-length analyses were carried out on reducing agarose gel-electrophoresis. The single-stranded RNA products were used in a fluorescent binding assay. The fluorescently labeled RNA sequence 5’6-carboxyfluorescein(6-FAM)/GAAGCUCUAUCUCCCCUCCAGGAGCCCAGCUAUGAAC

UCCUUCUCCACAAGCGCCUUCGGUCCAGUUGCCUUCUCCCUGGGGCUGCUCCUG GUGUUGCCUGCUGCCUUCCCUGCC-3', comprising positions 91–205 of the IL-6 (NM_000600.4) mRNA sequence, was produced by Integrated DNA Technologies.

An *in vitro* binding assay was carried out on a Monolith NT.115 Pico MicroScale Thermophoresis instrument (NanoTemper GmbH, Germany), in nuclease-free water, at 25°C, with 80% Laser Power, 10% LED Power, by 2bind GmbH, Regensburg, Germany. Fluorescence enhancement of the 6-FAM labeled specific truncated IL-6 RNA sequence or a 6-FAM labeled unspecific DNA, as negative control, was measured after addition of PRINS or ΔPRINS. The concentration of fluorescently labeled molecules (c_A) was 10 nM constantly, while unlabeled RNA concentration (c_T) ranged from 126.75 nM to 61.9 pM. Initial fluorescence was analyzed for binding curves by the following formulation based on a 1:1 binding model:

$$A + T \leftrightarrow AT$$

$$F(c_T) = F_u + (F_b - F_u) * \frac{c_{AT}}{c_A}$$

$$\frac{c_{AT}}{c_A} = \frac{1}{2c_A} * \left(c_T + c_A + K_d - \sqrt{(c_T + c_A + K_d)^2 - 4c_T c_A}\right)$$

F_u fluorescence intensity in unbound state

F_b fluorescence intensity in bound state

c_A concentration of fluorescent molecule (constant)

c_T concentration of

c_{AT} concentration of formed complex

K_d dissociation constant

$\frac{c_{AT}}{c_A}$ fraction bound

3.9 ELISA

Cell supernatants were centrifuged (5000 rpm, 4 min, 4°C) to pellet cell debris and the amount of cytokines IL-1α, IL-1β, IL-6, IL-8, CCL-5 and TNF-α was determined by ELISA (IL-1α Duo Set, IL-1β ELISA Duo Set and TNF-α Duo Set, Biotechne; Human IL-6 Mini

TMB ELISA Development Kit, Human IL-8 TMB ELISA Development Kit, Human RANTES ATBS ELISA Development Kit, PeproTech, Rocky Hill, NJ, USA), according to the manufacturer's instructions.

3.10 Statistical analysis

Experiments were carried out in triplicates with at least three biological repeats, as indicated in Figure legends. For statistical analysis, one-way or two-way repeated measurement analysis of variance (ANOVA) was used to compare more than two groups, and one-tailed, paired T-test was used to compare two groups, as indicated in the Figure legend. In case of a non-normal distribution, nonparametric tests were used (Kruskal-Wallis test and Wilcoxon-test). Statistical analysis was carried out using R software Ver 3.2.2. and Sigma Plot Ver. 13.0, the significance level was set at $p \leq 0.05$.

4. Results

4.1 Keratinocytes respond to cytosolic nucleotide exposure with increased cytokine expression

Initially we compared dsRNA- and dsDNA-induced cytokine-expression profiles in three keratinocyte cell types: normal human epidermal keratinocytes (NHEKs), the HaCaT cell line (69) and the HPV-KER cell line (70). These results helped us to determine which cell type is best suited to study keratinocyte inflammatory reactions, and provided us details about the kinetics of poly(I:C) and poly(dA:dT) induced cytokine expression. Poly(I:C) strongly induced IL-6 and TNF-α expression in all three cell types, and poly(dA:dT) induced expression in all cell types with slightly different kinetics as well as expression that was an order of magnitude lower than that observed with poly(I:C) (Fig. 5.). Peak expression was observed 3 to 6 hours after poly(I:C) transfection, whereas peak expression after poly(dA:dT) transfection occurred 6 to 12 hours after treatment in all cell types studied. Reaction to poly(I:C) was much faster in HaCaT cells than the other cell types studied. In contrast, the kinetics of poly(dA:dT)-induced reaction did not differ in the three cell types. In agreement with our previous findings (70), the HPV-KER cell line and NHEK cells exhibited similar cytokine mRNA expression kinetics upon poly(I:C) and poly(dA:dT) induction.

Detailed studies on poly(I:C) induced inflammatory reactions of keratinocytes were previously conducted (11,20,30,75), but analysis of poly(dA:dT) induced reactions in keratinocytes was mostly reduced to the study of inflammasome activation (13,14,28). Therefore, we analyzed the expression and secretion of several inflammasome independent cytokines in NHEKs upon poly(dA:dT) treatment. We confirmed that poly(dA:dT) significantly induced the expression and secretion of IL-1β in keratinocytes (Fig. 6. D, I), as described previously (4). In addition, poly(dA:dT) induced very strong mRNA expression of IL-1α, IL-6, IL-8 and tumor necrosis factor (TNF)-α in NHEKs (Fig. 6. A-E). In a manner similar to mRNA expression, poly(dA:dT) transfection induced the secretion of the investigated cytokines (Fig. 6. F-J).

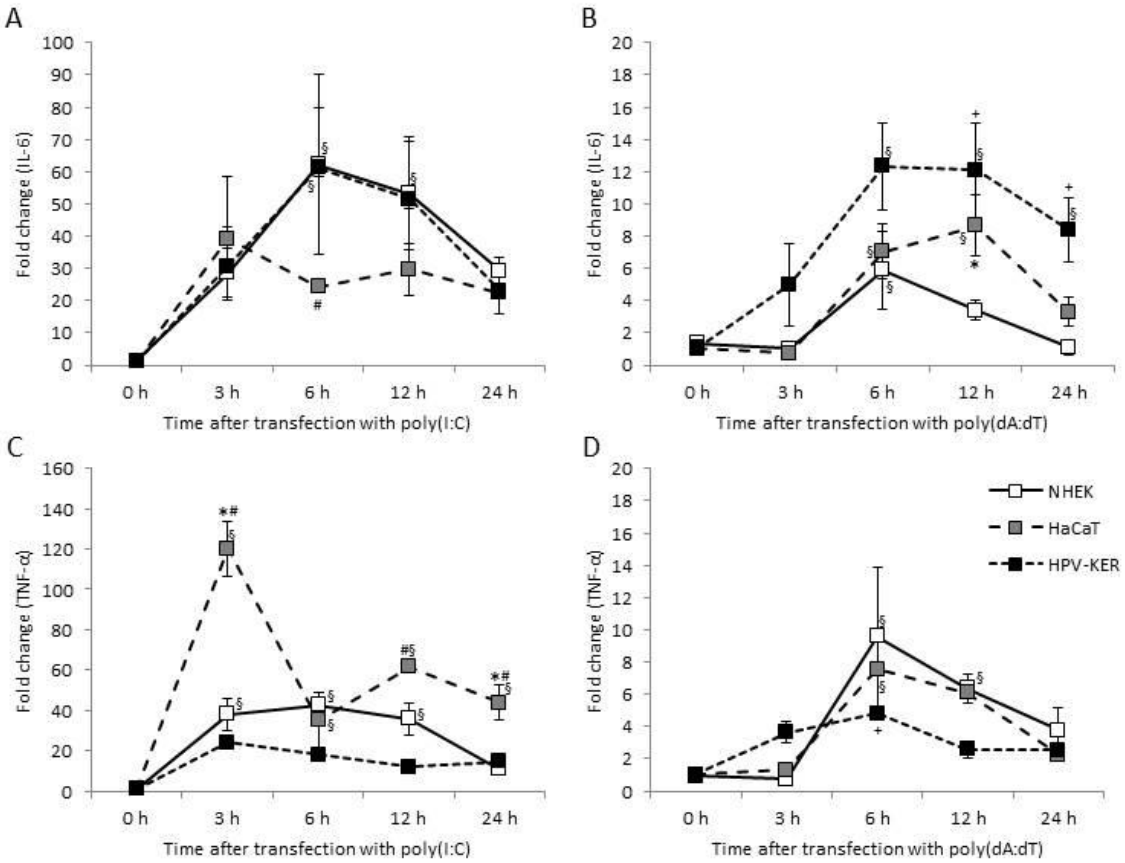

Figure 5. Kinetics of expression of the interleukin-6 (IL-6) and tumor necrosis factor α (TNF-α) cytokines in normal human epidermal keratinocytes (NHEK), HaCaT and HPV-KER cell lines upon transfection with 0.666 µg/ml poly(I:C) (A, C) and poly(dA:dT) (B, D). Relative expression was determined by the ΔΔCT method, normalized to 18S rRNA expression and compared to the expression of the untreated 0 hour samples. Data are presented as mean of three independent experiments ± standard error. Significance was determined by two-way repeated measurement ANOVA, *p<0.05 HaCaT vs NHEK; # p< HaCaT vs HPV-KER; + p<0.05 HPV-KER vs NHEK; § p<0.05 vs 0h samples within a cell type.

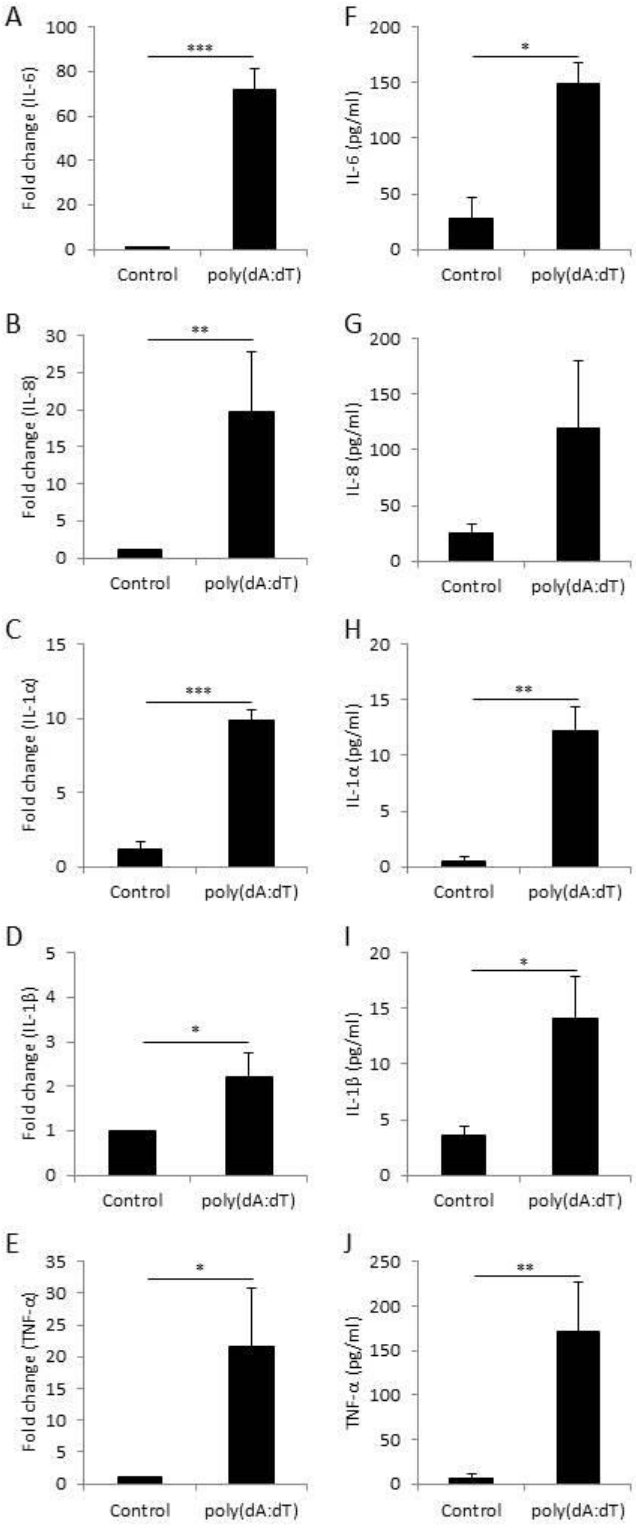

Figure 6. Poly(dA:dT) treatment induces the expression (A-E) and secretion (F-J) of inflammatory cytokines in NHEKs. NHEKs were transfected with 0.666 µg/ml poly(dA:dT) for 12 hours. mRNA expression was detected for cytokines by real-time RT-PCR, secretion of cytokines was determined by ELISA. Data are presented as mean ± SE of at least three independent experiments, significance was determined by Student's T-test * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

4.2 Poly(I:C) and poly(dA:dT) treatments induce NF-κB, MAP kinase and STAT activation in keratinocytes

NF-κB activation in HPV-KER keratinocytes was assessed by an NF-κB–luciferase reporter assay (Fig 7. A). The kinetic differences of NF-κB activation between poly(I:C) and poly(dA:dT) transfected cells resembled the corresponding cytokine expression differences: peak-activation occurred at 6 hours after poly(I:C) treatment, whereas the peak activation with poly(dA:dT) occurred 24 hours after treatment. The delayed NF-κB signaling response to poly(dA:dT) was confirmed with detection of phosphorylated IκBα by western blot analysis (Fig. 7. B and Fig. 8. A).

Western blot analysis of MAP kinase (Fig 7. C) and STAT (Fig 7. D) pathways showed that both poly(I:C) and poly(dA:dT) induced the phosphorylation of ERK1/2 and STAT-1 as well as STAT-3 signaling. Densitometric analysis showed a faster phosphorylation of STAT-1 and STAT-3 in poly(I:C) treated samples compared to poly(dA:dT) treatment (Fig. 8.). In addition, phosphorylation of p38 and JNK pathways were not affected at the studied time points, which was also confirmed by densitometric analysis (Fig. 8. C, D).

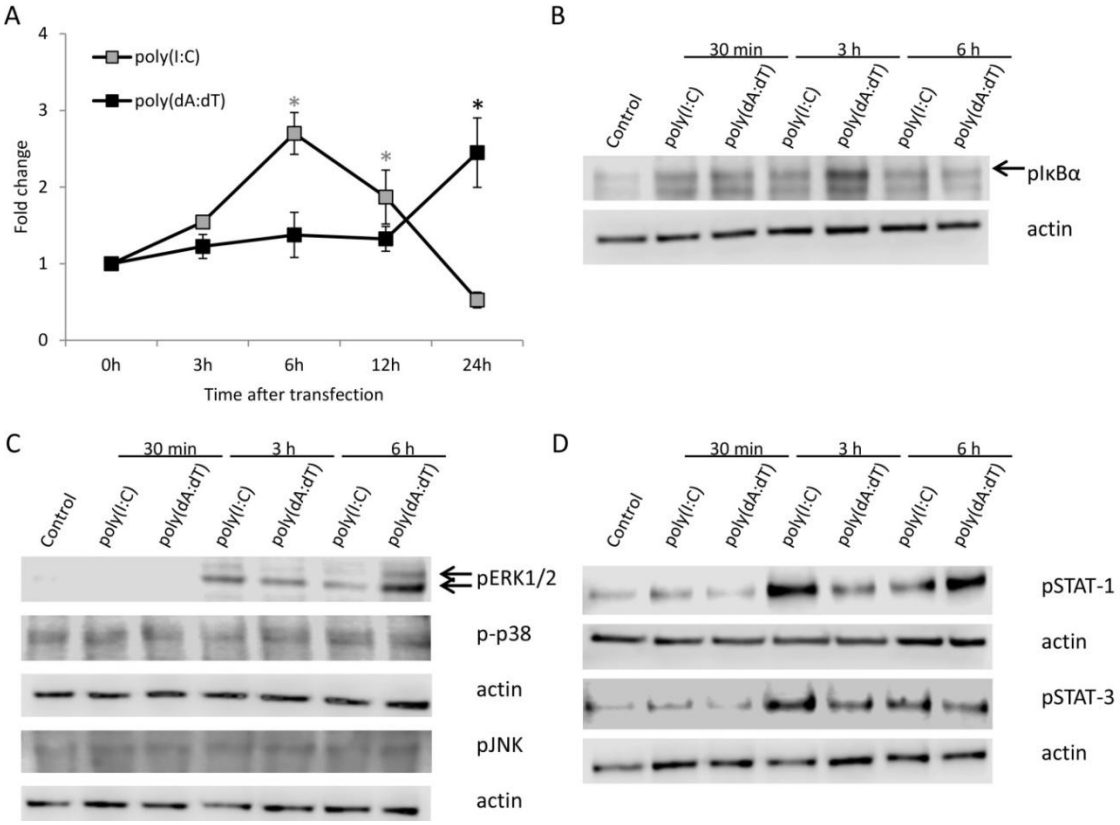

Figure 7. Activation of NF-κB, MAPK and STAT signal transduction pathways in HPV-KER cells upon poly(I:C) or poly(dA:dT) transfection assessed by NF-κB-luciferase reporter assay (A) and western blot analysis (B–D). (A) NF-κB luciferase reporter assay exhibited faster activation of NF-κB transcription factor upon poly(I:C) treatment than poly(dA:dT) treatment. Raw luminescence intensity values were normalized to the intrinsic control renilla activity, and compared to the 0 h untreated samples. Data are presented as mean of three independent experiments ± standard error; statistical significance was assessed by two-way repeated measurement ANOVA * $p < 0.05$, grey: poly(I:C) treatment compared to untreated 0 h samples, black: poly(dA:dT) treatment compared to untreated 0 h samples; (B) Increase in phosphorylated NF-κB inhibitor α (IκBα) after poly(I:C) or poly(dA:dT) treatment, peaking later after poly(dA:dT) treatment than after poly(I:C) treatment, arrow indicate the lane for phosphorylated IκBα; (C) Phosphorylation of ERK1/2 increases after poly(I:C) or poly(dA:dT) treatment, peaking later after poly(dA:dT) treatment than after poly(I:C) treatment, arrows indicate from top to bottom the lanes for phosphorylated ERK1 and ERK2. Phosphorylation of p38 and JNK was not observed upon poly(I:C) or poly(dA:dT) treatment; (D) Phosphorylation of both STAT-1 and STAT-3 occurs faster in poly(I:C) treated samples than in poly(dA:dT) treated samples. Western blot results are representative for at least three independent experiments. Actin was used as loading control.

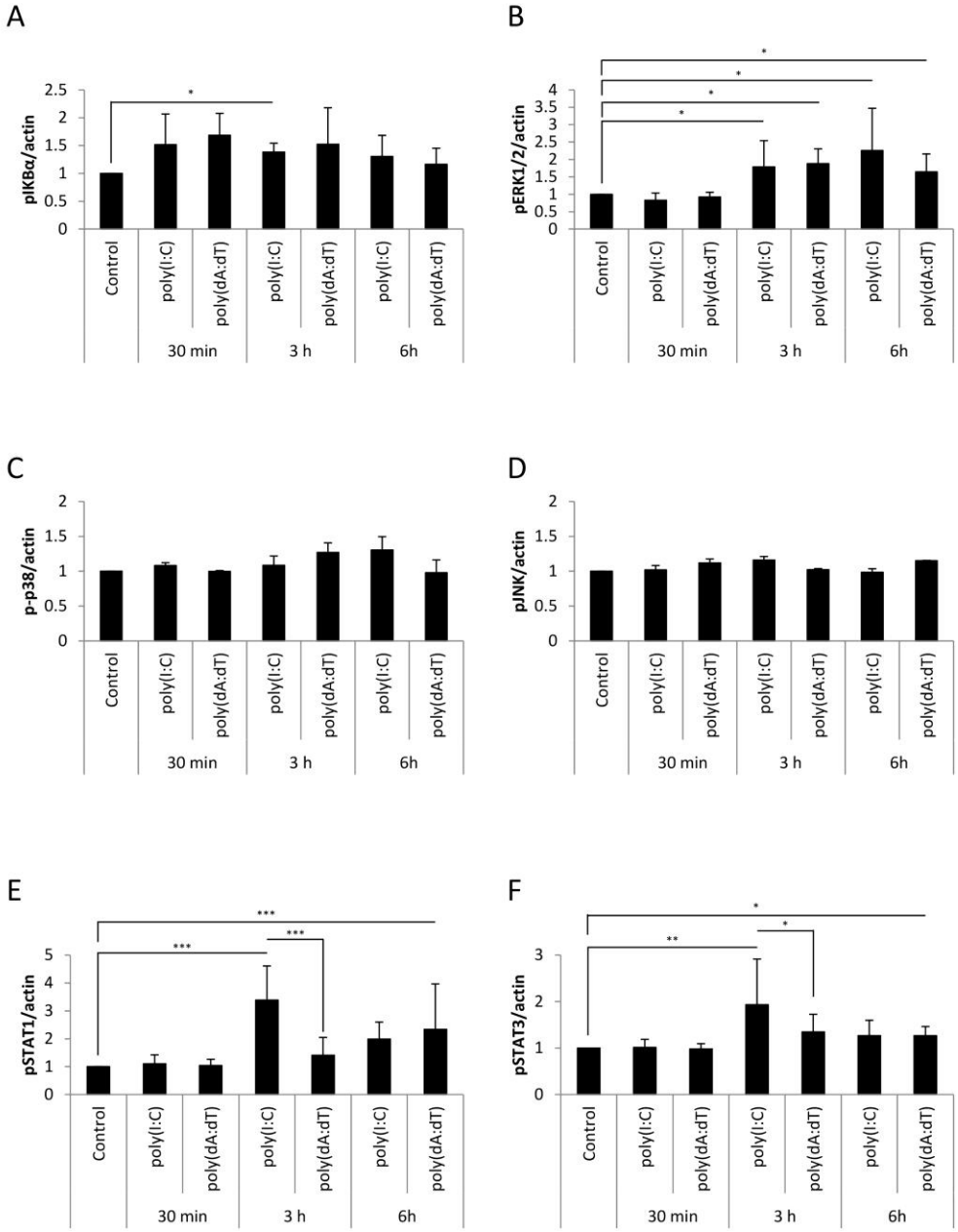

Figure 8. Densitometric analysis of Western blots on the phosphorylated forms IκBα (A), ERK1/2 (B), p38 (C), JNK (D), STAT-1 (E), STAT-3 (F) normalized to the level of the loading control actin. Densitometric analysis of the bands was carried out using Image Pro Plus software (Media Cybernetics Inc. Rockville, MD, USA). Intensity of bands from the protein of interest were normalized to the intensity of actin bands of the respective blots. Statistical analysis was carried out by two-way repeated measurement ANOVA. Data are presented as means ± SD of the densitometric analysis of n=5 (A,B,E,F) or n=3 (C,D) blots. *p<0.05; **p<0.01; ***p<0.001

4.3 Cytokine expression of keratinocytes upon poly(I:C) and poly(dA:dT) treatment relies on NF-κB, p38 and STAT signaling

To address the role of the activated signaling routes in poly(I:C)- and poly(dA:dT)-induced cytokine expression, keratinocytes were pre-incubated with the specific inhibitors of NF-κB (Bay 11-7085), MEK1/2 (PD95089), p38 (SB203580), JNK (SP600125), STAT-1 (Fludarabine) and STAT-3 (Stattic) for an hour before transfection with poly(I:C) for 6 hours or poly(dA:dT) for 12 hours. Time points of sample collection were determined with respect on the peak expression of cytokines (Fig. 5.). Inhibition of NF-κB nearly completely abolished both the poly(I:C)- and poly(dA:dT)-induced expression of IL-6 and TNF-α (Fig. 9. A).

Although activation could not be confirmed by our western blot results (Fig. 7. C), inhibition of p38 signaling resulted in significantly decreased IL-6 and TNF-α expression (Fig. 9. B). In contrast, the inhibition of JNK signaling did not affect cytokine expression (Fig. 9. C). The inhibition of MEK1/2 signaling significantly increased the poly(I:C)- and poly(dA:dT)-induced production of IL-6 (Fig. 9. D), suggesting a possible negative regulatory role of this pathway.

Specific inhibition of STAT-3 signaling significantly decreased both poly(I:C)- and poly(dA:dT)-induced cytokine expression (Fig. 9. F), whereas the inhibition of STAT-1 affected only IL-6 expression (Fig. 9. E).

Thus, whereas both poly(I:C)- and poly(dA:dT)-induced IL-6 expression was affected by most of the studied signaling routes, TNF-α expression was only affected by NF-κB, p38 and STAT-3.

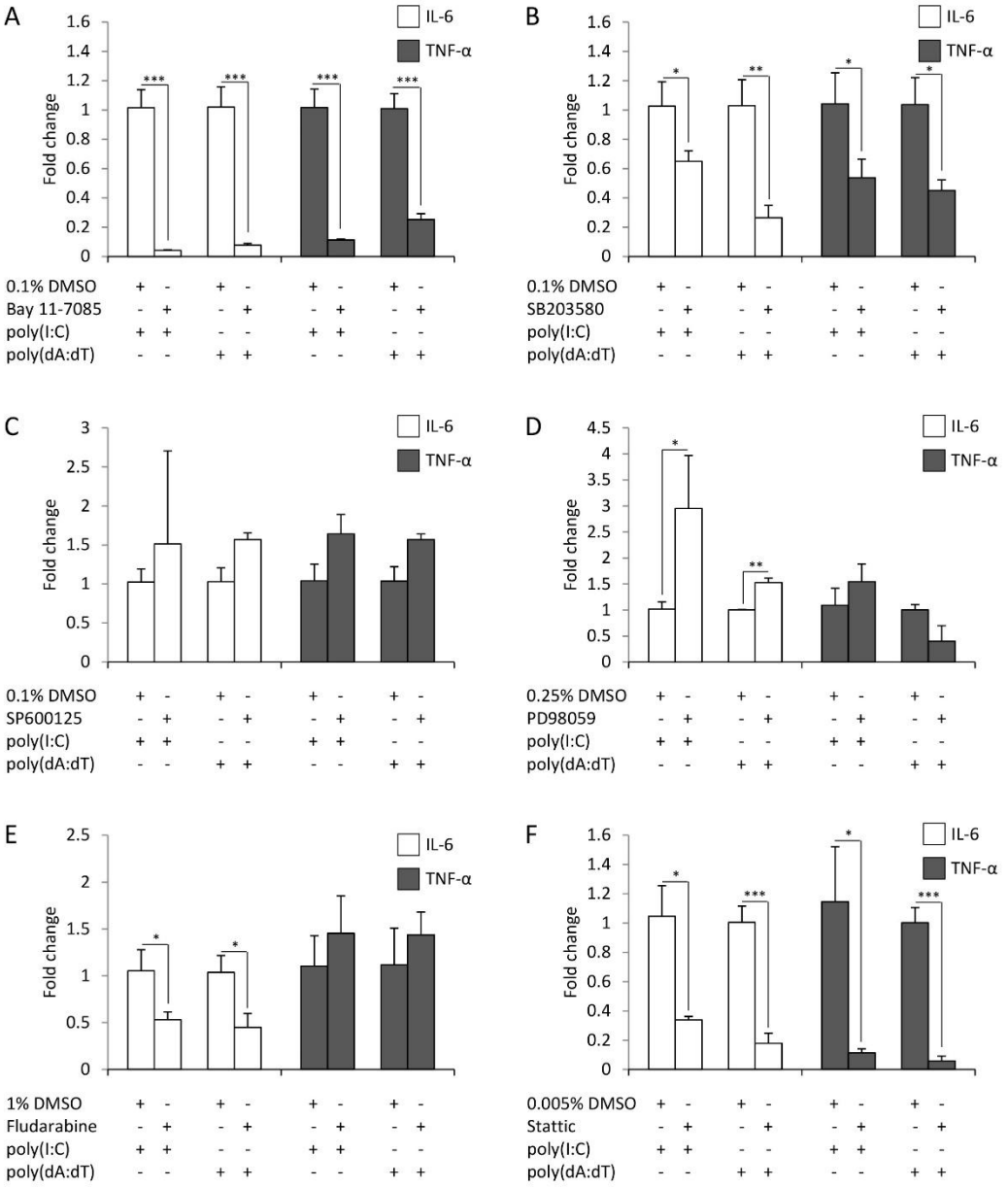

Figure 9. Inhibition of different signaling routes has divergent effects on the expression of the IL-6 (white bars) and TNF-α (grey bars) cytokines in keratinocytes. The effect of inhibition of NF-κB (A), p38 (B), JNK (C), MEK1/2 (D), STAT-1 (E) and STAT-3 (F) on poly(I:C) (6 hours after transfection) and poly(dA:dT) (12 hours after transfection) induction of IL-6 (white bars) and TNF-α (grey bars) expression in HPV-KER cells. Fold change of mRNA expression values were determined by the ΔΔCT method, normalized to 18S rRNA expression. As all inhibitors were dissolved in DMSO, the relative mRNA expression levels were compared to the expression levels in samples treated with DMSO + poly(I:C) or DMSO + poly(dA:dT), respectively. Poly(I:C) and poly(dA:dT) induction was in every case significant compared to the untreated control samples; no significant difference was observed between the cytokine-expression level of the samples treated with poly(I:C), poly(dA:dT), DMSO + poly(I:C) or DMSO + poly(dA:dT). Data are represented as the means of three independent experiments ± standard error; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ determined by Student's T-test.

4.4 PRINS expression decreases upon poly(dA:dT) treatment in NHEKs

We and others have demonstrated altered PRINS expression upon exposure to inflammatory molecules (56,67). Therefore we set out to examine PRINS expression upon poly(I:C) and poly(dA:dT) treatments. Poly(dA:dT) treatment significantly decreased the expression of PRINS in NHEKs (Fig. 10. A.), and a similar tendency was seen in the HPV-KER cell line (Fig. 10. C). Interestingly in HaCaT cells poly(dA:dT) treatment induced a trend of increased expression of PRINS (Fig. 10. B). Poly(I:C) treatment did not lead to significant PRINS expression changes (data not shown). Significant change in PRINS expression was only prevalent in NHEKs, thus NHEKs were used to determine whether PRINS plays a role in keratinocyte immune responses.

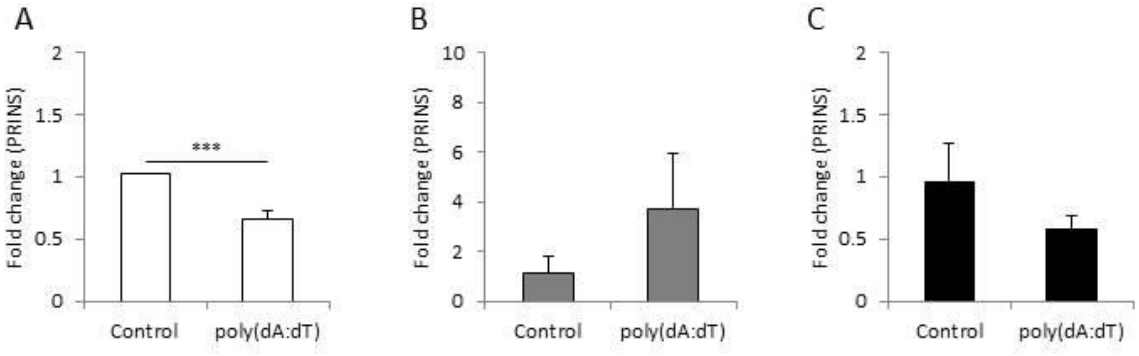

Figure 10. PRINS expression changed upon poly(dA:dT) transfection in NHEKs (A), HaCaT cells (B) and HPV-KER cells (C). Cells were transfected by poly(dA:dT) for 12 hours, and relative expression was determined by the ΔΔCT method, normalized to 18S rRNA expression and compared to the expression of the untreated samples. Data are presented as mean of three independent experiments ± SE. Significance was determined by Student's T-test, ***$p \leq 0.001$.

4.5 PRINS overexpression alters IL-6 and IL-8 levels in keratinocytes

The change in PRINS expression upon inflammatory stimuli suggests the possible contribution of PRINS to inflammatory responses of NHEK cells. To determine whether PRINS can regulate inflammatory cytokine expression, cells were transfected with a plasmid based construct for overexpressing PRINS, and the expression of cytokines was studied after poly(dA:dT) treatment. Unfortunately, in our initial experiments we found high intraindividual differences in the rate of poly(dA:dT) induced cytokine expression, which strongly inhibited the proper evaluation of the results. Upon these findings we introduced a model to increase inflammation by priming the cells with TNF-α and interferon (IFN)-γ for 24 hours before poly(dA:dT) transfection, which resulted in a similar decreased PRINS expression as poly(dA:dT) transfection alone (Fig. 11. A). The combined TNF-α and IFN-γ priming step is often applied before poly(dA:dT) treatment to gain pronounced inflammasome activation (14,28); however the cumulative effect of this treatment on the expression of other cytokines has not been examined thoroughly. Indeed, IL-1β secretion was enhanced by the TNF-α and IFN-γ priming compared to poly(dA:dT) treatment alone, but IL-1β mRNA expression was not affected by priming (Fig. 11. E, Fig. 12. D). Similarly, priming had no effect on TNF-α mRNA expression (Fig. 11. F). However, priming induced significantly higher mRNA expression of the other investigated cytokine genes (Fig. 11. B-D), and higher secretion of IL-1α, IL-1β and IL-8 (Fig. 12. B-D).

To analyze the role of PRINS in these inflammatory reactions, we overexpressed PRINS during the combined priming and poly(dA:dT) treatment. Expression and secretion of IL-6 and IL-8 were significantly decreased by PRINS overexpression (Fig. 13.), whereas mRNA expression and secretion of IL-1α, IL-1β and TNF-α were not affected (data not shown). These results suggest that PRINS can influence the regulation of other inflammatory processes.

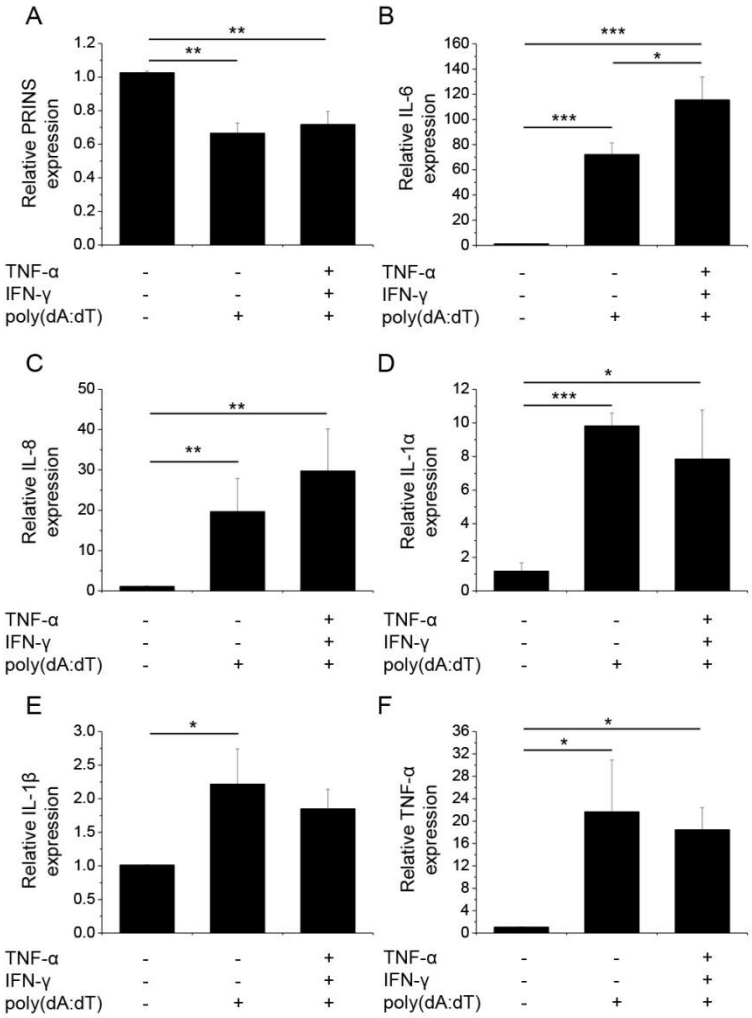

Figure 11. Poly(dA:dT) treatment decreased the expression of PRINS but induced the mRNA expression of cytokines. NHEKs were transfected with 0.666 μg/ml poly(dA:dT) for 12 hours with or without priming for 24 hours with 5 ng/ml TNF-α and IFN-γ. RNA expression was detected for PRINS (A) and cytokines IL-6 (B), IL-8 (C), IL-1α (D), IL-1β (E), TNF-α (F) by real-time RT-PCR. Data are presented as mean ± SE of five independent experiments * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

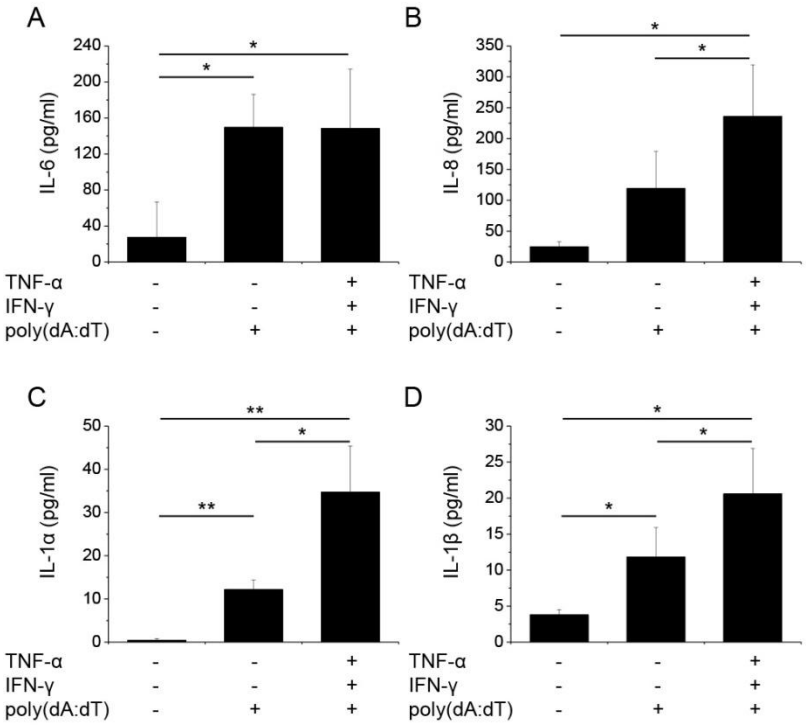

Figure 12. Poly(dA:dT) treatment induced the secretion of cytokines by NHEKs. NHEKs were transfected with poly(dA:dT) for 12 hours with or without priming for 24 hours with 5 ng/ml TNF-α and IFN-γ. Secretion of cytokines IL-6 (A), IL-8 (B), IL-1α (C), IL-1β (D) was detected by ELISA from cell supernatant. Data are presented as mean ± SE of five independent experiments. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

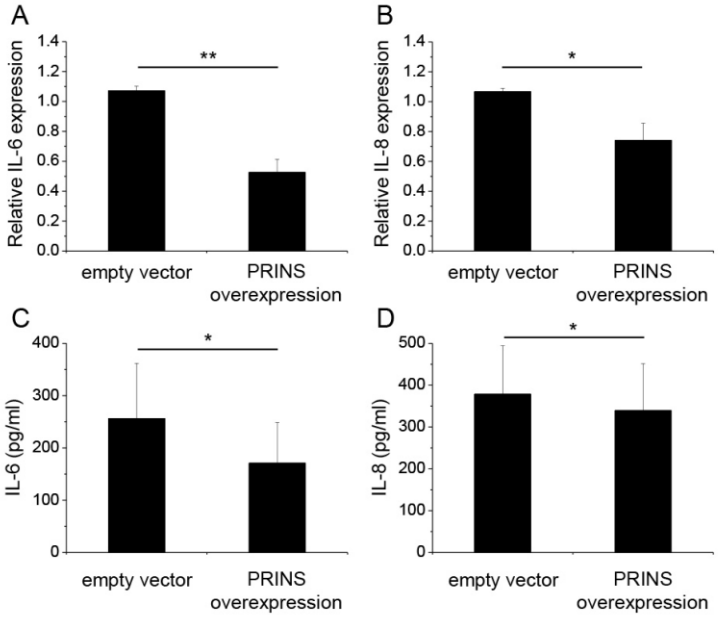

Figure 13. Overexpression of PRINS alters the expression and secretion of IL-6 and IL-8. In parallel to priming with 5 ng/ml TNF-α and IFN-γ, NHEKs were transfected with a pcDNA3.1(+) vector containing the PRINS cDNA; an empty pcDNA3.1(+) vector was used as control. After 24 hours, cells were transfected with poly(dA:dT). RNA expression of cytokines IL-6 (A), IL-8 (B) was detected by real-time RT-PCR. Secretion of cytokines IL-6 (C), IL-8 (D), was detected by ELISA from cell supernatants. Data are presented as mean ± SE of four independent experiments. * $p \leq 0.05$; ** $p \leq 0.01$.

4.6 *In silico* analysis revealed putative interacting sites between the PRINS lncRNA and the IL-6 mRNA

The mRNA of the chemokine CCL-5 was previously predicted to interact with PRINS; however it was not reported whether this interaction affects the stability of the CCL-5 mRNA (57). Therefore, we measured mRNA expression and secretion of CCL-5 during PRINS overexpression, and found that both decreased (Fig. 14.) in a manner similar to the changes observed for IL-6 and IL-8. The similarity of these expression profiles led us to hypothesize similar mechanism(s) for IL-6, IL-8 and CCL-5 regulation mediated by PRINS.

To predict interactions between PRINS (AK022045) and the mRNAs of IL-6 (NM_000600.4) and IL-8 (NM_000584.3), we performed an *in silico* analysis using the INTARNA (73,74) and the RIsearch (71) softwares. As sequence details of the CCL-5 mRNA and PRINS interaction have not been described in detail (57), we also included the CCL-5

mRNA (NM_002985.2) in the *in silico* analysis. As a control for the reliability of the prediction analyses, mRNA sequences of cytokines not affected by PRINS overexpression (IL-1α – M28983.1, IL-1β – NM_000576.2, TNF-α – NM_000594.3) were also included. The regions predicted by both algorithms were considered putative interacting sites. Putative interaction sites were not predicted for the cytokines not affected by PRINS overexpression (IL-1α, IL-1β, TNF-α) in this analysis. PRINS interaction regions were only predicted for the IL-6 and CCL-5 mRNAs (Fig. 15. B, C), two mediators affected by PRINS overexpression. A distance of approximately 200 nt separates the predicted interaction sites in the PRINS sequence, and the corresponding sites occur in the 5' untranslated region (UTR) of IL-6 and the 3' UTR of CCL-5 (Fig. 15. A). No interaction site was predicted for IL-8.

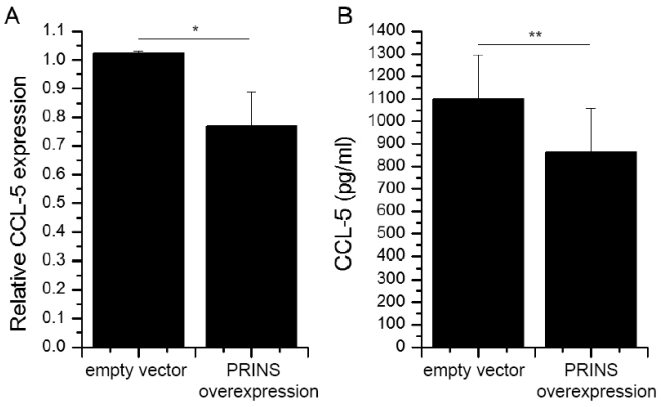

Figure 14. Overexpression of PRINS alters the expression and secretion of CCL-5. In parallel to priming with 5 ng/ml TNF-α and IFN-γ, NHEKs were transfected with a pcDNA3.1(+) vector containing the PRINS cDNA; an empty pcDNA3.1(+) vector was used as control. After 24 hours, cells were transfected with poly(dA:dT). RNA expression of CCL-5 was detected by real-time RT-PCR, secretion of CCL-5was measured by ELISA from cell supernatants. Data are presented as mean ± SE of four independent experiments. * $p \leq 0.05$; ** $p \leq 0.01$.

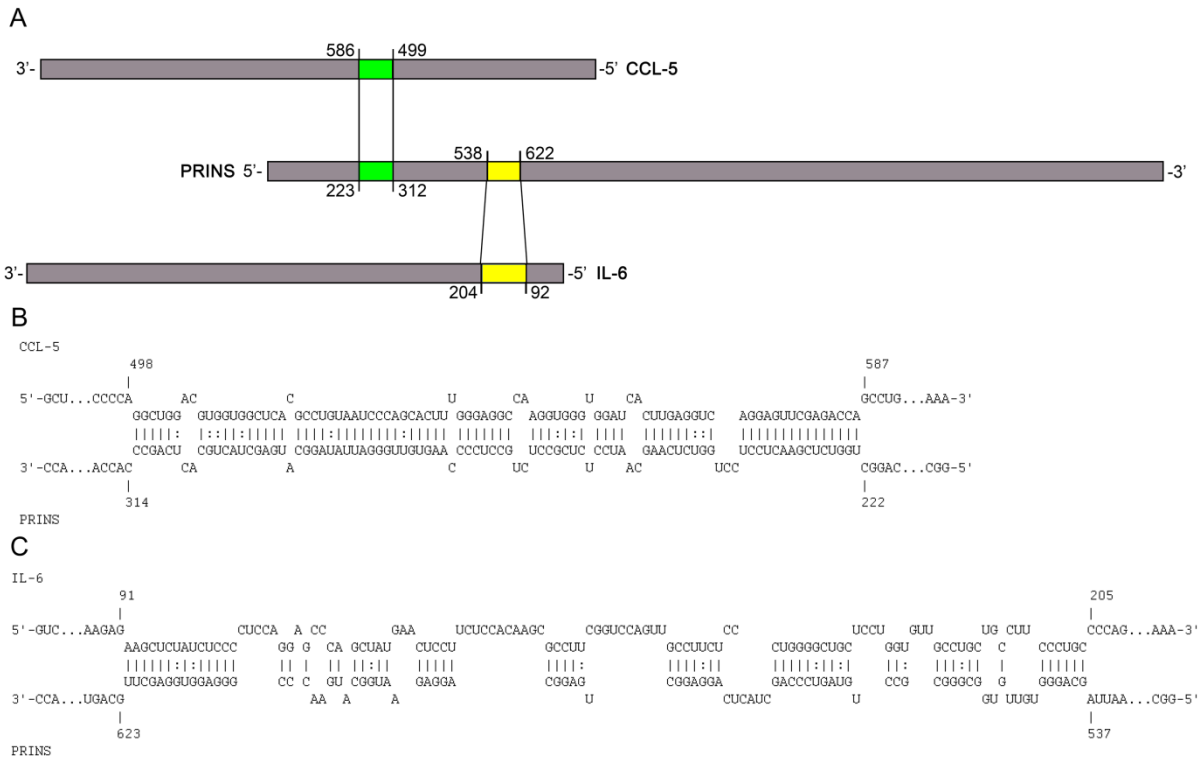

Figure 15. Putative interactions of PRINS lncRNA with CCL-5 and IL-6 mRNAs. Schematic representation of the *in silico* predicted interaction sites in the PRINS sequence for the CCL-5 and IL-6 mRNAs (A). PRINS sequences predicted to interact with CCL-5 mRNA (B) and IL-6 mRNA (C). Nucleotide positions are given based on the AK022045, NM_000600.4 and NM_002985.2 reference sequences.

4.7 PRINS binds to IL-6 mRNA through direct, sequence-specific interaction

To validate the predicted interaction site, an *in vitro* binding experiment was carried out using the PRINS lncRNA and the IL-6 mRNA. Binding affinity was determined using the single–stranded PRINS RNA and a fluorescently labeled, truncated IL-6 mRNA sequence containing the predicted interacting sequence. The ΔPRINS sequence, in which the predicted interaction site to IL-6 was replaced by scrambled sequence, was used as a control. As a negative control, a fluorescently labeled DNA sequence with no similarity to either PRINS or IL-6 RNAs was used. While PRINS exhibited a very high binding affinity to the labeled IL-6 mRNA (Fig. 16. A, Kd=10.3436 nM), specific binding for ΔPRINS (Fig. 16. B) or the unspecific labeled DNA could not be detected. This *in vitro* result confirms the specificity of

the *in silico* predicted regions (538–622 of PRINS, AK022045, and 92–204 of IL-6 mRNA, NM_000600.4).

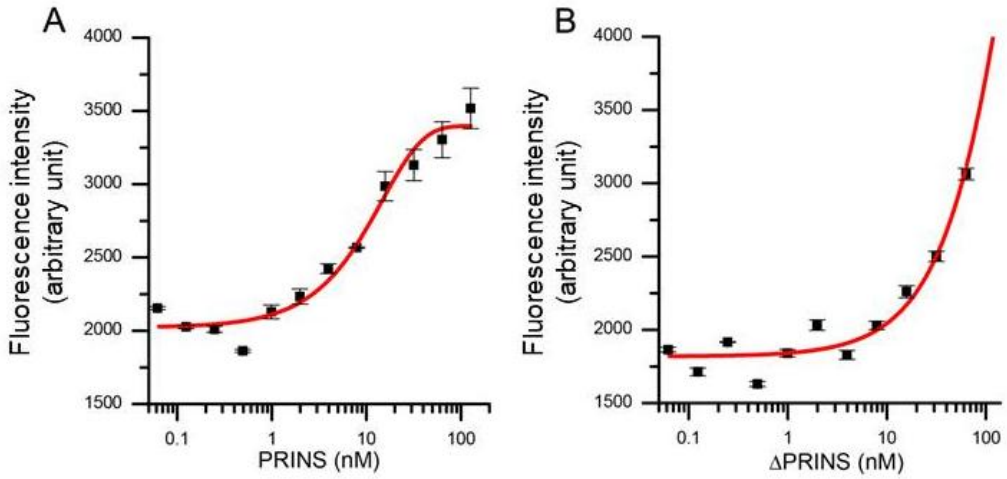

Figure 16. PRINS specifically binds to IL-6 mRNA. The binding affinity of PRINS (A) and ΔPRINS (B) to IL-6 mRNA was determined by analyzing the initial fluorescence enhancement caused by specific binding. The concentration of labeled IL-6 mRNA was constant (10nM), while the concentration of PRINS and ΔPRINS varied from 62 pM to 127 nM.

4.8 PRINS lncRNA regulates IL-6 expression in NHEKs through sequence-specific interaction

To validate the functionality of the *in silico* predicted and *in vitro* determined IL-6 mRNA interacting region in the PRINS sequence, we performed the overexpression experiments in NHEKs with vectors containing the wildtype PRINS or ΔPRINS (with scrambled IL-6 binding site) sequences, during the combined priming and poly(dA:dT) treatment. IL-6 expression was not affected by overexpression of ΔPRINS but was, in contrast, significantly decreased by PRINS overexpression (Fig. 17. A), and similar tendencies were seen in the amount of secreted IL-6 (Fig. 17. D). To confirm the specificity of this region in IL-6 regulation, the expression of CCL-5 was also studied. CCL-5 expression and secretion decreased similarly both in cells overexpressing ΔPRINS and in cells overexpressing PRINS (Fig. 17. B, E). IL-8 expression upon PRINS or ΔPRINS overexpression showed a similar tendency to IL-6 expression, although significant differences could not be detected (Fig. 17. C, F). These result demonstrated that the binding site in the PRINS sequence is essential and specific for the regulation of IL-6 expression.

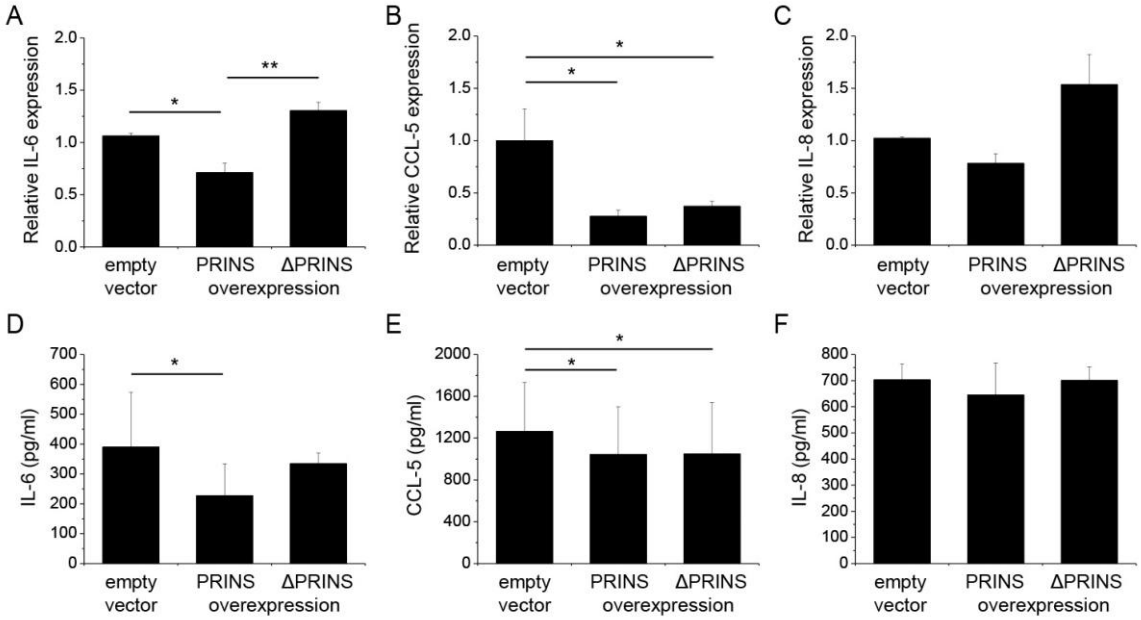

Figure 17. The 538–622 nt region of PRINS is required for the regulation of IL-6 expression. In parallel to priming with 5 ng/ml TNF-α and IFN-γ, NHEKs were transfected with a pcDNA3.1(+) vector containing the PRINS cDNA or the ΔPRINS cDNA (containing a scrambled version of the IL-6 binding site at positions 538–622 nt); the empty pcDNA3.1(+) vector served as control. Expression of cytokines IL-6 (A), CCL-5 (B) and IL-8 (C) was detected by real-time RT-PCR. Secretion of cytokines IL-6 (D), CCL-5 (E), IL-8 (F) was detected by ELISA from cell supernatants. Nucleotide positions based on the AK022045 reference sequence. Data are presented as mean ± SE of three independent experiments * $p \leq 0.05$; ** $p \leq 0.01$.

5. Discussion

RNA and DNA fragments are known as important PAMPs or DAMPs that induce innate immune processes of professional immune cells, such as macrophages and dendritic cells (DCs) (26,76–78), as well as non-professional immune cells, such as keratinocytes (20,21). Accumulation of nucleotide fragments in keratinocytes contributes to the pathogenesis of psoriasis leading to parakeratosis (34), as well as in the promotion of inflammation by activating DCs (18) and to the activation of inflammasomes in keratinocytes (14,28).

We have a deeper knowledge on RNA and DNA fragments induced processes in professional immune cells, much less is known for non-professional immune cells, such as keratinocytes. Thus, we aimed to study these reactions in keratinocytes. To study keratinocyte immune functions we applied treatments with the cytosolic dsRNA-analogue poly(I:C) and the cytosolic dsDNA-analogue poly(dA:dT), and characterized the immune responses of these cells with different approaches. The transcriptional regulation of signal transduction pathways and the posttranscriptional control by an lncRNA were also analyzed.

Our results are in agreement with recent reports describing inflammasome activation in keratinocytes (13,14,28) and IL-6, IL-8 and TNF-α induction in canine keratinocytes (21) and human melanocytes (23) upon poly(dA:dT) exposition.

Previously we found that the inflammatory responses of the widely used HaCaT cell line upon exposure to *Propionibacterium acnes* differ from that of NHEKs, while the HPV-KER cell line (established and characterized in our laboratory) shows similar inflammatory (70,79) and ultraviolet–B- irradiation-induced responses to NHEKs (64). Therefore, we compared the IL-6 and TNF-α expression of NHEK, HaCaT (69) and HPV-KER (70) cells after poly(I:C) and poly(dA:dT) exposure. In line with our previous findings (70), we found that the cytokine expression patterns in the HaCaT cells significantly differ from the responses of HPV-KER and NHEK cells. Moreover, HaCaT cells are thought to be less suitable to study inflammatory signaling pathways due to their constant NF-κB activation (80), therefore the HPV-KER cell line was selected to study the transcriptional regulation of nucleotide fragment induced cytokine expression.

NF-κB, MAPKs and STAT signaling have been reported to participate in nucleotide-fragment-induced inflammatory cytokine expression in several cell types (23,25,26,29,30);

however, limited information is available for these signaling events in keratinocytes upon nucleotide fragment induction (20,75). According to our results, poly(I:C) induces activation of the studied signaling pathways in a shorter time than poly(dA:dT), and a corresponding shift in cytokine expression peaks was observed. The difference in peak timing is likely due to direct activation of TLR3 signaling by poly(I:C) (81); while it has been shown that poly(dA:dT) must first be transcribed to RNA before activating NF-κB through RIG-I dependent sensing (26) (Fig. 18.).

In addition to poly(I:C)- and poly(dA:dT)-induced NF-κB activation, phosphorylation of ERK1/2, STAT-1 and STAT-3 was also observed; but the phosphorylation of other studied MAPKs (p38 and JNK) was not affected. In contrast, a previous study using keratinocytes reported poly(I:C) induction of p38 signaling but no induction of ERK1/2 signaling (20). In melanocytes, another epidermal cell type, poly(dA:dT)-induced phosphorylation of p38 and JNK signaling was observed without ERK1/2 activation (23). These differences might be due to differences in time points used: our study examined p38 phosphorylation 30 minutes after poly(I:C) and poly(dA:dT) transfection in HPV-KER cells, whereas the previous study examined p38 phosphorylation in NHEKs 15 minutes after treatment, observing a reduction after 30 minutes (20). These results suggest that poly(I:C)- and poly(dA:dT)-induced p38 phosphorylation might be a rapid event in keratinocytes. Although we could not confirm p38 phosphorylation, inhibition of p38 signaling during transfection with poly(I:C) or poly(dA:dT) resulted in decreased IL-6 and TNF-α expression, which is in agreement with a previous report on poly(I:C)-induced TNF-α expression in keratinocytes (20).

In monocytes and melanocytes, inhibition of ERK1/2 and JNK signaling pathways abolished nucleotide-induced IL-6 and TNF-α expression (23,82). In mouse models, the disruption of ERK1/2 signaling by the inhibition of dual specificity mitogen-activated protein kinase kinase 1 and 2 (MEK1/2) functions have been shown to have anti-inflammatory effects (82,83).

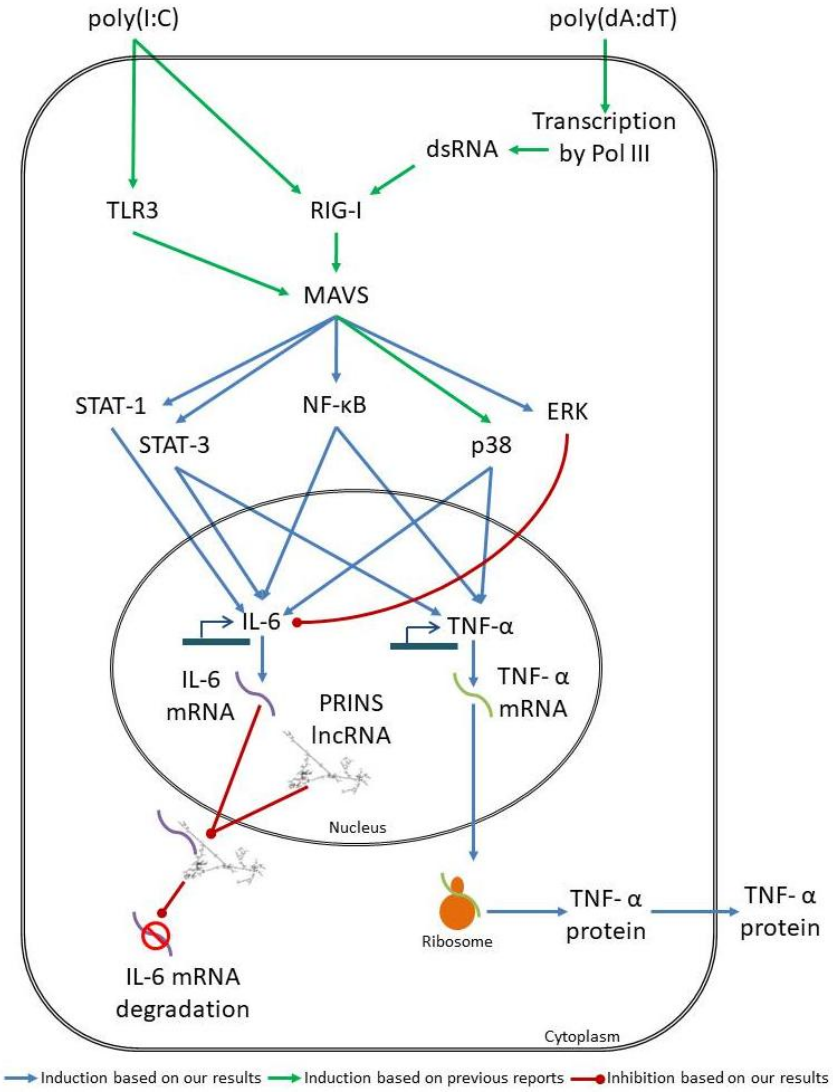

Figure 18. Schematic representation of poly(I:C) and poly(dA:dT) induced signaling events and posttranscriptional mRNA expression control in keratinocytes. Our results indicate that poly(I:C) and poly(dA:dT) induce the same inflammatory pathways; however the mode of sensing differs leading to the observed kinetic differences. The receptors for poly(I:C) sensing are toll-like receptor 3 (TLR3) (24) and retinoic acid induced gene I (RIG-I) (25). In contrast, poly(dA:dT) is transcribed to double-stranded (ds) RNA by RNA polymerase III (Pol III) and is subsequently sensed by RIG-I (26). The transcription step might be responsible for the delayed response to poly(dA:dT). The receptors activate the NF-κB, MAPK and STAT signaling routes through the adaptor molecule mitochondrial antiviral signaling protein (MAVS), and, thus, regulate the transcription of the cytokines. The mRNA of the cytokines either is translated by ribosomes and the protein is secreted by the cells, as in the case of TNF-α, or they undergo posttranscriptional degradation induced by the sequence specific binding of PRINS lncRNA, as in the case of IL-6

In contrast, in our experiments the disruption of ERK1/2 signaling through inhibition of MEK1/2 kinases increased the expression of the inflammatory mediator IL-6. These results are in agreement with previous *in vivo* findings that therapeutic inhibition of MEK1/2 in patients is often accompanied by an inflammatory skin rash (84). The results suggest that ERK1/2 signaling – in contrast to other cell types – has a negative regulatory function in inflammatory reactions in keratinocytes. Previous reports have already demonstrated similar results: ERK1/2 signaling was shown to negatively regulate NF-κB activation (85), and inhibition of MEK1/2 led to increased NF-κB, STAT-1 and interferon-regulatory factor signaling in human keratinocytes (86), although, we did not observe an increase in NF-κB activation upon inhibition of ERK1/2 signaling.

STAT signaling is known to be induced by inflammatory cytokines (87) and by poly(I:C) treatment (20,75). We found that in addition to poly(I:C), poly(dA:dT) also induced STAT-1 and STAT-3 signaling in keratinocytes. Moreover, similarly to the observed NF-κB activation, STAT-1 and STAT-3 activation both exhibited a delay after poly(dA:dT) transfection compared to poly(I:C) treatment. Poly(I:C)-induced STAT-1 activation has been shown to regulate TLR-3 and TNF-α expression (20). In our experiments, STAT-1 was found to regulate poly(I:C)- and poly(dA:dT)-induced IL-6 expression, whereas TNF-α was not affected. Inhibition of STAT-3 abolished both poly(I:C)- and poly(dA:dT)-induced IL-6 and TNF-α expression, showing the different regulatory functions of each STAT transcription factors.

Due to large-scale gene-expression studies the number of annotated human non-coding RNAs has increased rapidly (88), but functional roles have been assigned only to a few of them (89,90). PRINS was one of the first lncRNAs described to be dysregulated in a disease, namely in psoriasis (39). The expression of PRINS increases in response to various cellular stressors (56) in HaCaT cells, as well as to hypoxia in HK-2 cells (57) suggesting that PRINS contributes to the stress response of the cells. We aimed to study how PRINS expression is changed in response to nucleotide fragment induction in keratinocytes, and whether PRINS plays a role in the nucleotide fragment induced stress responses of keratinocytes. We found that poly(dA:dT) treatment decreased PRINS expression in NHEKs in line with previous reports in NHEKs and macrophages upon inflammatory stimuli (56,67). To gain a more pronounced and more uniform inflammatory cytokine expression in NHEKs, we introduced a

combined treatment. Since before poly(dA:dT) treatment a priming step with the cytokines TNF-α and IFN-γ – which were described as key elements in keratinocyte immune responses (40) – is often applied to induce a pronounced inflammasome activation (14), we analysed the effect of the priming step on the expression of various cytokines. Priming induced significantly enhanced IL-6 and IL-8 mRNA expression. In addition, cytokine secretion was also significantly increased compared to poly(dA:dT) treatment alone.

PRINS was recently described to potentially interact with the CCL-5 mRNA (57), but the functions of this interaction remained to be elucidated. In this study, we demonstrate that PRINS overexpression decreases the expression of IL-6, IL-8 and CCL-5 in keratinocytes upon poly(dA:dT) treatment. In addition, decreased IL-6 expression was observed upon PRINS overexpression in UVB treated samples as well. Using *in silico* analysis, regions in the PRINS sequence were predicted to interact with the CCL-5 and IL-6 mRNA. As inhibition of transcription by DNA:lncRNA triplex formation or posttranscriptional destabilization of the mRNA by mRNA-lncRNA duplex formation has been reported (89), we analyzed the sequences and found that the interacting site on IL-6 spans two exons, indicating an mRNA-lncRNA interaction. Moreover, PRINS demonstrated perinuclear localization in keratinocytes (64), making it possible to exert its effect at the posttranscriptional level. The mRNA-lncRNA interaction was validated *in vitro*: PRINS showed a very high binding affinity (Kd=10.3436 nM) to IL-6 mRNA, and the destruction of the predicted binding site abolished the ability of PRINS to bind to the IL-6 mRNA. The binding site of PRINS lies within the 5' UTR of the IL-6 mRNA, which is a rare phenomenon. The majority (~40%) of lncRNAs bind to the 3' UTR of their target mRNAs, while only around 5% of lncRNAs is able to bind to the 5' UTR of their target (91). The functionality of the mRNA-lncRNA interaction was also observed at the cellular level, as overexpression of ΔPRINS, in which the IL-6 interacting site is scrambled, did not decrease IL-6 levels. Thus, we demonstrated that PRINS is able to bind the IL-6 mRNA and this specific interaction destabilizes IL-6 expression and secretion in NHEKs (Fig. 18.).

Similarly to IL-6, PRINS overexpression decreased the expression of IL-8, but no interaction site could be predicted on IL-8 mRNA. We hypothesize that this is a secondary effect (92), however we cannot exclude the possibility of a yet unknown interaction site between PRINS and IL-8 mRNA.

Like many other non-coding RNAs, PRINS seems to have multiple cellular functions (43). It binds to nucleophosmin, a chaperon protein, and facilitates its transition from the nucleolus to the nucleoplasm upon UVB irradiation (64). Additionally, our recent results suggest that PRINS is involved in the regulation of inflammation by inhibiting cytokine expression.

In this study, we show the similarities as well as the differences in inflammatory signaling events of keratinocytes induced by dsRNA and dsDNA, moreover provide a novel model of posttranscriptional control of cytokine expression in these cells by an lncRNA. Although dsRNA and dsDNA are recognized by different sets of receptors, they induce the same inflammatory signaling pathways in keratinocytes, however with different kinetics and magnitude of activation. Our data revealed that transfection with the synthetic dsRNA and dsDNA analogues poly(I:C) and poly(dA:dT) induced activation of NF-κB and STAT signaling, which along with p38 signaling were also shown to be functional in inducing cytokine mRNA expression. Moreover, we showed the negative regulatory role of ERK1/2 signaling in nucleotide-induced cytokine mRNA expression. Another negative regulatory role was proven for PRINS lncRNA in poly(dA:dT) induced IL-6 and CCL-5 expression, through sequence specific binding to the mRNA of the cytokines. Based on our current and previous findings (39,56,57), we hypothesize that elevated PRINS expression in psoriatic uninvolved epidermis may contribute to downregulation of the inflammatory functions in psoriatic keratinocytes and maintenance of normal phenotype.

Studies of the last decade have highlighted disturbances in the signal transduction events in psoriasis that have led to the development of targeted therapeutics against specific signaling components. However, there is still a lack of knowledge on every pre- and posttranscriptional aspect of these mechanisms. Our studies were performed using a non-professional immune cell type, keratinocytes; however the same mechanisms might be also relevant in professional immune cells. These results deepen the existing knowledge and contribute to the understanding of these signaling events and the posttranscriptional control in keratinocytes, moreover widen our knowledge on the cellular functions of PRINS and, in general, about lncRNAs.

6. Summary, novel findings of the experimental work

In this study, we characterized the dsRNA and dsDNA induced immune responses in human keratinocytes by studying pre- and posttranscriptional regulation of cytokine expression.

- We compared the dsRNA and dsDNA induced cytokine expression in three keratinocyte cell types: NHEKs, HaCaT cells and HPV-KER cells. We found that transfection by the synthetic dsRNA and dsDNA analogue poly(I:C) and poly(dA:dT) induce the expression of IL-6 and TNF-α with notable kinetic differences and rate of induction.
- We found that poly(I:C) and poly(dA:dT) induce the same inflammatory signaling pathways in keratinocytes, albeit with different kinetics and magnitude of activation. Our data revealed that transfection with poly(I:C) and poly(dA:dT) induced activation of NF-κB and STAT signaling, which along with p38 signaling were also shown to be functional in inducing cytokine mRNA expression. We also showed the negative regulatory role of ERK1/2 signaling in nucleotide-induced IL-6 mRNA expression in keratinocytes, which seems to be a celltype specific event.
- By studying the expression pattern of PRINS lncRNA we found decreased expression upon poly(dA:dT) transfection in NHEKs, while poly(I:C) did not led to altered expression of PRINS.
- We have identified a potential negative regulatory role for PRINS lncRNA in poly(dA:dT) induced IL-6 and CCL-5 expression. In-depth analysis of this phenomenon revealed the sequence specific interaction between PRINS and the mRNA of IL-6 and CCL-5, which might be responsible for the posttranscriptional control of the mRNA expression. These results led us to hypothesize that elevated PRINS expression in psoriatic uninvolved epidermis may contribute to the downregulation of the inflammatory functions in psoriatic keratinocytes and maintenance of the normal phenotype.

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Differential Inflammatory-Response Kinetics of Human Keratinocytes upon Cytosolic RNA- and DNA-Fragment Induction

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Abstract: Keratinocytes are non-professional immune cells contributing actively to innate immune responses partially by reacting to a wide range of molecular patterns by activating pattern recognition receptors. Cytosolic nucleotide fragments as pathogen- or self-derived trigger factors are activating inflammasomes and inducing anti-viral signal transduction pathways as well as inducing expression of inflammatory cytokines. We aimed to compare the induced inflammatory reactions in three keratinocyte cell types—normal human epidermal keratinocytes, the HaCaT cell line and the HPV-KER cell line—upon exposure to the synthetic RNA and DNA analogues poly(I:C) and poly(dA:dT) to reveal the underlying signaling events. Both agents induced the expression of interleukin-6 and tumor necrosis factor α in all cell types; however, notable kinetic and expression level differences were found. Western blot analysis revealed rapid activation of the nuclear factor κB (NF-κB), mitogen activated protein kinase and signal transducers of activator of transcription (STAT) signal transduction pathways in keratinocytes upon poly(I:C) treatment, while poly(dA:dT) induced slower activation. Inhibition of NF-κB, p38, STAT-1 and STAT-3 signaling resulted in decreased cytokine expression, whereas inhibition of mitogen-activated protein kinase kinase 1/2 (MEK1/2) signaling showed a negative feedback role in both poly(I:C)- and poly(dA:dT)-induced cytokine expression. Based on our in vitro results nucleotide fragments are able to induce inflammatory reactions in keratinocytes, but with different rate and kinetics of cytokine expression, explained by faster activation of signaling routes by poly(I:C) than poly(dA:dT).

Keywords: cytosolic nucleotide fragments; keratinocyte; poly(I:C); poly(dA:dT); signal transduction pathway; interleukin-6; tumor necrosis factor α

1. Introduction

The skin provides the primary interface between the body and the environment and forms a physical barrier against invading pathogens. Keratinocytes—the main cell type of the epidermis—form the physical barrier of the stratum corneum and are immunocompetent cells as well, making the epidermis an active member of the immune system.

Keratinocytes express a wide range of pattern recognition receptors and are responsive to various pathogen associated molecular patterns [1–4], including RNA and DNA fragments, which have

been implicated in antiviral defense of keratinocytes [5,6]. Cytosolic RNA and DNA fragments are also known as pathogen- as well as damage-associated molecular patterns (PAMPs and DAMPs), which induce innate immune functions of professional and non-professional immune cells. In non-infectious skin diseases, such as psoriasis, receptors for RNA and/or DNA fragments [7–9], moreover their activators: self-derived RNA and DNA fragments and RNA:DNA duplexes are highly abundant in the lesional epidermis [10,11]. During normal cornification, keratinocytes express deoxyribonucleases (DNases) [12]; however, it was recently shown that reduced keratinocyte DNase activity in psoriasis results in suppressed DNA degradation and, as a consequence, parakeratosis [13] and the presence of excess DNA fragments in the cytosol. Similarly, disturbed ribonuclease activities were described in psoriatic skin [14,15], which might result in excess RNA fragments.

These self-derived fragments activate among others the absent in melanoma 2 (AIM2) inflammasome [10,16,17] and inflammatory cytokine expression through their receptors in keratinocytes [7,11,18] initiating inflammatory events. Nucleotide fragment induced reactions have been studied by using synthetic RNA analogue poly(I:C) and DNA analogue poly(dA:dT), which both induce type I interferon (IFN-α/β) and inflammatory cytokine expression in keratinocytes [7,18–20]. Poly(I:C) is recognized primarily by toll-like receptor 3 (TLR3) [21], although, TLR3-independent sensing of poly(I:C) has also been observed with involvement of retinoic acid induced gene I (RIG-I) and melanoma differentiation-associated gene 5 [22]. Poly(dA:dT) recognition partially overlaps with poly(I:C) recognition, since RIG-I serves as a receptor after poly(dA:dT) has been transcribed by RNA polymerase III into double-stranded (ds) RNA molecules [23]. Cyclic GMP-AMP synthase (cGAS), a newly described cytosolic DNA receptor implicated in antiviral responses, binds dsDNA sequences independently and activates interferon regulatory factor 3 in cooperation with interferon-γ-inducible protein 16 [6].

The basal expression of most inflammatory cytokines is low and is regulated in response to stimuli at the transcriptional level, mediated by transcription factors of the nuclear factor κB (NF-κB), mitogen activated protein kinases (MAPK) and signal transducers of activator of transcription (STAT) signal transduction pathways [18], which have been reported to participate in nucleotide-induced inflammatory cytokine expression in several cell types [23–26]. Poly(I:C) was found to induce NF-κB, p38 and STAT-1 signaling in keratinocytes, whereas, in melanocytes, poly(dA:dT) induces NF-κB, p38 and c-Jun N-terminal kinase (JNK) signaling, which differentially regulates cytokine expression [18,24].

Although Cheng and coworkers have reported that sensing of poly(I:C) or poly(dA:dT) and the induced inflammatory reactions after exposure to these molecules partially overlap [22]; no comprehensive data is available for these reactions in keratinocytes. We aimed to compare poly(I:C)- and poly(dA:dT)-induced inflammatory reactions in keratinocytes and the underlying signal transduction events. We found that poly(I:C) and poly(dA:dT) induce similar signal transduction events in keratinocytes; however, the kinetics are faster and the rate of cytokine induction is higher in response to poly(I:C). Moreover, our results suggest a negative feedback role for the activation of extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) signaling in keratinocytes, for both poly(I:C)- and poly(dA:dT)-induced inflammatory signaling.

2. Results

2.1. Keratinocytes Respond to Poly(I:C) and Poly(dA:dT) with Increased Interleukin-6 (IL-6) and Tumor Necrosis Factor α (TNF-α) Expression

To study cytosolic RNA- and DNA-induced cytokine-expression profiles in keratinocytes, we used three keratinocyte cell types: normal human epidermal keratinocytes (NHEKs), the HaCaT cell line [27] and the HPV-KER cell line [28]. Poly(I:C) strongly induced IL-6 and TNF-α expression in all three cell types, and poly(dA:dT) induced expression in all cell types with slightly different kinetics as well as expression that was an order of magnitude lower than that observed with poly(I:C) (Figure 1). Peak expression was observed 3 to 6 h after poly(I:C) transfection, whereas peak expression after poly(dA:dT) transfection occurred 6 to 12 h after treatment in all cell types studied. Reaction to

poly(I:C) in HaCaT cells differed significantly from the other cell types (Figure 1A,C). In contrast, poly(dA:dT)-induced reaction differed in all three cell types (Figure 1B,D). Previously we found that the HPV-KER cell line and NHEK cells exhibited similar cytokine mRNA expression [28], which agreed with our finding on the expression kinetics upon poly(I:C) induction.

To study the induced signaling pathways in keratinocytes, we used only the HPV-KER cell line. HPV-KER cells previously showed similar reactions to NHEKs [28] and HaCaT cells exhibited a slightly different cytokine-expression profile, moreover HaCaT cells are known to exhibit constant activation of inflammatory signaling [29], while high intra-individual differences were observed in the inflammatory inductions of NHEKs.

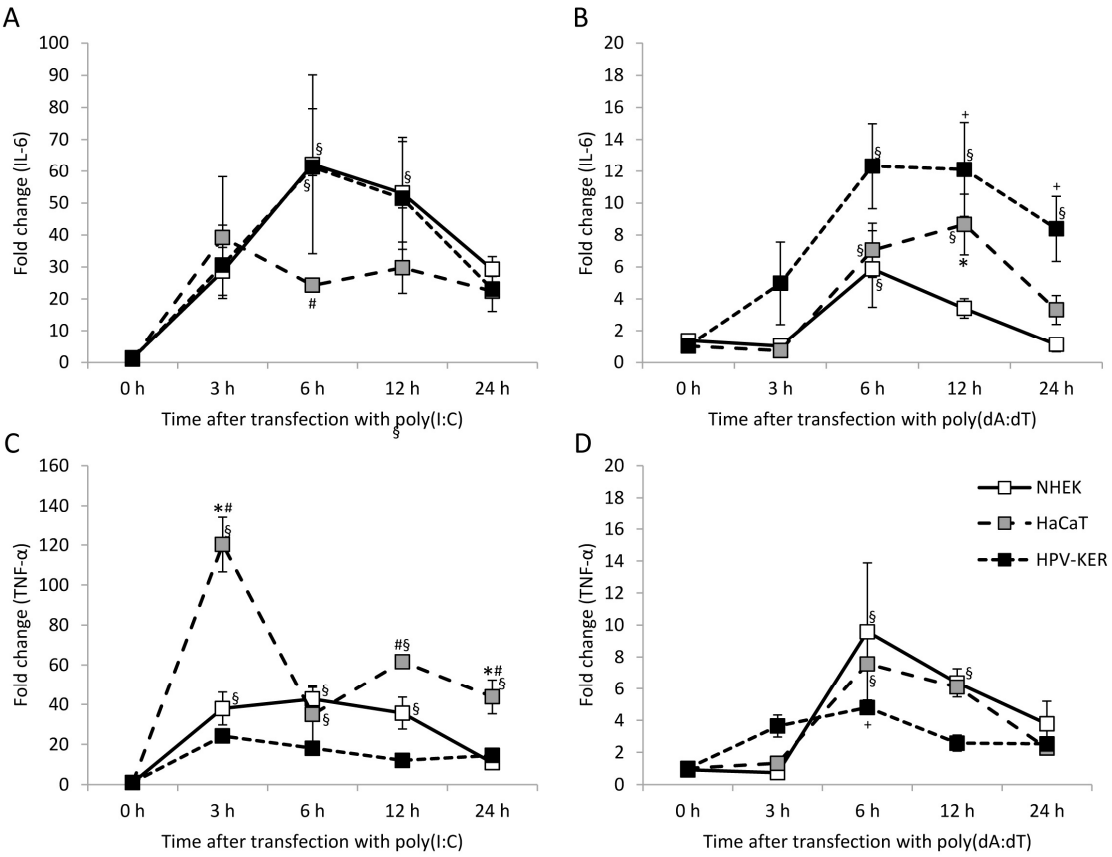

Figure 1. Kinetics of expression of the interleukin-6 (IL-6) and tumor necrosis factor α (TNF-α) cytokines in normal human epidermal keratinocytes (NHEK) and HaCaT and HPV-KER cell lines upon transfection with 0.666 µg/mL poly(I:C) (**A**,**C**) and poly(dA:dT) (**B**,**D**). Relative expression was determined by the $\Delta\Delta C_t$ method, normalized to 18S rRNA expression and compared to the expression of the untreated 0 h samples. Data are presented as mean of three independent experiments ± standard error. Significance was determined by two-way repeated measurement analysis of variance (ANOVA), * $p < 0.05$ HaCaT vs. NHEK; # $p <$ HaCaT vs. HPV-KER; + $p < 0.05$ HPV-KER vs. NHEK; § $p < 0.05$ vs. 0 h samples within a cell type.

2.2. Poly(I:C) and Poly(dA:dT) Treatment Induces Nuclear Factor κB (NF-κB), Mitogen Activated Protein Kinase (MAPK) and Signal Transducers of Activator of Transcription (STAT) Activation in Keratinocytes

NF-κB activation in HPV-KER keratinocytes was assessed by an NF-κB–luciferase reporter assay (Figure 2A). The kinetic differences of NF-κB activation between poly(I:C) and poly(dA:dT) transfected cells resembled the corresponding cytokine expression differences: peak-activation occurred at 6 h after poly(I:C) treatment, whereas the peak activation with poly(dA:dT) occurred 24 h after treatment.

The delayed NF-κB signaling response to poly(dA:dT) was confirmed with detection of phosphorylated NF-κB inhibitor α (IκBα) by western blot analysis (Figure 2B and Figure S1A).

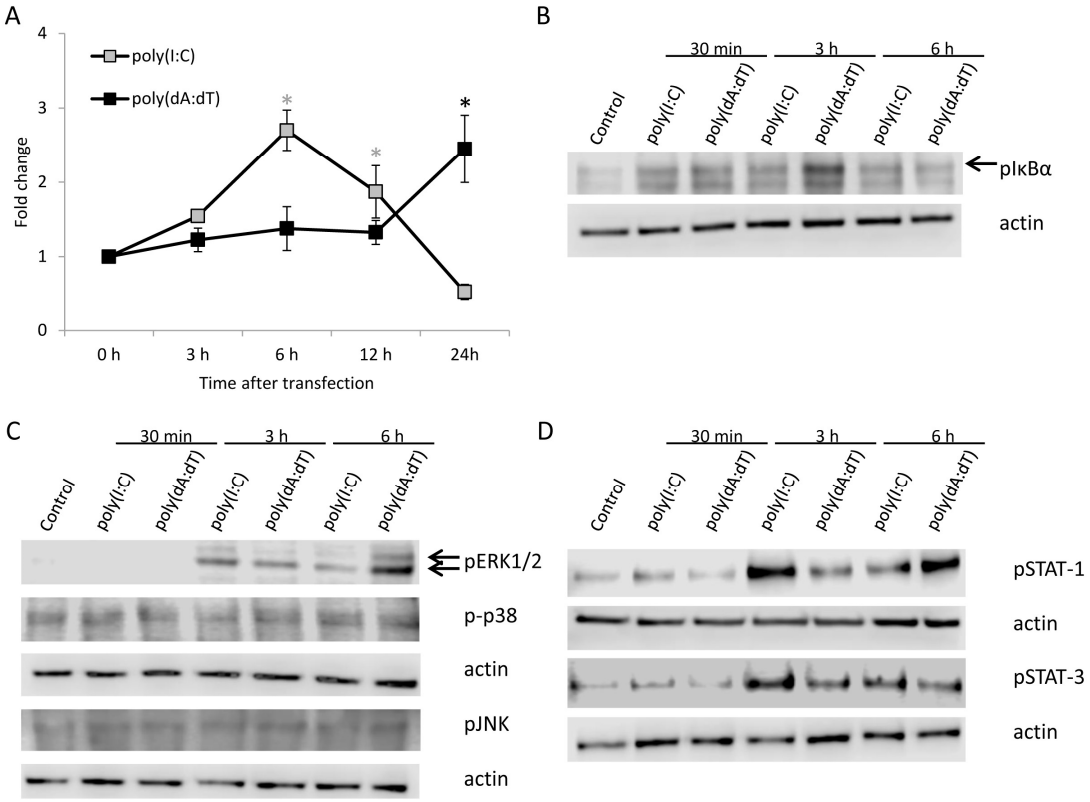

Figure 2. Activation of Nuclear Factor κB (NF-κB), Mitogen Activated Protein Kinase (MAPK) and Signal Transducers of Activator of Transcription (STAT) signal transduction pathways in HPV-KER cells upon poly(I:C) or poly(dA:dT) transfection assessed by NF-κB-luciferase reporter assay (**A**) and western blot analysis (**B**–**D**). (**A**) NF-κB luciferase reporter assay exhibited faster activation of NF-κB transcription factor upon poly(I:C) treatment than poly(dA:dT) treatment. Raw luminescence intensity values were normalized to the intrinsic control renilla activity, and compared to the 0 h untreated samples. Data are presented as mean of three independent experiments ± standard error; statistical significance was assessed by two-way repeated measurement ANOVA * $p < 0.05$, grey: poly(I:C) treatment compared to untreated 0 h samples, black: poly(dA:dT) treatment compared to untreated 0 h samples; (**B**) Increase in phosphorylated NF-κB inhibitor α (IκBα) after poly(I:C) or poly(dA:dT) treatment, peaking later after poly(dA:dT) treatment than after poly(I:C) treatment, arrow indicate the lane for phosphorylated IκBα; (**C**) Phosphorylation of ERK1/2 increases after poly(I:C) or poly(dA:dT) treatment, peaking later after poly(dA:dT) treatment than after poly(I:C) treatment, arrows indicate from top to bottom the lanes for phosphorylated ERK1 and ERK2. Phosphorylation of p38 and JNK was not observed upon poly(I:C) or poly(dA:dT) treatment; (**D**) Phosphorylation of both STAT-1 and STAT-3 occurs faster in poly(I:C) treated samples than in poly(dA:dT) treated samples. Western blot results are representative for at least three independent experiments. Actin was used as loading control.

Western blot analysis of MAP kinase (Figure 2C) and STAT (Figure 2D) pathways showed that both poly(I:C) and poly(dA:dT) induced the phosphorylation of ERK1/2 and STAT-1 as well as STAT-3 signaling. Densitometric analysis showed a faster phosphorylation of STAT-1 and STAT-3 in poly(I:C) treated samples compared to poly(dA:dT) treatment (Figure S1). In addition, phosphorylation of p38 MAPK and JNK pathways were not affected at the studied time points, which was also confirmed by densitometric analysis (Figure S1C,D).

2.3. Cytokine Expression of Keratinocytes upon Poly(I:C) and Poly(dA:dT) Treatment Relies on NF-κB, p38 and STAT Signaling

To address the role of the activated signaling routes in poly(I:C)- and poly(dA:dT)-induced cytokine expression, keratinocytes were pre-incubated with the specific inhibitors of NF-κB (Bay 11-7085), dual specificity mitogen-activated protein kinase kinase1 and 2 (MEK1/2) (PD95089), p38 (SB203580), JNK (SP600125), STAT-1 (fludarabine) and STAT-3 (Stattic) for an hour before transfection with poly(I:C) or poly(dA:dT).

Time points of sample collection were determined with respect on the peak expression of cytokines (Figure 1). Inhibition of NF-κB nearly completely abolished both the poly(I:C)- and poly(dA:dT)-induced expression of IL-6 and TNF-α (Figure 3A).

Although activation could not be confirmed by our western blot results (Figure 2C), inhibition of p38 signaling resulted in significantly decreased IL-6 and TNF-α expression (Figure 3B). In contrast, the inhibition of JNK signaling did not affect cytokine expression (Figure 3C). The inhibition of MEK-1 signaling significantly increased the poly(I:C)- and poly(dA:dT)-induced production of IL-6 (Figure 3D), suggesting a possible negative regulatory role of this pathway.

Specific inhibition of STAT-3 signaling significantly decreased both poly(I:C)- and poly(dA:dT)-induced cytokine expression (Figure 3F), whereas the inhibition of STAT-1 affected only IL-6 expression (Figure 3E). Thus, whereas both poly(I:C)- and poly(dA:dT)-induced IL-6 expression was affected by most of the studied signaling routes, TNF-α expression was only affected by NF-κB, p38 and STAT-3.

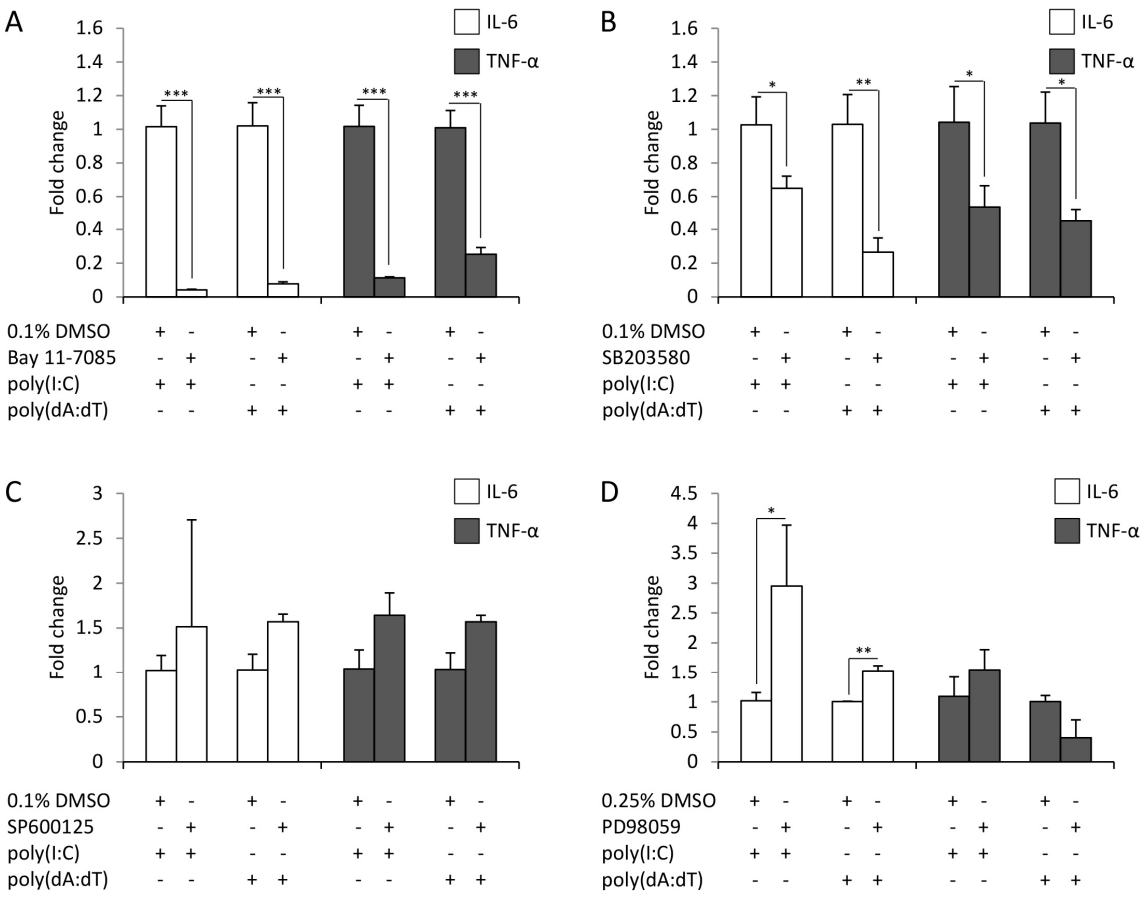

Figure 3. *Cont.*

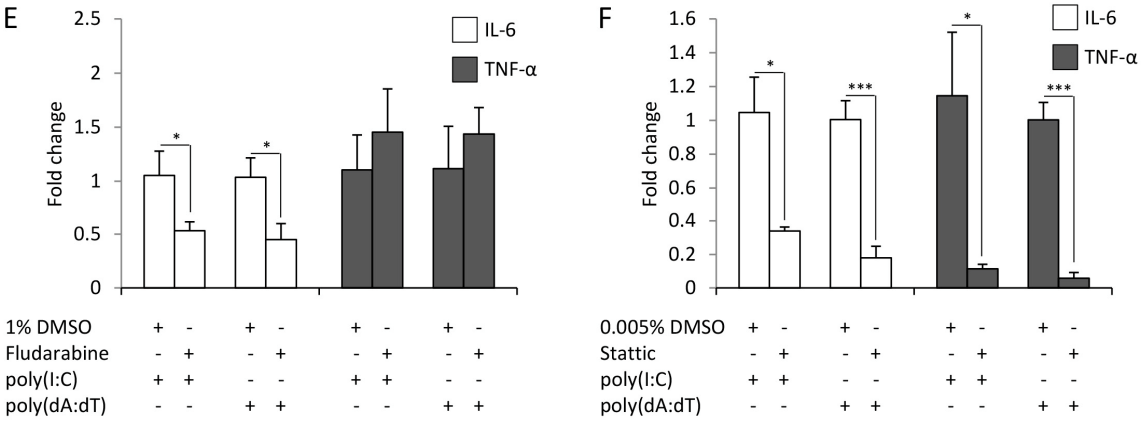

Figure 3. Inhibition of different signaling routes has divergent effects on the expression of the IL-6 (white bars) and TNF-α (grey bars) cytokines in keratinocytes. The effect of inhibition by NF-κB (**A**), p38 (**B**), c-Jun N-terminal kinase (JNK) (**C**), mitogen-activated protein kinase kinase 1 and2 (MEK1/2) (**D**), STAT-1 (**E**) and STAT-3 (**F**) on poly(I:C) (6 h after transfection) and poly(dA:dT) (12 h after transfection) induction of IL-6 (white bars) and TNF-α (grey bars) expression in HPV-KER cells. Fold change of mRNA expression values were determined by the $\Delta\Delta C_t$ method, normalized to 18S rRNA expression. As all inhibitors were dissolved in dimethyl sulfoxide (DMSO), the relative mRNA expression levels were compared to the expression levels in samples treated with DMSO + poly(I:C) or DMSO + poly(dA:dT), respectively. Poly(I:C) and poly(dA:dT) induction was in every case significant compared to the untreated control samples; no significant difference was observed between the cytokine-expression level of the samples treated with poly(I:C), poly(dA:dT), DMSO + poly(I:C) or DMSO + poly(dA:dT). Data are represented as the means of three independent experiments ± standard error; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ determined by Student's *t*-test.

3. Discussion

RNA and DNA fragments are known as important PAMPs or DAMPs that induce innate immune processes of professional immune cell types, such as macrophages and dendritic cells [23,30–32], as well as non-professional immune cells, such as keratinocytes [18]. Accumulation of nucleotide fragments in keratinocytes is involved in the pathogenesis of psoriasis leading to parakeratosis [13], as well as in the promotion of inflammation by activating dendritic cells [33] and in the activation of inflammasomes in keratinocytes [10,17]. However, the induced inflammatory signaling pathways and how they contribute to cytokine expression in keratinocytes were not previously studied.

In this study, we characterized innate immune responses of human keratinocytes to the cytosolic exposure of the dsRNA analogue poly(I:C) and the dsDNA analogue poly(dA:dT). We compared the IL-6 and TNF-α expression of NHEK, HaCaT [27] and HPV-KER [28] cells after poly(I:C) and poly(dA:dT) exposure. Previously we found that the inflammatory responses of the widely used HaCaT cell line upon exposure to *Propionibacterium acnes* differ from that of NHEKs, while the HPV-KER cell line (established and characterized in our laboratory) shows similar inflammatory [28,34] and ultraviolet-B irradiation-induced responses to NHEKs [35]. In line with our previous findings [28], we found that the cytokine expression patterns in the HaCaT cells differ significantly from the responses of HPV-KER and NHEK cells. Moreover, HaCaT cells are thought to be less suitable to study inflammatory signaling pathways due to their constant NF-κB activation [29].

NF-κB, MAPKs and STAT signaling have been reported to participate in nucleotide-fragment-induced inflammatory cytokine expression in several cell types [22–26]; however, limited information is available for these signaling events in keratinocytes upon nucleotide fragment induction [18,36]. According to our results, poly(I:C) induces activation of the studied signaling pathways in a shorter time than poly(dA:dT), and a corresponding shift in cytokine expression peaks was observed. The difference in peak timing is likely due to direct activation of TLR3 signaling by poly(I:C) [37]; while it

has been shown that poly(dA:dT) must first be transcribed to RNA before activating NF-κB through RIG-I dependent sensing [23] (Figure 4).

In addition to poly(I:C)- and poly(dA:dT)-induced NF-κB activation, phosphorylation of ERK1/2, STAT-1 and STAT-3 was also observed; but phosphorylation of other studied MAPKs (p38 and JNK) was not affected. In contrast, a previous study using keratinocytes reported poly(I:C) induction of p38 signaling but no induction of ERK1/2 signaling [18]. In melanocytes, another epidermal cell type, poly(dA:dT)-induced phosphorylation of p38 and JNK signaling was observed without ERK1/2 activation [24]. These differences might be due to differences in time points used: our study examined p38 phosphorylation 30 min after poly(I:C) and poly(dA:dT) transfection in HPV-KER cells, whereas the previous study examined p38 phosphorylation in NHEKs 15 min after treatment, observing a reduction after 30 min [18]. These results suggest that poly(I:C)- and poly(dA:dT)-induced p38 phosphorylation might be a rapid event in keratinocytes. Although we could not confirm p38 phosphorylation, inhibition of p38 signaling during transfection with poly(I:C) or poly(dA:dT) resulted in decreased IL-6 and TNF-α expression, which is in agreement with a previous report on poly(I:C)-induced TNF-α expression in keratinocytes [18].

In monocytes and melanocytes, inhibition of ERK1/2 and JNK signaling pathways abolished nucleotide-induced IL-6 and TNF-α expression [24,38]. In mouse models, the disruption of ERK1/2 signaling by the inhibition of MEK1/2 functions have been shown to have anti-inflammatory effects [39,40]. In contrast, in our experiments the disruption of ERK1/2 signaling through inhibition of MEK1/2 kinases increased the expression of the inflammatory mediator IL-6. These results agree with previous in vivo findings that therapeutic inhibition of MEK1/2 in patients is often accompanied by an inflammatory skin rash [41]. These results suggest that ERK1/2 signaling—in contrast to other cell types—has a negative regulatory function in inflammatory reactions in keratinocytes. Previous reports have already demonstrated similar results: ERK1/2 signaling was shown to negatively regulate NF-κB activation [42], and inhibition of MEK1/2 led to increased NF-κB, STAT-1 and interferon-regulatory factor signaling in human keratinocytes [43], altough, we did not observe an increase in NF-κB activation upon inhibition of ERK1/2 signaling.

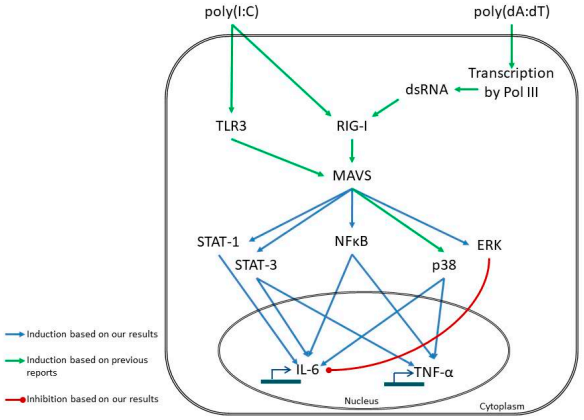

Figure 4. Proposed model of poly(I:C)- and poly(dA:dT)-induced signaling events leading to IL-6 and TNF-α expression in keratinocytes, based on our results and results of previous reports [21–23,44]. Our results indicate that poly(I:C) and poly(dA:dT) induce the same inflammatory pathways; however, the mode of sensing differs, leading to the observed differences in kinetic. The receptors for poly(I:C) sensing are toll-like receptor 3 (TLR3) [21] and retinoic acid induced gene I (RIG-I) [22]. In contrast, poly(dA:dT) is transcribed to double-stranded (ds) RNA by RNA polymerase III (Pol III) and is subsequently sensed by RIG-I [23]. The transcription step might be responsible for the delayed response to poly(dA:dT). The receptors activate the NF-κB, MAPK and STAT signaling routes through the adaptor molecule mitochondrial antiviral signaling protein (MAVS), and, thus, regulate the transcription of the cytokines.

STAT signaling is known to be induced by inflammatory cytokines [45] and by poly(I:C) treatment [18,36]. We found that, in addition to poly(I:C), poly(dA:dT) also induced STAT-1 and STAT-3 signaling in keratinocytes, and that the induction exhibited a delay in activation after poly(dA:dT) transfection similar to those observed with other pathways. Poly(I:C)-induced STAT-1 activation has been shown to regulate TLR3 and TNF-α expression [18]. In our experiments, STAT-1 was found to regulate poly(I:C)- and poly(dA:dT)-induced IL-6 expression, whereas TNF-α was not affected. Inhibition of STAT-3 abolished both poly(I:C)- and poly(dA:dT)-induced IL-6 and TNF-α expression, showing the different regulatory functions of each STAT transcription factors.

In this study, we show the similarities as well as the differences in inflammatory signaling events of keratinocytes induced by dsRNA and dsDNA. Our data revealed that transfection with the synthetic dsRNA and dsDNA analogues poly(I:C) and poly(dA:dT) induced activation of NF-κB and STAT signaling, both of which were also shown to be functional in inducing cytokine expression. Moreover, we showed the negative regulatory role of ERK1/2 signaling in nucleotide-induced cytokine expression. Although dsRNA and dsDNA are recognized by different sets of receptors, they induce the same inflammatory signaling pathways in keratinocytes, albeit with different kinetics and magnitude of activation.

Studies of the last decade have highlighted disturbances in the signal transduction events in psoriasis that have led to the development of targeted therapeutics against specific signaling components. However there is still a lack of knowledge on every aspect of these mechanisms. Our results deepen the existing knowledge and contribute to the understanding of these signaling events induced in keratinocytes.

4. Materials and Methods

4.1. Cell Culture

NHEK cells, the HaCaT cell line [27] and the HPV-KER cell line established in our laboratory [28] were used for the experiments. After obtaining written informed consent from investigated individuals, skin speciments from the Plastic Surgery Unit of our Department were used to separate NHEKs, as described previously. Investigations were carried out in accordance with the rules of the Helsinki Declaration, and prior study, the study design was approved by the Human Investigation Review Board of the University of Szeged (PSO-EDAFN-002, 23 February2015, Szeged, Hungary). The epidermis was separated from the dermis with overnight incubation in Dispase (Roche Diagnostics, Manheim, Germany), and keratinocytes were obtained after maceration in 0.25% trypsin. All cell types were grown in 75 cm 2 cell culture flasks. NHEKs and HPV-KER cells were maintained in keratinocyte serum-free medium containing epidermal growth factor and bovine pituitary factor (Gibco Keratinocyte SFM Kit; Life Technologies, Copenhagen, Denmark) and supplemented with 1% antibiotic/antimycotic solution (PAA Laboratories GmBH, Pasching, Austria) and 1% L-glutamine (PAA Laboratories). HaCaT cells were grown in DMEM with 4.5 g/L glucose supplemented with 1% antibiotic/antimycotic solution, 1% L-glutamine and 10% fetal bovine serum at 37 °C in a humidified atmosphere with 5% CO_2. The medium was changed every 2 days.

4.2. Stimulation of the Cells

HPV-KER cells, HaCaT cells or third passage NHEKs were seeded into 6-well plates. After 24 h, cells were transfected with 0.666 μg/mL polydeoxyadenylic acid-polydeoxythymidylic acid double-stranded homopolymer (poly(dA:dT)) (InvivoGene, San Diego, CA, USA) or with 0.666 μg/mL polyinosinic-polycytidylic acid (poly(I:C)) (Sigma Aldrich, Saint Louis, MO, USA) using the X-tremeGene 9 transfection reagent (Roche Diagnostics). Cells were harvested at indicated time points.

For inhibition studies, cells were incubated 1 h prior to poly(dA:dT)/poly(I:C) transfection with inhibitors for NF-κB (Bay 11-7085, 10 μM; MedChem Express, Monmouth Junction, NJ, USA), STAT-1

(Fludarabine, 10 μM; Sigma Aldrich), STAT-3 (Stattic, 5 μM; Sigma Aldrich), MEK1 (PD98059, 20 μM; Sigma Aldrich), JNK (SP600125, 10 μM; Tocris Bioscience, Bristol, UK) and p38 (SB203580, 10 μM; Tocris Bioscience).

4.3. RNA Isolation and RT-PCR

Cells were harvested in TRIzol® Reagent (Invitrogen Corp., Carlsbad, CA, USA) and total RNA was isolated following the manufacturer's instructions. Potential genomic DNA contamination was removed by using the Turbo DNA-free Kit (Ambion, Life Technologies) according to the manufacturer's instructions. 1 μg total RNA was reverse transcribed into cDNA by 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 TaqMan probes (Roche Diagnostics) and qPCRBIO Probe Mix Lo-ROX (PCR Biosystem Ltd., London, UK) on a C1000 Touch Thermal Cycler (Bio-Rad Laboratories), using primers reported previously [20]. The expression of each gene was normalized to the expression of the 18S rRNA gene. Relative mRNA levels were calculated by the $\Delta\Delta C_t$ method.

4.4. Detection of NF-κB Induction

Luciferase assays were performed to determine the NF-κB activity in response to poly(dA:dT) treatment. The HPV-KER cell line was transfected with the NF-κB reporter construct vector pNF-κB-luc *Cis*-Reporter Plasmid (Stratagene, La Jolla, CA, USA) and the pGL4.75 [hRluc/CMV] plasmid (Promega, Madison, WI, USA) with the use of the X-tremeGene9 transfection reagent. The treated cells were washed twice with PBS, lysed with passive lysis buffer (Biotium, Hayward, CA, USA) and the luciferase activities in the lysates were measured using the Firefly & Renilla Dual Luciferase Assay Kit (Biotium) and Thermo Luminoskan Ascent (Thermo Scientific, Rockford, IL, USA), according to the manufacturer's instructions. All samples were measured three times and the luciferase activity derived from the NF-κB-luc plasmid was normalized to the activity of the Renilla luciferase activity from pGL4.75 [hRluc/CMV] plasmid.

4.5. Western Blot Analysis

Cells were harvested at indicated time points after poly(dA:dT) or poly(I:C) transfection and lysed in lysis buffer supplemented with 0.5% SDS and 1% Halt™ Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific). Equal amounts of protein in ProTrack Loading Buffer (Lonza, Basel, Switzerland) were separated on a 7.5% TGX Fast Cast Gel and transferred to nitrocellulose membrane (0.45 μm; Bio-Rad Laboratories). After blocking the membrane in 5% non-fat milk in Tris-Buffered Saline containing 0.2% Tween-20, primary antibodies were incubated overnight at 4 °C with constant agitation. HRP-conjugated secondary antibodies were incubated for 60 min at room temperature. Signal was visualized with SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Scientific) on a C-Digit Blot Scanner (LI-COR Corp., Lincoln, NE, USA). Primary antibodies used were phospho-IκBα (Santa Cruz Biotechnology, Dallas, TX, USA), phospho-ERK1/2 (BioLegend, San Diego, CA, USA), phospho-STAT-1 (Ser727; Cell Signaling Technology, Danvers, MA, USA), phospho-STAT-3 (Ser727; Cell Signaling Technology), phospho-JNK (T183/Y185; Bio-Techne, Abingdon, UK) and phospho-p38 alpha (T180/Y182; Bio-Techne).

4.6. Statistical Analysis

Two-way repeated measurement analysis of variance (ANOVA) was used to compare more than two groups, and one-tailed, paired Student's *t*-test was used to compare two groups, as indicated in the figure legend. Based on at least three independent biological repeats, data are presented as mean ± standard error. Statistical analysis was carried out using Sigma Plot Ver. 13.0, (Systat Software Inc. Erkrath, Germany) the significance level was set at $p \leq 0.05$.

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/1422-0067/19/3/774/s1.

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Author Contributions: Judit Danis and Márta Széll conceived and designed the experiments and wrote the manuscript, Judit Danis, Luca Janovák, Barbara Gubán and Anikó Göblös performed the experiments and analyzed the data. Kornélia Szabó, Lajos Kemény and Zsuzsanna Bata-Csörgő contributed reagents and cells, helped with evaluation of the results and critically revised the manuscript. All authors have revised and approved the final version.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

IL-6	interleukin-6
TNF-α	tumor necrosis factor α
PAMP	pathogen associated molecular pattern
DAMP	damage associated molecular pattern
DNase	deoxyribonuclease
AIM2	absent in melanoma 2
IFN	interferon
TLR	toll like receptor
RIG-I	retinoic acid induced gene I
cGAS	cyclic GMP-AMP synthase
ds	double-stranded
poly(dA:dT)	polydeoxyadenylic acid-polydeoxythymidylic acid double-stranded homopolymer
poly(I:C)	Polyinosinic-polycytidylic acid
MAPK	mitogen activated protein kinase
JNK	c-Jun N-terminal kinase
ERK1/2	extracellular signal-regulated protein kinase 1 and 2
MEK1/2	dual specificity mitogen-activated protein kinase kinase 1 and 2
NF-κB	nuclear factor κB
IκBα	NF-κB inhibitor α
STAT	signal transducer and activator of transcription
NHEK	normal human epidermal keratinocyte

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II.

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ORIGINAL RESEARCH
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PRINS Non-Coding RNA Regulates Nucleic Acid-Induced Innate Immune Responses of Human Keratinocytes

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Cytosolic DNA fragments are recognized as pathogen- and danger-associated molecular patterns that induce a cascade of innate immune responses. Moreover, excessive cytosolic DNA can enhance chronic inflammation, predominantly by activating inflammasomes, and thereby contributing to the pathogenesis of chronic diseases, such as psoriasis. Psoriasis associated non-protein coding RNA induced by stress (PRINS) is a long non-coding RNA, which has been shown to be associated with psoriasis susceptibility and cellular stress responses; however, the precise mechanism of its action has not been determined. Here, we provide evidence that, in addition to inflammasome activation, cytosolic DNA induces intracellular inflammatory reactions while decreasing PRINS gene expression. Furthermore, PRINS overexpression can ameliorate the inflammatory-mediator production of keratinocytes induced by cytosolic DNA. Overexpression of PRINS resulted in decreased interleukin-6 (IL-6) and chemokine (C–C motif) ligand 5 (CCL-5) expression and secretion. *In silico* analysis predicted direct binding sites between PRINS and the mRNAs, which was confirmed by an *in vitro* binding assay and on cellular level. Our results indicated that PRINS binds directly to the mRNAs of IL-6 and CCL-5 at specific binding sites and eventually destabilizes these mRNAs, leading to a decrease in their expression and secretion of the corresponding proteins. These results may indicate a restrictive role for PRINS in inflammatory processes.

Keywords: PRINS, long non-coding RNA, interleukin-6, CCL-5, keratinocyte, poly(dA:dT)

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INTRODUCTION

The skin provides the first line of defense against a variety of environmental, chemical, and physical stimuli and acts as an active member of the innate immune system. Double-stranded DNA (dsDNA) fragments are known to induce antiviral responses in keratinocytes (1, 2) as well as induce inflammasome activation and subsequent interleukin (IL)-1β release in these cells (3, 4). Moreover, keratinocytes express a wide range of pattern recognition receptors (PRR) for nucleotide fragments (5, 6), mainly implicated in antiviral reactions (2, 6). These PRRs are required for immune response in an acute infection and might also lead to the exacerbation of chronic inflammatory inherited multifactorial diseases, such as psoriasis (7). Psoriasis is caused by the interplay of professional immune cells and keratinocytes. Cytosolic nucleotide fragments are highly abundant in psoriatic skin (3), leading to chronic activation of professional immune cells (8), and are thought to be an initiator factor in the disease. In particular, cytosolic nucleotide fragments do not lead to antiviral reaction, but instead activate the AIM2 inflammasome signaling in psoriatic keratinocytes (3), leading to prolonged inflammation.

In the last decade, genome-scale transcription studies have uncovered non-coding RNAs as previously unrecognized players in the dysregulation of inflammatory reaction in psoriatic skin (9, 10). The contribution of both microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) to psoriasis has been extensively studied (11, 12). We were first to identify a lncRNA potentially contributing to disease susceptibility: PRINS, the psoriasis susceptibility-related non-coding RNA induced by stress, which is highly expressed in psoriatic non-lesional epidermis compared both to lesional or healthy epidermis (13). The expression of PRINS is modified by a diverse set of cellular stressors, including starvation, ultraviolet B (UVB) irradiation, translation inhibition (14), and hypoxia (15). Moreover, microbial stimuli decreased PRINS expression in macrophages (16) and normal human epidermal keratinocytes (NHEKs) (14), and PRINS was recently shown to interact with chemokine (C–C motif) ligand 5 (CCL-5, also known as RANTES) in kidney epithelial cells (15). CCL-5 is a chemokine attracting leukocytes to the site of inflammation. Under inflammatory conditions, keratinocytes produce large amount of CCL-5 to attract antigen-presenting cells (17) and T-cells into the epidermis (18); moreover, CCL-5 is overexpressed in atopic dermatitis and psoriatic lesions (19). Based on these findings, we aimed to investigate whether the PRINS lncRNA contributes to keratinocyte innate immune responses.

According to our results, PRINS can ameliorate dsDNA-induced keratinocyte immune responses. Bioinformatic analysis of the PRINS lncRNA sequence revealed putative interaction sites for interleukin-6 (IL-6) and CCL-5 mRNAs. Destruction of the putative interacting region resulted in the loss of the ability of PRINS to bind to the IL-6 mRNA. In addition, our functional studies confirmed the regulatory role of the interaction between PRINS and IL-6 mRNA. Our results suggest a general anti-inflammatory function for PRINS and provide insight to the role of high PRINS expression in psoriatic non-lesional skin.

MATERIALS AND METHODS

Cell Culture and Inflammatory Stimuli

Normal human epidermal keratinocytes used for the experiments were separated from skin specimens obtained from the Plastic Surgery Unit of our department. Written informed consent was obtained from all investigated individuals. The study was approved by the Human Investigation Review Board of the University of Szeged and complied with the ethical standards of research and in accordance with the Helsinki Declaration.

Epidermis was separated from the dermis 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, containing EGF and BPE (Gibco Keratinocyte SFM Kit; Life Technologies, Copenhagen, Denmark) and 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 medium was changed every 2 days.

Third passage keratinocytes were seeded into 6-well plates. Cells were primed in supplement-free medium by addition of 5 ng/ml tumor necrosis factor-α (TNF-α) and 5 ng/ml interferon-γ (IFN-γ). After 24 h, cells were transfected with 0.666 μg/ml polydeoxyadenylic acid–polydeoxythymidylic acid double-stranded homopolymer [poly(dA:dT), Sigma Aldrich, Saint Louis, MO, USA] using the X-tremeGENE 9 DNA transfection reagent (Roche Diagnostics). Cells were harvested 12 h after poly(dA:dT) transfection.

Gene-Specific Overexpression

For overexpressing PRINS, the AK022045 cDNA sequence [Biological Resource Center (NBRC) National Institute of Technology and Evaluation, Chiba, Japan] was cloned into a pcDNA3.1(+) vector. The empty pcDNA3.1(+) vector served as a control.

The ΔPRINS construct was created by replacing the AK022045 region (position 538–622) with the following scrambled sequence: GTGCGTGGCGGAGACGTGGTGGTAGAC CGAATTGAGGAGGATCCGAAGGTTAGACGT AGGCGATCGCCGCTTCGGACGCGGTCGC. The ΔPRINS sequence was created by GeneArt gene synthesis (Thermo Scientific), and cloned into a pcDNA3.1(+) vector.

Transient transfection of NHEK cells was carried out at approximately 70% confluency in parallel to cytokine priming, using the X-tremeGENE HP DNA transfection protocol, as described by the manufacturer (Roche Diagnostics). The transfection efficiency was 85% on average, as determined by the transfection of a GFP reporter construct (Lonza, Basel, Switzerland) and analysis of GFP expression by flow cytometry. The effectiveness of overexpression was investigated with real-time RT-PCR (Figure S1 in Supplementary Material).

In Silico Prediction of Interacting Sites

Sequence complementarity between PRINS (AK022045) and the mRNA of IL-1α (M28983.1), IL-1β (NM_000576.2), IL-6 (NM_000600.4), IL-8 (NM_000584.3), TNF-α (NM_000594.3), and CCL-5 (NM_002985.2) was analyzed using two algorithms: RIsearch (20), which uses a simplified nearest-neighbor energy model, and INTARNA (21–23), which calculates the free-energy values of the interaction based on predicted global and local structures of mRNAs. The regions predicted by both programs were considered as putative interaction sites.

RNA Isolation and RT-PCR

Total RNA was isolated from cells using TRIzol® Reagent (Invitrogen Corp., Carlsbad, CA, USA), following the manufacturer's instructions. Turbo DNA-free Kit (Ambion, Life Technologies) was used for the removal of contaminating DNA. cDNA was synthesized from 1 μg total RNA using 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 (Roche Diagnostics) using a C1000 Touch Thermal Cycler (Bio-Rad Laboratories) and the primers listed in Table S1 in Supplementary Material. The expression of each gene was normalized to the 18S rRNA gene. Relative mRNA levels were calculated by the ΔΔCt method.

ELISA

Cell supernatants were centrifuged (5,000 rpm, 4 min, 4°C) to pellet cell debris, and the amount of cytokines IL-1α, IL-1β, IL-6, IL-8, CCL-5, and TNF-α was determined by ELISA (IL-1α Duo Set, IL-1β ELISA Duo Set, and TNF-α Duo Set, R&D Systems, Minneapolis, MN, USA; Human IL-6 Mini TMB ELISA Development Kit, Human IL-8 TMB ELISA Development Kit, Human RANTES ATBS ELISA Development Kit, PeproTech, Rocky Hill, NJ, USA), according to the manufacturer's instructions.

In Vitro Transcription and *In Vitro* Binding Assay

PRINS and ΔPRINS RNA sequences were produced by *in vitro* transcription from pcDNA3.1(+) containing the AK022045 or ΔPRINS cDNA sequence, using Transcript Aid T7 *In Vitro* Transcription Kit (Thermo Scientific). Products were purified by the GeneJET RNA Purification Kit (Thermo Scientific). Quality and sequence-length analyses were carried out on reducing agarose gel-electrophoresis. The single-stranded RNA products were used in a fluorescent binding assay. The fluorescently labeled RNA sequence 5′6-carboxyfluorescein(6-FAM)/GAAGCUCUAU CUCCCCUCCAGGAGCCCAGCUAUGAACUCCUUCUCC ACAAGCGCCUUCGGUCCAGUUGCCUUCUCCCCU GGGGCUGCUCCUGGUGUUGCCUGCUGCCUUCCCU GCC-3′, comprising positions 91–205 of the IL-6 (NM_000600.4) mRNA sequence, was produced by Integrated DNA Technologies.

An *in vitro* binding assay was carried out on a Monolith NT.115 Pico MicroScale Thermophoresis instrument (NanoTemper GmbH, Germany), in nuclease-free water, at 25°C, with 80% Laser Power, 10% LED Power, by 2bind GmbH, Regensburg, Germany. Fluorescence enhancement of the 6-FAM labeled specific truncated IL-6 RNA sequence or a 6-FAM labeled unspecific DNA, as negative control, was measured after addition of PRINS or ΔPRINS. The concentration of fluorescently labeled molecules was 10 nM constantly, while unlabeled RNA concentration ranged from 126.75 nM to 61.9 pM. Initial fluorescence was analyzed for binding curves; the formulation used is displayed in Figure S2 in Supplementary Material.

Statistical Analysis

Experiments were carried out in triplicate with at least three biological repeats, as indicated in figure legends. For statistical analysis, one-way ANOVA was used to compare more than two groups, and one-tailed, paired *t*-test was used to compare two groups. Statistical analysis was carried out using R software, version 3.2.2., and the significance level was set at $p \leq 0.05$.

RESULTS

Poly(dA:dT) Treatment of Keratinocytes Induces the Expression of Inflammatory Cytokines while Decreasing the Expression of PRINS

To study how dsDNA influences NHEKs immune function, we analyzed the expression (**Figure 1**) and secretion (**Figure 2**) of several inflammatory cytokines and the expression of PRINS upon poly(dA:dT) exposure.

We confirmed that poly(dA:dT) significantly induced the expression and secretion of IL-1β in keratinocytes, as described previously (4). In addition, poly(dA:dT) induced very strong mRNA expression of IL-1α, IL-6, IL-8, and TNF-α in NHEKs (**Figures 1B–F**).

To gain pronounced inflammatory reaction in NHEKs, a priming step using TNF-α and IFN-γ is often applied before poly(dA:dT) treatment (3, 4); however, the cumulative effect of this treatment on the expression of other cytokines has not been examined thoroughly. When cells were primed for 24 h before poly(dA:dT) treatment, IL-1β secretion was enhanced by the combination of TNF-α and IFN-γ compared to poly(dA:dT) treatment alone. In contrast, IL-1β mRNA expression was not affected by priming (**Figures 1E** and **2D**). Similarly, priming had no effect on TNF-α mRNA expression (**Figure 1F**). However, priming induced significantly higher mRNA expression of the other investigated cytokine genes (**Figures 1B–D**).

In a manner similar to mRNA expression, poly(dA:dT) transfection induced the secretion of the investigated cytokines, and the priming step significantly enhanced the amount of secreted IL-1α, IL-1β, and IL-8 in the keratinocyte supernatant (**Figure 2**).

As we and others have demonstrated altered PRINS expression upon exposure to inflammatory molecules (14, 16), we examined PRINS expression upon poly(dA:dT) treatment. Poly(dA:dT) alone or combined with cytokine priming decreased the expression of PRINS in NHEKs (**Figure 1A**). The expression of PRINS returned to the initial level after 24 h (data not shown), whereas the expression of inflammatory cytokines declined during this period.

PRINS Overexpression Decreased IL-6 and IL-8 Levels in Keratinocytes

The change in PRINS expression upon inflammatory stimuli suggests the possible contribution of PRINS to inflammatory responses of NHEK cells. To determine whether PRINS can regulate inflammatory cytokine expression, cells were transfected with a construct for overexpressing PRINS during the combined priming and poly(dA:dT) treatment. Expression and secretion of IL-6 and IL-8 was significantly decreased by PRINS overexpression (**Figure 3**), whereas mRNA expression and secretion of IL-1α, IL-1β, and TNF-α were not affected (data not shown). These results suggest that PRINS does not influence inflammasome activation, but instead influences the regulation of other inflammatory processes.

In Silico Analysis Revealed Putative Interacting Sites between the PRINS lncRNA and the IL-6 mRNA

The mRNA of the chemokine CCL-5 was previously predicted to interact with PRINS; however, it was not reported whether this interaction affects the stability of the CCL-5 mRNA (15). Therefore, we measured mRNA expression and secretion of CCL-5 during PRINS overexpression, and found that both decreased (Figure S3 in Supplementary Material) in a manner similar to the changes observed for IL-6 and IL-8. The similarity of these expression

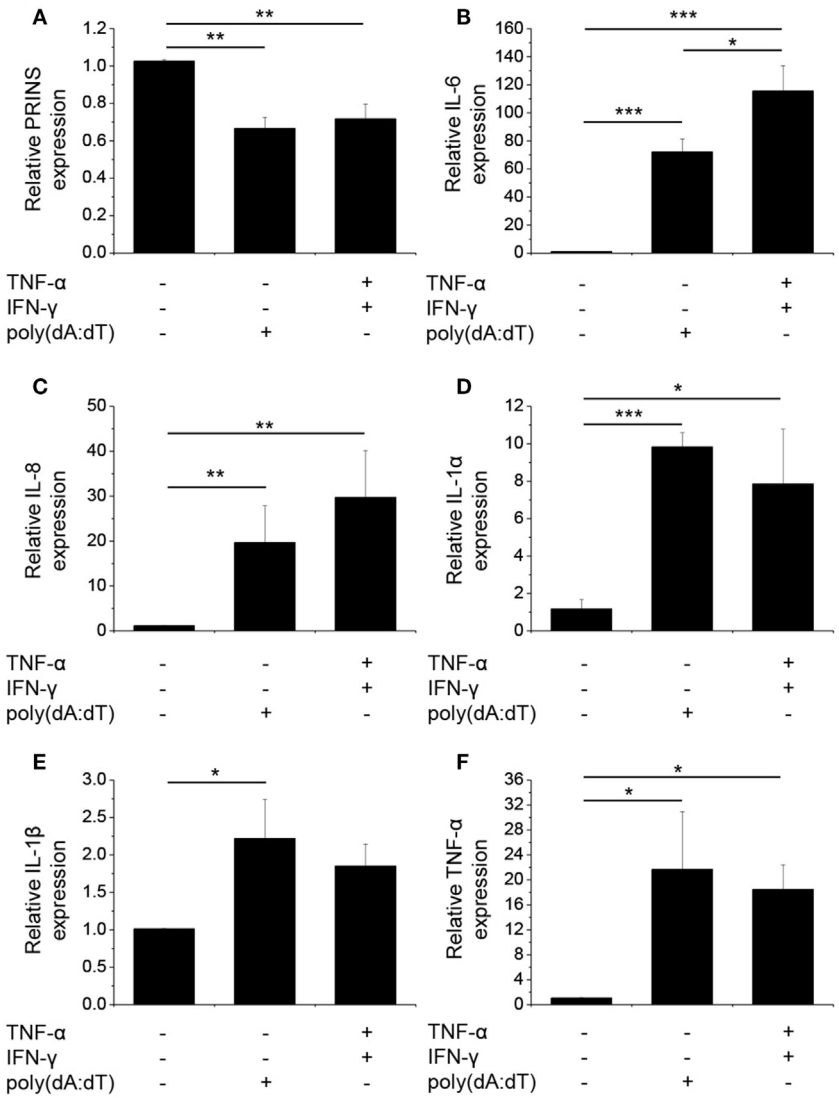

FIGURE 1 | Poly(dA:dT) treatment decreased the expression of PRINS but induced the expression and secretion of cytokines. Normal human epidermal keratinocytes were transfected with poly(dA:dT) for 12 h with or without priming for 24 h with 5 ng/ml tumor necrosis factor-α (TNF)-α and interferon-γ (IFN-γ). RNA expression was detected for PRINS **(A)** and cytokines interleukin-6 (IL-6) **(B)**, IL-8 **(C)**, IL-1α **(D)**, IL-1β **(E)**, TNF-α **(F)** by real-time RT-PCR. Data are presented as mean ± SE of five independent experiments (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$).

profiles led us to hypothesize similar mechanism(s) for IL-6, IL-8, and CCL-5 regulation mediated by PRINS.

To predict interactions between PRINS (AK022045) and the mRNAs of IL-6 (NM_00600.4) and IL-8 (NM_000584.3), we performed an *in silico* analysis using INTARNA (22, 23) and RIsearch (20) software. As sequence details of the CCL-5 mRNA and PRINS interaction have not been described in detail (15), we also included the CCL-5 mRNA (NM_002985.2) in the *in silico* analysis. As a control for the reliability of the prediction analyses, mRNA sequences of cytokines not affected by PRINS overexpression (IL-1α—M28983.1, IL-1β—NM_000576.2, TNF-α—NM_000594.3) were also included. The regions predicted by both algorithms were considered putative interacting sites. Putative interaction sites were not predicted for the cytokines not affected by PRINS overexpression (IL-1α, IL-1β, TNF-α) in this analysis. PRINS interaction regions were only predicted for the IL-6 and CCL-5 mRNAs (**Figures 4B,C**), two mediators affected by PRINS overexpression. A distance of approximately 200 nt separates the predicted interaction sites in the PRINS sequence, and the corresponding sites occur in the 5′ untranslated region (UTR) of IL-6 and the 3′ UTR of CCL-5 (**Figure 4A**). No interaction site was predicted for IL-8.

PRINS Binds to IL-6 mRNA through Direct, Sequence-Specific Interaction

To validate the predicted interaction site, an *in vitro* binding experiment was carried out using the PRINS lncRNA and the IL-6 mRNA. Binding affinity was determined using the

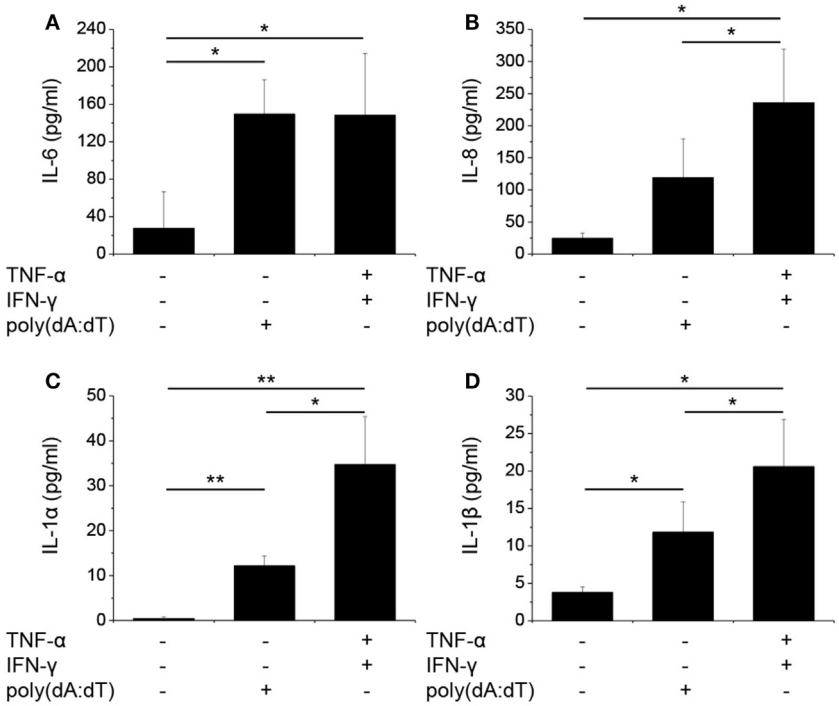

FIGURE 2 | Poly(dA:dT) treatment induced the secretion of cytokines by normal human epidermal keratinocytes (NHEKs). NHEKs were transfected with poly(dA:dT) for 12 h with or without priming for 24 h with 5 ng/ml tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ). Secretion of cytokines interleukin-6 (IL-6) **(A)**, IL-8 **(B)**, IL-1α **(C)**, IL-1β **(D)** was detected by ELISA from cell supernatant. Data are presented as mean ± SE of five independent experiments (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$).

single-stranded PRINS RNA and a fluorescently labeled, truncated IL-6 mRNA sequence containing the predicted interacting sequence. The ΔPRINS sequence, in which the predicted interaction site to IL-6 was replaced by scrambled sequence, was used as a control. As a negative control, a fluorescently labeled DNA sequence with no similarity to either PRINS or IL-6 RNAs was used. While PRINS exhibited a very high binding affinity to the labeled IL-6 mRNA (**Figure 5A**, Kd = 10.3436 nM), specific binding for ΔPRINS and the unspecific labeled DNA could not be detected (**Figure 5B**). This result confirms *in vitro* the specificity of the regions predicted *in silico* (538–622 of PRINS, AK022045 and 92–204 of IL-6 mRNA, NM_000600.4).

PRINS Decreases IL-6 Expression in NHEKs through Sequence-Specific Interaction

To further validate the functionality of the *in silico* predicted and *in vitro* determined IL-6 mRNA interacting region in the PRINS sequence, we performed the overexpression experiments in NHEKs with vectors containing the wild-type PRINS or ΔPRINS (with scrambled IL-6 binding site) sequences, during the combined priming and poly(dA:dT) treatment. IL-6 expression was not affected by overexpression of ΔPRINS but was, in contrast, significantly decreased by PRINS overexpression (**Figure 6A**), and similar tendencies were seen in the amount of secreted IL-6 (**Figure 6D**). To confirm the specificity of this region in IL-6 regulation, the expression of CCL-5 was also studied. CCL-5 expression and secretion decreased similarly both in cells overexpressing ΔPRINS and in cells overexpressing PRINS (**Figures 6B,E**). IL-8 expression upon PRINS or ΔPRINS overexpression showed a similar tendency to IL-6 expression, although significant differences could not be detected (**Figures 6C,F**). These result demonstrated that the binding site in the PRINS sequence is essential and specific for the regulation of IL-6 expression.

DISCUSSION

Due to large-scale gene-expression studies the number of annotated human non-coding RNAs has increased rapidly (24), but functional roles have been assigned only to a few of them (25, 26). PRINS was one of the first lncRNAs described to be dysregulated in a disease, namely in psoriasis (13). PRINS is located in an intron of the KIAA1217 gene on the sense strand (27), and, thus, it can be considered as a long intronic non-coding RNA. The expression of PRINS increases in response to various cellular stressors (14) in HaCaT cells, as well as to hypoxia in HK-2 cells (15) suggesting that PRINS contributes to the stress response of the cells. Recent reports on PRINS showing decreased expression upon inflammatory stimuli in both NHEKs and macrophages (14, 16), and the potential interaction between PRINS and the mRNA of CCL-5 (15) has led us to study its potential contribution to keratinocytes immune responses.

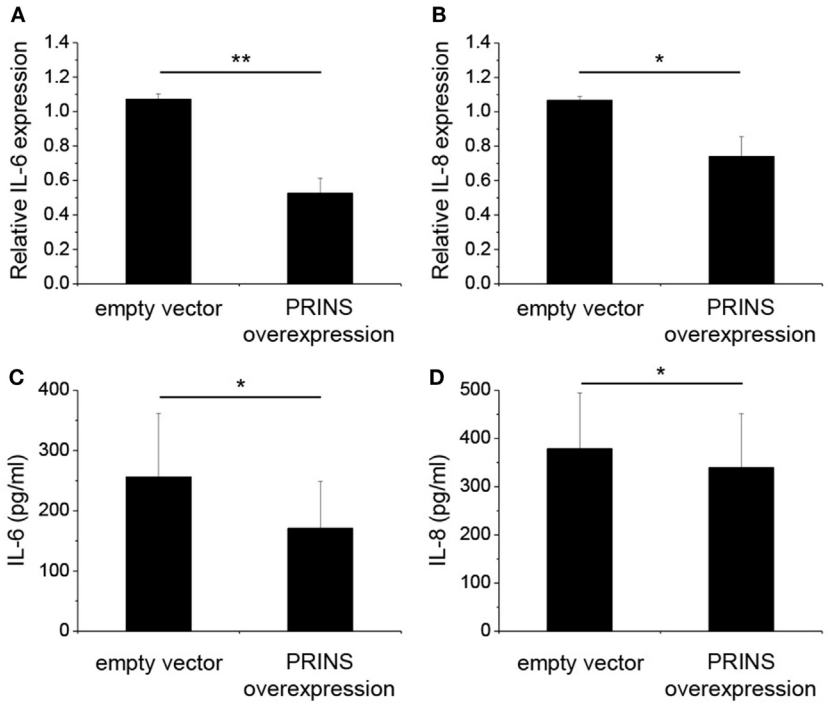

FIGURE 3 | Overexpression of PRINS regulates the expression and secretion of interleukin-6 (IL-6) and IL-8. In parallel to priming with 5 ng/ml tumor necrosis factor-α and interferon-γ, normal human epidermal keratinocytes were transfected with a pcDNA3.1(+) vector containing the PRINS cDNA; an empty pcDNA3.1(+) vector was used as control. After 24 h, cells were transfected with poly(dA:dT). RNA expression of cytokines IL-6 **(A)** and IL-8 **(B)** was detected by real-time RT-PCR. Secretion of cytokines IL-6 **(C)** and IL-8 **(D)**, was detected by ELISA from cell supernatants. Data are presented as mean ± SE of four independent experiments (*$p \leq 0.05$; **$p \leq 0.01$).

To study keratinocyte immune functions, we applied a treatment with the cytosolic DNA-analog poly(dA:dT), which was reported to induce inflammasome activation in these cells (3, 28) and induce the expression of several cytokines through a RIG-I-dependent mechanism in professional immune cells (29). Before poly(dA:dT) treatment, a priming with the cytokines TNF-α and IFN-γ was applied, which were described to be key elements in keratinocyte immune responses (30) and induce a pronounced inflammasome activation (4). Our results are in agreement with recent reports describing inflammasome activation in keratinocytes (3, 4, 28) and IL-6, IL-8, and TNF-α induction in canine keratinocytes (6) and human melanocytes (31) upon poly(dA:dT) exposition. Priming induced significantly enhanced mRNA expression of IL-6 and IL-8. In addition, cytokine secretion significantly increased compared to poly(dA:dT) treatment alone. In contrast to cytokine expression, poly(dA:dT) treatment decreased PRINS expression in NHEKs in line with previous reports in NHEKs and macrophages (14, 16).

PRINS was recently described to potentially interact with the CCL-5 mRNA (15), but its function remains to be elucidated. In this study, we demonstrate that PRINS overexpression decreases the expression of IL-6, IL-8, and CCL-5 in keratinocytes upon poly(dA:dT) treatment. In addition, decreased IL-6 expression was observed upon PRINS overexpression in UVB-treated samples as well (data not shown). Using *in silico* analysis, regions in the PRINS sequence were predicted to interact with CCL-5 and IL-6 mRNA. As inhibition of transcription by DNA:lncRNA triplex formation or posttranscriptional destabilization of the mRNA by mRNA–lncRNA duplex formation has been reported (25), we analyzed the sequences and found that the interacting site on IL-6 spans two exons, indicating an mRNA–lncRNA interaction. Moreover, PRINS demonstrated perinuclear localization in keratinocytes (32), making it possible to exert its effect at the posttranscriptional level. The mRNA–lncRNA interaction was validated *in vitro*: PRINS showed a very high binding affinity (Kd = 10.3436 nM) to IL-6 mRNA, and the destruction of the predicted binding site abolished the ability of PRINS to bind to the IL-6 mRNA. The binding site of PRINS lies within the 5′ UTR of the IL-6 mRNA, which is a rare phenomenon. The majority (~40%) of lncRNAs bind to the 3′ UTR of their target mRNAs, while only around 5% of lncRNAs is able to bind to the 5′ UTR of their target (33). The functionality of the mRNA–lncRNA interaction was also observed at the cellular level, as overexpression of ΔPRINS, in which the IL-6 interacting site is scrambled, did not decrease IL-6 levels. Thus, we demonstrated that PRINS is able to bind the IL-6 mRNA and this specific interaction destabilizes IL-6 expression and secretion in NHEKs.

Similar to IL-6, PRINS overexpression decreased the expression of IL-8, but no interaction site could be predicted. We hypothesize that this is a secondary effect (34); however, we cannot exclude the possibility of a yet unknown interaction site between PRINS and IL-8 mRNA.

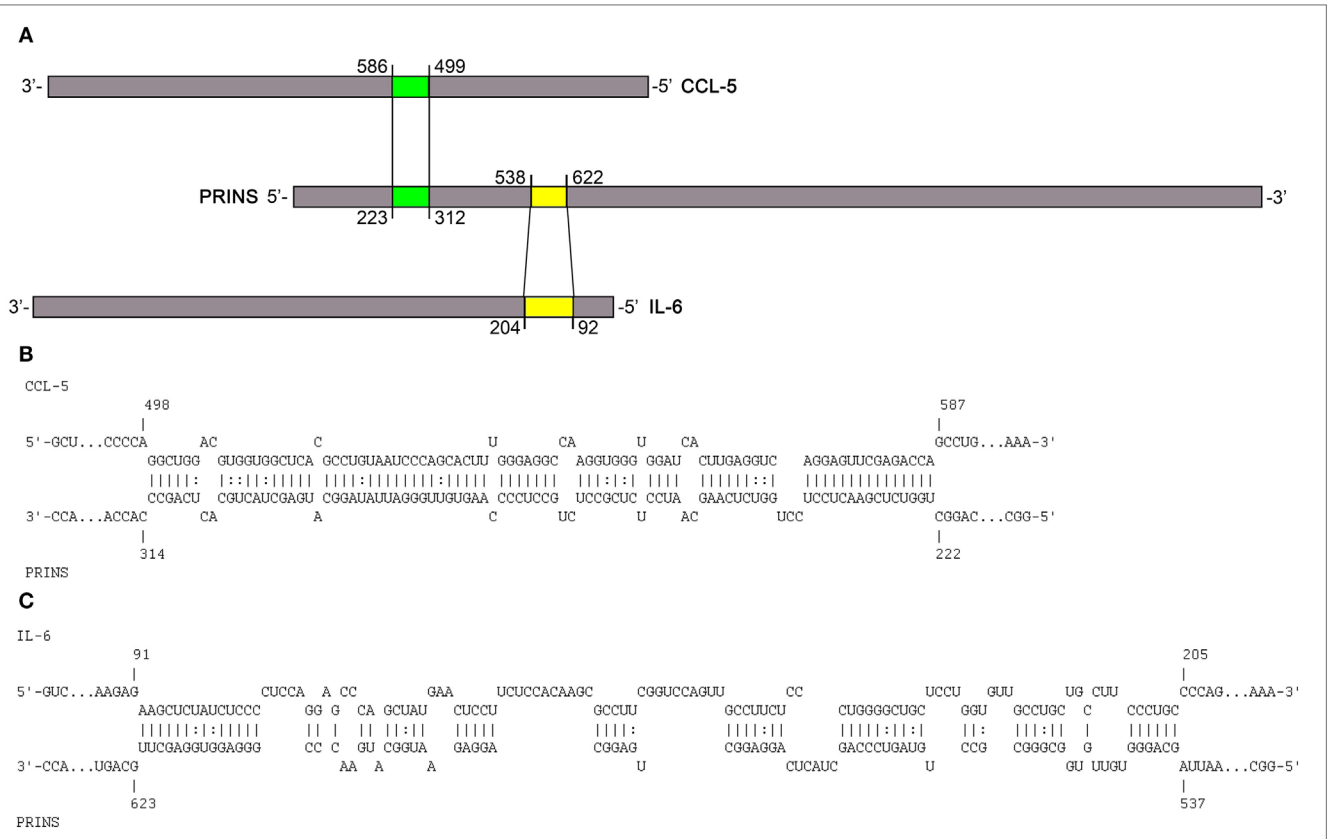

FIGURE 4 | Putative interactions between PRINS long non-coding RNA and chemokine (C-C motif) ligand 5 (CCL-5) and interleukin-6 (IL-6) mRNAs. Schematic representation of the *in silico* predicted interaction sites in the PRINS sequence for the CCL-5 and IL-6 mRNAs **(A)**. PRINS sequences predicted to interact with CCL-5 mRNA **(B)** and IL-6 mRNA **(C)**. Nucleotide positions are given based on the AK022045, NM_000600.4, and NM_002985.2 reference sequences.

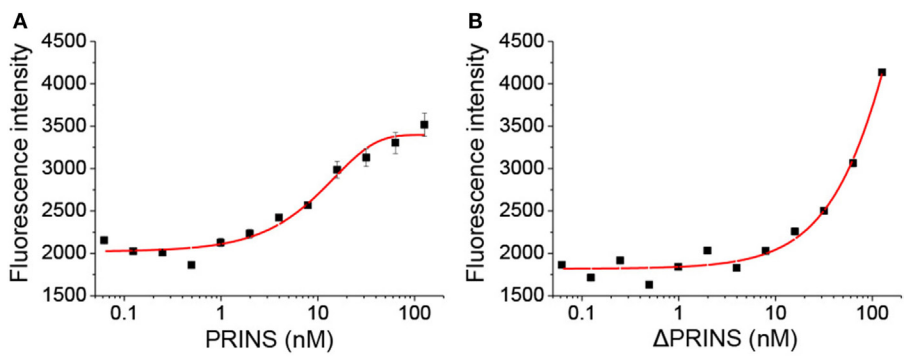

FIGURE 5 | PRINS specifically binds to interleukin-6 (IL-6) mRNA. The binding affinity of PRINS **(A)** and ΔPRINS **(B)** to IL-6 mRNA was determined by analyzing the initial fluorescence enhancement caused by specific binding. The concentration of labeled IL-6 mRNA was constant (10 nM), while the concentration of PRINS and ΔPRINS varied from 62 pM to 127 nM.

Many lncRNAs which were reported to regulate mRNA expression were found to act as primary transcripts for miRNAs (35). We have found no evidence that PRINS is cleaved to smaller miRNA precursors, but the IL-6 binding site of PRINS is 96% similar to the miR5585, which is predicted by TargetMiner to have IL-6 mRNA as a target (36). miR5585 is located on Chromosome 7, whereas PRINS is located on Chromosome 10, so their high similarity and overlapping functions might originate from gene duplication.

Like many other non-coding RNAs, PRINS seems to have multiple cellular functions (37). It binds to nucleophosmin, a chaperon protein, and facilitates its transition from the nucleolus to the nucleoplasm upon UVB irradiation (32). Additionally, our recent results suggest that PRINS is involved with inflammation

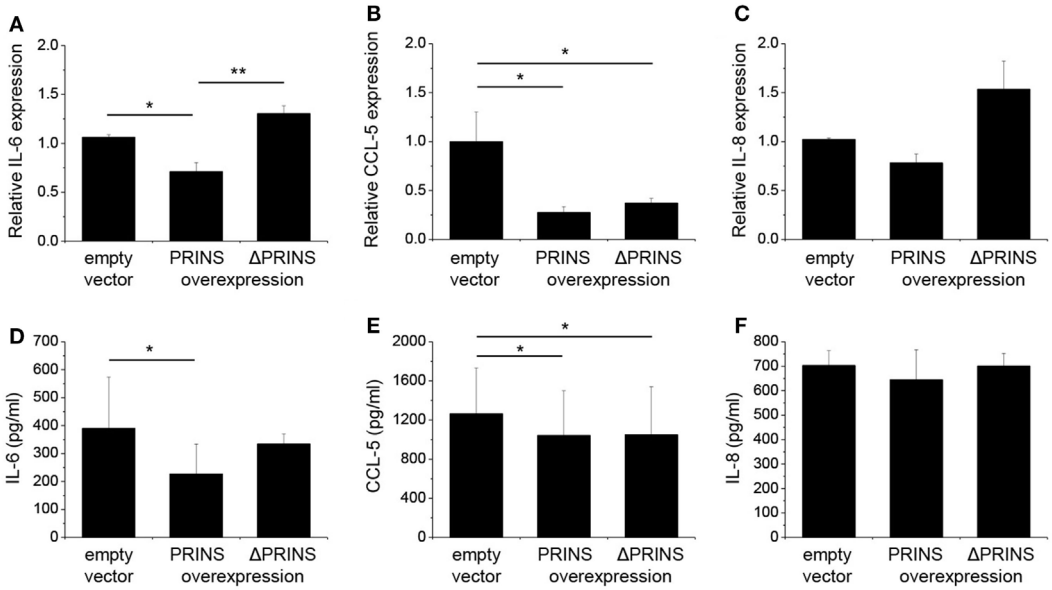

FIGURE 6 | The 538–622 nt region of PRINS is required for the regulation of interleukin-6 (IL-6) expression. In parallel to priming with 5 ng/ml tumor necrosis factor-α and interferon-γ, normal human epidermal keratinocytes were transfected with a pcDNA3.1(+) vector containing the PRINS cDNA or the ΔPRINS cDNA (containing a scrambled version of the IL-6 binding site at positions 538–622 nt); the empty pcDNA3.1(+) vector served as control, after 24 hours cells were transfected by poly(dA:dT). Expression of cytokines IL-6 **(A)**, CCL-5 **(B)**, and IL-8 **(C)** was detected by real-time RT-PCR. Secretion of cytokines IL-6 **(D)**, CCL-5 **(E)**, and IL-8 **(F)** was detected by ELISA from cell supernatants. Nucleotide positions based on the AK022045 reference sequence. Data are presented as mean ± SE of three independent experiments (*$p \leq 0.05$; **$p \leq 0.01$).

by inhibiting cytokine expression. Based on our current and previous findings (13–15), we hypothesize that elevated PRINS expression in psoriatic uninvolved epidermis may contribute to downregulation of the inflammatory functions in psoriatic keratinocytes and maintenance of normal phenotype.

Our studies were performed using a non-professional immune cell type, keratinocytes; however, the same mechanisms might be also relevant in professional immune cells. Further studies on the same PRINS-related cellular events upon additional stressors such as UVB or microbial components may widen our knowledge on the cellular functions of PRINS and, in general, about lncRNAs.

AUTHOR CONTRIBUTIONS

JD and MS designed the study, JD and AG prepared laboratory work and analyzed the data. DJ, AG, ZB-C, LK, and MS interpreted the data and drafted the manuscript. All authors approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.01053/full#supplementary-material.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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INVITED REVIEW

PRINS, a primate-specific long non-coding RNA, plays a role in the keratinocyte stress response and psoriasis pathogenesis

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Abstract In the last few years with the recent emergence of high-throughput technologies, thousands of long non-coding RNAs (lncRNAs) have been identified in the human genome. However, assigning functional annotation and determining cellular contexts for these RNAs are still in its infancy. As information gained about lncRNA structure, interacting partners, and roles in human diseases may be helpful in the characterization of novel lncRNAs, we review our knowledge on a selected group of lncRNAs that were identified serendipitously years ago by large-scale gene expression methods used to study human diseases. In particular, we focus on the Psoriasis-susceptibility-Related RNA Gene Induced by Stress (PRINS) lncRNA, first identified by our research group as a transcript highest expressed in psoriatic non-lesional epidermis. Results gathered for PRINS in the last 10 years indicate that it is conserved in primates and plays a role in keratinocyte stress response. Elevated levels of PRINS expression in psoriatic non-lesional keratinocytes alter the stress response of non-lesional epidermis and contribute to disease pathogenesis. Finally, we propose a categorization for the PRINS lncRNA based on a recently elaborated system for lncRNA classification.

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Keywords Psoriasis · lncRNA · Keratinocyte stress response · PRINS · Primate specific

Introduction

One of the biggest surprises at the completion of the Human Genome Project [24] was the discovery of the low protein-coding capacity of the completed sequence: only approximately 2 % of our genome encodes proteins, corresponding to roughly 20,000 genes. Is it a matter of wastefulness that "Mother Nature" maintains at least 234 genes (more than 1 % of protein encoding genes) [41] to keep our genome in a good shape when only 2 % of it has any meaning? Now, it is clear that this repair machinery is not working leanly, as approximately 80 % of our DNA content is functional and most of it is transcribed into RNA [20, 22]. Understanding this only opened new questions: what is the function of this large amount of RNA? Will this understanding bring us closer to resolving questions about cell physiology? Moreover, will it bring new understanding of the still unknown mechanisms of genetically determined diseases that afflict us? Thanks to recently developed molecular biology tools, such as tiling array technologies [46] and RNA-Seq [13] as well as bioinformatics tools [8], we know that tens of thousands of functional RNA molecules are transcribed from our genome with varying length, genomic content, and functions. Concomitant with the growing number of characterized RNA molecules, the need for effective classification has increased. Although many attempts have been made to define subclasses of RNA function and various classification criteria have been proposed by multiple authors [62], RNA size has remained the primary aspect amenable for classification. From the start of the classification attempts, it was suspected that microRNAs (miRNAs, approximately 22 nucleotides in length) comprised

a distinct class of molecules within non-coding RNAs. Now, it is clear that their function, as well as their size, distinguishes miRNA. To date (http://www.mirbase.org/cgi-bin/mirna_summary.pl?org=hsa), 1881 distinct human miRNAs have been identified, and since 2001, extensive research has uncovered the function of many miRNAs in physiologically normal and disease conditions. miRNAs are known to be involved in the regulation of all aspects of intracellular events and the mechanism by which they act has been clarified [23].

The function of another distinct group, the long non-coding RNAs (lncRNA, >200 nucleotides), is not as well known. Thousands of lncRNAs have been identified in the recent years [74], and it is generally accepted that this group of RNAs has much more diverse functions than miRNAs.

Already at the beginning of the molecular biology revolution, it was relatively easy to identify protein-coding genes within human sequences by identifying open reading frames (ORF). No such sequence analysis tools are available for identifying non-coding genes. Since the 1990s, when the importance of lncRNAs was uncovered, many attempts have been made to develop computational approaches for identifying lncRNAs within the human genome [25, 75]. Through the efforts of the GENCODE Project, a good estimate for the frequency, the structure, the evolution, and the expression of lncRNAs is available [17]. An analysis of lncRNA annotation by Darrien et al. (2012) concluded that the human genome includes approximately 14,000 lncRNA genes, a number that are much higher than those estimated for miRNA genes and relatively close to the number of protein-coding genes. Although much information has been gathered about the functions of proteins and miRNAs in health and in disease, we are still at the beginning of our journey in exploring the functions of lncRNAs.

The great past and bright future of lncRNAs unexpectedly identified by large-scale gene expression experiments in human diseases

In order to understand the functions of thousands of lncRNAs, we must characterize them one by one, understand their molecular and cellular contexts, and discover their contribution to human diseases. Before the "big boom" of omics research, sporadic reports of the identification and functional characterization of lncRNAs were available. Interestingly, most of these early reports came from attempts to identify differentially expressed transcripts in human diseases using relatively old-fashioned methods of large-scale gene expression, such as complementary DNA (cDNA) λ phage library screening [16], cDNA subtractive hybridization [33], and differential display [61]. These studies identified the first lncRNAs, such as BC200, MALAT-1, PCGEM, and DD3. In subsequent systematic studies, much information has been gathered on structures, interaction partners, subcellular localizations, and functions of these lncRNAs. Moreover, data clearly indicated that these gene products might serve as novel diagnostic tools and therapeutic targets for treating human diseases. Clearly, lncRNA research is not just an "l'art pour l'art" activity of biomedical specialists but is expected to have clinical utility.

Two important lncRNAs early identified were BC1, a neuron-specific rodent lncRNA [16], and its human orthologue, BC200 [70]. The in-depth study of these two orthologues revealed interesting data on their functions, long before the upheaval brought by the advent of systematic large-scale bioinformatics and next-generation techniques. These lncRNAs are expressed in neurons, specifically in the post-synaptic area of dendrites. Both gene products are part of a cytoplasmic ribonucleoprotein complex and bind to the poly(A)-binding protein (PABP) [43, 48]. PABP plays an important role in translation regulation in eukaryotic cells, along with translation factors, such as eIF4F, eRF3, and Paip proteins [36, 57]. Furthermore, it has been also reported that BC1 and BC200 bind the fragile X mental retardation protein, the product of the FMRP disease-causing gene [79]. Dysfunction(s) of BC1/BC200-FMRP binding leads to abnormal translation in the post-synaptic area and contributes to the phenotype seen in fragile X mental retardation syndrome [80]. BC1 and BC200 were the first lncRNAs to be used in knock-out experiments: BCI knock-out mice exhibited behavioral changes and shorter life spans compared to controls [45]. BC200 was also shown to have a role in aging and Alzheimer's disease: Mus et al. (2007) [50] have demonstrated that normal aging is associated with decreased BC200 expression in the brain, while BC200 expression was significantly upregulated in the brains of Alzheimer's patients, where abnormal localization to the perykarial region was observed. Under normal conditions, BC200 is expressed exclusively in neurons; however, BC200 was found to be expressed in human neoplasms [10]. Recently, De Leener et al. [15] proposed BC200 as a potential biomarker for diagnosing early-stage breast cancer.

BC200 is not the only early-identified lncRNA that holds promise as a diagnostic marker in human cancer. The prostate-specific PCGEM1 and DD3 lncRNAs were identified by differential display as early as 2000 [61]. PCGEM1 was found to have ethnic-specific expression, being much higher in the prostate epithelial cells of African-American prostate cancer patients compared to Caucasian patients. Moreover, PCGEM was found to be upregulated in normal prostates of individuals with relatives who had been diagnosed with prostate cancer. The pathogenic role of PCGEM1 in prostate cancer was further indicated by its ectopic expression in LNCaP and NIH3T3 cells, which resulted in hyperproliferation [53]. Some years later, PCGEM1 was patented as a promising biomarker for prostate cancer [32]. In a recent paper, it was suggested that PCGEM may serve as a reliable biomarker for the

assessment of drug efficacy during prostate cancer treatment [29]. While PCGEM is expressed both in healthy and in diseased prostates, the DD3 lncRNA, identified in the same differential display experiment [61], exhibits strictly specific expression for prostate cancer, providing the possibility of developing a highly specific diagnostic tool for the disease. Based on this observation, Tinzl et al. (2004) [71] developed a nucleic acid-based diagnostic tool that can be used to detect DD3 lncRNA from urine with high specificity and sensitivity. The product, Progensa, has been on the market since 2011 [21]. In a recent advance, Ding et al. [19] developed an oncolytic viral vector, Ad.DD3.E1A.E1B(Δ55)-(PTEN), under the control of the prostate-specific DD3 promoter and proved its apoptotic effect in prostate cancer cell lines.

MALAT-1 lncRNA was first identified in a cDNA subtractive hybridization experiment from metastasizing lung adenocarcinoma in 2000 [33]. MALAT-1 is possibly the most extensively studied of the early-identified lncRNAs, as it is expressed in a wide range of tissue types and its overexpression has been detected in many human malignancies. Extensive studies have also shed light on the cellular function of MALAT-1: Hutchinson et al. (2007) [31] reported that MALAT-1 co-localizes with SC35 splicing domains, suggesting that MALAT-1 may be a component of the splicing machinery. *Cis* and *trans* regulatory factors for the localization of the MALAT-1 lncRNA to nuclear speckles have been identified [47]. Functional studies performed on cell lines from different types of human malignancies revealed that MALAT-1 is indeed key for the maintenance of hyperproliferation and metastasizing potential [26, 34, 56, 69, 76, 77, 81, 83]. In addition to being a promising biomarker for the diagnosis of a wide range of human malignancies, MALAT-1 proved to be a putative target for siRNA-mediated therapy, as recently demonstrated by Ren et al. [54].

Taken together, the above examples of lncRNAs demonstrate well that several gene products incidentally identified by large-scale gene expression studies have been scientifically and medically interesting, and their study has not only lead to a better understanding of human pathologies but has uncovered potential diagnostic tools and therapeutic targets. Next, we discuss the example of an lncRNA identified by differential display in a study of psoriasis. We describe its role in keratinocyte physiology and psoriasis pathogenesis.

Identification of PRINS, an lncRNA involved in psoriasis pathogenesis

Psoriasis, affecting approximately 2–4 % of the population, is a classic multifactorial skin disease. The interplay of multigenic susceptibility as well as environmental and lifestyle factors leads to the development of symptoms, characterized by epidermal hyperproliferation and inflammation [18]. Intensive research of the last few decades revealed that abnormally functioning keratinocytes and skin-infiltrating professional immune cells are responsible for the disease phenotype [4–6]. As yet, it is still unknown whether abnormal keratinocyte functions of normal-appearing epidermis or aberrant lymphocyte activation is the primary trigger for development of the disease. Accumulating evidence suggests that altered skin tissue homeostasis, especially keratinocyte-specific alterations of the normal-appearing skin of psoriatic patients, is key in the initiation of the disease phenotype. The “immune era” of psoriasis research unquestionably brought breakthroughs for new, targeted therapies of the disease [28]. Nonetheless, to identify novel targets for intervention and possibly for prevention, we must understand the role of aberrant keratinocyte function in the course of the disease.

To this end, 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. We previously performed a differential display experiment to compare gene expression in non-lesional psoriatic epidermis and control healthy epidermis. In 2000, several differentially expressed protein-coding transcripts in the psoriatic non-lesional epidermis were identified, and of these, we focused on the expression of EDA+ fibronectin. We were first to demonstrate that, upon cytokine induction, keratinocytes of the non-lesional epidermis are able to produce this form of cellular fibronectin and, thus, maintain an autocrine loop resulting in keratinocytes hyperproliferation [66]. This finding confirmed our a priori hypothesis that not only professional immune cells, but also keratinocyte-derived factors contribute to disease susceptibility.

In addition to protein-coding transcripts differentially expressed in psoriatic non-lesional epidermis, we also identified a transcript that was unlikely to encode a protein but, nevertheless, exhibited 100 % sequence identity to the 3′ end of a cDNA (GenBank accession number AK022045) previously identified in a cDNA library derived from a 10-week-old human embryo. In parallel with sequence similarity searches, in vitro experiments were performed to determine the expression pattern of the identified transcript during proliferation and differentiation of keratinocytes. The highest expression of the non-coding RNA was found in serum-starved, contact-inhibited keratinocytes as well as in these cells immediately after serum re-addition; however, when the cells began to proliferate, the expression of the AK022045 transcript dropped dramatically. With this compelling result, we decided to turn our focus to the in-depth characterization of this transcript, and since then, we have been engaged in parallel but manifold interconnected characterization of its role in psoriasis and in keratinocyte stress response.

Extensive sequence similarity searches and the determination of the 5′ end of the transcript allowed us to draw a putative structure for the newly identified gene. The full-length transcript is 3.6 kb, and a putative TFIIB transcription factor binding site was identified in the genomic sequence proximal to the putative transcription start site. The transcript contains two exons, both harboring *Alu* elements, and shows a high degree of similarity to a heat shock element in a small non-coding RNA, *G8*. Based on these findings, we supposed that this transcript is an lncRNA and named it Psoriasis-susceptibility-Related RNA Gene Induced by Stress (PRINS). The full-length sequence is available to the scientific community (http://www.ncbi.nlm.nih.gov/gene/?term=PRINS[sym]) [58].

By using quantitative real-time PCR (Q-RT-PCR) [58] and in situ hybridization (ISH) methods [65], PRINS expression was determined in human tissue types. These two experimental approaches revealed that PRINS expression varied in different human tissue types: the highest level of expression was observed in the veins, high levels were found in tissues derived from female and male gonads and lung, moderate expression was detected in tissue types derived from the gastrointestinal tract, and no apparent expression was present in the brain. Both ISH and Q-RT-PCR revealed a relatively high level of basal PRINS expression in healthy epidermis.

The contribution of PRINS to the pathogenesis of psoriasis susceptibility was further indicated in experiments of organotypic skin cultures. Organotypic skin cultures from healthy volunteers and from the non-lesional skin of psoriatic patients were co-incubated with a T cell lymphokine mix previously shown to induce the proliferation of non-lesional psoriatic epidermal keratinocytes but not keratinocytes derived from normal healthy epidermis [5, 6]. PRINS expression differed in the two systems: while the treatment decreased PRINS expression in the non-lesional psoriatic epidermis, it was unchanged in the normal healthy epidermis. This result suggested that PRINS may contribute to psoriasis susceptibility as a modifier gene and may be part of the inherently altered molecular network of non-lesional epidermal keratinocytes contributing to disease pathogenesis [58].

To identify PRINS interacting partners and targets, in vitro binding assays [65] and cDNA microarray experiments [64] were performed. In the latter, PRINS expression was silenced in keratinocytes and the genes with altered expressions were studied in detail. G1P3, one of the identified genes to be under the control of PRINS, had been previously shown to play a pathogenic role in human malignancies with anti-apoptotic effects, and it is regulated by interferon-α [11, 67]. These two features of G1P3 are also important in psoriasis pathogenesis [40, 52]. We found that the mRNA expression of PRINS-regulated G1P3 was upregulated 400-fold in lesional and 9-fold in non-lesional psoriatic epidermis, compared to healthy epidermis. In vitro experiments revealed that the down-regulation of G1P3 inhibited the spontaneous apoptosis of keratinocytes, indicating that its high expression might contribute to the altered apoptotic features of psoriatic keratinocytes and, thus, to disease pathogenesis. Taken together, these results indicate that the deregulation of the PRINS lncRNA contributes to psoriasis pathogenesis at least partially by altering the expression of G1P3 and leading to decreased sensitivity of keratinocytes toward spontaneous apoptosis [64]. In another set of experiments, an in vitro binding assay identified the nucleophosmin (NPM) protein as a direct interacting partner of the PRINS lncRNA. To determine whether this interaction had any relevance to psoriasis pathogenesis, the expression of NPM was studied in both healthy and psoriatic non-lesional epidermises. No apparent difference was found in the level or pattern of expression. Our finding was in agreement with a previous study examining nuclear staining for NPM in epithelial cells [7]. Additional evidence that NPM and the PRINS lncRNA might be direct interacting partners came from staining patterns in cultured keratinocytes: ISH staining of PRINS and immunohistochemical (IH) staining of NPM showed for both a predominant nuclear localization. In psoriatic lesional epidermis, however, the staining pattern of NPM was dramatically changed in the different layers of the epidermis: the highest level of NPM expression was found in basal and immediate suprabasal keratinocytes. Moreover, keratinocytes showing the highest level of NPM expression exhibited a marked cytoplasmic immunopositivity, revealing that, in keratinocytes of lesional psoriatic epidermis, both the level and the intracellular pattern of NPM expression were changed [65].

Thus, altered expression of proteins that are either interacting partners of PRINS or are under the control of PRINS indicates that, indeed, this lncRNA plays an important role in psoriasis and that, by altering normal keratinocyte function(s), it contributes to disease pathogenesis.

The role of PRINS in keratinocyte stress response

The first outstanding finding about the possible cellular functions of PRINS was the contrast between the high expression found in serum-starved, contact-inhibited keratinocytes and the very low levels of expression in highly proliferating keratinocytes [58], suggesting that PRINS may have a key role in the keratinocyte stress response. To test this, the survival of keratinocytes was studied during down-regulation of PRINS. No effects of PRINS down-regulation were found on the survival of keratinocytes under favorable culturing conditions; however, when serum was withdrawn from the PRINS down-regulated keratinocytes, the survival rate decreased significantly. This result confirmed that elevated PRINS expression in stressed keratinocytes is not an epiphenomenon but that, indeed, this lncRNA contributes to the cellular stress

response. Further in vitro experiments revealed that other stress factors, including microbial components (viral and bacterial), translation inhibition with cyclohexamide, and UV-B irradiation, are able to induce high-level PRINS expression in keratinocytes. The UV-B results were especially intriguing, since the PRINS interaction partner NPM has a well-documented role in cellular UV-B response: the predominantly nucleolar localization of NPM is changed upon UV-B irradiation in fibroblasts, and in cancer cells [44, 78], translocation to the nucleoplasm and, to some extent, to the cytoplasm occurs. In vitro experiments were performed to determine whether the same phenomenon occurs in epidermal keratinocytes and whether PRINS has any role in it. PRINS expression was down-regulated in UV-B-irradiated keratinocytes, and intracellular re-distribution of NPM occurred subsequently. Thus, NPM was localized to the nucleus in keratinocytes in which PRINS was down-regulated, indicating that PRINS physically interacted with NPM as well as functionally regulated NPM in this cellular stress response [65]. Taken together, our in vitro results suggested that PRINS lncRNA contributes to both stress responses and apoptosis signaling in keratinocytes, and relevantly, its role in psoriasis pathogenesis involves altering these pathways.

Nuclear factor kappa B (NF-κB) signaling is known to be altered in psoriatic keratinocytes [73], and this may be a link between T cell-mediated and keratinocyte-mediated processes in disease pathogenesis. To determine whether PRINS has a role in NF-κB signaling, PRINS expression was altered in keratinocytes and the activity of the NF-κB pathway was examined after lipopolysaccharide (LPS) induction and priming with psoriasis-related cytokines and detected using a luciferase-based approach. LPS induction of the NF-κB pathway is well known [49], and it has been shown that LPS can upregulate the expression of PRINS in keratinocytes. Neither the down-regulation [3] nor the robust upregulation [14] of PRINS had an effect on NF-κB activity, indicating that PRINS is not involved in this signaling pathway in keratinocytes, although other influences were not excluded.

We are currently working on identifying the signaling pathways in keratinocytes by which PRINS affects cell functions and contributes to disease pathogenesis in psoriasis.

Classification of PRINS, a novel non-coding RNA conserved in primates

In the 1990s and early 2000s—when lncRNAs were identified accidently rather than by systematic search—the need to classify non-coding RNAs was not particularly compelling. Although the reports of novel lncRNAs caught the interest of the scientific community at scientific meetings, the general response was to consider them too eccentric to be taken seriously. However, this attitude has dramatically changed in the last few years as a result of two developments: on one hand, a large body information about lncRNAs has accumulated from large-scale gene expression studies in the last 15–20 years [35], and on the other hand, thousands of novel human lncRNA genes have been identified using high-throughput methods in the last few years [27]. Systematic annotation of these newly identified lncRNA transcripts was necessary to aid researchers in their search of the understanding the functions of lncRNAs.

In the last few years, several attempts have been made to address this need, resulting in a rather complicated system for categorizing aspects [62] as well as an "easier-to-follow" system for categorizing lncRNAs [38]. The varying—and we believe—still evolving taxonomy of lncRNA reflects the novelty of the field.

To provide a guide to the lncRNA classification suggested by Kapusta et al. [38], we use this system to apply an initial classification for the PRINS lncRNA.

According to the Kapusta classification, the first aspect to consider when categorizing an lncRNA is its genomic context. The first report of PRINS lncRNA [58] provided its location to be on the short arm of human chromosome 10 (map position 10p12.31) and indicated that it is composed of two exons and an intron of approximately 7 kb in length. The entire PRINS lncRNA resides in an intron of the recently annotated KIAA1217 gene, also known as SKT, which is involved in early stages of embryogenesis [63]. Proximal to KIAA1217 is the OUT deubiquitinase 1 gene, whereas distal to KIAA1217 is the Rho GTPase-activating protein 21. Interestingly, the miR603 miRNA is located in a KIAA1217 intron, 3′ of the PRINS coding region.

The second aspect to consider when categorizing an lncRNA is its chromatin context. A transcription start site was identified 6 kb proximal to the putative 5′ end of the PRINS gene using the ENCODE database. This region is marked by a high density of binding sites for several transcription factors, including GATA2, Fos, HDAC2 and STAT3, and histone modification sites associated with active transcription, such as mono- and tri-methylation of lysine 4 of histone H3 (H3K4me1/3) and acetylation of lysine 9 and 27 (H3K9Ac, H3K27Ac), suggesting that a strongly regulated active promoter might be associated with the lncRNA. The region adjacent to the 3′ end of the PRINS lncRNA gene also contains histone modification sights, which, due to the close 3′ proximity to the PRINS lncRNA gene, might be an enhancer element. Of all cells examined to date, the highest signals were found in normal human keratinocytes, indicating that the PRINS lncRNA is indeed expressed in keratinocytes and its expression is regulated by epigenetic factors.

The third and possibly most informative aspect to consider is the subcellular localization of the lncRNA. From our experimental results, we found that the PRINS lncRNA is mainly localized in the nucleolus of normal human cultured

keratinocytes, although moderate perinuclear and cytoplasmic expression was also detected. This is in good agreement with reports that non-coding RNAs localize mainly to the nucleus [37], which suggests a role in the temporal–spatial regulation of nuclear organization and/or regulation of gene expression. The intracellular localization of the NPM protein, which was identified to be physically and functionally interacting with PRINS, is indicative of a nucleolar/nuclear role for PRINS. As early as 1984 [60], NPM was reported to be localized in the nucleolus and shown to shuttle between the nucleolus and the cytoplasm [9]. As both NPM [2] and PRINS [68] demonstrate strict regulation during cell growth and during cellular stress response and, additionally, have functionally overlapping features during these processes [65], we postulate that, indeed, these two molecules interact in these processes.

Since sequence conservation of lncRNAs is rare, it is assumed that their biological activities are dependent on structure. The putative secondary structure of the PRINS lncRNA was determined from the primary sequence by computational prediction (Fig. 1a). In addition to strong structural features, the PRINS sequence might also determine its cellular localization, as it includes the AGCCC pentamer with the sequence restrictions at positions −8 (T or A) and −3 (G or C) of a motif which was reported to be crucial for nuclear localization of lncRNAs [82]. Our ISH results are in good agreement with this sequence-based analysis, as the highest level of PRINS expression was detected in the nucleolus of keratinocytes, with moderate staining in the nucleoplasm and the cytoplasm.

Intriguingly, the PRINS lncRNA sequence contains three *Alu* elements. It is well established that transposable elements (TEs) have been very important in the evolution of lncRNAs [39]. According to some estimates, approximately two thirds of functioning human lncRNAs contain at least one TE-derived element, which are seldom found in protein-coding genes. These elements very often make up a relatively large portion of the lncRNA genes [42]. This is also true for the PRINS lncRNA, which contains three *Alu* elements comprising approximately one third of its sequence (Fig. 1b). The possible contribution of TE elements to lncRNA evolution and function is extensively reviewed by Kapusta et al. [38].

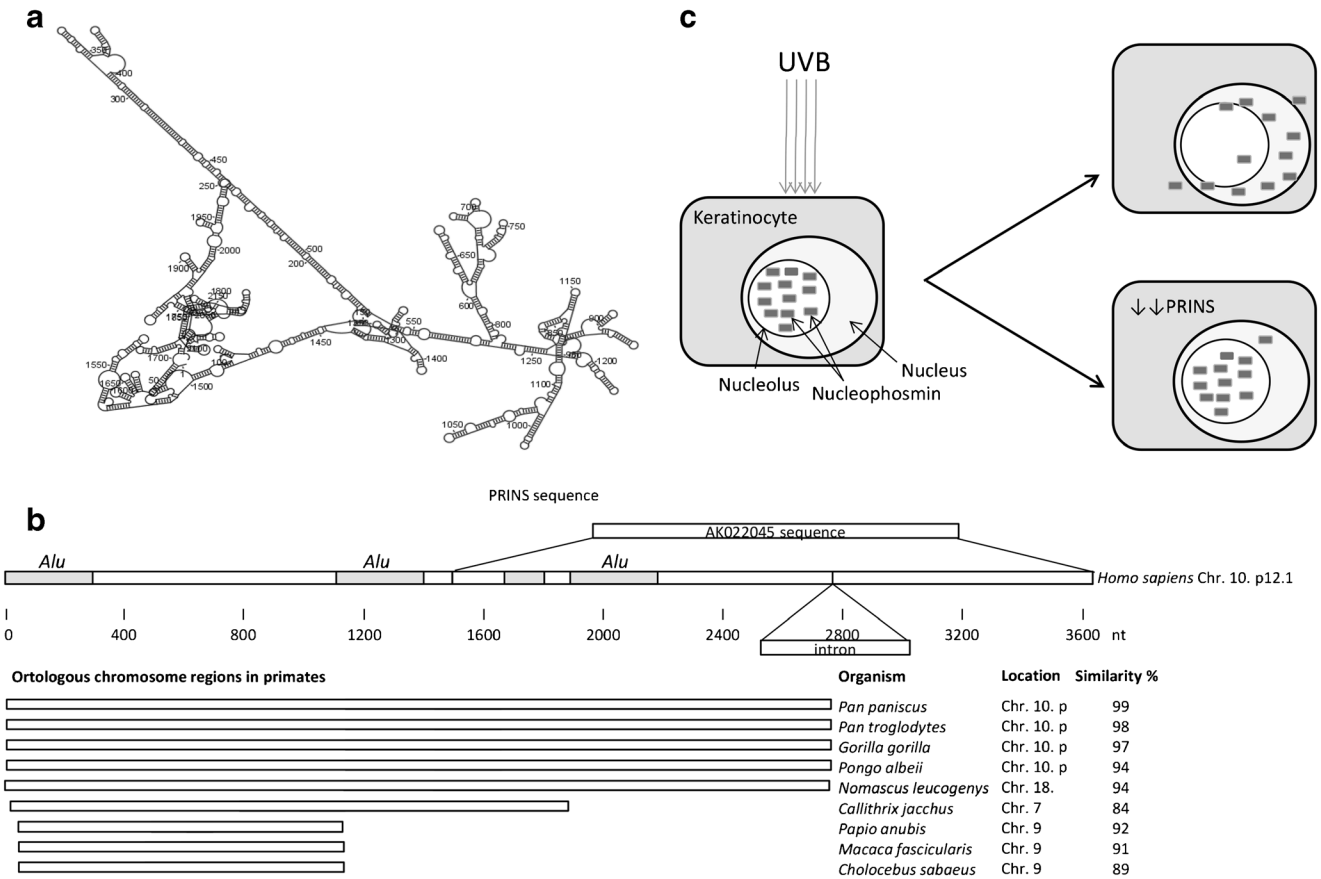

Fig. 1 Major characteristics of the PRINS lncRNA. **a** Putative secondary structure of the PRINS lncRNA. **b** Similarity search identified three *Alu* elements within the PRINS lncRNA sequence. The PRINS gene is localized on the p12.1 arm of human Chr. 10, which is highly conserved in human and four other primate species. Although partial sequence similarity was found, it is largely due to the conservation of *Alu* elements. In other primate species, the PRINS sequence was distributed on other chromosomes. **c** UVB irradiation induces the shuttling of nucleophosmin (NPM) from the nucleolus to the nucleoplasm. Silencing of the PRINS lncRNA, which physically interacts with NPM, results in the retention in the nucleolus

It has been estimated that only 21 % of known lncRNAs occurring in primates have orthologues in other orders and only 3 % of the primate lncRNAs have an orthologue in tetrapods [51]. The results obtained with the PRINS lncRNA sequence agree well with these estimates, in that it could be found only in the genomes of primates with variations in the extent of similarity (Fig. 1b). PRINS and the orthologues with the highest similarity among primate species reside on the short arm of chromosome 10. Taken together, these data suggest that the PRINS lncRNA gene is most probably a primate-specific sequence and transposition was the major mechanism of its origin.

As many lncRNAs serve as sources for miRNAs [55], it was interesting to examine the PRINS lncRNA for miRNA pre-sequences. However, no pre-miRNA sequences were identified using the ever-growing mirbase database (http://www.mirbase.org/). It is interesting to note, however, that the intron in which the PRINS lncRNA is located harbors a miRNA. Analysis of miRNA harboring lncRNAs has shown it to be an evolutionary conserved group [12]. Thus, as PRINS is a primate-specific lncRNA, it is not surprising that it does not harbor any miRNA sequences.

The final criterion for classifying lncRNAs is the most challenging: function. However, this aspect is the most likely to advance the understanding of the role of lncRNAs in health and disease. Because the highest PRINS expression was observed in the nucleolus of normal human keratinocytes and the sequence contains a nuclear-specific motif, it is reasonable to assume its functions are in the nucleolus or nuclei. Our in vitro binding assays identified NPM as the most prominent interaction partner of PRINS, and the highest NPM expression was found in the nucleolus [60]. These results together with the results from the PRINS IHS and binding assay support the hypothesis that PRINS is a physical partner of NPM in the nucleolus and the complex formed by their binding contributes to the cellular stress response (Fig. 1c). Whether PRINS is interacting with proteins other than NPM and how it functions in concert with other keratinocyte-expressed lncRNAs [30] and/or miRNAs [59] are still challenging questions to answer. Moreover, the data cited above have been obtained from experiments performed in normal human keratinocytes and keratinocyte cell lines, leaving open the question whether PRINS is expressed with the same intracellular pattern and functions the same way in other cell types.

Together, the results from our experiments indicate that the evolutionarily young, primate-specific PRINS is one of the lncRNAs that is differentially expressed in psoriasis [72], that it plays a role in keratinocyte stress response, and that its elevated expression in psoriatic non-lesional epidermis contributes to the altered stress response of psoriatic keratinocytes and, thus, to disease pathogenesis. Psoriasis is a human-specific multifactorial skin disease, which has not yet been identified in other primates. It remains to be determined whether any association exists between the primate/human lncRNAs and the special susceptibility to certain multifactorial diseases that exist only in humans.

Conclusions

There is no doubt that one of the greatest (and previously unforeseen) achievements of genome programs was the discovery of novel layers of cell regulation represented by lncRNAs. The importance of these transcripts is underscored by the fact that they are almost equal in number with protein-coding genes in the human genome [17]. We are still far from being able to comprehensively place these novel regulatory molecules into regulatory networks, similarly to what are currently known of protein–protein and protein–DNA interactions based on experimental data. A tremendous amount of experimental work, spanning decades, was necessary to gain sufficient information about individual proteins and DNA elements to describe these regulatory networks. Although highly developed in silico methods may speed this process for regulatory RNAs—including lncRNAs—it is reasonable to assume that, unless newly identified lncRNAs are experimentally characterized, it will not be possible to identify their cellular contexts. Classification of lncRNAs is crucial for their annotation, and information already available for the well-known, serendipitously identified lncRNAs is likely to have great importance in this work. According to some estimates, there are approximately 130 human lncRNAs extensively characterized to date [1] and they may serve well in the development of classification and annotation of thousands of identified but not yet characterized lncRNAs. In addition to understanding the cellular functions of these molecules, their contribution to human diseases will have to be elucidated, promising insights into the missing heritable and yet unrevealed mechanisms of human diseases.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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