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

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Summary of the PhD Thesis

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Szeged

2018

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)
IF:6.429*
(Independent citation: 0 Self citation: 1 Cumulative: 1)

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)
IF:3.156
(Independent citation: 6 Self citation: 1 Cumulative: 7)

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)
IF:3.236
(Independent citation: 3 Self citation: 2 Cumulative: 5)

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)
(Independent citation: 1 Self citation: 1 Cumulative: 2)

1. Introduction

1.1 Immune functions of keratinocytes

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. Anatomically the skin can be divided into three layers, the epidermis, the dermis and the subcutaneous tissue.

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 keratinocytes are also immunocompetent cells. They express several pattern recognition receptors (PRRs) and are responsive to various pathogen and danger associated molecular patterns (PAMPs and DAMPs). The PRRs make keratinocytes the first immune sentinels against pathogens. Keratinocytes express different Toll-like receptors (TLR), RIG-like receptors, and NOD-like receptors recognizing a wide range of microbial compounds.

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. 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, which are recognized as DAMPs, leading to a prolonged inflammation in chronic inflammatory diseases.

Nucleotide fragment 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) and inflammatory cytokine expression in keratinocytes. Poly(I:C) is recognized primarily by TLR3, but the involvement of RIG-I and melanoma differentiation-associated gene 5 (MDA5) was also described. 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, but it can be also sensed by cyclic GMP-AMP synthase.

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 nuclear factor κB (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.

1.2 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, the interplay of environmental and life-style factors in individuals carrying multigenic susceptibility loci leads to the development of the symptoms: scaly, red, well-demarcated plaques on the skin.

One of the endogenous triggering factors thought to initiate the disease are self-derived cytosolic nucleotide fragments. Receptors for RNA and DNA fragments, moreover their activators, the self-derived RNA and DNA fragments and RNA:DNA duplexes are highly abundant in the lesional epidermis. In the initiation phase of the disease, an antimicrobial peptide cathelicidine (LL-37) forms 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} and 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. We previously performed a differential display and a cDNA microarray experiment comparing the gene expression of psoriatic non-involved and healthy epidermis, to characterize these abnormal molecular patterns in the psoriatic non-lesional epidermis. These experiments revealed deregulated extracellular matrix expression, identified a yet unknown long non-coding RNA, named PRINS, revealed splicing disturbances and showed altered expression of inflammatory mediators IL-1β and IL-23 and the inflammasome regulator caspase recruitment domain family member (CARD) 18.

1.3 PRINS long 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. Non-coding RNAs larger than 200 nucleotides are referred to as long ncRNAs (lncRNAs). The number of lncRNA genes (>70 000) in the human genome

outnumbers protein-coding genes (<20 000). They are markedly heterogeneous in size and cellular function, thus enormous effort is still needed to determine their specific functions. Currently, functional annotation and detailed mechanism of action was described for less than 0.1% of all predicted lncRNAs.

Our research group was the first to identify an lncRNA by a differential display experiment as highly expressed in psoriatic non-lesional epidermis compared to healthy epidermis and potentially contributing to psoriasis susceptibility: PRINS, the *psoriasis susceptibility related non-coding RNA induced by stress*. The expression of PRINS was found to be modified by a diverse set of cellular stressors, including starvation, ultraviolet B (UVB) irradiation, translation inhibition and hypoxia, and silencing of PRINS in HaCaT cells during stress exposure decreased cellular viability. These results suggested its contribution to cellular stress responses.

Using various bioinformatical tools, we have analyzed genomic features of the *PRINS* gene, 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. The entire *PRINS* gene resides in an intron of the recently annotated *KIAA1217* gene, also known as *SKT*, which is involved in early stages of embryogenesis. A transcription start site marked by a high density of binding sites for several transcription factors and histone modification sites associated with active transcription was identified 6 kb proximal to the putative 5' end of the *PRINS* gene using the ENCODE database, suggesting that a strongly regulated active promoter might be associated with the lncRNA. 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. This is also true for the *PRINS* lncRNA gene, which contains three *Alu* elements comprising approximately one third of its sequence. *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 the highest similarity on the short arm of chromosome 10.

To identify interacting partners of PRINS lncRNA, a cDNA microarray and an *in vitro* protein binding assay 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. 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. 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. The sequence of PRINS might also determine its cellular localization, as it includes a motif which was reported to be crucial for nuclear localization of lncRNAs.

Together, the results from our previous experiments indicate that the evolutionarily young, primate- specific PRINS is one of the lncRNAs differentially expressed in psoriasis, and it plays a role in keratinocyte stress response.

Recent publications on PRINS non-coding RNA supported its involvement in inflammatory cellular processes. Microbial stimuli decreased PRINS expression in macrophages and normal human epidermal keratinocytes (NHEKs). 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. PRINS was also shown to be involved in the resistance against TNF-α induced apoptosis of colorectal cancer cells. 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

- Normal human epidermal keratinocytes (NHEKs), the HaCaT cell line and the HPV-KER cell line were used for the experiments.
- Cells were transfected with 0.666 μg/ml polyinosinic–polycytidylic acid [poly(I:C)] or with 0.666 μg/ml polydeoxyadenylic acid-polydeoxythymidylic acid double-stranded homopolymer [poly(dA:dT)] using the X-tremeGene 9 transfection reagent, with or without priming by addition of 5ng/ml tumor necrosis factor-α (TNF-α) and 5ng/ml interferon-γ (IFN-γ) for 24 hours. Cells were harvested at indicated time points.
- For inhibition studies, cells were incubated 1 hour prior to poly(I:C)/poly(dA:dT) transfection with inhibitors for NF-κB (Bay11-7085, 10 μM), STAT-1 (Fludarabine, 10 μM), STAT-3 (Stattic, 5 μM), MEK1/2 (PD98059), JNK (SP600125, 10μM) and p38 (SB203580, 10 μM).
- Total RNA was isolated from cells using TRIzol® Reagent following the manufacturer's instructions, and DNase treated by Turbo DNA-free Kit. cDNA was synthesized from 1 μg total RNA using the iScript cDNA Synthesis Kit.
- Real-time RT-PCR experiments were carried out with the Universal Probe Library system using a C1000 Touch Thermal Cycler. Relative mRNA levels were calculated by the ΔΔCt method.
- NF-κB luciferase reporter assays were performed to determine the NF-κB activity in response to poly(I:C)/poly(dA:dT) treatment, by transfection pNFκB-luc Cis-Reporter Plasmid and the pGL4.75 [hRluc/CMV] plasmid.
- HPV-KER cells were harvested after poly(I:C)/poly(dA:dT) transfection and lysed in lysis buffer. Equal amounts of protein were separated by SDS-PAGE and transferred to nitrocellulose membrane. After blocking primary antibodies were incubated overnight at 4°C with constant agitation. HRP-conjugated secondary antibodies were incubated for 60 minutes at room temperature. Signal was visualized with SuperSignal™ West Pico Chemiluminescent Substrate on a C-Digit Blot Scanner.
- For overexpressing PRINS, the AK022045 cDNA sequence was cloned into a pcDNA3.1(+) vector. In the ΔPRINS construct the position 538–622 in the AK022045 sequence was replaced by the following scrambled sequence, and cloned into a pcDNA3.1(+) vector: 5'-GTGCGTGGCGGAGACGTGGTGGTAGAC CGAATTGAGGAGGATCCGAAGGTTAGACGTAGGCGATCGCCGCTTCGGA

CGCGGTCGC-3'. The empty pcDNA3.1(+) vector served as a control. X-tremeGENE HP DNA transfection reagent was used for transfection of NHEKs.

- Interaction 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 RIsearch and INTARNA. The regions predicted by both programs were considered as putative interaction sites.
- PRINS and ΔPRINS RNA sequences were produced by *in vitro* transcription using Transcript Aid T7 In Vitro Transcription Kit. The single-stranded RNA products were used in a fluorescent binding assay.
- The fluorescently labeled RNA sequence 5'6-carboxyfluorescein(6-FAM)/GAAGCU CUAUCUCCCCUCCAGGAGCCCAGCUAUGAACUCCUUCUCCACAAGCGCC UUCGGUCCAGUUGCCUUCUCCCUGGGGCUGCUCCUGGUGUUGCCUGCU GCCUUCCCUGCC-3', comprising positions 91–205 of the IL-6 (NM_000600.4) mRNA sequence, was produced by Integrated DNA Technologies.
- *In vitro* binding assay was carried out on a Monolith NT.115 Pico MicroScale Thermophoresis instrument in nuclease-free water, at 25°C, with 80% Laser Power, 10% LED Power, by 2bind GmbH, Regensburg, Germany. Initial fluorescence was analyzed for binding curves by the following formulation based on a 1:1 binding model.
- The amount of cytokines IL-1α, IL-1β, IL-6, IL-8, CCL-5 and TNF-α was determined by ELISA in cell supernatants.
- Experiments were carried out in triplicates with at least three biological repeats. For statistical analysis, 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. 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 nucleic acid exposure with increased cytokine expression and NF-κB, MAP kinase and STAT activation

We compared dsRNA- and dsDNA-induced cytokine-expression profiles in three keratinocyte cell types: NHEKs, the HaCaT and the HPV-KER cell line. 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). Peak expression was observed 3 to 6 hours after poly(I:C) transfection, and 6 to 12 hours after poly(dA:dT) transfection. Reaction to poly(I:C) was faster in HaCaT cells than the other cell types studied. The kinetics of poly(dA:dT)-induced reaction did not differ in the three cell types. In the subsequent experiment we used the HPV-KER cell line.

NF-κB activation in HPV-KER keratinocytes was assessed by an NF-κB–luciferase reporter assay. 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.

Western blot analysis 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, with a faster phosphorylation of STAT-1 and STAT-3 in poly(I:C) treated samples compared to poly(dA:dT) treatment. Phosphorylation of p38 and JNK pathways were not affected.

4.2 Poly(I:C) and poly(dA:dT) induced cytokine expression of keratinocytes 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, HPV-KER keratinocytes were pre-incubated with the specific inhibitors of NF-κB, MEK1/2, p38, JNK, STAT-1 and STAT-3 for an hour before transfection with poly(I:C) or poly(dA:dT). Inhibition of NF-κB nearly completely abolished both the poly(I:C)- and poly(dA:dT)-induced expression of IL-6 and TNF-α. Although activation could not be confirmed by our western blot, inhibition of p38 signaling resulted in significantly decreased IL-6 and TNF-α expression. In contrast, the inhibition of JNK

signaling did not affect cytokine expression. The inhibition of MEK1/2 signaling significantly increased the poly(I:C)- and poly(dA:dT)-induced production of IL-6, suggesting a possible negative regulatory role of this pathway. Inhibition of STAT-3 signaling significantly decreased both poly(I:C)- and poly(dA:dT)-induced cytokine expression, whereas the inhibition of STAT-1 affected only IL-6 expression.

In conclusion, 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.

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

We examined PRINS expression upon poly(I:C) and poly(dA:dT) treatments. Poly(dA:dT) treatment significantly decreased the expression of PRINS in NHEKs, while poly(I:C) treatment did not led to significant changes. Significant change in PRINS expression was only prevalent in NHEKs, thus NHEKs were used in the subsequent experiments.

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. Since in NHEKs high intra-individual differences in the rate of poly(dA:dT) induced cytokine expression was found, we introduced a model to increase inflammation by priming the cells with TNF-α and IFN-γ for 24 hours before poly(dA:dT) transfection, which resulted in a similar decreased PRINS expression as poly(dA:dT) transfection alone, with higher expression and secretion of cytokines.

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, whereas mRNA expression and secretion of IL-1α, IL-1β and TNF-α were not affected. These results suggest that PRINS can influence the regulation of other inflammatory processes.

4.4 *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. We measured mRNA expression and secretion of CCL-5 during PRINS overexpression, and found that both decreased 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) and CCL-5 (NM_002985.2) we performed an *in silico* analysis using the INTARNA and the RIsearch software. 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. 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. No interaction site was predicted for IL-8.

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

An *in vitro* binding experiment was carried out using the PRINS lncRNA and the IL-6 mRNA to validate the predicted interaction. 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 (Kd=10.3436 nM), specific binding for ΔPRINS and the unspecific labeled DNA could not be detected. This *in vitro* result confirms the specificity of the *in silico* predicted regions.

4.6 PRINS regulates IL-6 expression 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. IL-6 expression was not affected by overexpression of ΔPRINS but was, in contrast, significantly decreased by PRINS overexpression, and similar tendencies were seen in the amount of secreted IL-6. CCL-5 expression and secretion decreased similarly both in cells overexpressing ΔPRINS and in cells overexpressing PRINS, which confirmed the specificity of the interacting region for IL-6. IL-8 expression upon PRINS or ΔPRINS overexpression showed a similar tendency to IL-6 expression, although significant differences could not be detected. These result demonstrated that the binding site in the PRINS sequence is essential and specific for the regulation of IL-6 expression.

5. Discussion

RNA and DNA fragments are known as important PAMPs or DAMPs that induce innate immune processes of the cells. 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 keratinocytes with different approaches. The transcriptional regulation through signal transduction pathways and the posttranscriptional control by lncRNAs were also analyzed.

Previously we found that the inflammatory responses of the widely used HaCaT cell line differ from that of NHEKs, while the HPV-KER cell line (established and characterized in our laboratory) shows similar inflammatory and ultraviolet–B- irradiation-induced responses to NHEKs. In line with these previous findings, 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, 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; however, limited information is available for these signaling events in keratinocytes upon nucleotide fragment induction. 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); while it has been shown that poly(dA:dT) must first be transcribed to RNA before activating subsequent signaling through RIG-I dependent sensing. NF-κB, p38, STAT-1 and STAT-3 signal transduction were found to induce cytokine expression by poly(I:C) and poly(dA:dT) in keratinocytes.

In monocytes and in mouse models, inhibition of ERK1/2 and JNK signaling pathways were shown to have anti-inflammatory effects and abolish nucleotide-induced IL-6 and TNF-α expression. In contrast, in our experiments the disruption of ERK1/2 signaling 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. These results suggest that ERK1/2 signaling in keratinocytes has a negative regulatory function in inflammatory reactions.

Due to large-scale gene-expression studies the number of annotated human non-coding RNAs has increased rapidly, but functional roles have been assigned only to a few of them, thus we aimed to study, whether PRINS can regulate inflammatory functions of keratinocytes.

PRINS overexpression decreased the poly(dA:dT) induced expression of IL-6, IL-8 and CCL-5 in NHEKs. Using *in silico* analysis, specific regions in the PRINS sequence were predicted to interact with CCL-5 and IL-6 mRNA. The interaction site on the IL-6 sequence spans two exons, indicating an mRNA-lncRNA interaction. 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. In contrast to the majority (~40%) of lncRNAs, which bind to the 3' UTR of their target mRNAs, the binding site of PRINS lies within the 5' UTR of the IL-6 mRNA. The functionality of the mRNA-lncRNA interaction was also observed at the cellular level, as overexpression of ΔPRINS, harboring a scrambled IL-6 interacting site, did not decrease IL-6 levels, while CCL-5 expression was decreased both by PRINS and ΔPRINS overexpression. Thus, we demonstrated that PRINS is able to bind the IL-6 mRNA and this specific interaction is required to destabilize IL-6 expression and secretion in NHEKs. Based on these current findings and previous results, 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. The 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.

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6. Summary

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 downregulation of the inflammatory functions in psoriatic keratinocytes and maintenance of the normal phenotype.

7. Acknowledgement

I would like to express my deepest gratitude to my supervisor, Professor Márta Széll, for her constant support and supervision of my work. She always stood by, when I needed her guidance and support, but also provided me enough independence during my scientific work.

I would like to thank Professor Lajos Kemény for the opportunity to be able to join the MTA-SZTE Dermatological Research Group and perform my studies here.

It also gives me a pleasure to thank Professor Zsuzsanna Bata-Csörgő for her ideas and comments related to my work and during the preparation of the manuscripts.

I would like to express my special thanks to Kornélia Szabó for her support and valuable suggestions, and her continuous help with technical questions and issues during my work.

I am grateful to my colleagues Anikó Göblös, Lilla Erdei, Beáta Szilvia Bolla, Barbara Konczné Gubán and Renáta Bozó for introducing and helping me with the research techniques and for their friendship and support during the years. I also thank all of my colleagues, who have helped me at any stages at the Department of Dermatology and Allergology and at the MTA-SZTE Dermatological Research Group.

During my work at the research group, I was lucky to supervise the work of two talented and enthusiastic undergraduate students: Letizia La Rosa form the University of Catania and Luca Janovák from the University of Szeged. They both have contributed to the manual work which led to the results of this Thesis, and through the supervision of their laboratory work and Bachelor's Thesis preparation I had a great opportunity to learn about research management and improve my skills on how to introduce and guide students in their scientific research.

My special thanks go to my friends and family, for their patience, constant support, encouragement and endless love. And last but not least, thank you Gergő for your love. Without you and your special support in the last two years I would not be here now. Thank you!

This study was supported by several research grants from the National Research, Development and Innovation Office (OTKA K105985, K111885, GINOP-2.3.2-15-2016-00015) and by the National Talent Program (NTP-NFTÖ-17-B-0164) provided by the Hungarian Ministry of Human Capacities.