Mechanism of cytosolic nucleic acid induced IL-23 expression of human keratinocytes

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PhD Thesis

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List of publications

Scientific papers included in this thesis:

I. **Kelemen E**., Ádám É., Sági S.M., Göblös A., Kemény L., Bata-Csörgő Z., Széll M. and Danis J.: Psoriasis-Associated Inflammatory Conditions Induce IL-23 mRNA Expression in Normal Human Epidermal Keratinocytes, *Int J Mol Sci* 23(1):540 (2022)

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IF:2.281

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Abbreviations:

CCL5: chemokine ligand-5

cGAS: cyclic GMP-AMP synthase

DAMP: danger associated molecular patterns

DNase: dezoxyribonuclease

ds: double stranded

ERK: extracellular signal regulated protein kinase

IFN: interferon

IL-12: interleukin-12

IL-17: interleukin-17

IL-1β: interleukin-1-beta

IL-23: interleukin-23

IL-6: interleukin-6

JNK: c-Jun-N terminal kinase

lncRNA: long noncoding RNA

MAPK: mitogen activated protein kinase

MDA-5 (IFIH1): melanoma differentiation-associated protein-5

MEK1/2: dual specificity mitogen-activated protein kinase kinase 1 and 2

miRNA: microRNA

mtDNA: mitochondrial DNA

ncRNA: noncoding RNA

NET: neutrophil extracellular trap

NF-kB: nuclear factor-kappa B

NHEK: normal human epidermal keratinocyte

NPM: nucleophosmin

p38: phosphorylated-p38 mitogen activated protein kinase

PAMP: pathogen associated molecular patterns

poly(dA:dT): poly deoxyadenilic acid-poly deoxythymidylic acid double-stranded homopolymer

poly(I:C): polyinosinc-polycytidilic acid

PRINS: psoriasis-related noncoding RNA induced by stress

PRR: pathogen recognition receptors

RIG-1: retionic acid induced gene 1

SKT: Sickle Tail Protein homolog

ss: single stranded

STAT-1: signal transducer and activator of transcription-1

STAT-3 signal transducer and activator of transcription-3

TH-cells: T-helper cells

TLR3: Toll-like receptor 3

TNFα: tumor necrosis factor-alpha

1. Introduction

1.1 The skin

The skin is the largest organ of the human body, which provides the first line of defense against environmental, chemical and physical injury and infection by acting as an active participant of the innate immune system. An important role of the skin is to maintain proper physiological conditions for the host by preventing fluid loss or allowing synthesis of vitamins and hormones [1,2]. Anatomically the skin is structured into three layers (Fig. 1A). The lowest part is the hypodermis composed of subcutaneous tissue, above it the dermis can be found which consists of connective tissue and harbours mechanoreceptors to provide the sense of touch and heat trough nociceptors and thermoreceptors [3]. The outermost layer of the skin is the epidermis separated from the dermis by the basement membrane. It is mainly responsible for the barrier function and protection of lower layers from enviromental pathogens, oxidative stress, UV light and chemical compounds [1–3].

1.1.1. Epidermis

The upper layer of the skin is the epidermis. It's key function is to form a protective interface separating the individual from the external environment. The epidermis has a multilayered epithelium structure - mainly composed by keratinocytes-, the interfollicular epidermis and associated structures such as hair follicles, sebaceous-and sweat glands [3]. The thickness of the interfollicular epidermis differs in different body sites, based on this the skin can be divided into thick or thin skin. Five-layered epidermis is only found on the palms, soles and fingers, while four layered epidermis covers most of the body (Fig. 1.). The outermost layer called stratum corneum contains terminally differentiated keratinocytes (corneocytes) which die and are shed from the body, and also continuously retrieved from the stratum basale [1–4]. The next thin, clear layer of dead keratinocytes is the stratum lucidum, only present in palms and soles [2]. The stratum granulosum is found between the stratum corneum and stratum spinosum. In this layer the keratinocytes loose their nucleus and create a lipid layer while release lamellar bodies, and keratohyalin granules are also clearly identified in these keratinocytes. Stratum spinosum is formed by constantly proliferating keratinocytes from the stratum basale layer. The lowest layer is the stratum basale, made up by keratinocytes which are still adherent to the basement membrane by hemidesmosomes [2,5]. In the different layers of the epidermis we can observe different cell types beside the keratinocytes. In the stratum spinosum immune cells, Langerhans-cells, and lymphocytes are observed, while in the stratum basale melanocytes and Merkel cells (cells of the pheripheral nervous system) can be found. [1,3].

In chronic inflammatory skin diseases the structure of the epidermal layers shows remarkable alterations. In psoriasis the mitotic rate and premature maturation of keratinocytes are increased leading to epidermal thickening (acanthosis), and incompletely cornified cells result in the retention of nuclei in stratum corneum (parakeratosis) [6]. Because of these alterations the epithelial cells overreact to environmental factors, and the produced inflammatory mediators activate and recruit professional immune cells (Fig. 1B). Here their mediators also stimulate excessive proliferation and abnormal differentiation of the epithelial cells. Thus the keratinocytes and professional immune cells can reinforce each others functions in a self-exciting vicious circle [6].

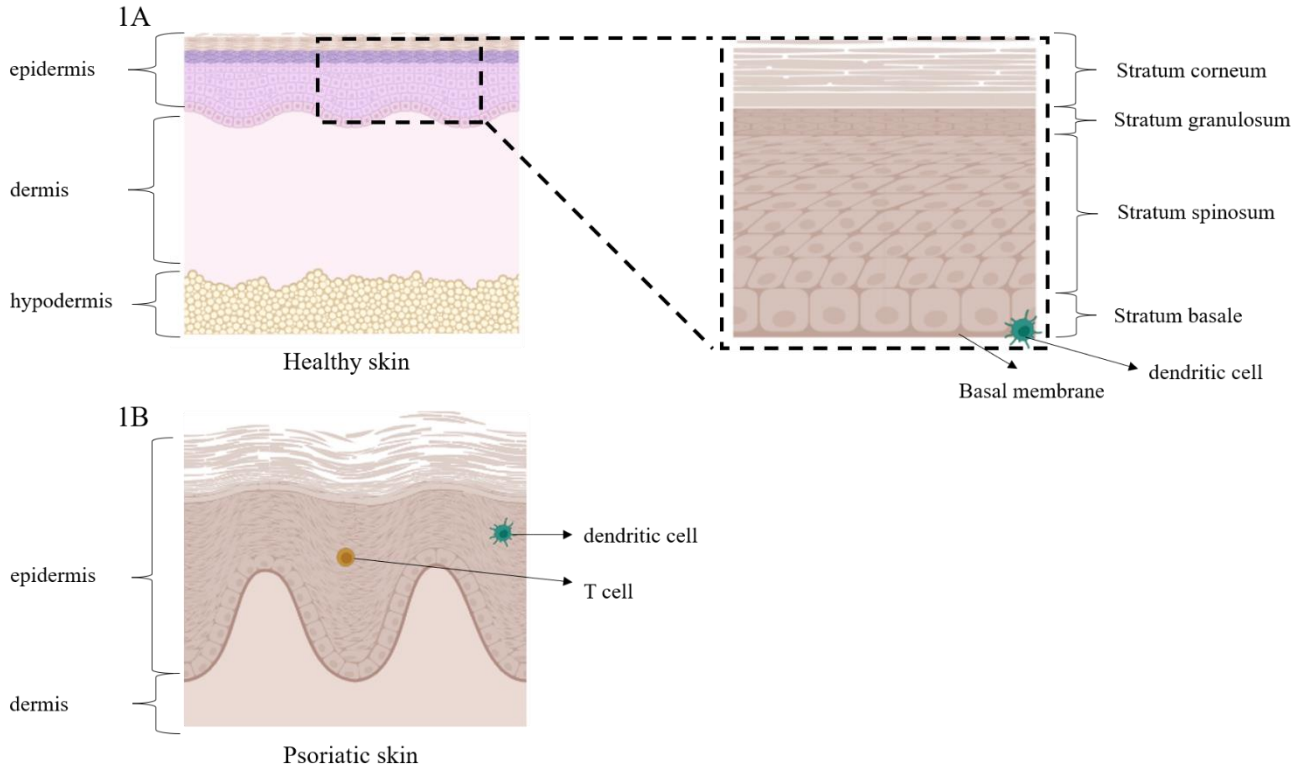

Figure 1. The healthy skin and the layers of the healthy epidermis (A) and the structure of the psoriatic skin (B). (A) The left side of the upper picture shows the layers of the healthy skin, and on the right side we can observe the layers of the enlarged epidermis. (B) The lower picture shows the structure of the layers in psoriatic skin: we can observe a thickened epidermis in the lesional psoriatic skin of the patients, while the non-lesional area's phenotype is similar to healthy epidermis.

1.2 Immune functions of keratinocytes

Keratinocytes are the major cell type of the epidermis, they actively participate in the innate immune response, but can also modulate the operation of the adaptive immune response. These cells form an effective barrier against the invading patogens and minimize moisture loss. Keratinocytes are often called non-professional immune cells, because they can recognize

harmful effects with their pattern recognition receptors (PRR), moreover they are responsive to various pathogen and danger associated molecular patterns (PAMPs and DAMPs). Among PRRs they express Toll-like receptors (TLRs), which are located on the cell surface (TLR1, TLR2, TLR4, TLR5 and TLR6) or in endosomes (TLR3 and TLR9). The expression of these receptors might be crucial for promoting skin immune responses, as activation of these receptors leads to a predominant TH1 type immune response and the production of type I interferons (IFNs) [7,8]. Furthermore, keratinocytes are the main source of the proinflammatory cytokines of the IL-1 family. These cytokines are produced as inactive precursors (pro-IL-1β and pro-IL-18) and are activated by inflammatory stimuli by the multimolecular inflammasome complexes containing caspases [9–11]. Keratinocytes also produce antimicrobial peptides at damaged epithelium surfaces, recruit the immune cells of the host and modulate their cytokine production, thus preventing the host from microbial invasion by killing the pathogens [12].Consequently, keratinocytes are able to perform and initiate immune effector functions. They can present antigens in some circumstances, and can control the migration of immune cells into the skin through the expression of surface adhesion molecules, which — once in the epidermis — induce inflammation and eliminate pathogens. If these processes get out of control it can lead to chronic inflammatory skin diseases such as psoriasis [6].

1.3 Psoriasis

Psoriasis is a multifactorial, chronic, inflammatory skin disease affecting approximately 2% of the population worldwide and has been known since ancient times. Both genetic predisposition and environmental factors play a role in its development. Raised, well-demarcated, erythematous oval plaques with silvery scales are the prominent signs of the most common form, the plaque-type psoriasis. This symptom is caused by the abnormal proliferation and differentiation of basal keratinocytes and their dysregulated interplay with professional immune cells. The plaques mostly occur on elbows, knees and the scalp, but they can also affect any part of the body [6,13]. At different stages of the disease, a variety of innate and adaptive immune cells and proinflammatory mediators are involved. The abberrant immune and epidermal response seen in psoriasis is maintained by pathogenic crosstalk between epithelial and immune cells, and it is primarily driven by proinflammatory molecules, such as TNF-α, IL-23 and IL-17 (Fig. 2.). In recent years, therapeutic targeting of these mediators has proven to be clinically effective [1,14].

1.3.1.The psoriatic non-lesional skin

Plaques are sorrunded by clinically healthy-looking skin, which is referred to as non-lesional or uninvolved skin. Despite the fact that the uninvolved skin of psoriasis patients appears macroscopically identical to normal skin, it contains molecular, cellular and extracellular alterations and, in several aspects, has a pre-psoriatic phenotype [15–19]. Although there is no clear infiltration of immune cells in non-lesional skin, compared with the healthy skin, an increase in T helper and suppressor cells, $CD11b^+$ cells, and some subsets of dendritic cells can be observed [20,21]. Whereas the psoriatic lesional epidermis has a strong type I cytokine profile, the non-lesional epidermis also exhibits significant mRNA expression of several cytokines (e.g., IL-1α, IL-1β, IL-17, TNF-α, and IFN-γ) in contrast to the healthy epidermis [22]. The TNFα/IL-23/IL-17 axis has a crucial role in maintaining the psoriatic plaques [23], however initiation of plaque formation might depend on other cytokines as well. These findings suggest altered proinflammatory conditions in the non-lesional skin, which might be responsible for the initiation of psoriatic lesions [24,25].

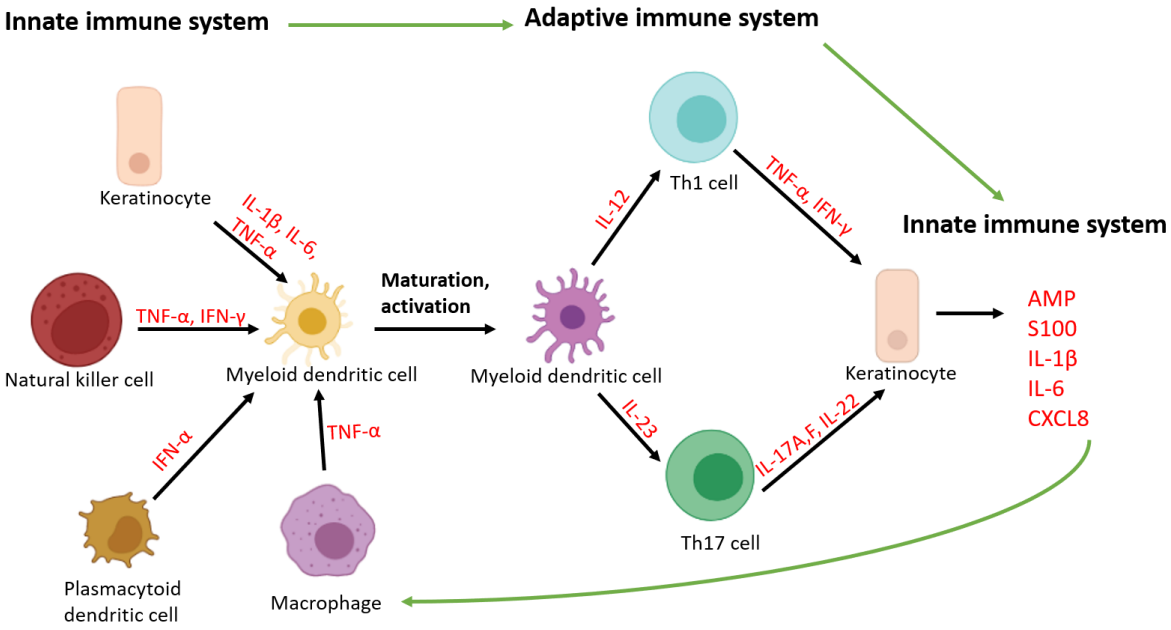

Figure 2. Innate and adaptive immunity in psoriasis. Myeloid dendritic cells can be activated by innate immune cells, which produce key cytokines (TNF-α, IFN-α, IFN-γ, IL-1β and IL-6). The differentiation of Th1 and Th17 cells requires activated dendritic cells to present antigens and secrete mediators (IL-12 and IL-23). T-cells also secrete mediators, such as IL-17A and F, IL-22, which activate keratinocytes and induce the production of antimicrobial peptides (LL-37, cathelicidin and β-defensins), proinflammatory cytokines (TNF-α, IL-1β, and Il-6), chemokines and S100 proteins. This is a rich interface between effectors of the innate and adaptive immune system, which shapes the psoriatic inflammatory processes. The figure is based on Nestle et.al, 2009 [6].

1.3.2. Free nucleic acid induced immune responses in the skin and in psoriasis

After physical trauma or infection, keratinocytes release LL-37, which is a cationic antimicrobial peptide that binds DNA and RNA fragments released by damaged skin cells. LL-37 and self-derived nucleic acids form a complex found in psoriatic lesions, and this complex activates TLR7/9 bearing plasmacytoid dendritic cells, which are normally absent from healthy skin [26–28]. These cells release proinflammatory cytokines (IL-1β, IL-6 and TNF) and interferons (IFNs) and through these have an important role in the development of the disease also by activating keratinocytes and myeloid dendritic cells (Fig. 2.). The number of myeloid dermal dendritic cells is elevated in psoriatic skin [29], and the mature cells migrate to skin-draining lymph nodes to present antigen to naive T-cells. T-cells are also critical for the initiation phase of the disease as their interaction with activated dermal dendritic cells is a central event in the development of plaque formation and the resulting creation of an IL-23/IL-17 inflammatory environment. In this environment, IL-23 derived from dendritic cells and macrophages promotes the effector functions of T helper 17 (Th17) and cytotoxic cells [13]. Taken together, these data indicate that cytosolic nucleic acid fragments, which are recognized as PAMPs and DAMPs, are highly abundant in the psoriatic skin and their presence can lead to the chronic activation of professional immune cells. This in turn, leads to the thickening of the epidermis and the formation of inflammed plaques [30].

The role of nucleic acid fragments in psoriasis is supported by the elevated levels of cell-free DNA found in blood of psoriasis patients, but the exact source of the nucleic acids associated with the initial inflammatory events is not yet known [31]. Reduced deoxyribonuclease activity in keratinocytes and disturbed ribonuclease activity in psoriatic skin have been observed [32,33]. Increased serum mitochondrial DNA (mtDNA) levels originating from mitochondrial dysregulation can act as a DAMP [34,35]. In psoriatic skin, neutrophils and neutrophil extracellular traps (NETs) might be bound by LL37, and these complexes activate inflammatory reactions that could possibly cause a self-exciting cycle contributing to chronic inflammation in psoriasis [36]. Interferon-γ (IFN-γ), produced by Th1 and Th17 cells can also contribute to the recognition of nucleic acids. The expression of IFN-γ is elevated in psoriatic lesional skin and in the serum of the patients [37], thereby, this cytokine is able to prime nucleic-acid-induced inflammatory responses in keratinocytes and drive IL-23 expression [38].

1.3.3. The role of IL-23 in psoriasis

Many recent publications suggests that the Th17/interleukin-23 axis plays a dominant role in the development of psoriasis, as it promotes chronic inflammation. IL-23 is responsible for

the development of Th17 cells, leading to a production of IL-17 and IL-22 cytokines, which are involved in the pathogenesis of psoriasis. Keratinocytes and activated antigen-presenting cells (Langerhans cells, macrophages and dendritic cells) all produce IL-23, the expression of which is elevated in psoriatic lesional skin, leading to an increased number of Th17 cells [39,40]. Recently IL-23 targeting therapies for psoriasis have become widely used.

IL-23 is a heterodimeric cytokine consisting of a unique p19 and p40 subunit, the latter shared with IL-12. Increased levels of these two subunits and the IL-23 receptor were found in psoriatic skin of patients, suggesting that IL-23 — rather than IL-12 — plays a role in disease pathogenesis [41]. Ustekinumab, a monoclonal antibody against the common p40 subunit is a highly effective treatment for psoriasis, and the antibodies targeting directly the IL-23 specific p19 subunit (guselkumab, tildrakizumab and risankizumab) also show promising impact for symptom amelioration [13,42].

1.4 Non-coding RNAs

With the completion of the human genom project it became clear, that only approximately 2% of the genome encode proteins, however the majority of it is transcribed into RNA. At least 90% of the genome is actively transcribed into RNAs, which have no protein coding potency, called as non-coding RNAs. Non-coding RNAs were previously considered evolutionary „junk molecules" or transcriptional noise, however many studies suggest that they are important regulators of diverse cellular processes and diseases [43,44]. The non-coding RNAs can be divided into two major classes according to their size: small non-coding RNAs are smaller than 200 nucleotides and long non-coding RNAs are longer than 200 nucleotides. MicroRNAs (19-22 nucleotides) are the most intensively studied subgroup of small non-coding RNAs, which regulate the expression of many genes and signaling pathways. Long non-coding RNAs (lncRNA) have many diverse functions. They can also participate in many cellular processes, such as cell proliferation, immune responses and apoptosis [43–45], by interacting with proteins, DNA and RNA.

Since lncRNAs are markedly heterogenous in size and cellular function, they can be classified according to their genomic location: enhancer lncRNAs, intergenic lncRNAs, promoter-associated lncRNAs, natural antisens lncRNAs, 3' or 5' untranslated region overlapping lncRNAs, sense-overlapping lncRNAs and intronic lncRNAs [45].

The latest genome-scale transcription studies described that these RNAs may play a role in different cellular processes like dysregulation of inflammatory functions and disease pathogenesis. More than 10.000 non-coding RNAs have been described so far, but for most of

them the exact biological role is not known yet. Clarifying their function could contribute to better understanding of various diseases and revealing their tissue- and disease-specific expression might lead to recognize them as ideal biomarkers and therapeutic targets [44,46,47].

1.4.1 PRINS long non-coding RNA

At the beginning of the 2000s a long non-coding RNA referred to as psoriasis-susceptibility-related RNA gene induced by stress (PRINS) was identified by a differential display experiment in our research group. The 3' end of PRINS is 100% similar to the AK022045 RNA sequence. PRINS exhibited higher levels in the uninvolved skin of psoriatic patients than in lesional or healthy epidermis, which suggested its possible contribution to psoriasis susceptibility [18]. Different sets of cellular stressors like ultraviolet-B (UV-B) irradiation, starvation, translation inhibition [48] and hypoxia [49] modified the expression of PRINS. Furthermore, silencing of PRINS in HaCaT cells during stress exposure decreased cellular viability. These results suggested that PRINS has a protective role in cells exposed to stress [18].

By using bioinformatical tools, it was found that PRINS gene is located on the short arm of human chromose 10 (10p12.31 map position) and is composed of two exons with an approximately 7 kb long intron in between (Fig.3 A) [18].

PRINS is an intronic lncRNA, becuase it is inserted in an intron of the SKT gene (KIAA1217), involved in early stages of embryogenesis [50]. The OUT deubiquitinase 1 gene is proximal to SKT, whereas the Rho GTPase activating protein 21 is distal to SKT. 3' of the PRINS coding region, the miR603 miRNA is located in an SKT intron. Using the ENCODE database, a transcription start site was identified 6 kb proximal to the putative 5' end of the PRINS gene [51]. We hypothesize that a strongly regulated active promoter might be associated with PRINS, because this region is marked by a high density of binding sites of several transcription factors, 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) [52]. The region neighbouring to the 3' end of the PRINS lncRNA gene also carries histone modification sights, which due to the close 3' proximity to the PRINS lncRNA gene might be an enhancer element (Fig.3 A). The PRINS lncRNA contains three *Alu* elements (the most abundant transposable elements), and it is well known that transposable elements have been very important in the evolution of lncRNAs [53,54]. PRINS lncRNA gene is a primate specific sequence and its orthologues could be found only in the genomes of primates with variations in the extent of similarity (Fig.3 B) [52].

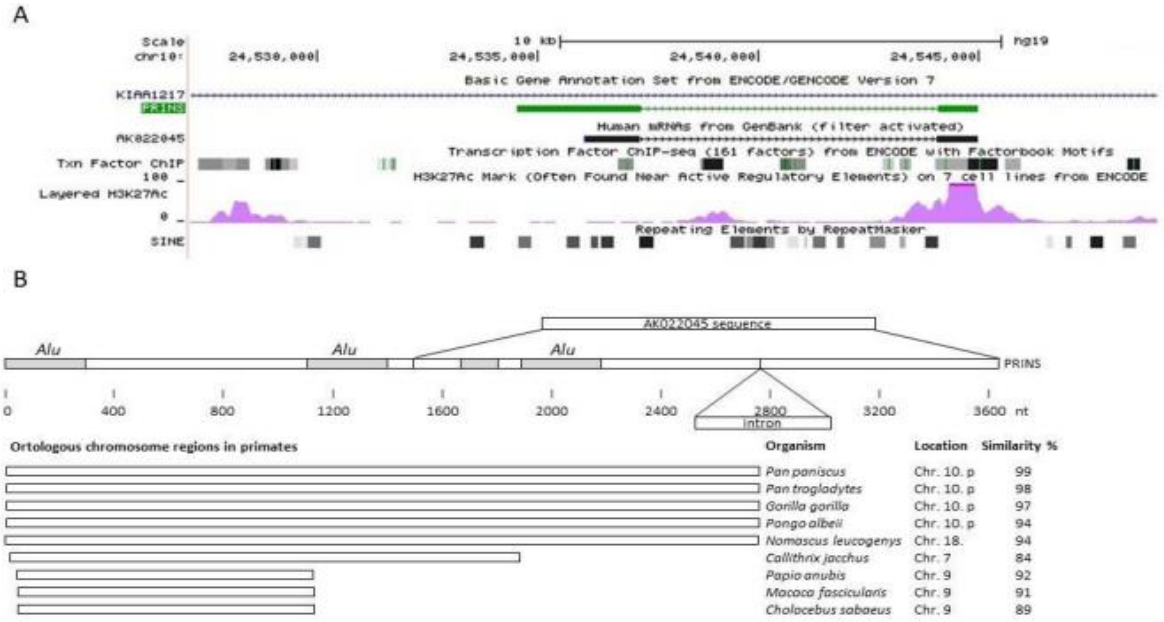

Figure 3. Charactheristic of the PRINS lncRNA identified by USCS Genom Browser (A) and NCBI BLAST (B). (A) PRINS gene is located in an intron of the SKT gene on chromosome 10. The 3' end of the PRINS gene sequence show 100% similarity with the AK022045 RNA sequence. Acetylation of Histone H3 lysine K27 and high density of transcription factor binding sites was identified in normal human epidermal keratinocytes approximately 6 kb upstream from the PRINS gene and at the 3' end of the gene, suggesting an active transcription. (B) The PRINS gene is located on the p12.31 arm of human chromosome 10, which is highly conserved in human and other primate species [52].

Previously, our group carried out a cDNA microarray [55] and an *in vitro* protein binding assay [56] to identify potential interacting partners of PRINS. In the cDNA microarray G1P3 was identified (also known as interferon-α-inducible protein 6), as downregulated in HeLa cells by PRINS silencing [55]. From the *in vitro* protein binding assay nucleophosmin (NPM) was identified as a binding partner of PRINS. It is known that NPM is an ubiquitously expressed nucleolar phosphoprotein, which shuttles to the nucleoplasm after UV-B irradiation in fibroblasts, cancer cells and cultured keratinocytes. The retention of NPM in the nucleolus has been observed after gene-specific silencing of PRINS [56].

According to our current knowledge we hypothesize that the evolutionary young, primate specific long non-coding RNA PRINS is differentially expressed in psoriasis, plays a role in keratinocyte stress response and thus in disease pathogenesis. Previously we have shown that PRINS binds directly to the mRNAs of IL-6 and CCL-5 (RANTES) at specific binding sites and eventually destabilizes these mRNAs, leading to a decrease in their accumulation [30,49], (Fig.4.). All these data together indicate that PRINS also has a restrictive role in inflammatory processes.

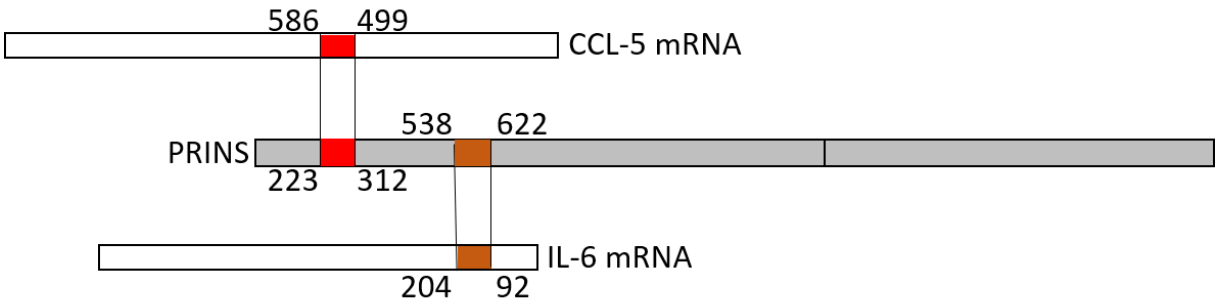

Figure 4. Putative binding sites identified by bioinformatics tools between inflammatory molecules (IL-6 and CCL5) and PRINS long non-coding RNA [30]**.**

2. Aims

The aim of our research group is to clarify the details of nucleic acid induced innate immune mechanisms in keratinocytes. In the frame of this project a qPCR array (containing 84 psoriasis relevant genes) was carried out to identify genes showing altered expression as a result of nucleic acid analogue treatment. One of the genes showing the highest change in transcription was the IL-23 cytokine. Therefore we aimed to:

- reveal details of IL-23 expression pathways in keratinocytes by analyzing the free nucleic acid induced signal transduction pathways and pattern recognition receptors, and study how they contribute to IL-23 expression in human keratinocytes
- examine whether PRINS long non-coding RNA has a regulatory role in nucleic acid induced IL-23 production in human keratinocytes

3. Materials and Methods

3.1 Cell culture

For the experiments, we used the HPV-KER cell line [57] and normal human epidermal keratinocytes (NHEKs) isolated from skin samples retrieved from the Plastic Surgery Unit of our Department after informed written consent was obtained from the volunteers. The experiments were approved by the Human Investigation Review Board of the University of Szeged (PSO-EDAFN-002, 23 February 2015, Szeged, Hungary) and investigations were carried out in accordance with the rules of the Helsinki Declaration. The isolation of the NHEKs from skin samples was described earlier [58]. Keratinocytes were grown in Keratinocyte-SFM Medium (Gibco, Thermo Fischer Scientific, Waltham, MA, USA), supplemented with EGF (Epidermal Growth Factor), BPE (Bovine Pituitary Extract), 1% L-glutamine and 1% antibiotic-antimycotic solution. Medium was changed every second day until the third passage.

3.2 Stimulation of the cells

HPV-KER cells and third-passage NHEK cells were seeded into 6-well plates at a density of 200,000 cells/ml. After twenty-four hours, medium was changed to supplement-free medium and cells were transfected with 1 μg/ml poly deoxyadenylic acid-poly deoxythymidylic acid double-stranded homopolymer (poly(dA:dT)) (InvivoGen, Sand 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, Basel, Switzerland), according to the manufacturer's instructions. Cells were harvested at indicated time points.

For PRINS overexpression, the AK022045 cDNA sequence (Biological Research Center, National Institute of Technology and Evaluation, Chiba, Japan) was cloned into a pcDNA3.1(+) vector as described previously [30]. As a control, the empty pcDNA3.1(+) vector was used. One μg of plasmid DNA was used for the transfection of the NHEK cells using the X-tremeGene HP transfection reagent (Roche Diagnostics, Basel, Switzerland), according to the manufacturer's instructions.

For siRNA-mediated gene silencing of PRRs, the ON-TARGETplus SMARTpool TLR3, RIG-1, IFIH-1, cGAS siRNAs or ON-TARGETplus Non-Targeting Pool (Dharmacon, Lafayette, USA) constructs were used at a final concentration of 40 nM for transfection using the X-tremeGene siRNA transfection reagent (Roche Diagnostics, Basel, Switzerland), according to the manufacturer's instructions.

For inhibition experiments, cells were incubated 1 hour prior to poly(dA:dT)/poly(I:C) transfection with specific inhibitors of NF-κB (Bay 11-7085, 10 μM; MedChemExpress, Monmouth Junction, NJ, USA), STAT-1 (Fludarabine, 10 μM; Sigma Aldrich), STAT-3 (Stattic, 5 μM; Sigma Aldrich), JNK (SP600125, 10 μM; Tocris Bioscience, Bristol, UK), MEK-1 (PD98059, 20 μM; Sigma Aldrich) and p38 (SB203580, 10 μM; Tocris Bioscience). Since all of the signal transduction pathway inhibitors were solved in DMSO to different concentrations, the same amount of DMSO was applied as control.

3.3 RNA isolation and RT-PCR

At indicated time points after transfection, 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 using the Turbo DNA-free Kit (Ambion, Thermo Fischer Scientific, Waltham, MA, USA), according to the manufacturer's instructions. During cDNA synthesis, 1 μg total RNA was reverse transcribed by the EvoScript Universal cDNA master mix (Roche, Indianapolis, USA). Real-time RT-PCR experiments were carried out with the Universal Probe Library (Roche Diagnostics, Basel, Switzerland) or the TaqMan Gene expression Assay (Thermo Scientific, Rockford, USA) and qPCRBIO Probe Mix Lo-ROX (PCR Biosystem Ltd., London, UK) on a C1000 Touch Thermal Cycler (Bio-Rad Laboratories). The expression of each gene was normalized to the GAPDH mRNA, and relative mRNA levels were calculated using the $\Delta\Delta C_t$ method. Primers and Probes are listed in Table 1.

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

Manufacturer	mRNA	Strand	Primer sequences	Probe.no
Roche Diagnostics-Universal Probe Library	GAPHD	Forward	AGC CAC ATC GCT CAG ACA C	60
		Reverse	GCC CAA TAC GAC CAA ATC C	
	IL-6	Forward	CAG GAC CCC AGC TAT GAA CT	45
		Reverse	GAA GGC AGC AGG CAA CAC	
	TLR-3	Forward	AGA GTT GTC ATC GAA TCA AAT TAA AG	80
		Reverse	AAT CTT CCA ATT GCG TGA AAA	
	RIG-1	Forward	GTG GGC AAT GTC ATC AAA A	6
		Reverse	GAA GCA CTT GCT ACC TCT TCG	
	MDA-5 (IFIH1)	Forward	AGG CAC CAT GGG AAG TGA T	36
		Reverse	GGT AAG GCC TGA GCT GGA G	
Thermo Fischer Scientific	PRINS	TaqMan Gene Expression Assays		Hs03671803_s1
	IL-23	TaqMan Gene expression Assays		Hs00900828_g1

3.4 In silico prediction of interacting sites

Using INTARNA [59], sequence complementarity between PRINS (AK022045) and the mRNA of IL-23 (NM_016584.3) were analyzed. INTARNA calculates the free-energy values of interaction based on predicted global and local structures of mRNAs. This *in silico* analysis revealed a binding site on the IL-23A mRNA that partly overlaps with the previously identified IL-6 binding sequences [30].

3.5 pmirGLO dual-luciferase miRNA target expression vector

The *in silico* identified putative PRINS binding site sequence of IL-23 cDNA was synthesized and inserted into the pmirGLO vector (Promega) between the *Dra* I and *Xba* I restriction sites, resulting the pmirGLO-IL23BS construct (Supplementary Fig.4). HEK293 cells were transiently transfected by the PRINSpcDNA3.1 or pmirGLO plasmid as controls. Cells were also co-transfected with 0.5 μg pmirGLO-IL23BS or empty pmirGLO plasmid together with the PRINSpcDNA3.1 vector. In each transfection experiment 0.025 μg Renilla-luciferase-expressing pGL4.75 hRluc/CMV (Promega) plasmid was used as internal control. Cells were harvested after 24 hours, washed with PBS (Phosphate-buffered saline), lysed with passive lysis buffer (Biotium, Hayward, CA, USA), and luciferase activities were measured

using the Firefly & Renilla Dual Luciferase Assay Kit (Biotium) and SYNERGY/HTX Multi-Mode reader (Bio Tek Instruments, Winooski, VT, USA), according to the manufacturer's instructions. The luciferase activity derived from the pmirGLO plasmids was normalized to the activity of the Renilla-luciferase activity.

Table 2 The sequence of the oligonucleotides used for cloning the binding site

IL-23 binding site Fwd: 5'-AAACTAGGATCCAGCTTCATGCCTCCCTACTGGGCCTCAGCCAACTCCTGCT-3
IL-23 binding site Rev: 5'-CTAGAGCAGGAGTTGGCTGAGGCCCAGTAGGGAGGCATGAAGCTGGATCCTAGTTT-3

3.6 Statistical analysis

Experiments were carried out in duplicates with at least three biological repeats. For statistical analysis, a one-tailed, paired Students's t-test was used with correction for multiple comparisons. The significance level was set at $p \leq 0.05$. Pearson's correlation calculation was used to analyze correlation between IL-23 mRNA induction and PRINS overexpression mediated IL-23 mRNA depletion.

4. Results

4.1 Free nucleic acid analogues induce increased IL-23 mRNA expression in human keratinocytes

4.1.1. Psoriasis-relevant gene expression pattern is modulated by free nucleic acid analogues

To identify genes responding with altered expression in NHEKs to treatment with the synthetic nucleic acid analogue poly(I:C) and poly(dA:dT), a qPCR array containing 84 psoriasis-relevant genes including cytokines, chemokines, antimicrobial peptides and cytoplasmic receptors was performed. In this experiment, we observed that 14 genes were not expressed by the cells, whereas the expression of 15 additional genes, mostly antimicrobial peptides, was not altered after treatment with either poly(I:C) or poly(dA:dT). We identified 37 genes that were induced by both treatments, including cytokines, chemokines and pattern recognition receptors (PRRs). Increased expression in response only to poly(I:C) treatment was observed for 7 additional genes (Fig.5.A, B).

Most importantly, we observed that in a manner similar to other inflammatory cytokines, such as IL-6 and TNF-α (results previously published in [58] and Fig.6.B), IL-23 expression increased in response to both poly(dA:dT) and poly(I:C) treatment. Since IL-23 is known to play an important role in psoriasis pathogenesis, we designed further experiments to examine the molecular mechanism of free nucleic acid induced IL-23 transcription in human keratinocytes.

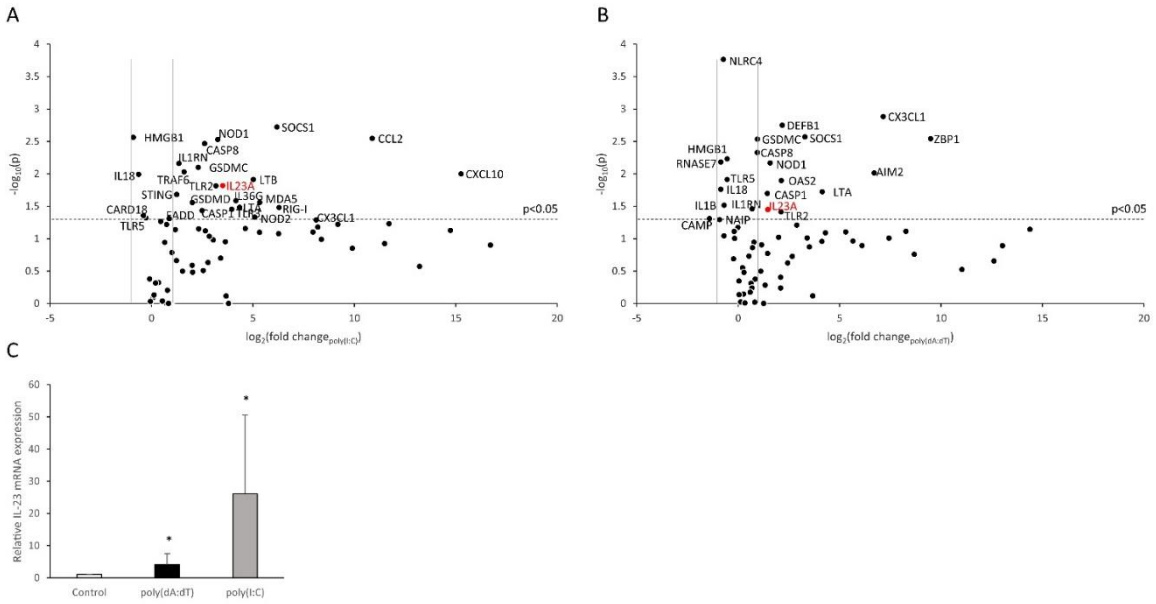

Figure 5. Changes in keratinocyte gene expression induced by poly(I:C) (A) and poly(dA:dT) (B). RT-PCR validation on independent samples revealed that NHEKs respond to poly(I:C) and poly(dA:dT) with increased IL-23 mRNA expression(C). To assess multiple gene expression changes upon free nucleic acid stimuli, a qPCR array of inflammatory mediators and receptors was carried out on normal human epidermal keratinocyte samples transfected by 0.666 μg/mL poly(I:C) (A) or 1 μg/mL poly(dA:dT) for 12 hours (B) and expression changes were compared to mock transfected samples. Results are presented as volcano plots, significant changes ($p<0.05$) are displayed above the horizontal dashed line. Vertical lines indicate a ±2-fold change in expression compared to mock transfected control samples. Experiments were carried out in four independent experiments, statistical significance was calculated by Student's t-test with correction for multiple comparisons (A and B). For validation NHEKs were transfected with 0.666 μg/mL poly(I:C) and 1 μg/mL poly(dA:dT), samples were collected 12 hours after treatments. Relative IL-23 mRNA expression was determined by the ΔΔCt method, normalized to GAPDH mRNA expression and compared to the expression of the time-matched mock treated (Contol) samples. Data are presented as mean of six independent experiments ±SD. Significance was determined by one tailed, paired Student's-test with correction, *$p<0.05$ compared to control (C).

In the qPCR array, mRNA samples derived from four healthy donors were used, and the first validation experiments were carried out on the same mRNA set. These results confirmed that poly(I:C) induced significant increase in IL-23 mRNA, whereas poly(dA:dT) induced an upward trend in expression (Fig.6.A). To expand the validation of the array, we included additional independent donors (n=6) and observed significantly higher IL-23 expression after both treatments, however, the treatment with poly(I:C) had a more pronounced effect in this experiment as well (Fig.5.C).

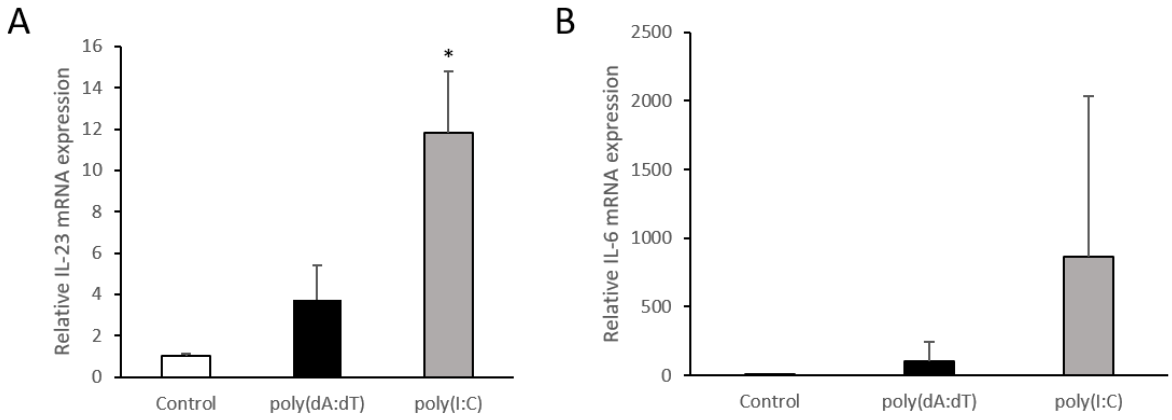

Figure 6. RT-PCR validation of the array results using the same total RNA set demonstrated that the NHEKs respond to poly(I:C) and poly(dA:dT) with increased IL-23 (A) and IL-6 (B) mRNA expression. NHEKs were transfected with 0.666 μg/mL poly(I:C) and 1 μg/mL poly(dA:dT). for 12 hours. Relative IL-23 and IL-6 mRNA expressions were determined by the ΔΔCt method, normalized to the GAPDH mRNA expression and compared to the expression of mock-treated (Contol) 24 h samples. Data are presented as mean of four independent experiments ± SD. Significance was determined by one-tailed, paired Student's t-test,*$p<0.05$ compared to control.

4.1.2. Poly(I:C) and poly(dA:dT) induce IL-23 mRNA expression with different kinetics

Previously, we identified kinetic differences in poly(I:C)- and poly(dA:dT)-induced inflammatory responses in keratinocytes [58]. To clarify this in the case of IL-23, we measured IL-23 mRNA levels at different time points after nucleic acid treatments. Compared to the previously investigated inflammatory mediators, such as IL-6 or TNF-α, which peak at 6 to 12 hours after poly(I:C) or (dA:dT) treatment [58] (Fig.7.), peak expression of IL-23 was observed later, at 24 hours after poly(I:C) transfection, while a slowly rising tendency was detected after poly(dA:dT) treatment, and these levels were lower than those observed after poly(I:C) treatment (Fig.8.). To further confirm our results, HPV-KER cells were also included in our experiments. In the HPV-KER cells — similarly what we detected in NHEKs — we also observed a peak expression of IL-23 mRNA after 24 hours of poly(I:C) treatment, but after poly(dA:dT) transfection, we detected a rising tendency (Fig.9.).

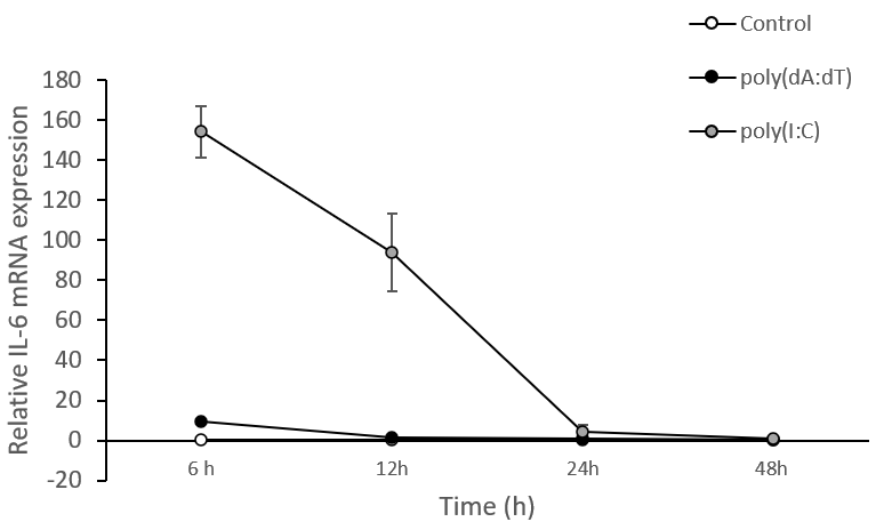

Figure 7. Kinetics of the IL-6 mRNA expression in NHEKs upon nucleic acid induction. Cells were transfected with 0.666 μg/mL poly(I:C) and 1 μg/mL poly(dA:dT), and samples were collected at 6, 12, 24, 48 h after transfection. Relative IL-6 mRNA expression was determined by the ΔΔCt method, normalized to GAPDH mRNA expression and compared to the expression of the untreated 0 hour (Control) samples. Data are presented as means of three independent experiments ± SD.

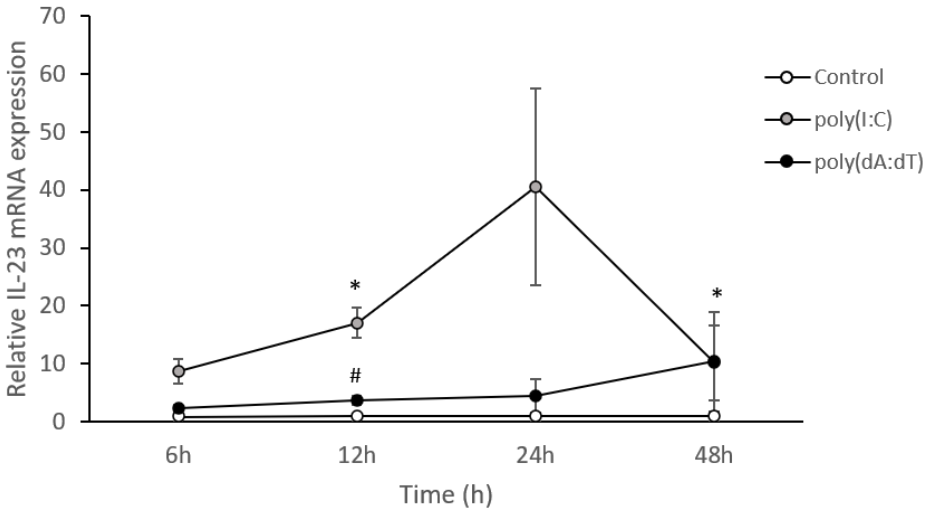

Figure 8. Kinetics of the IL-23 mRNA expression in NHEKs upon nucleic acid induction. Cells were transfected with 0.666 μg/mL poly(I:C) and 1 μg/mL poly(dA:dT), and samples were collected at 6, 12, 24, 48 h after transfection. Relative IL-23 mRNA expression was determined by the ΔΔCt method, normalized to GAPDH mRNA expression and compared to the expression of the untreated (Control) 0 hour samples. Data are presented as means of three independent experiments ± SD. Sinificance was determined by one tailed, paired Sutdent's t-test, *$p<0.05$ poly(I:C) treated vs. time-matched control samples, # $p<0.05$ poly(dA:dT) treated vs. time matched control samples.

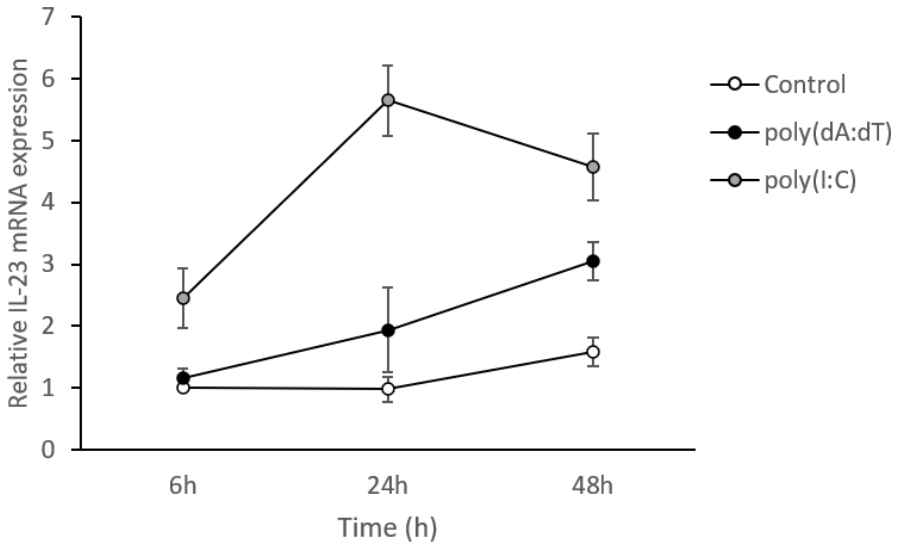

Figure 9. Kinetics of IL-23 mRNA expression in HPV-KER cells upon nucleic acid induction. Cells were transfected with 0.666 μg/mL poly(I:C) and 1 μg/mL poly(dA:dT), and samples were collected at 6, 12, 24, 48 h after transfection. Relative IL-23 mRNA expression was determined by the ΔΔCt method, normalized to GAPDH mRNA expression and compared to the expression of the untreated (Control) 0 hour samples. Data are presented as means of three biological replicates ± SD.

4.1.3. Psoriasis-specific stimuli induce IL-23 mRNA expression

In psoriatic skin, keratinocytes are exposed not only to free nucleic acids but to numerous cytokines, which shape the inflammatory response of keratinocytes. To compare the effects of psoriasis relevant specific cytokines with the effects of nucleic acids on IL-23 mRNA expression in keratinocytes, we treated the cells with IL-17A, IL-12, TNF-α and IL-23, and synthetic nucleic acid analogues imiquimod (IMQ), poly(dA:dT) and poly(I:C), since these molecules have long been known to have a role in psoriasis pathogenesis. The highest IL-23 expression was induced by poly(I:C), whereas poly(dA:dT) and TNF-α treatment also elevated IL-23 transcription but to a lesser extent (Fig.10.). Other psoriasis-relevant cytokines and IMQ, which is used to induce psoriasis in mouse models [60], did not affect IL-23 mRNA expression in keratinocytes.

Interestingly, although IL-23 is thought to originate from professional immune cells, in our experiments THP-1 macrophages and Jurkat T-cells failed to exceed the level of IL-23 mRNA production of keratinocytes in response to poly(I:C), poly(dA:dT) and TNFα (data not shown). These results indicate that synthetic nucleic acids modelling free cellular nucleic acids — especially poly(I:C) — play an important role in the psoriasis-associated inflammatory processes by inducing elevated IL-23 levels in human keratinocytes.

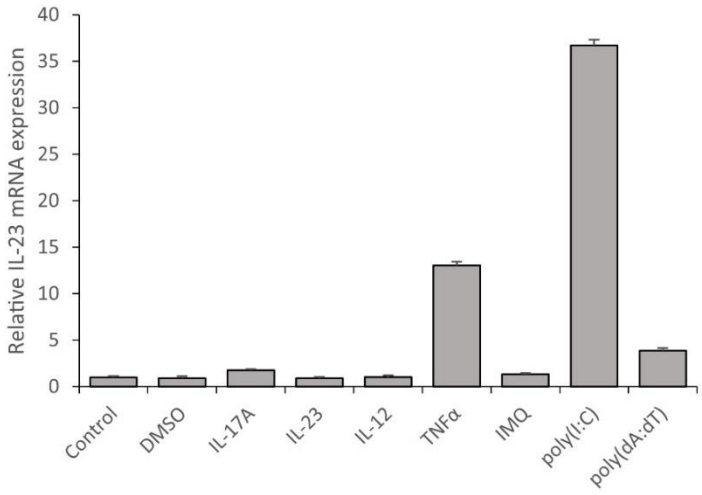

Figure 10. Synthetic nucleic acid analogues and TNFα induce IL-23 mRNA expression in NHEKs. Cells were transfected with 0.666 μg/mL poly(I:C), 1 μg/mL poly(dA:dT), 2 μl DMSO, 1 μg/mL IL-17A, 1 μg/mL IL-23, 1 μg/mL IL-12, 0.5 μg/mL TNFα and 1.33 μg/mL IMQ. Relative IL-23 mRNA expression was determined by the ΔΔCt method, normalized to GAPDH mRNA expression and compared to the expression of the untreated control samples. Results of one representative experiment are shown, data are presented as the average of three technical repetitions ± SD.

4.2 Free nucleic acids act through specific receptors and signaling pathways to modulate IL-23 mRNA expression levels

4.2.1. TLR3 is the main nucleic acid sensing receptor conveying IL-23 mRNA expression in NHEKs

In psoriasis, nucleic acid fragments originating from the tissue or pathogens are present and recognized as danger signals for disease development [31,33,35]. Several pathogen recognition receptors (PRRs) that recognize these RNA and DNA fragments and induce inflammatory mechanisms have already been identified [61–63], and many of them are expressed both in keratinocytes and professional immune cells. We aimed to examine which of these receptors plays an important role in mediating nucleic acid induced IL-23 mRNA expression in keratinocytes. To this end, siRNA-mediated silencing was carried out to decrease the levels of TLR-3, RIG-I, IFIH1(MDA-5) and cyclic GMP-AMP synthase (cGAS). The effect of silencing was verified by qPCR (Fig.11). Our results showed that poly(I:C)-induced IL-23 mRNA expression is mediated primarily by TLR3 in NHEKs. Silencing none of the receptors in our experiments did affect the influence of poly(dA:dT) on IL-23 transcription (Fig.12, Supplementary fig.1A,B,C,D). The same experiments carried out in HPV-KER cells revealed that besides TLR3, several other PRRs like RIG-1, IFIH, cGAS might also regulate poly(I:C) and poly(dA:dT) induced IL-23 mRNA expression in these cells(Fig.13.).

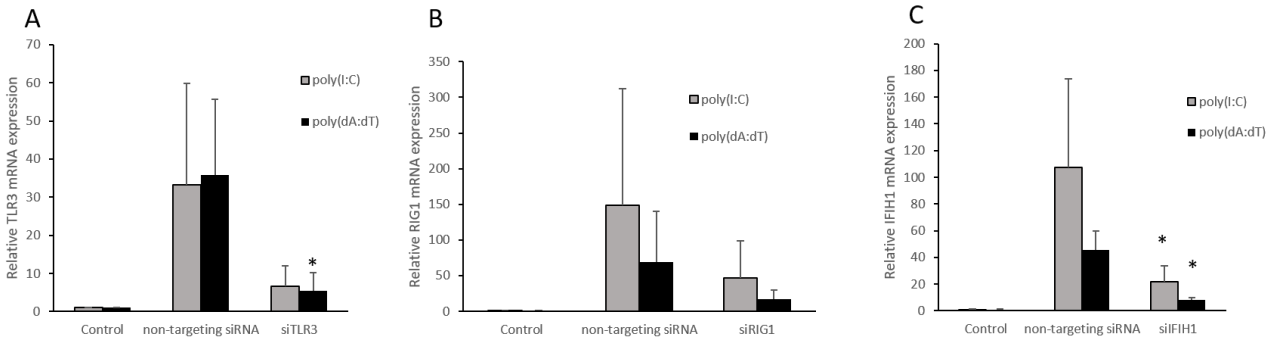

Figure 11. The effect of silencing on the mRNA expression level of the pattern recognition receptors TLR3 (A), RIGI (B) and IFIH1 (C) in NHEKs Pattern recognition receptors were silenced with siRNA mediated inhibiton for 24 hours and the cells were subseqently transfected with 0.666 μg/mL poly(I:C) and 1 μg/mL poly(dA:dT). Relative expressions of the receptors were determined by the $\Delta\Delta C_t$ method, normalized to GAPDH mRNA expression and compared to the expression of the untreated control samples. Data are presented as the mean of three experiments ± SD. Significance was determined by one tailed, paired Student's-test *p<0.05, compared to the non-targeting siRNA transfected samples.

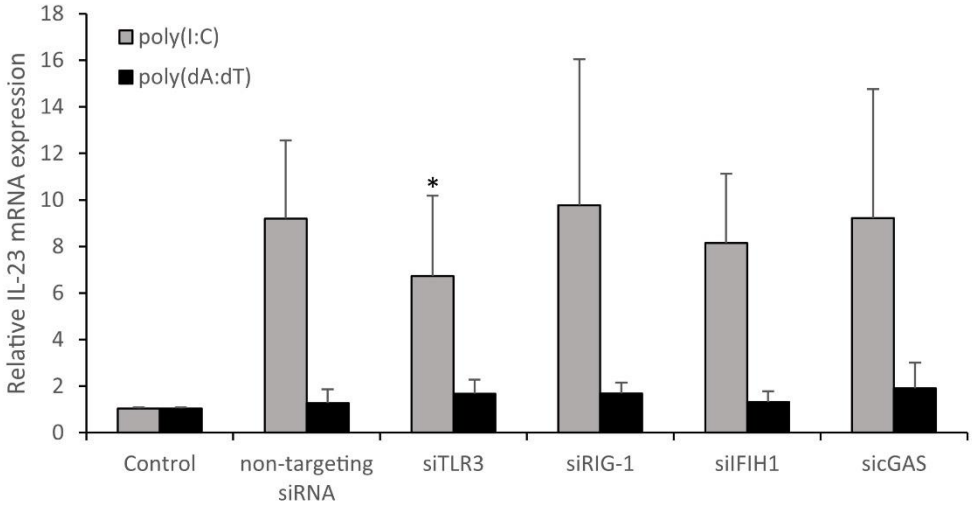

Figure 12. Silencing of TLR3 pattern recognition receptor affects poly(I:C) induced IL-23 mRNA expression of NHEKs. Expression of pattern recognition receptors were silenced with siRNA mediated inhibiton for 24 hours and subsequently the cells were transfected with 0.666 μg/mL poly(I:C) and 1 μg/mL poly(dA:dT). Relative IL-23 mRNA expression was determined by the ΔΔCt method, normalized to GAPDH mRNA expression and compared to the expression of the non-treated control samples. Data are presented as means of four independent experiments ± SD. Significance was determined between siRNA transfected samples and the non-targeting siRNA-transfected samples by the one tailed paired Student's t-test with correction for multiple comparisons *p<0.05

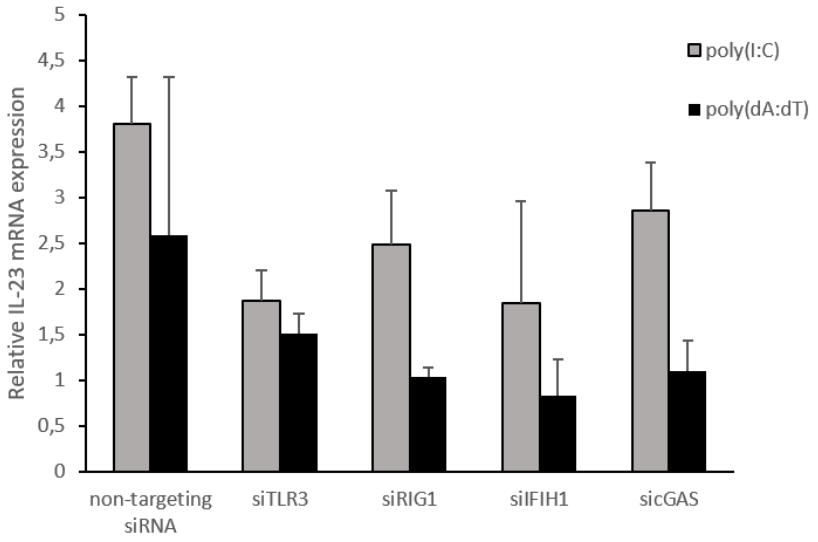

Figure 13. The IL-23 mRNA expression level upon silencing of the pattern recognition receptors in HPV-KER cells. Pattern recognition receptors were silenced with siRNA mediated inhibiton for 24 hours and the cells were subseqently transfected with 0.666 μg/mL poly(I:C) and 1 μg/mL poly(dA:dT). Relative expression of IL-23 mRNA was determined by the $\Delta\Delta C_t$ method, normalized to GAPDH mRNA expression and compared to the expression of the non-targeting siRNA control samples. Data are presented as a mean of three biological replicates ± SD. Significance was determined between siRNA transfected samples and the non-targeting siRNA-transfected samples by the one tailed paired Student's t-test

4.2.2. Multiple pathways transmit free nucleic acid signals to mediate IL-23 mRNA expression

Our next aim was to identify the downstream signaling pathways through which elevated nucleic acid levels lead to increased IL-23 mRNA synthesis in keratinocytes. In our studies, we examined six pathways that were previously shown to be affected by nucleic acids in other cell types [64]. The activity of a major component of each of these pathways was decreased by applying specific inhibitors, such as Bay 11-7085 for NF-κB, PD95089 for dual specificity MEK1/2, SB203580 for p38, SP600125 for JNK (c-Jun-N terminal kinase, fludarabine for STAT-1 (signal transducer and activator of transcription-1) and Stattic for STAT-3. Keratinocytes were preincubated with the inhibitor for an hour before transfection with poly(I:C) or poly(dA:dT). Our results showed that poly(I:C)-induced IL-23 mRNA expression is mediated by JNK, ERK1/2, NF-κB and STAT3 pathways, downstream of TLR3 receptor (Fig.14, Supplementary fig. 2A, B, C). However, inhibition of either of these pathways had no effect on poly(dA:dT)-induced IL-23 mRNA expression, which might also be due to the low IL-23 transcription induction triggered by poly(dA:dT). Furthermore, we also observed that

similarly to NHEKs poly(I:C) induced IL-23 mRNA expression is mediated by ERK1/2 and NF- κB pathways in HPV-KER cells (Fig.15.).

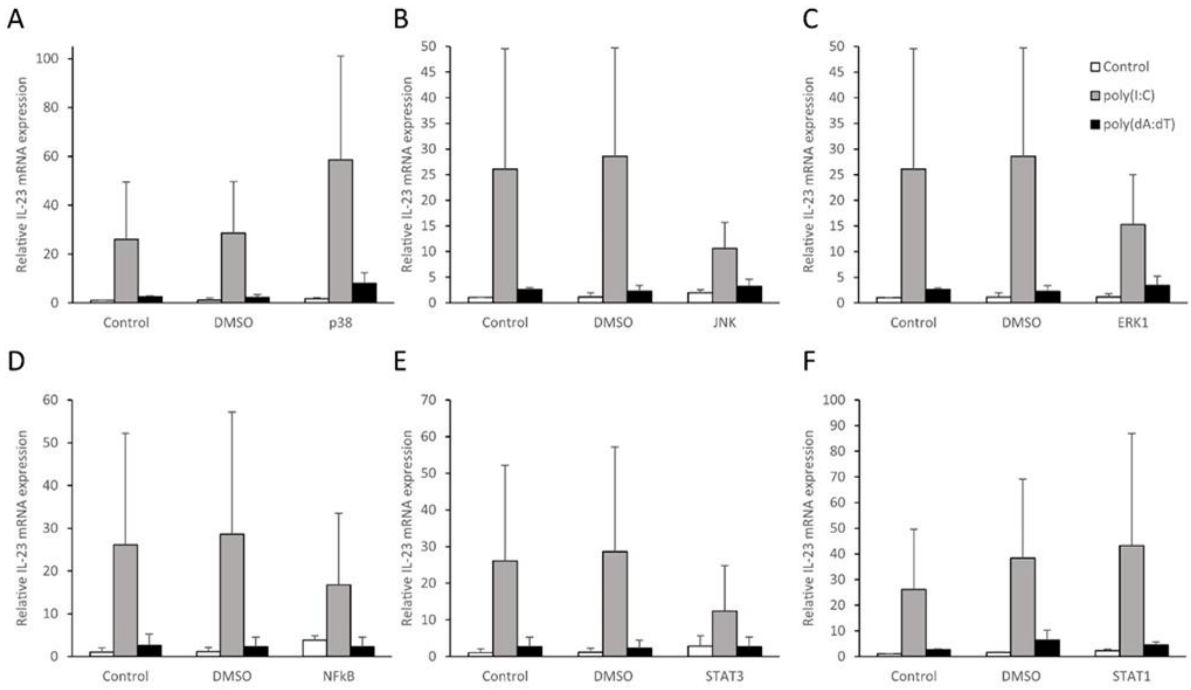

Figure 14. Inhibition of JNK, ERK1, NF-κB and STAT3 pathway components had profound effect on the nucleic acid induced IL-23 mRNA expression in NHEK cells. Specific inhibiton of signaling pathway components were used for 1 hour with the cells being subsequently transfected with 0.666 μg/mL poly(I:C) and 1 μg/mL poly(dA:dT) for 24 hours. Equal concentration of DMSO was used as solvent-control for each inhibitor, demonstrating no difference from non-pretreated control samples. Relative expression was determined by the ΔΔCt method, normalized to GAPDH mRNA expression and compared to the expression of the untreated control samples. Data are presented as mean of three independent experiments ± SD. Significance was tested by one tail, paired T-test comparing to DMSO-treated solvent-control samples. In spite of obvious differencies in the values, no significance could be demonstrated among them due to high standard deviations of the independent experiments performed on NHEKs derived from independent volunteers.

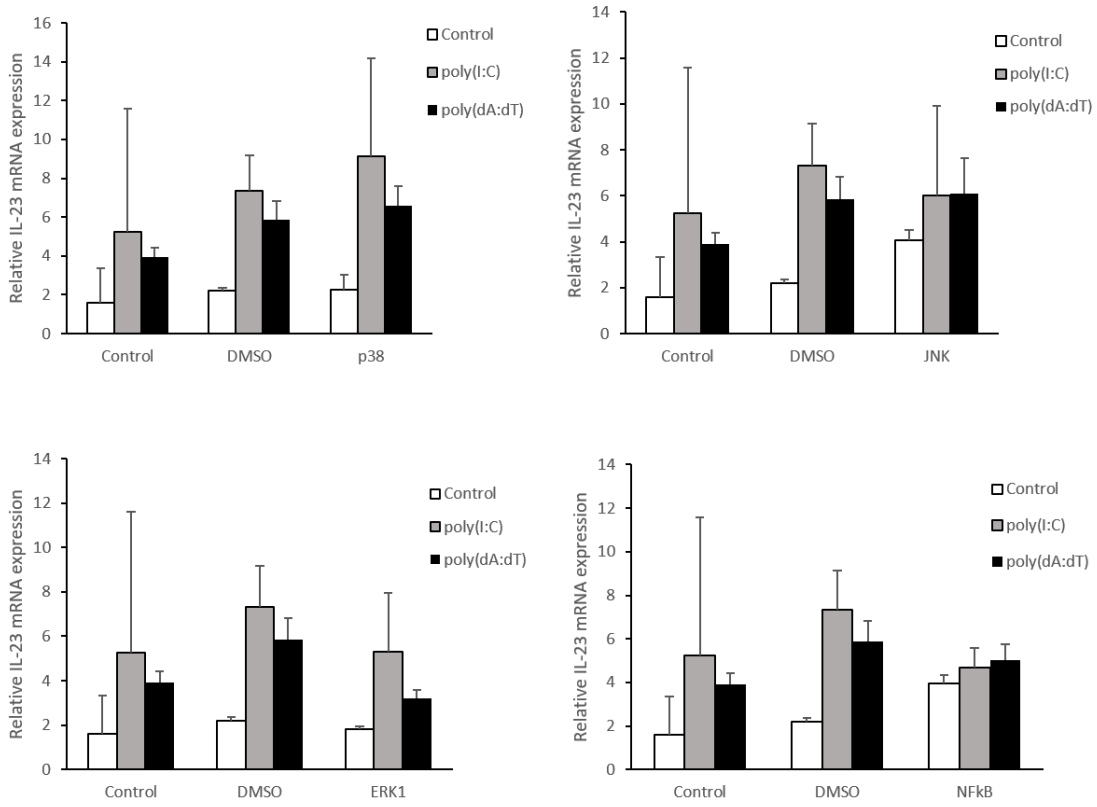

Figure 15. Inhibition of the ERK1 and NF-κB pathway components had an effect of the nucleic acid induced IL-23 mRNA expression of HPV-KER cells. Specific inhibiton of signaling pathway components were used for 1 hour with the cells being subsequently transfected with 0.666 μg/mL poly(I:C) and 1 μg/mL poly(dA:dT) for 24 hours. Equal concentration of DMSO was used as solvent-control for each inhibitor, demonstrating no difference from non-pretreated control samples. Relative expression was determined by the ΔΔCt method, normalized to GAPDH mRNA expression and compared to the expression of the untreated control samples. Data are presented as mean of three biological replicates ± SD. Significance was tested by one tail, paired T-test comparing to DMSO-treated solvent-control samples.

4.3 The effect of the PRINS long non-coding RNA shows a great inter-individual difference on nucleic acid analogue induced IL-23 mRNA expression in human keratinocytes

Connection between PRINS and psoriasis has been described in several earlier studies [18,19,48,56]; such as regulation of certain inflammatory mediators (IL-6 and CCL5) by PRINS in nucleic-acid-induced inflammatory reactions. To identify additional targets for PRINS-mediated regulation in NHEKs, a qPCR-array was used to identify genes showing altered expression as a consequence of nucleic acid challenge and PRINS overexpression. A PRINS overexpressing construct was transiently transfected into NHEK cells, and the cells were

subsequently treated with poly(I:C) or poly(dA:dT) to identify how PRINS overexpression alters the nucleic acid induced gene expression in keratinocytes. In the qPCR-array impaired upregulation of inflammatory genes including cytokines, chemokines, receptors and effector molecules was observed. Among others, NLRP1, GSDMC, CX3CL1, SOCS1 exhibited the most prominent changes in expression (Fig.16A, B). We have previously shown that PRINS binds to the mRNA of IL-6 and CCL-5 leading to their degradation [30]. These mRNAs were also downregulated in the qPCR-array, validating our results. The qPCR array identified another psoriasis-related transcript, IL-23 mRNA that was downregulated in the PRINS overexpressing keratinocytes. We confirmed this result by validating the array data with keratinocytes from the same donors as used for the qPCR array (Fig.16C).

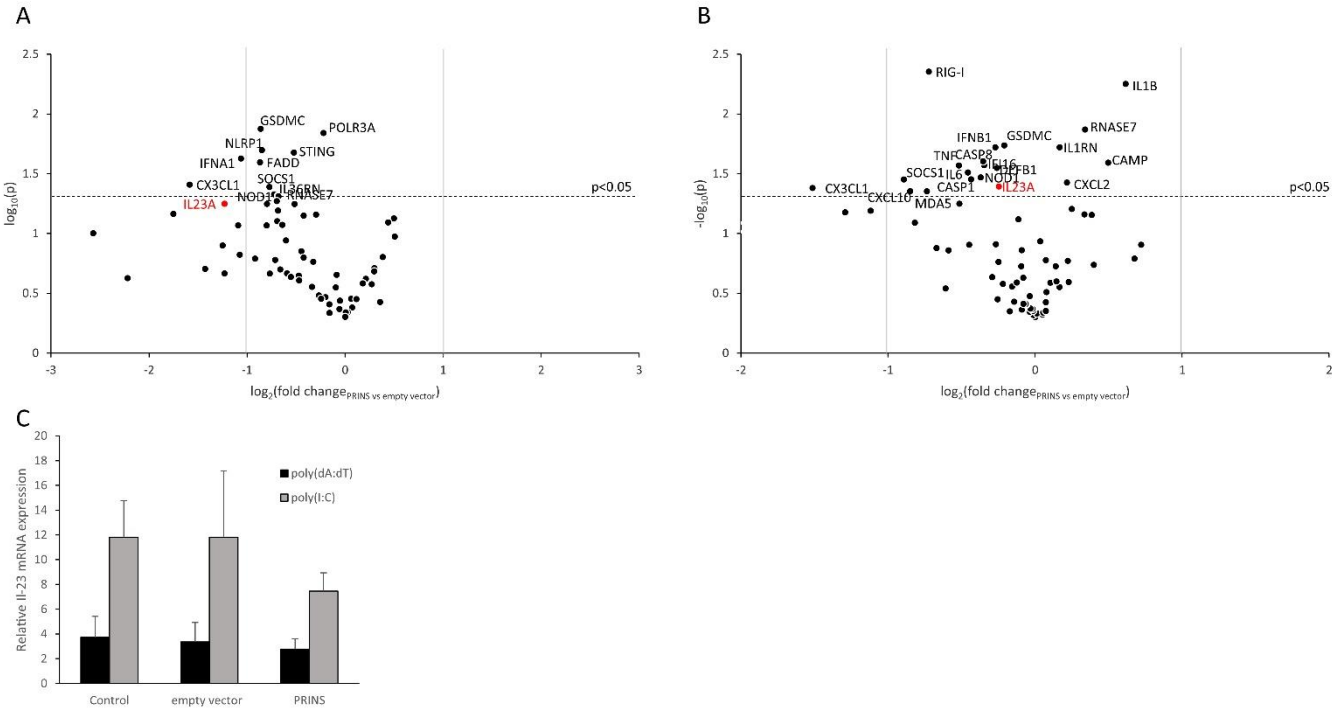

Figure 16. Gene expression changes upon the overexpression of the PRINS long non-coding RNA in NHEKs transfected with poly(I:C) (A) and poly(dA:dT) (B). RT-PCR validation of the array results regarding nucleic acid induced IL-23 mRNA expression upon PRINS overexpression in NHEKs (C). To assess multiple gene expression changes, a qPCR array of inflammatory mediators and receptors was carried out on NHEK cells transfected by a pcDNA3.1(+) vector containing the PRINS sequence or the empty vector. 24 hours later PRINS or emtpy vector carrying cells were transfected by 0.666 μg/mL poly(I:C) (A) or 1 μg/mL poly(dA:dT) (B) for 12 hours. Expression changes in PRINS overexpressing samples were compared to expression values of empty vector transfected samples. Results are presented as volcano plots, significant changes ($p<0.05$) are displayed above the horizontal dashed line. Vertical lines indicate a ±2-fold change in expression compared to empty vector transfected cells. Experiments were carried out on four independent samples derived from healthy voluteers, statistical significance was

calculated by Student's t-test with correction for multiple comparisons (A and B). Cells overexpressing PRINS were transfected with 0.666 μg/mL poly(I:C) and 1 μg/mL poly(dA:dT). Relative IL-23 mRNA expression was determined by the ΔΔCt method, normalized to GAPDH mRNA expression and compared to the expression of the untreated (Control) samples. For the RT-PCR experiment we used the same total mRNA sample set (n=4) as for the array experiment. Data are presented as mean of four independent experiments ± SD. Statistical significance was not found as assesed by one tail, paired Student's t-test (C).

To further support the role of PRINS in IL-23 mRNA regulation, we examined the effect of the silencing or overexpression of PRINS (Fig. 18) on the nucleic acid induced IL-23 mRNA expression in NHEK cells derived from additional individuals. These experiments confirmed that PRINS overexpression significantly reduced IL-23 mRNA expression after poly(dA:dT) treatment, whereas a decreasing trend was observed after poly(I:C) treatment (Fig. 17 A, C). We observed an opposite tendency when PRINS expression was decreased by silencing (Fig. 17 B, D), which suggests that PRINS might have a role in the nucleic acid induced IL-23 mRNA expression of keratinocytes. However, statistical significance was not reached in many cases, since large inter-individual differences among the samples derived from independent donors could be observed (Fig.19.).

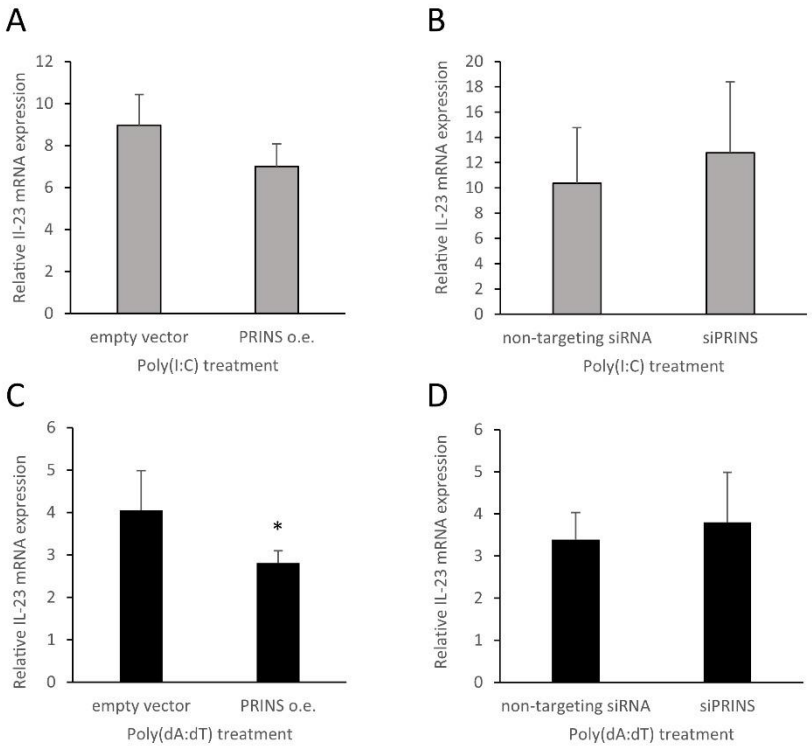

Figure 17. The effect of PRINS overexpression (A, C) and silencing (B, D) on the IL-23 mRNA expression upon poly(I:C) (A, B) and poly(dA:dT) (C, D) treatment. PRINS overexpressing (A, C) or silenced (B, D) cells were transfected with 0.666 μg/mL poly(I:C) or 1 μg/mL poly(dA:dT) Relative

IL-23 mRNA levels were determined by the ΔΔCt method, normalized to GAPDH mRNA expression and compared to mock transfected samples. Data are presented as means of eight (A) fifteen (C) and three (B,D) independent experiments ± SD. Statistical significance was assessed by one-tailed, paired Student's t-test *p<0.05.

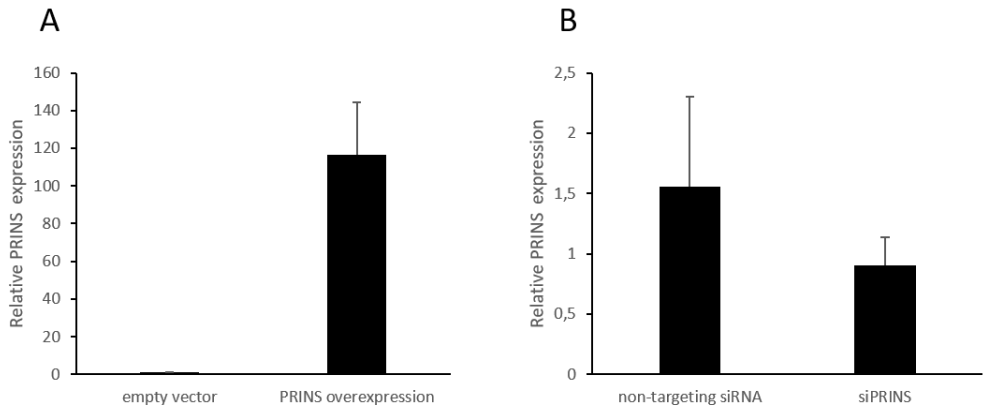

Figure 18. The effect of PRINS overexpression (A) and silencing (B) in PRINS expression. Cells were transfected by the empty vector pcDNA3.1(+) and PRINS-overexpressing construct (A), or transfected by non-targeting siRNA or PRINS-targeting siRNA. Relative PRINS RNA levels were determined by the $\Delta\Delta C_t$ method, normalized to GAPDH mRNA expression, compared to the mock transfected control samples. Data are presented as means of four independent experiments ± standard deviation. Significance was determined by one tailed, paired Student's t-test.

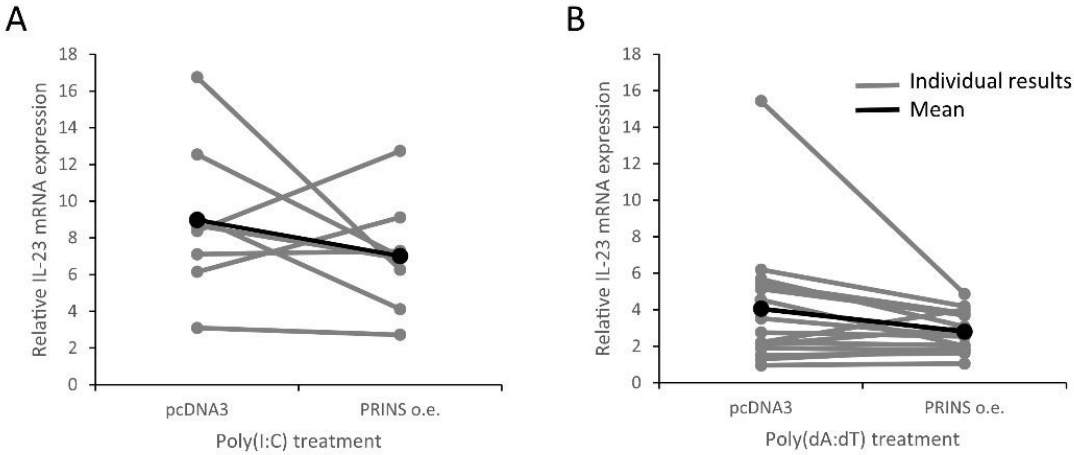

Figure 19. The effect of PRINS overexpression on poly(I:C) (A) and poly(dA:dT) (B) induced IL-23 mRNA expression in NHEKs isolated from the epidermis of 8 (A) and 15 (B) healthy volunteers. PRINS overexpressing cells were transfected with 0.666 μg/mL poly(I:C) (A) n=8 or with 1 μg/mL poly(dA:dT) (B) ; n=15. Relative IL-23 mRNA expression was determined by the ΔΔCt method, normalized to GAPDH mRNA expression and compared to mock-transfected samples. Expression values of individual samples are shown in gray, the mean values of the individual results is shown in black.

Bioinformatic analysis of IL-6 and CCL-5 predicted an mRNA-lncRNA binding site and our functional studies confirmed the physical link between these molecules [30]. Thus we attempted to determine whether a PRINS-interaction site is present in IL-23 mRNA. The predicted binding site in PRINS to the IL-23 mRNA (Fig.20.) partly overlaps the previously identified IL-6 mRNA binding sequence. Since IL-23 is an extremely polymorphic gene, we hypothesized that a reason for the large inter-individual differences observed between the donors might be due to single-nucleotide variants (SNPs) at the putative PRINS interaction site in IL-23, which could interfere with PRINS binding. Moreover, certain SNPs of the IL-23 gene are linked to psoriasis susceptibility [65–67]. Searching the NCBI (National Center for Biotechnology Information) database for polymorphisms at the putative binding site, 13 SNPs were identified on the IL-23 gene (https://www.ncbi.nlm.nih.gov/nuccore/NM_016584.3). We sequenced the putative binding site of the IL-23 gene from each donor and compared the sequences. Sequence variants of the gene could not be associated with the IL-23 mRNA expression response to PRINS overexpression. Subsequently, all exons of the IL-23 gene were sequenced for the enrolled donors, and again no association between haplotype and the differential effect of PRINS on IL-23 mRNA abundance was observed (data not shown).

To confirm the putative binding between PRINS and the IL-23 mRNA identified *in silico*, we used a luciferase-based vector carrying cloning sites at the 3' end of the luciferase gene. The putative PRINS interacting sequence of the IL-23 cDNA (Table 2.) was inserted into this vector (pmirGLO-IL23BS). In the absence of any binding partner, transfected cells exhibited luciferase activity. If a binding event were to occur between PRINS and the IL-23 mRNA sequence, the luciferase mRNA would be destabilized, and luciferase activity would not be detected. Upon co-transfection the cells with pmirGLO-IL23BS- and PRINS-expressing plasmids, we observed hardly any difference in luciferase activity in the presence or absence of the interaction site, indicating no physical interaction through this sequence between IL-23 mRNA and PRINS (Fig.21.).

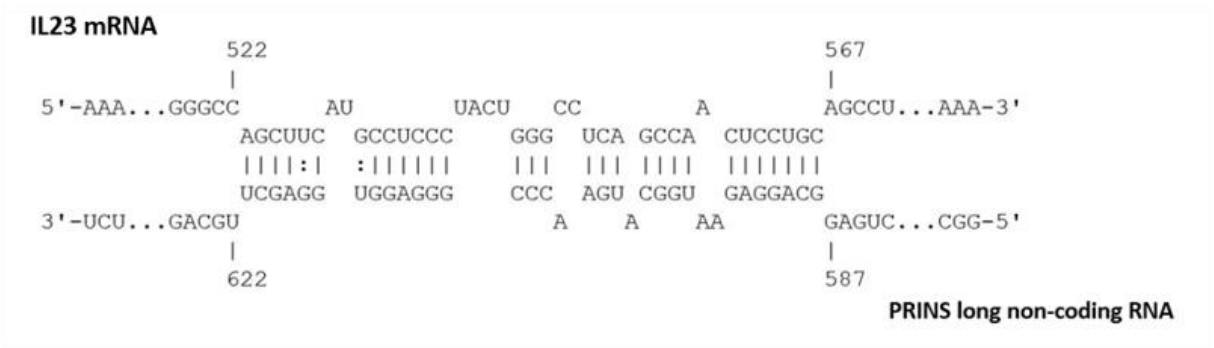

Figure 20 The putative IL-23 mRNA sequence for PRINS binding.

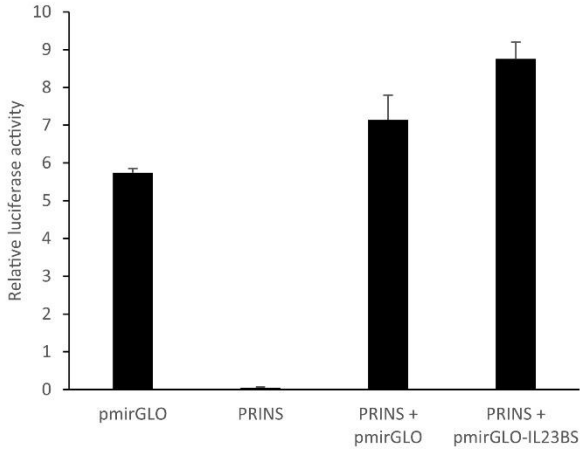

Figure 21. In vitro binding assay revealed no interaction between the putative PRINS interacting sequence on IL-23 mRNA and PRINS long non-coding RNA. Cells were transfected with PRINS pcDNA3.1 and pmirGLO plasmids as controls or co-transfected with the PRINS pcDNA3.1 plasmid in combination with pmirGLO or pmirGLO-IL23BS vectors. The pGL4.75 [hRluc/CMV] plasmid (Promega) was used as internal control. Cells were harvested after 24 h and luciferase activities in the lysates were measured using the Firefly & Renilla Dual Luciferase Assay Kit and SYNERGY/HTX Multi-Mode reader following the manufacturer's instructions. The luciferase activity derived from the pmirGLO plasmid was normalized to the activity of the Renilla luciferase activity. Results of a representative experiment are shown with 3 technical repetitions.

However, we noticed that IL-23 mRNA downregulation by PRINS overexpression was more apparent in those donor samples where initial induction of IL-23 mRNA transcription by nucleic acids was the highest (Fig. 19.), suggesting a correlation. Pearson's correlation analysis confirmed a significant, moderate negative correlation between the extent of IL-23 mRNA expression by nucleic acid induction and the rate of decrease upon PRINS overexpression (Fig. 22.). This suggest that an adequately high level of IL-23 mRNA is required for the achievement of regulatory effect of PRINS on IL-23 mRNA expression.

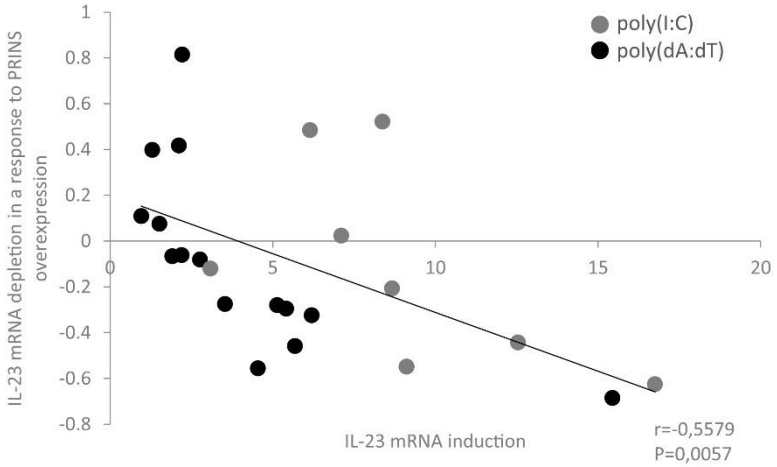

Figure 22. Analysis of the association between nucleic acid induced IL-23 mRNA abundance and the effect of PRINS overexpression on it shows a significant moderate negative correlation. IL-23 mRNA depletion was determined as the relative change of PRINS overexpressing samples compared to pcDNA3.1 transfected samples and displayed as a function of the poly(I:C) or poly(dA:dT) induced IL23 mRNA expression of each sample.

5. Discussion

Nucleic acid fragments are important PAMPs or DAMPs that induce innate immune processes of professional and non-professional immune cells [64,68]. Activation of inflammasomes in keratinocytes and dendritic cells mediates the promotion of inflammation [26], and the accumulation of RNA and DNA fragments in keratinocytes has pathogenic role in the psoriatic parakeratosis [69,70]. Several studies suggested that the Th17/IL-23 axis plays a role in disease pathogenesis [60,71,72]. Increased expression of the subunits of IL-23 and its receptor were found in psoriatic lesional skin, which suggest that this cytokine — and not IL-12 — plays a role in psoriatic inflammation [42,73,74]. However, there are no available data about the possible connection between the effect of these inflammatory agents.

In this study we aimed to investigate the mechanism of how nucleic acid challenge regulates IL-23 mRNA expression in keratinocytes and in HPV-KER cell line. In our previous experiments we found that upon exposure to *Propionibacterium acnes*, HPV-KER cell line shows similar inflammatory [75,76] and ultraviolet-B irradiation-induced responses to NHEK cells [56]. We also found that NHEK cells and the HPV-KER cell line exhibited similar cytokine mRNA expression [75], but high intra-individual differences were observed in the inflammatory inductions of NHEKs [58]. Our results showed that both poly(I:C) and poly(dA:dT) induce IL-23 mRNA expression in keratinocytes, but to a different degree and with different kinetics. The difference might originate from differences in recognition of RNA and DNA by PRRs. Specific PRRs that are capable of sensing nucleic acids in keratinocytes have already been wildly described. These receptors recognize RNA and DNA fragments from both native and pathogenic origin and induce inflammatory mechanisms. TLR3 recognizes dsRNAs from viruses as well as poly(I:C), and it is essential for the production of the IL-12p40 subunit [61]. TLR3 was also shown to induce IL-23p19 expression through interferon regulatory factor 6 (IRF6) [38]. RIG-I and IFIH1 belong to the RIG-like receptors [61]. RIG-I predominantly recognizes short dsRNA, while IFIH1 senses long dsRNA from viruses. However, it was previously shown that poly(dA:dT) is transcribed into dsRNA by RNA polymerase III before recognized by the RIG-I receptor [58,77], which, as suggested previously, might explain the kinetic differences between poly(I:C) and poly(dA:dT) treatments [58]. Recently, cGAS was described as the major PRR to recognize cytosolic DNA fragments [62,63]. To date, the involvement of these receptors in the production of IL-23 by keratinocytes has not been addressed. Our results demonstrated that, whereas poly(I:C)-mediated IL-23 mRNA expression was decreased by silencing of TLR3 in both NHEKs and HPV-KER cells, in line with previous reports [38], silencing of none of the studied receptors had an effect on

poly(dA:dT)-induced IL-23 mRNA expression in NHEKs. Nevertheless, we emphasize that the level of induction after poly(dA:dT)-treatment never approached the level induced by poly(I:C) treatment, which might explain the negative results in the silencing experiments. However in HPV-KER cells we observed higher IL-23 expression levels upon poly(dA:dT) treatment. Our recptor silencing experiments were carried out on NHEK cells isolated from four healthy donors and even in this small data set, we observed differences in the reactions of the individuals, especially in case of donors NHK49 and 51 where IFIH1 silencing resulted in a remarkable decrease in the IL-23 mRNA level (Supplementary fig. 1). These results drew our attention to the importance of differential sensitivity of individuals in responses to different agents. We could also observe differences between NHEKs and HPV-KER cells: in NHEKs only TLR3 seems to participate in nucleic acid induced IL-23 production, while in HPV-KER cells silencing several receptors caused a decrease in the level of nucleic acid induced IL-23 transcription.

The nuclear-factor (NF)-κB signaling pathway affects cell survival, proliferation and anti-apoptotic effects of lymphocytes and keratinocytes and it is known that TNF-α induces Th17 to produce pro-inflammatory cytokines through this pathway in psoriatic lesions [39]. In eukaryotic cells, three mitogen-activated protein kinase (MAPK) cascades have been identified: ERK, JNK and p38. The ERK signaling pathway plays an important role in cell proliferation and differentiation, while JNK and p38 are mainly related to the stress response and apoptosis of cells [78]. The JAK/STAT signaling pathway, also known as the IL-6 signaling pathway, is involved in many biological processes, such as cell proliferation, differentiation and apoptosis and is also closely related to many immune and inflammatory diseases [79]. It has been shown that, in human keratinocytes, poly(I:C) induces NF-κB, p38 and STAT-1 signaling [64], whereas poly(dA:dT) treatment activated NF-κB, p38 and JNK signaling in human melanocytes [80]. Recent studies show that increased levels of oxidative products activate keratinocytes, Th1 and Th17 cells through the MAPK, NF-κB and JAK-STAT pathways, resulting in the production of several cytokines which are involved in psoriatic inflammation [81–83].

According to our results inhibition of the ERK-1, JNK, NF-κB and STAT3 pathways resulted in a decrease in poly(I:C)-induced IL-23 mRNA expression of keratinocytes, suggesting that all of these pathway components play a role in the RNA-induced production of IL-23 mRNA under inflammatory circumstances. Blockage of these pathways did not result in complete decay of IL-23 expression, showing the involvement of other, related pathways including the known role of TLR3 signaling through IRF6 [38]. These suggest that poly(I:C) induced IL-23 mRNA expression in NHEKs is mainly mediated by TLR3 driven activation of several parallel pathways. Interestingly, similarly to silencing of the major nucleic acid sensing

PRRs, inhibition of these major pathways did not have an effect on poly(dA:dT)-induced IL-23 mRNA expression, suggesting the importance of alternative receptors and pathways in DNA-induced IL-23 mRNA expression that have not yet been identified. The involvement of other, less exposed receptors or pathways in poly(dA:dT)-induced IL-23 mRNA expression would also explain the observed lower expression compared to poly(I:C)-induced expression levels.

PRINS long non-coding RNA has been characterized as a contributor to the anti-inflammatory state of the epidermis by binding directly to inflammatory molecules, specifically to IL-6 and CCL-5 mRNAs [30,48]. To identify additional target transcripts of PRINS under inflammatory conditions, nucleic acid induction was applied during PRINS overexpression. PRINS overexpression led to a decrease in nucleic acid induced IL-23 mRNA expression, and a putative lncRNA-mRNA binding site was identified *in silico*. Although initially we confirmed the decrease in IL-23 mRNA expression upon PRINS overexpression, increasing the number of independent samples lead us to conclude that there were large inter-individual differences among the donors. Since a direct lncRNA–mRNA interaction could be affected by SNPs, their presence was assessed; however, sequencing the putative binding site on the IL-23 gene of the different donors revealed no correlation with the presence of sequence variants and the IL-23 mRNA response to PRINS overexpression. Interaction could also not be confirmed with an experimental approach to evaluate the direct interaction between the IL-23 mRNA putative binding site and the PRINS lncRNA molecule using a luciferase-based binding assay. In further studies, the responses of the donors seemed to be related to the level of the nucleic acid induced IL-23 mRNA expression. A correlation analysis was carried out and confirmed the possible negative correlation between the nucleic acid induced IL-23 mRNA expression levels and the decrease upon PRINS overexpression, suggesting the need for sufficiently high levels of IL-23 mRNA for PRINS regulation to be detected. However, even in cases when induction of IL-23 mRNA expression was sufficiently high, PRINS might act indirectly, rather than by direct interaction with the IL-23 mRNA.

Although IL-23 in psoriasis is predominantly thought to be released by professional immune cells, our results indicated that this cytokine might originate from keratinocytes as well. Nucleic acids activate several PRRs in these cells, leading to the upregulation of several inflammatory molecules, including IL-23. Thus, keratinocyte-derived nucleic acids in the skin might also contribute to the development of psoriasis by elevating the IL-23 levels in keratinocytes through an autocrine mode. Taken together, our data suggest that individual differences in sensitivity towards the levels of nucleic acids in the tissue might lead to different IL-23 levels. These differences might in turn be related to the different reactions towards biological agents used in

psoriasis treatment. Detailed analysis of these processes will help us to identify new biomarkers for the development of personalized treatments for the disease.

It has long been debated by the scientific community of psoriasis research, whether altered immune cells play an exlusive major role in the development of psoriasis, with keratinocytes acting as subordinate passive contributors [13,84]. However, several publications [85,86] and our own results [15,58,87] support the idea that keratinocytes are also active participants in these processes, as they not only play a role in the innate immune response, but also modulate the course of the acquired immune response. Moreover, in our recent review we [88] also suggested that we may consider the psoriatic non-lesional epidermis as a battlefield of constant fight between susceptibility and protective molecular factors in which keratinocyte innate immune processes also play a key role.

6. Summary, novel findings of the experimental work

In this study we characterized the dsRNA and dsDNA induced IL-23 expression in human epidermal keratinocytes and HPV-KER cells by studying the transcriptional regulation of this cytokine.

- We compared the dsRNA and dsDNA induced IL-23 expression in human epidermal keratinocytes. We found that transient transfection by the synthetic dsRNA and dsDNA analogue poly(I:C) and poly(dA:dT) induced IL-23 transcription in keratinocytes, but to different degree and with different kinetics.
- Silencing of the pattern recognition receptors showed that TLR3 is the main receptor mediating the poly(I:C) specific regulation of IL-23 mRNA expression levels in both NHEKs and HPV-KER cells, however in HPV-KERs other receptors also contribute to this process.
- We showed that the inhibition of the ERK-1, JNK, NF-kB and STAT3 pathways resulted in a decrease in the nucleic acid induced induction of IL-23 mRNA expression in keratinocytes.
- Our results indicate that free nucleic acids contribute to the development of psoriasis by elevating the IL-23 levels through specific receptors and signaling pathways in keratinocytes.
- According to our qPCR array nuclecic acid induced IL-23 expression can be changed by elevated PRINS levels. We confirmed this results by increasing the number of donors and by increasing or decreasing the levels of PRINS in keratinocytes.
- Since we observed very big differences in the samples derived from different individuals, we sequenced the putative bindig site of the donors and found no correlation with the appearance of the variants and the IL-23 response to PRINS overexpression. We also could not detect interaction between the IL-23 putative binding site and the PRINS lncRNA molecule in our luciferase based assay.

Taken together, our data suggest that individual differences in sensitivity towards the levels of nucleic acids in the tissue might lead to different IL-23 levels. These differences might in turn be related to the different reactions towards biological agents used in psoriasis treatment. Detailed analysis of these processes will help us to identify new biomarkers for the development of personalized treatments for the disease.

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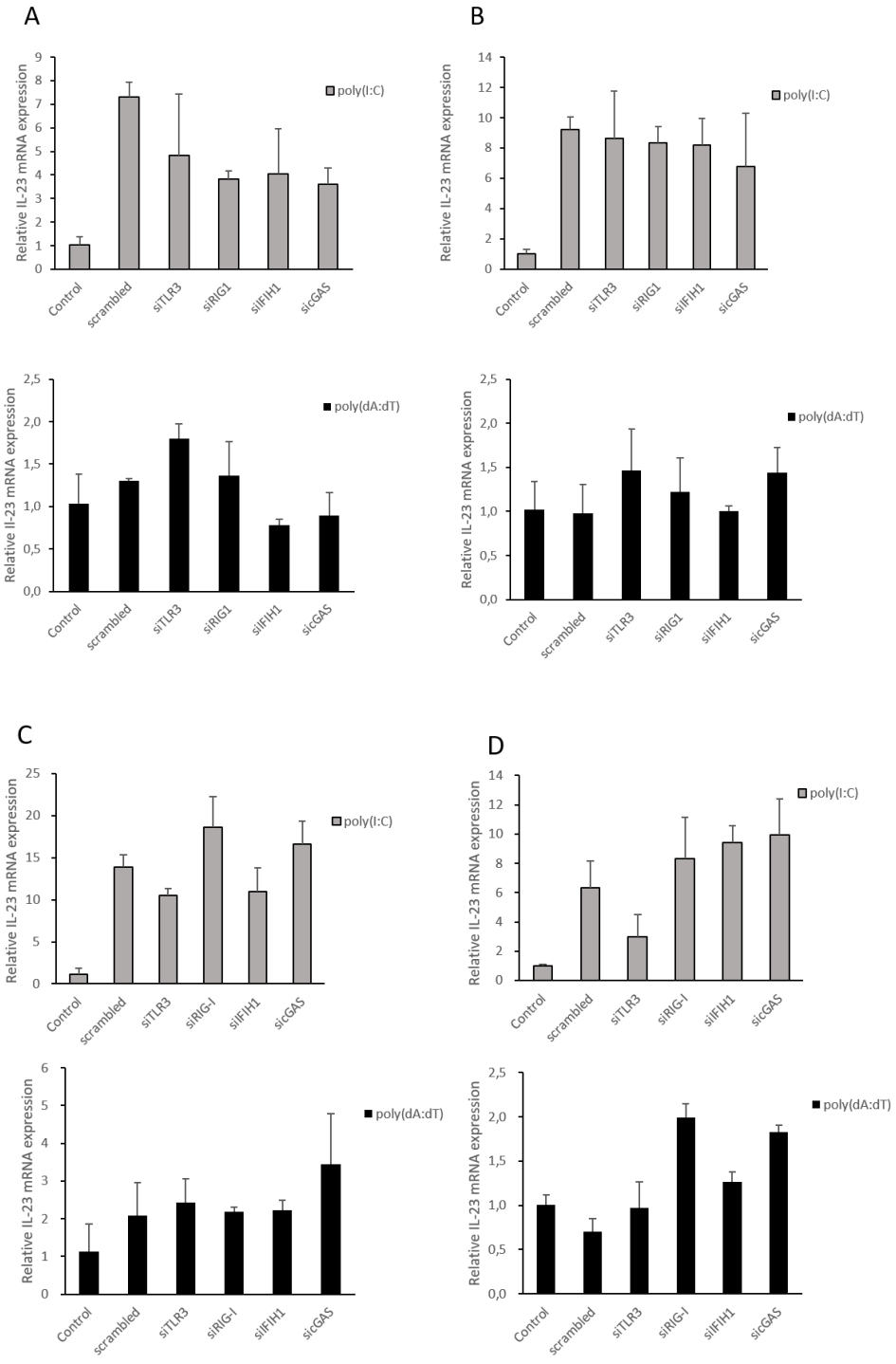
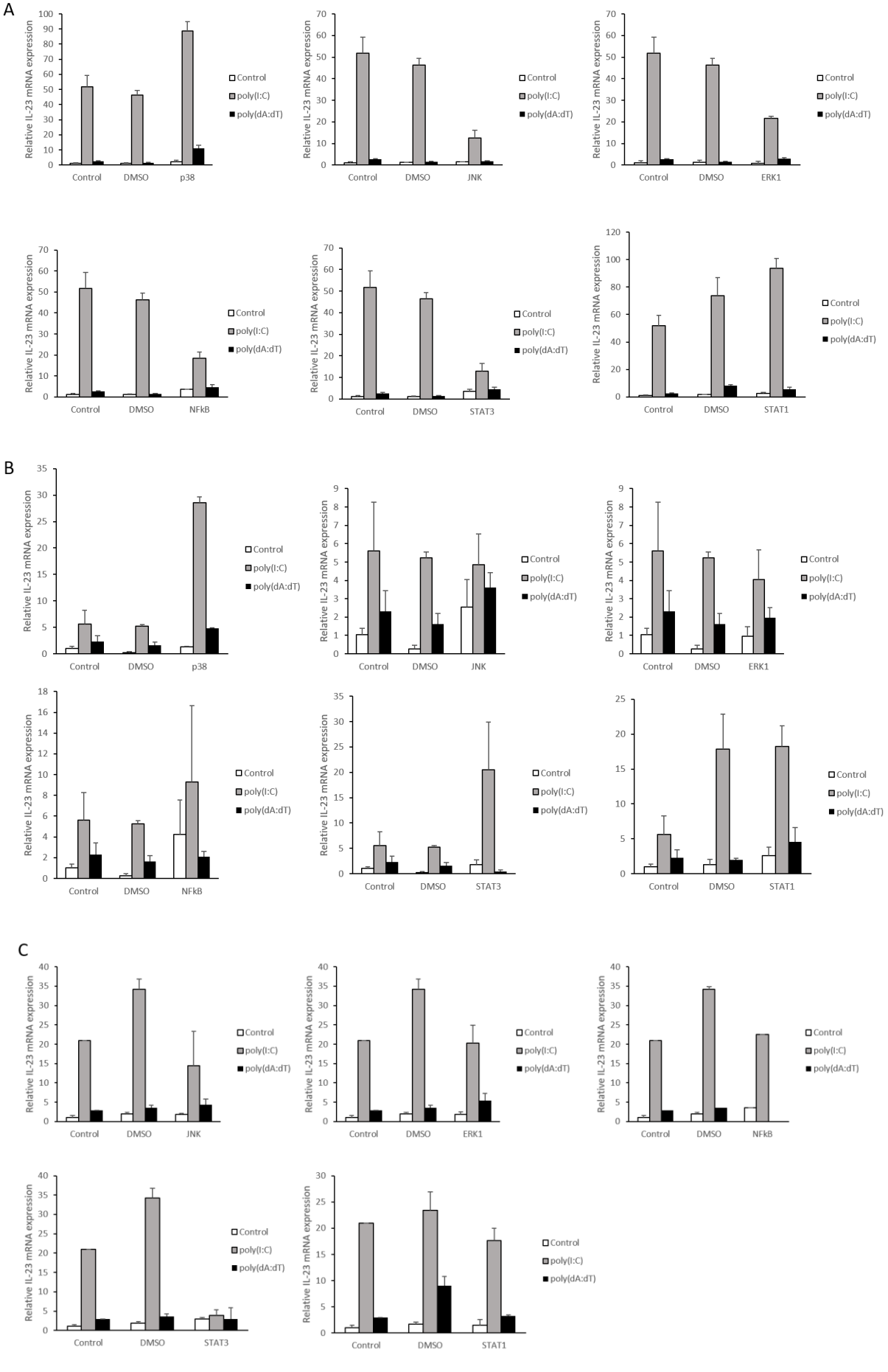
9. Supplementary figures



Supplementary figure 1 A, B, C, D The effect of the silencing of the pattern recognition receptors on the nucleic acid induced IL-23 mRNA expression in NHEKs isolated from four independent healthy volunteers Pattern recognition receptors were silenced with siRNA mediated inhibiton in for 24 hours and the cells were subseqeuently transfected with 0.666 μg/mL poly(I:C) and 1 μg/mL poly(dA:dT). Experiments on cells derived from each donor were carried out in triplicates. Relative IL-23 expression was determined by the $\Delta\Delta C_t$ method, normalized to GAPDH mRNA expression and compared to the expression of the untreated control samples. Data are presented as the average of triplicates.


A
B
C
Relative IL-23 mRNA expression
Control
poly(I:C)
poly(dA:dT)
Control
DMSO
p38
JNK
ERK1
NFkB
STAT3
STAT1

Supplementary Figure 2 A, B, C Inhibition of the signaling pathway components has an effect of the nucleic acid induced IL-23 expression of NHEKs isolated from three independent healthy volunteers. Isolates NHEK53 (A), NHEK59 (B), NHEK61 (C) Specific inhibiton of signaling pathway components were administered for 1 hours and subseqeuntly cells were transfected with 0.666 μg/mL poly(I:C) and 1 μg/mL poly(dA:dT) for 24 hours. Relative expression was determined by the $\Delta\Delta C_t$ method, normalized to GAPDH mRNA expression and compared to the expression of the control, untreated samples. Experiments on cells derived from each donor were carried out in triplicates. Data are presented as the average of triplicates ± standard deviation.

I.

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Article

Psoriasis-Associated Inflammatory Conditions Induce IL-23 mRNA Expression in Normal Human Epidermal Keratinocytes

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Abstract: Psoriasis is a multifactorial, chronic inflammatory skin disease, the development of which is affected by both genetic and environmental factors. Cytosolic nucleic acid fragments, recognized as pathogen- and danger-associated molecular patterns, are highly abundant in psoriatic skin. It is known that psoriatic skin exhibits increased levels of IL-23 compared to healthy skin. However, the relationship between free nucleic acid levels and IL-23 expression has not been clarified yet. To examine a molecular mechanism by which nucleic acids potentially modulate IL-23 levels, an in vitro system was developed to investigate the IL-23 mRNA expression of normal human epidermal keratinocytes under psoriasis-like circumstances. This system was established using synthetic nucleic acid analogues (poly(dA:dT) and poly(I:C)). Signaling pathways, receptor involvement and the effect of PRINS, a long non-coding RNA previously identified and characterized by our research group, were analyzed to better understand the regulation of IL-23 in keratinocytes. Our results indicate that free nucleic acids regulate epithelial IL-23 mRNA expression through the TLR3 receptor and specific signaling pathways, thereby, contributing to the development of an inflammatory milieu favorable for the appearance of psoriatic symptoms. A moderate negative correlation was confirmed between the nucleic-acid-induced IL-23 mRNA level and the rate of its decrease upon PRINS overexpression.

Keywords: psoriasis; nucleic acid analogues; interleukin-23; qPCR array

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1. Introduction

Psoriasis is a multifactorial, chronic inflammatory skin disease affecting 2% of the population and has been known since ancient times. Both congenital predisposition and environmental factors play a role in its development. Raised, well-demarcated, erythematous oval plaques with silvery scales are the prominent signs of the most common form, the plaque-type psoriasis. This symptom is caused by the abnormal proliferation and differentiation of basal keratinocytes and their dysregulated interplay with professional immune cells. The plaques mostly occur on elbows, knees and the scalp, but they can also affect any other part of the body [1,2]. Plaques are surrounded by clinically healthy-looking skin, which is referred to as non-lesional or uninvolved skin. Despite the fact that the uninvolved skin of psoriasis patients appears macroscopically identical to normal skin, it contains molecular, cellular and extracellular alterations and, in several aspects, has a pre-psoriatic phenotype [3–7].

At different stages of the disease, a variety of innate and adaptive immune cells and proinflammatory mediators are involved. The aberrant immune and epidermal response

seen in psoriasis is maintained by pathogenic crosstalk between epithelial and immune cells, and it is primarily driven by proinflammatory molecules, such as TNF-α, IL-23 and IL-17. In recent years, therapeutic targeting of these mediators has been proven to be clinically effective [8,9].

After physical trauma or infection, keratinocytes release LL-37, which is a cationic antimicrobial peptide that binds DNA and RNA fragments released by damaged skin cells. LL-37 and self-derived nucleic acids form a complex found in psoriatic lesions, and this complex activates TLR7/9-bearing plasmacytoid dendritic cells, which are normally absent from healthy skin [10–12]. These cells release proinflammatory cytokines (IL-1β, IL-6 and TNF) and interferons (IFNs) and through these have an important role in the development of the disease also by activating keratinocytes and myeloid dendritic cells. The number of myeloid dermal dendritic cells is elevated in psoriatic skin [13], and the mature cells migrate to skin-draining lymph nodes to present antigen to naive T-cells. T-cells are also critical for the initiation phase of the disease as their interaction with activated dermal dendritic cells is central to the development of plaque formation and the resulting creation of an IL-23/IL-17 inflammatory environment. In this environment, IL-23 derived from dendritic cells and macrophages promotes the effector functions of T helper 17 (Th17) and cytotoxic cells [1]. Taken together, these data indicate that cytosolic nucleic acid fragments, which are recognized as pathogen- and danger-associated molecular patterns (PAMPs and DAMPS), are highly abundant in the psoriatic skin and their presence can lead to the chronic activation of professional immune cells. These professional immune cells migrate into the epidermis, and their mediators stimulate excessive proliferation and abnormal differentiation of epithelial cells. This, in turn, leads to a thickening of the epidermis and the formation of inflamed plaques [14].

The role of nucleic acid fragments in psoriasis is supported by the elevated levels of cell-free DNA found in the blood of psoriasis patients, but the exact source of the nucleic acids associated with the initial inflammatory events is not yet known [15]. Reduced deoxyribonuclease activity in keratinocytes and disturbed ribonuclease activity in psoriatic skin have been observed [16,17]. Increased serum mtDNA levels originating from mitochondrial dysregulation can act as a DAMP [18,19]. In psoriatic skin, neutrophils and neutrophil extracellular traps (NETs) have also been reported as sources of nucleic acids. RNA associated with NETs might be bound by LL37, and these activate inflammatory reactions that could possibly cause a self-exciting cycle contributing to chronic inflammation in psoriasis [20]. Interferon-γ (IFN-γ), produced by Th1 and Th17 cells can also contribute to the recognition of nucleic acids. The expression of IFN-γ is elevated in psoriatic lesional skin and in the serum of the patients [21], thereby, this cytokine is able to prime nucleic-acid-induced inflammatory responses in keratinocytes and drive IL-23 expression [22].

A number of results suggested that the Th17/interleukin-23 axis plays a dominant role in the disease, as it promotes chronic inflammation. IL-23 is responsible for the development of Th17 cells, leading to a production of IL-17 and IL-22 cytokines, which are involved in the pathogenesis of psoriasis. Keratinocytes and activated antigen-presenting cells (Langerhans cells, macrophages and dendritic cells) all produce IL-23, the expression of which is elevated in psoriatic lesional skin, leading to an increased number of Th17 cells [23,24]. Recently, IL-23 targeting therapies for psoriasis have become widely used. IL-23 is a heterodimeric cytokine consisting of a unique p19 and a p40 subunit, the latter is shared with IL-12. Increased levels of these two subunits and the IL-23 receptor were found in psoriatic skin of patients, suggesting that IL-23, rather than IL-12, plays a role in disease pathogenesis [25]. Ustekinumab, a monoclonal antibody against the common p40 subunit is a highly effective treatment for psoriasis, and the antibodies targeting the p19 subunit (guselkumab, tildrakizumab and risankizumab) also show promising impact for symptom amelioration [1,26].

Although the literature reports high levels of IL-23 and free nucleic acids in psoriatic skin, it is not known yet whether there is an association between these participants in the development of the disease or whether they act independently.

To address this question, an in vitro system was developed to investigate IL-23 production of normal human epidermal keratinocytes (NHEKs) under psoriasis-like circumstances established with the introduction of the synthetic nucleic acid analogues poly(dA:dT) and poly(I:C). Signaling pathways, receptor involvement and the effect of the long non-coding RNA PRINS were analyzed to examine the regulation of this cytokine in keratinocytes.

At the beginning of the 2000s, our research group identified a long non-coding RNA, referred to as psoriasis-susceptibility-related RNA gene induced by stress (PRINS), which exhibited higher levels in the uninvolved skin of psoriatic patients than in lesional or healthy epidermis. PRINS has a protective role in cells exposed to stress [5]. It has also been shown that PRINS binds directly to the mRNAs of IL-6 and CCL-5 (RANTES) at specific binding sites and eventually destabilizes these mRNAs, leading to a decrease in their accumulation. Based on this, we conclude that PRINS has a restrictive role in inflammatory processes [5,14,27]. Our preliminary in silico data also suggested that PRINS contains an IL-23 mRNA binding site. However, there are currently no data in the literature about the possible connection between the high IL-23 level in psoriatic skin and the protective role of PRINS in inflammatory conditions.

Our results indicate that free nucleic acids regulate epithelial IL-23 production through the TLR3 receptor and specific signaling pathways, thereby, contributing to the development of the inflammatory milieu favorable for the appearance of psoriatic symptoms. Additionally, we demonstrated that PRINS, which was previously identified in the uninvolved epidermis of psoriatic patients as a protective factor, contributes to these processes with a high interindividual variability.

2. Results

2.1. Free Nucleic Acid Analogues Induce Increased IL-23 mRNA Expression in Human Keratinocytes

2.1.1. Psoriasis-Relevant Gene Expression Pattern Is Modulated by Free Nucleic Acid Analogues

To identify genes responding with altered expression in NHEKs to treatment with the synthetic nucleic acid analogues poly(I:C) and poly(dA:dT), a qPCR array containing 84 psoriasis-relevant genes (see list of genes as Supplementary Table S1), including cytokines, chemokines, antimicrobial peptides and cytoplasmic receptors, was performed. In this experiment, we observed that 14 genes were not expressed by the cells, whereas the expression of 15 additional genes, mostly antimicrobial peptides, was not altered after treatment with either poly(I:C) or poly(dA:dT). We identified 37 genes that were induced by both treatments, including cytokines, chemokines and pattern recognition receptors (PRRs). Increased expression only in response to poly(I:C) treatment was observed for seven additional genes (Figure 1A,B).

Most importantly, we observed that, in a manner similar to other inflammatory cytokines, such as IL-6 and TNF-α (results previously published in [28], and Supplementary Figure S1B) IL-23 expression increased in response to both poly(dA:dT) and poly(I:C) treatment. Since IL-23 is known to play an important role in psoriasis pathogenesis, we designed further experiments to examine the molecular mechanism of free nucleic acid–induced IL-23 transcription in human keratinocytes.

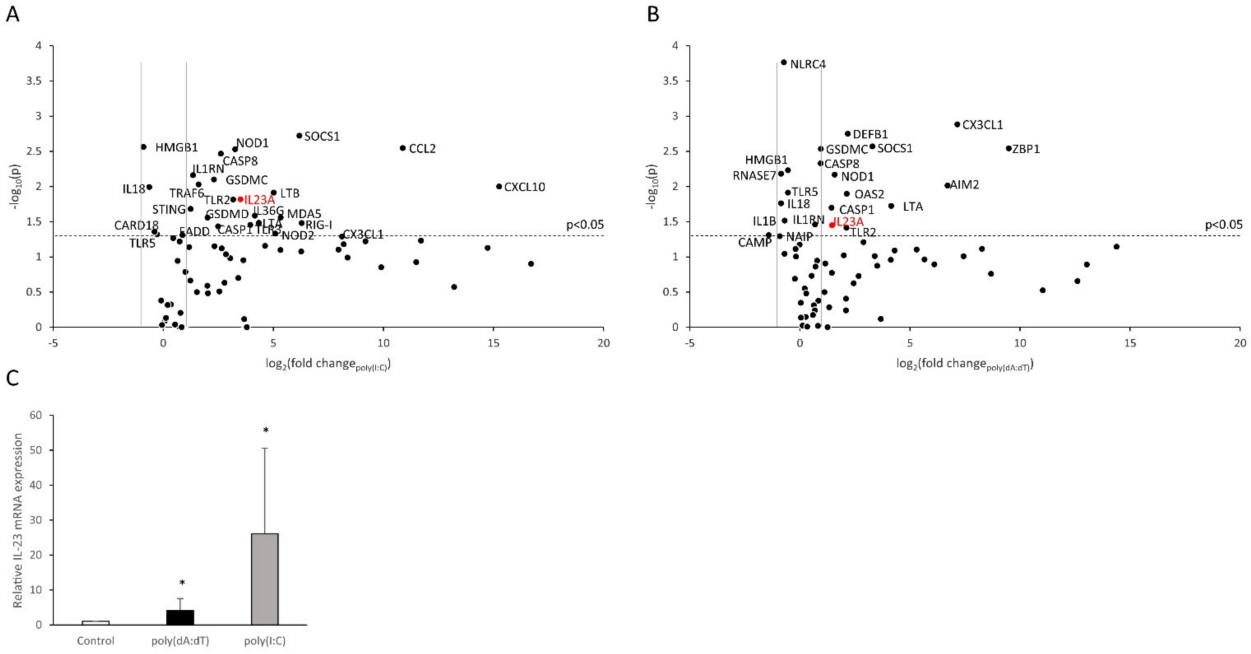

Figure 1. Changes in keratinocyte gene expression induced by poly(I:C) (**A**) and poly(dA:dT) (**B**). RT-PCR validation on independent samples revealed that NHEKs respond to poly(I:C) and poly(dA:dT) with increased IL-23 mRNA expression (**C**). To assess multiple gene expression changes upon inflammatory stimuli, a qPCR array of inflammatory mediators and receptors was carried out on normal human epidermal keratinocyte samples transfected by 0.666 μg/mL poly(I:C) (**A**) or 1 μg/mL poly(dA:dT) for 12 h (**B**) and expression changes were compared to mock-transfected samples. Results are presented as volcano plots, significant changes ($p < 0.05$) are displayed above the horizontal dashed line. Vertical lines indicate a ±2-fold change in expression compared to mocktransfected control samples. Four independent experiments were carried out, statistical significance was calculated by Student's *t*-test with correction for multiple comparisons (**A**,**B**). For validation, NHEKs were transfected with 0.666 μg/mL poly(I:C) and 1 μg/mL poly(dA:dT), samples were collected 12 h after treatments. Relative IL-23 mRNA expression was determined by the ΔΔCt method, normalized to GAPDH mRNA expression and compared to the expression of the time-matched mock-treated (Control) samples. Data are presented as mean of six independent experiments ± SD. Significance was determined by one-tailed, paired Student's-test with correction, * $p < 0.05$ compared to control (**C**).

In the qPCR array, mRNA samples derived from four healthy donors were used, and the first validation experiments were carried out on the same mRNA set. These results confirmed that poly(I:C) induced significant increase in IL-23 mRNA, whereas poly(dA:dT) induced an upward trend in expression (Supplementary Figure S1A,B). To expand the validation of the array, we included additional independent donors ($n = 6$) and observed significantly higher IL-23 expression after both treatments; however, the treatment with poly(I:C) had a more pronounced effect in this experiment as well (Figure 1C).

2.1.2. Poly(I:C) and Poly(dA:dT) Induce IL-23 mRNA Expression with Different Kinetics

Previously, we identified kinetic differences in poly(I:C)- and poly(dA:dT)-induced inflammatory responses in keratinocytes [28]. To clarify this in the case of IL-23, we measured IL-23 mRNA levels at different time points after nucleic acid treatments. Compared to the previously investigated inflammatory mediators, such as IL-6 or TNF-α, which peak at 6 to 12 h after poly(I:C) or (dA:dT) treatment [28] (Supplementary Figure S2), peak expression of IL-23 was observed later, at 24 h after poly(I:C) transfection, while a slowly rising tendency was detected after poly(dA:dT) treatment and the level was lower than that observed after poly(I:C) treatment (Figure 2).

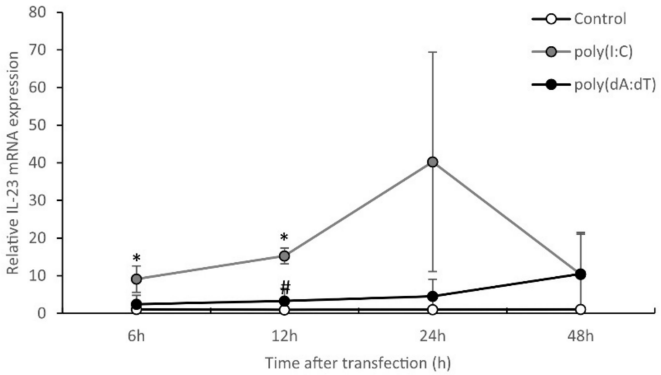

Figure 2. Kinetics of the IL-23 mRNA expression in NHEKs upon nucleic acid induction. Cells were transfected with 0.666 μg/mL poly(I:C) and 1 μg/mL poly(dA:dT), and samples were collected at 6, 12, 24 and 48 h after transfection. Relative IL-23 mRNA expression was determined by the ΔΔCt method, normalized to GAPDH mRNA expression and compared to the expression of the untreated (Control) 0 h samples. Data are presented as means of three independent experiments ± SD. Significance was determined by one-tailed, paired Student's *t*-test, * $p < 0.05$ poly(I:C)-treated vs. time-matched control samples, # $p < 0.05$ poly(dA:dT)-treated vs. time-matched control samples.

2.1.3. Psoriasis-Specific Stimuli Induce IL-23 mRNA Expression

In psoriatic skin, keratinocytes are exposed not only to free nucleic acids but to numerous cytokines, which shape the inflammatory response of the keratinocytes. To compare the effects of psoriasis-specific-cytokines with the effects of nucleic acids on IL-23 mRNA expression in keratinocytes, we used psoriasis-relevant cytokines, such as IL-17A, IL-12, TNF-α and IL-23, and synthetic nucleic acid analogues imiquimod (IMQ), poly(dA:dT) and poly(I:C), since these molecules have long been known to have a role in psoriasis pathogenesis. The highest IL-23 expression was induced by poly(I:C), whereas poly(dA:dT) and TNF-α treatment also elevated IL-23 transcription but to a lesser extent (Figure 3). Other psoriasis-relevant cytokines and IMQ, which is used to induce psoriasis in mouse models [29], did not affect IL-23 mRNA expression in keratinocytes.

Interestingly, although IL-23 is thought to originate from professional immune cells [30], in our experiments THP-1 macrophages and Jurkat T-cells failed to exceed the level of IL-23 mRNA production of keratinocytes in response to poly(I:C), poly(dA:dT) and TNFα (data not shown). These results indicate that synthetic nucleic acids modeling free cellular nucleic acids—especially poly(I:C)—play an important role in the psoriasis-associated inflammatory processes by inducing elevated IL-23 levels in human keratinocytes.

2.2. Free Nucleic Acids Act through Specific Receptors and Signaling Pathways to Modulate IL-23 mRNA Expression Levels

2.2.1. TLR3 Is the Main Nucleic Acid Sensing Receptor Conveying IL-23 mRNA Expression

In psoriasis, nucleic acid fragments originating from tissue or pathogens are present and recognized as pathogenic factors for disease development [15,17,19]. Several PRRs that recognize these RNA and DNA fragments and induce inflammatory mechanisms have already been identified [31–33], and many of these are expressed in both keratinocytes and professional immune cells. We aimed to examine which of these receptors plays an important role in mediating nucleic-acid-induced IL-23 mRNA expression in keratinocytes. To this end, siRNA-mediated silencing was carried out to decrease the level of TLR-3, RIG-I, IFIH1(MDA-5) and cyclic GMP-AMP synthase (cGAS) mRNA (Supplementary Figure S3). Our results showed that poly(I:C)-induced IL-23 mRNA expression is mediated primarily by TLR3. The silencing of the other receptors in our experiments did not affect the influence of poly(dA:dT) on IL-23 transcription (Figure 4 and Supplementary Figure S4A–D).

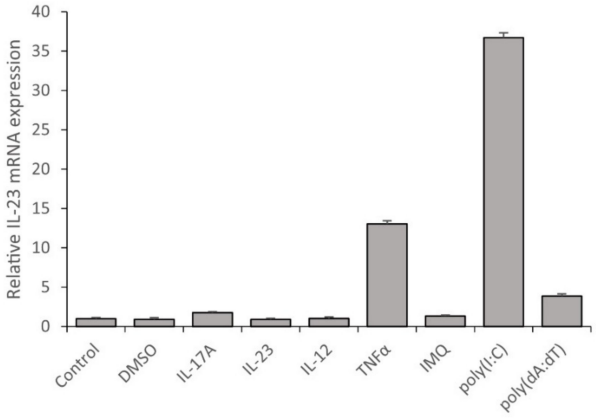

Figure 3. Synthetic nucleic acid analogues and TNFα induce IL-23 mRNA expression in NHEKs. Cells were transfected with 0.666 µg/mL poly(I:C), 1 µg/mL poly(dA:dT), 2 µL DMSO, 1 µg/mL IL-17A, 1 µg/mL IL-23, 1 µg/mL IL-12, 0.5 µg/mL TNFα and 1.33 µg/mL IMQ. Relative IL-23 mRNA expression was determined by the ΔΔCt method, normalized to GAPDH mRNA expression and compared to the expression of the untreated control samples. Results of one representative experiment are shown, data are presented as the average of three technical repetitions ± SD.

2.2.2. Multiple Pathways Transmit Free Nucleic Acid Signals to Mediate IL-23 mRNA Expression

Our next aim was to identify the downstream signaling pathways through which elevated nucleic acid levels lead to increased IL-23 mRNA synthesis in keratinocytes. In our studies, we examined six pathways that were previously shown to be affected by nucleic acids [34]. The activity of a major component of each of these pathways was decreased by applying specific inhibitors, such as Bay 11-7085 for NF-κB, PD95089 for dual-specificity mitogen-activated protein kinase kinase1 and 2, SB203580 for p38, SP600125 for JNK, fludarabine for STAT-1 and Stattic for STAT-3. Keratinocytes were preincubated with the inhibitor for an hour before transfection with poly(I:C) or poly(dA:dT). Our results showed that poly(I:C)-induced IL-23 mRNA expression is mediated by JNK, ERK1/2, NF-κB and STAT3 pathways, downstream of TLR3 receptor (Figure 5 and Supplementary Figure S5A–C). However, the inhibition of either of these pathways had no effect on poly(dA:dT)-induced IL-23 expression, which might also be due to the low IL-23 induction triggered by poly(dA:dT).

2.3. The Effect of the PRINS Long Non-Coding RNA Shows a Great Interindividual Difference on Nucleic Acid Analogue–Induced IL-23 mRNA Expression in Human Keratinocytes

Connection between PRINS and psoriasis has been described in several earlier studies [5,7,27,35]; for example, through the regulation of certain inflammatory mediators (IL-6 and CCL5) by PRINS in nucleic-acid-induced inflammatory reactions. To identify additional targets for PRINS-mediated regulation in NHEKs, a qPCR array was used to identify genes showing altered expression as a consequence of nucleic acid challenge and PRINS overexpression (see list of genes included in the qPCR-array as Supplementary Table S1). A PRINS overexpressing construct was transiently transfected into NHEK cells, and the cells were subsequently treated with poly(I:C) or poly(dA:dT) to identify how PRINS overexpression alters the nucleic-acid-induced gene expression in keratinocytes. In the qPCR-array, impaired upregulation of inflammatory genes, including cytokines, chemokines, receptors and effector molecules, was observed. Among others, NLRP1, GSDMC, CX3CL1 and SOCS1 exhibited the most prominent changes in expression (Figure 6A,B). We have previously shown that PRINS binds to the mRNA of IL-6 and CCL-5, leading to their degradation [14]. These mRNAs were also downregulated in the qPCR array, validating our results. The qPCR array identified another psoriasis-related transcript, IL-23 mRNA, that was downregulated in the PRINS-overexpressing keratinocytes. We confirmed this

result by validating the array data with keratinocytes from the same donors as used for the qPCR array (Figure 6C).

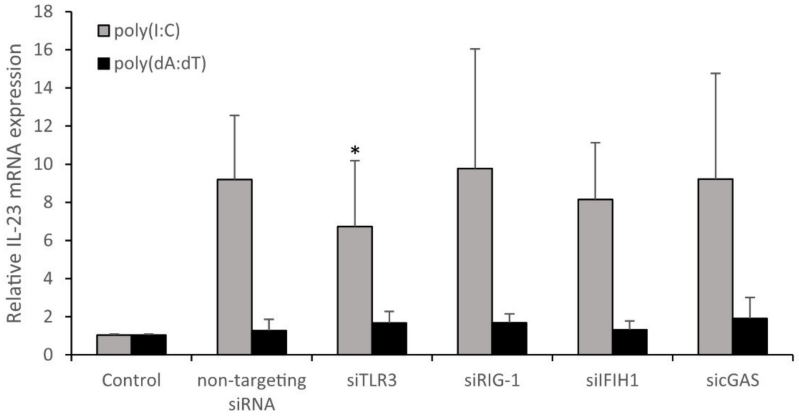

Figure 4. Silencing of the TLR3 pattern recognition receptor affects poly(I:C)-induced IL-23 mRNA expression of NHEKs. Expression of pattern recognition receptors were silenced with siRNA-mediated inhibiton for 24 h, and subsequently, the cells were transfected with 0.666 μg/mL poly(I:C) and 1 μg/mL poly(dA:dT). Relative IL-23 mRNA expression was determined by the ΔΔCt method, normalized to GAPDH mRNA expression and compared to the expression of the non-treated control samples. Data are presented as means of four independent experiments ± SD. Significance was determined between siRNA-transfected samples and the non-targeting siRNA-transfected samples by the one-tailed, paired Student's *t*-test with correction for multiple comparisons * $p < 0.05$.

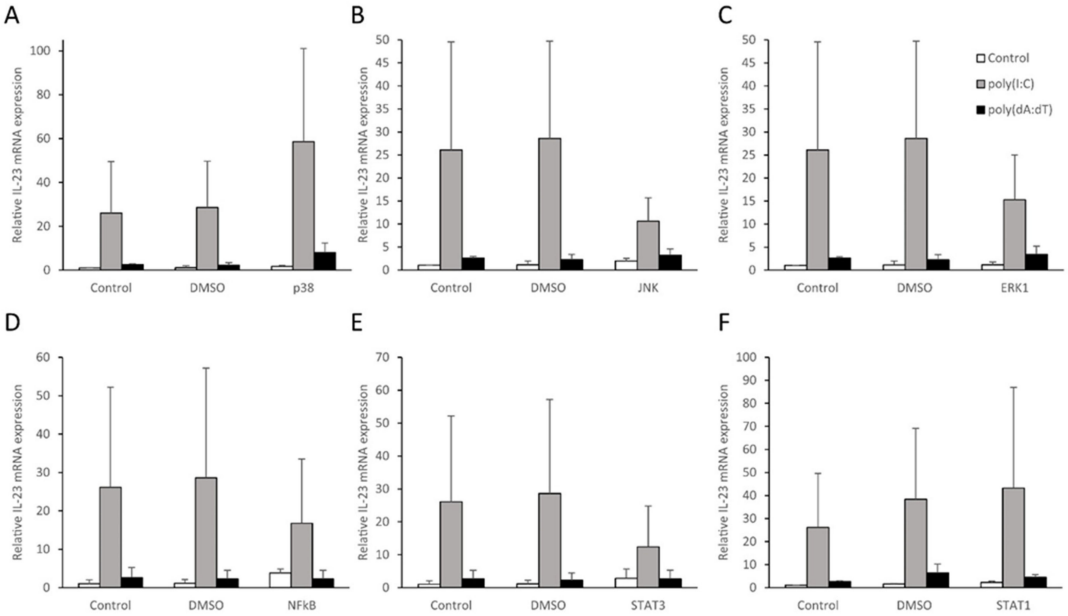

Figure 5. Inhibition of the JNK, ERK1, NF-κB and STAT3 pathway components had an effect on the nucleic-acid-induced IL-23 mRNA expression of NHEK cells (**A**–**F**). Specific inhibiton of signaling pathway components was used for 1 h with the cells being subsequently transfected with 0.666 μg/mL poly(I:C) and 1 μg/mL poly(dA:dT) for 24 h. Equal concentration of DMSO was used as solvent control for each inhibitor, demonstrating no difference from non-pretreated control samples. Relative expression was determined by the ΔΔCt method, normalized to GAPDH mRNA expression and compared to the expression of the untreated control samples. Data are presented as mean of three independent experiments ± SD. Significance was tested by one-tailed, paired *T*-test comparing to DMSO-treated solvent-control samples. In spite of obvious differencies in the values, no significance could be demonstrated among them due to high standard deviations of the independent experiments performed on NHEKs derived from independent volunteers.

To further support the role of PRINS in IL-23 mRNA regulation, we examined the effect of the silencing or overexpression of PRINS on the nucleic-acid-induced IL-23 mRNA expression in NHEK cells derived from additional individuals. These experiments confirmed that PRINS overexpression significantly reduced IL-23 mRNA expression after poly(dA:dT) treatment, whereas a decreasing trend was observed after poly(I:C) treatment (Figure 7A,C). We observed an opposite tendency when PRINS expression was decreased by silencing (Figure 7B,D and Supplementary Figure S6A,B), which suggests that PRINS might have a role in the nucleic-acid-induced IL-23 expression of keratinocytes. However, statistical significance was not reached in many cases, since large interindividual differences between the samples derived from independent donors can be observed (Figure 8).

Bioinformatic analysis of IL-6 and CCL-5 predicted an mRNA–lncRNA binding site [14]. Therefore, we attempted to determine whether a PRINS interaction site is present in IL-23 mRNA. The predicted binding site in PRINS to the IL-23 mRNA partly overlaps the previously identified IL-6 mRNA binding sequence (Supplementary Figure S4). Since IL-23 is an extremely polymorphic gene, we hypothesized that a reason for the large interindividual differences observed between the donors might be due to single-nucleotide variants (SNPs) at the putative PRINS interaction site in IL-23, which could interfere with PRINS binding. Moreover, SNPs of the *IL-23* gene are linked to psoriasis susceptibility [36–38]. Searching the NCBI (National Center for Biotechnology Information) database for polymorphisms at the putative binding site, 13 SNPs were identified on the *IL-23* gene (https://www.ncbi.nlm.nih.gov/nuccore/NM_016584.3 accessed on 1 September 2018). We sequenced the putative binding site of the *IL-23* gene from each donor and compared the sequences. Sequence variants did not correlate with the IL-23 response to PRINS overexpression. Subsequently, all exons of the *IL-23* gene were sequenced for the enrolled donors, and again no association between haplotype and the differential effect of PRINS on IL-23 mRNA abundance was observed (data not shown).

To confirm the putative binding between PRINS and the IL-23 mRNA identified in silico, we used a luciferase-based vector carrying cloning sites at the 3′ end of the luciferase gene. The putative PRINS-interacting sequence of the IL-23 cDNA (Supplementary Figure S7) was inserted into this vector (pmirGLO-IL23BS). In the absence of any binding partner, transfected cells exhibit luciferase activity. If a binding event were to occur between PRINS and the IL-23 mRNA sequence, the luciferase mRNA would be destabilized, and luciferase activity would not be detected. Upon co-transfection of the cells with pmirGLO-IL23BS- and PRINS-expressing plasmids, we observed hardly any difference in luciferase activity in the presence and absence of the interaction site, indicating no physical interaction through this sequence between IL-23 mRNA and PRINS (Figure 9).

However, we noticed that IL-23 mRNA downregulation by PRINS overexpression was more apparent in those donor samples where initial induction of IL-23 by nucleic acids was the highest (Figure 8), suggesting a correlation. Pearson's correlation analysis confirmed a significant, moderate, negative correlation between the extent of IL-23 mRNA expression by nucleic acid induction and the rate of decrease upon PRINS overexpression (Figure 10). This suggests that an adequately high level of IL-23 mRNA is required for the regulatory effect of PRINS on IL-23 mRNA expression.

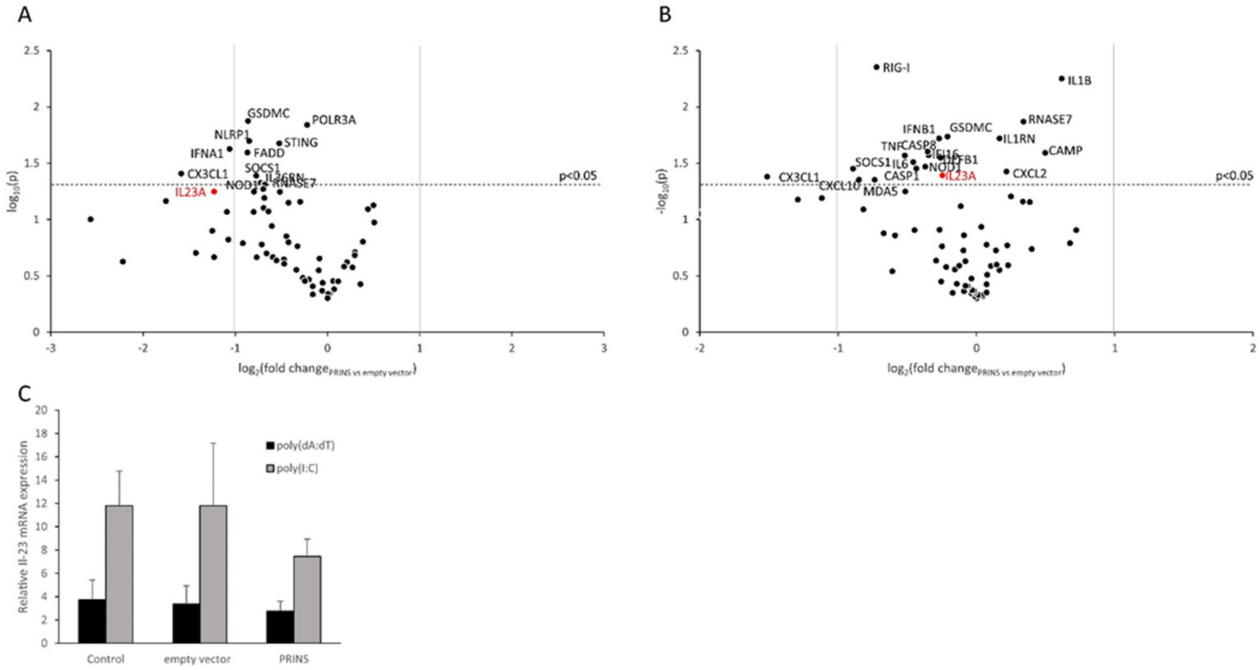

Figure 6. Gene expression changes upon the overexpression of the PRINS long non-coding RNA in NHEKs transfected with poly(I:C) (**A**) and poly(dA:dT) (**B**). RT-PCR validation of the array results regarding nucleic-acid-induced IL-23 mRNA expression upon PRINS overexpression in NHEKs (**C**). To assess multiple gene expression changes, a qPCR array of inflammatory mediators and receptors was carried out on NHEK cells transfected by a pcDNA3.1(+) vector containing the PRINS sequence or the empty verctor. Subsequently, 24 h later, PRINS or emtpy-vector-carrying cells were transfected by 0.666 μg/mL poly(I:C) (**A**) or 1 μg/mL poly(dA:dT) (**B**) for 12 h. Expression changes in PRINS-overexpressing samples were compared to expression values of empty-vector-transfected samples. Results are presented as volcano plots, significant changes ($p < 0.05$) are displayed above the horizontal dashed line. Vertical lines indicate a ±2-fold change in expression compared to empty-vector-transfected cells. Experiments were carried out on four independent samples derived from healthy voluteers, statistical significance was calculated by Student's *t*-test with correction for multiple comparisons (A and B). Cells overexpressing PRINS were transfected with 0.666 μg/mL poly(I:C) and 1 μg/mL poly(dA:dT). Relative IL-23 mRNA expression was determined by the ΔΔCt method, normalized to GAPDH mRNA expression and compared to the expression of the untreated (Control) samples. For the RT-PCR experiment, we used the same total mRNA sample set ($n = 4$) as for the array experiment. Data are presented as mean of four independent experiments ± SD. Statistical significance was not found as assesed by one-tailed, paired Student's *t*-test (**C**).

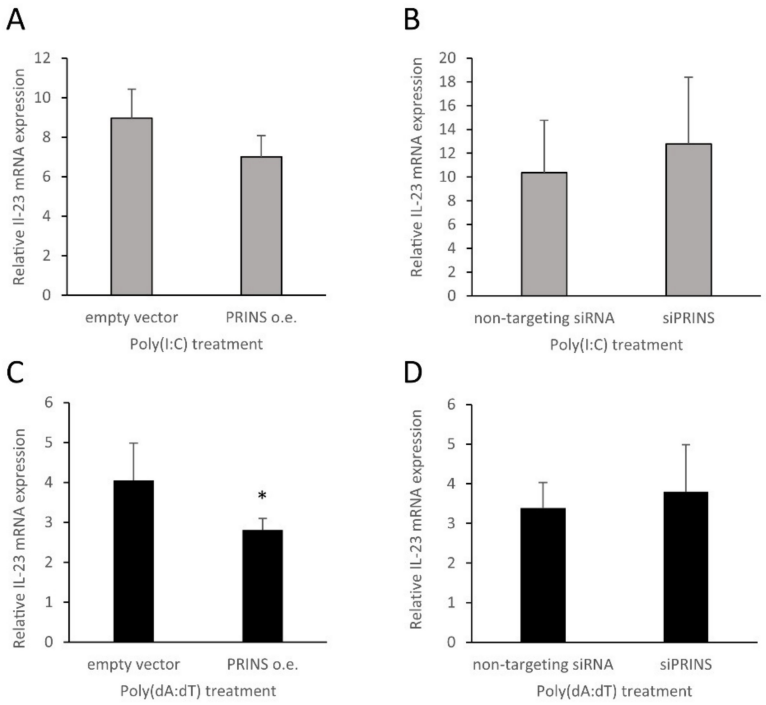

Figure 7. The effect of PRINS overexpression (**A**,**C**) and silencing (**B**,**D**) on the IL-23 mRNA expression upon poly(I:C) (**A**,**B**) and poly(dA:dT) (**C**,**D**) treatment. PRINS-overexpressing (**A**,**C**) or -silenced (**B**,**D**) cells were transfected with 0.666 μg/mL poly(I:C) or 1 μg/mL poly(dA:dT) Relative IL-23 mRNA levels were determined by the ΔΔCt method, normalized to GAPDH mRNA expression and compared to mock-transfected samples. Data are presented as means of eight (**A**), fifteen (**C**) and three (**B**,**D**) independent experiments ± SD. Statistical significance was assessed by one-tailed, paired Student's *t*-test, * $p < 0.05$.

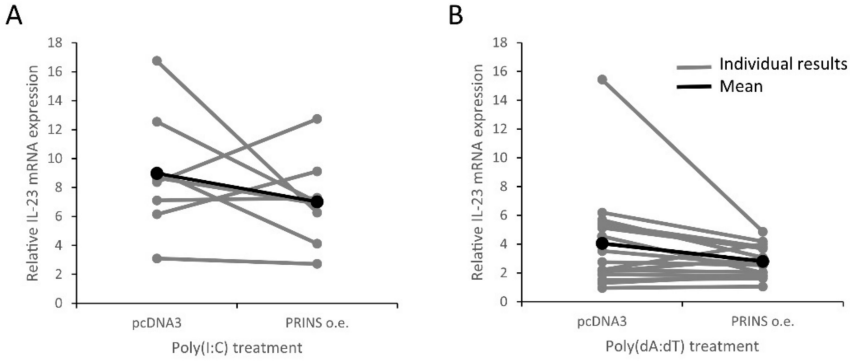

Figure 8. The effect of PRINS overexpression on poly(I:C)- (**A**) and poly(dA:dT)- (**B**) induced IL-23 mRNA expression in NHEKs isolated from the epidermis of 8 (**A**) and 15 (**B**) healthy volunteers. PRINS overexpressing cells were transfected with 0.666 μg/mL poly(I:C) (**A**), $n = 8$, or with 1 μg/mL poly(dA:dT) (**B**), $n = 15$. Relative IL-23 mRNA expression was determined by the ΔΔCt method, normalized to GAPDH mRNA expression and compared to mock-transfected samples. Expression values of individual samples are shown in gray, the mean values of the individual results is shown in black.

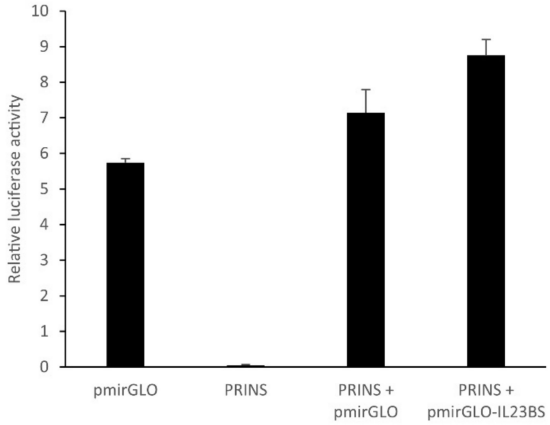

Figure 9. An in vitro binding assay revealed no interaction between the putative PRINS-interacting sequence on IL-23 and PRINS long non-coding RNA. Cells were transfected with PRINS pcDNA3.1 and pmirGLO plasmids as controls or co-transfected with the PRINS pcDNA3.1 plasmid in combination with pmirGLO or pmirGLO-IL23BS vectors. The pGL4.75 [hRluc/CMV] plasmid (Promega) was used as internal control. Cells were harvested after 24 h, and luciferase activities in the lysates were measured using the Firefly and Renilla Dual Luciferase Assay Kit and SYNERGY/HTX Multi-Mode reader following the manufacturer's instructions. The luciferase activity derived from the pmirGLO plasmid was normalized to the renilla luciferase activity. Results of a representative experiment are shown with three technical repetitions.

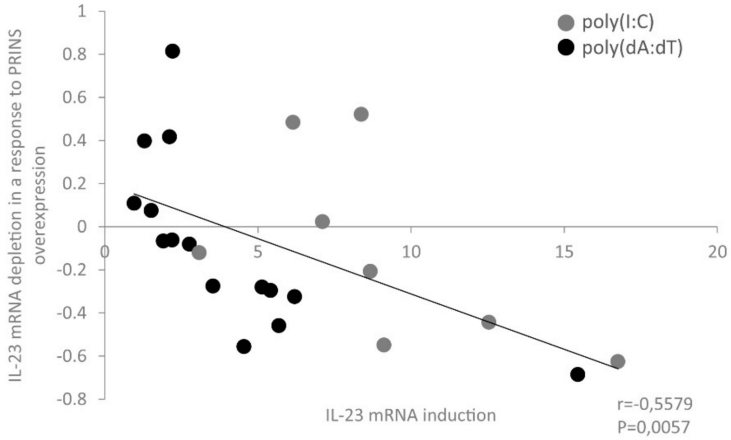

Figure 10. Analysis of the association between nucleic-acid-induced IL-23 mRNA abundance and the effect of PRINS overexpression on it shows a significant moderate negative correlation. The IL-23 mRNA depletion was determined as the relative change of PRINS-overexpressing samples compared to pcDNA3.1-transfected samples and displayed as a function of the poly(I:C)- or poly(dA:dT)-induced IL-23 mRNA expression of each sample.

3. Discussion

Nucleic acid fragments are important PAMPs or DAMPs that induce the innate immune processes of professional and non-professional immune cells [34,39]. Activation of inflammasomes in keratinocyte dendritic cells mediates the promotion of inflammation [10], and the accumulation of RNA and DNA fragments in keratinocytes has a pathogenic role in the psoriatic parakeratosis [40,41]. Several studies suggested that the Th17/IL-23 axis plays a role in disease pathogenesis [29,42,43]. Increased expression of the subunits of IL-23 and its receptor were found in psoriatic lesional skin, which suggest that this cytokine—and not IL-12—play a role in psoriatic inflammation [26,44,45]. However, there are no available data about the possible connection between the effect of these inflammatory agents.

In this study, we aimed to investigate the mechanism of how nucleic acid challenge regulates IL-23 mRNA expression in keratinocytes. Our results showed that both poly(I:C)

and poly(dA:dT) induce IL-23 mRNA expression in keratinocytes but to a different degree and with different kinetics. The difference might originate from differences in th e recognition of RNA and DNA by PRRs. Specific PRRs that are capable of sensing nucleic acids in keratinocytes have already been widely described. These receptors recognize RNA and DNA fragments of both native and pathogenic origin and induce inflammatory mechanisms. TLR3 recognizes dsRNAs from viruses as well as poly(I:C), and it is essential for the production of the IL-12p40 subunit [31]. TLR3 was also shown to induce IL-23p19 expression through interferon regulatory factor 6 (IRF6) [22]. RIG-I and IFIH1 belong to the RIG-like receptors [31]. RIG-I predominantly recognizes short dsRNA, while IFIH1 senses long dsRNA from viruses. However, it was previously shown that poly(dA:dT) is transcribed into dsRNA by RNA polymerase III before recognition by the RIG-I receptor [28,46], which, as suggested previously, might explain the kinetic differences between poly(I:C) and poly(dA:dT) treatment [28]. Recently, cGAS was described as the major PRR to recognize cytosolic DNA fragments [32,33]. To date, the involvement of these receptors in the production of IL-23 by keratinocytes has not been addressed. Our results demonstrated that, whereas poly(I:C)-mediated IL-23 mRNA expression was decreased by silencing TLR3, in line with previous reports [22], silencing of none of the studied receptors had an effect on poly(dA:dT)-induced IL-23 mRNA expression. Nevertheless, we emphasize that the level of induction after poly(dA:dT) treatment never approached the level induced by poly(I:C) treatment, which might explain the negative results in the silencing experiments. Our experiments were carried out on NHEK cells isolated from four healthy donors, and average values are depicted in Figure 4. However, even in this small dataset, we observed differences in the reactions of the individuals, especially in the case of donors NHK49 and 51, in which IFIH1 silencing resulted in a remarkable decrease in the IL-23 mRNA level (Supplementary Figure S2A–D). These results drew our attention to the importance of the differential sensitivity of individuals in responses to different agents.

The nuclear-factor (NF)-κB signaling pathway affects cell survival, proliferation and anti-apoptotic effects of lymphocytes and keratinocytes, and it is known that TNF-α induces Th17 to produce pro-inflammatory cytokines through this pathway in psoriatic lesions [23]. In eukaryotic cells, three mitogen-activated protein kinase (MAPK) cascades have been identified: ERK, JNK and p38. The ERK signaling pathway plays an important role in cell proliferation and differentiation, while JNK and p38 are mainly related to stress and apoptosis of cells [47]. The JAK/STAT signaling pathway, also known as the IL-6 signaling pathway, is involved in many biological processes, such as cell proliferation, differentiation and apoptosis, and is also closely related to many immune and inflammatory diseases [48]. It has been shown that, in human keratinocytes, poly(I:C) induces NF-κB, p38 and STAT-1 signaling [34], whereas poly(dA:dT) treatment activated NF-κB, p38 and JNK signaling in human melanocytes [49]. Recent studies show that increased levels of oxidative products activate keratinocytes, Th1 and Th17 cells through the MAPK, NF-kB and JAK-STAT pathways, resulting in the production of several cytokines which are involved in psoriatic inflammation [50–52].

Inhibition of the ERK-1, JNK, NF-κB and STAT3 pathways resulted in a decrease in poly(I:C)-induced IL-23 mRNA expression of keratinocytes, suggesting that all of these pathway components play a role in the RNA-induced production of IL-23 mRNA under inflammatory circumstances. Blockage of these pathways did not result in complete decay of IL-23 expression, showing the involvement of other, related pathways including the known role of TLR3 signaling through IRF6 [22]. These suggests that poly(I:C)-induced IL-23 expression in keratinocytes is mainly mediated by TLR3-driven activation of several parallel pathways. Interestingly, similarly to the silencing of the major nucleic-acid-sensing PRRs, inhibition of these major pathways did not have an effect on poly(dA:dT)-induced IL-23 mRNA expression, suggesting the importance of alternative receptors and pathways in DNA-induced IL-23 mRNA expression that have not yet been identified. The involvement of other, less exposed receptors or pathways in poly(dA:dT)-induced IL-23 mRNA expres-

sion would also explain the observed lower expression compared to poly(I:C)-induced expression levels.

PRINS long non-coding RNA has been characterized as a contributor to the anti-inflammatory state of the epidermis by binding directly to inflammatory molecules, specifically to IL-6 and CCL-5 mRNAs [14,35]. To identify additional target transcripts of PRINS under inflammatory conditions, nucleic acid induction was applied during PRINS overexpression. PRINS overexpression led to a decrease in nucleic-acid-induced IL-23 mRNA expression, and a putative lncRNA–mRNA binding site was identified in silico. Although initially we confirmed the decrease in IL-23 mRNA expression upon PRINS overexpression, increasing the number of samples led us to conclude that there were large interindividual differences among the independent donors. Since a direct lncRNA–mRNA interaction could be affected by SNPs, their presence was assessed; however, sequencing the putative binding site of the different donors revealed no correlation with the presence of sequence variants and the IL-23 mRNA response to PRINS overexpression. Interaction could not be confirmed with an experimental approach to evaluate the direct interaction between the IL-23 mRNA putative binding site and the PRINS lncRNA molecule using a luciferase-based binding assay. In further studies, the responses of the donors seemed to be related to the level of the nucleic-acid-induced IL-23 mRNA expression. A correlation assay was carried out and confirmed the possible negative correlation between the nucleic-acid-induced IL-23 mRNA expression levels and the decrease upon PRINS overexpression, suggesting the need for sufficiently high levels of IL-23 mRNA for PRINS regulation to be detected. However, even in cases when the induction of IL-23 expression was sufficiently high, PRINS might act indirectly rather than by direct interaction with the IL-23 mRNA.

Although IL-23 in psoriasis is predominantly thought to be released by professional immune cells, our results indicated that this cytokine might originate from keratinocytes as well. Nucleic acids activate several PRRs in these cells, leading to the upregulation of several inflammatory molecules, including IL-23. Thus, keratinocyte-derived nucleic acids in the skin might also contribute to the development of psoriasis by elevating the IL-23 levels in keratinocytes through an autocrine mode. Taken together, our data suggest that individual differences in sensitivity toward the levels of nucleic acids in the tissue might lead to different IL-23 levels. These differences might in turn be related to the different reactions toward biological agents used in psoriasis treatment. Detailed analysis of these processes will help us to identify new biomarkers for the development of personalized treatments for the disease.

4. Materials and Methods

4.1. Cell Culture

For these experiments, we used NHEKs isolated from skin samples retrieved from the Plastic Surgery Unit of our department after informed written consent was obtained from the volunteers. The experiments were approved by the Human Investigation Review Board of the University of Szeged (PSO-EDAFN-002, 23 February 2015, Szeged, Hungary), and investigations were carried out in accordance with the rules of the Helsinki Declaration. The isolation of the NHEKs from skin samples was described earlier [28]. Keratinocytes were grown in Keratinocyte-SFM Medium (Gibco, Thermo Fischer Scientific, Waltham, MA, USA), supplemented with EGF (epidermal growth factor), BPE (bovine pituitary extract), 1% L-glutamine and 1% antibiotic–antimycotic solution. The medium was changed every second day until the third passage.

4.2. Stimulation of the Cells

Third-passage NHEK cells were seeded into six-well plates at a density of 200,000 cells/mL. After 24 h, the medium was changed to supplement-free medium, and cells were transfected with 1 µg/mL poly deoxyadenylic acid–poly deoxythymidylic acid double-stranded homopolymer (poly(dA:dT)) (InvivoGen, 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, Basel, Switzerland), according to the manufacturer's instructions. Cells were harvested at indicated time points.

For PRINS overexpression, the AK022045 cDNA sequence (Biological Research Center, National Institute of Technology and Evaluation, Chiba, Japan) was cloned into a pcDNA3.1(+) vector as described previously [14]. As a control, the empty pcDNA3.1(+) vector was used. One microgram of plasmid DNA was used for the transfection of the NHEK cells using the X-tremeGENE HP transfection reagent (Roche Diagnostics), according to the manufacturer's instructions.

For siRNA-mediated gene silencing of PRRs, the ON-TARGETplus SMARTpool TLR3, RIG-1, IFIH-1, cGAS siRNAs or ON-TARGETplus Non-Targeting Pool (Dharmacon, Lafayette, CO, USA) constructs were used at a final concentration of 40 nM for transfection using the X-tremeGENE siRNA transfection reagent (Roche Diagnostics), according to the manufacturer's instructions.

For inhibition experiments, NHEK cells were incubated 1 h prior to poly(dA:dT)/poly(I:C) transfection with specific inhibitors for NF-κB (Bay 11-7085, 10 μM; MedChemExpress, Monmouth Junction, NJ, USA), STAT-1 (Fludarabine, 10 μM; Sigma Aldrich), STAT-3 (Stattic, 5 μM; Sigma Aldrich), JNK (SP600125, 10 μM; Tocris Bioscience, Bristol, UK), MEK-1 (PD98059, 20 μM; Sigma Aldrich) and p38 (SB203580, 10 μM; Tocris Bioscience). Since all of the signal transduction pathway inhibitors were solved in DMSO (Sigma Aldrich) to different concentrations, the same amount of DMSO was applied as control.

4.3. RNA Isolation and RT-PCR

At indicated time points after transfection, 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 using the Turbo DNA-free Kit (Ambion, Thermo Fischer Scientific), according to the manufacturer's instructions. During cDNA synthesis, 1 μg total RNA was reverse transcribed by the EvoScript Universal cDNA master mix (Roche Diagnostics). Real-time RT-PCR experiments were carried out with the TaqMan Gene expression Assay (PRINS: Hs03671803_s1; IL-23p19: Hs00900828_g1, GAPDH: Hs01548420_m1) (Thermo Fisher Scientific) using the qPCRBIO Probe Mix Lo-ROX (PCR Biosystem Ltd., London, UK) on a C1000 Touch Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA). The expression of each gene was normalized to the GAPDH mRNA, and relative mRNA levels were calculated using the ΔΔCt method.

4.4. In Silico Prediction of Interacting Sites

Using INTARNA [53], sequence complementarity between PRINS (AK022045) and the mRNA of IL-23 (NM_016584.3) was analyzed. INTARNA calculates the free-energy values of interaction based on predicted global and local structures of mRNAs. This in silico analysis revealed a binding site on the IL-23A mRNA that partly overlaps with the previously identified IL-6 binding sequences [14].

4.5. pmirGLO Dual-Luciferase miRNA Target Expression Vector

The sequence of the putative PRINS binding site of IL-23 cDNA that was identified in silico was synthesized and inserted into the pmirGLO vector (Promega, Madison, WI, USA) between the *Dra* I and *Xba* I restriction sites, resulting in the pmirGLO-IL23BS construct (Supplementary Figure S4). HEK293 cells were transiently transfected by the PRINSpcDNA3.1 or pmirGLO plasmid as controls. Cells were also co-transfected with 0.5 μg pmirGLO-IL23BS or empty pmirGLO plasmid together with the PRINSpcDNA3.1 vector. In each transfection experiment, 0.025 μg renilla-luciferase-expressing pGL4.75 hRluc/CMV (Promega) plasmid was used as internal control. Cells were harvested after 24 h, washed with PBS (phosphate-buffered saline) and lysed with passive lysis buffer (Biotium, Hayward, CA, USA), and luciferase activities were measured using the Firefly and Renilla Dual Luciferase Assay Kit (Biotium) and SYNERGY/HTX Multi-Mode reader (Bio Tek Instruments, Winooski, VT, USA), according to the manufacturer's instructions.

The luciferase activity derived from the pmirGLO plasmids was normalized to the renilla-luciferase activity.

4.6. Statistical Analysis

Experiments were carried out in duplicate with at least three biological repeats. For statistical analysis, a one-tailed, paired Student's *t*-test was used with correction for multiple comparisons. The significance level was set at $p \leq 0.05$. Pearson's correlation calculation was used to analyze the correlation between IL-23 mRNA induction and PRINS-overexpression-mediated IL-23 mRNA depletion.

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II.

The Psoriatic Nonlesional Skin: A Battlefield between Susceptibility and Protective Factors

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In the last two decades, large-scale gene-expression studies on psoriatic skin samples revealed that even though nonlesional skin is macroscopically identical to healthy skin, it harbors several molecular differences. Originally, these molecular differences were thought to represent susceptibility factors for plaque formation. However, we review in this paper the several factors of immune regulation and structural alteration that are specific for the nonlesional skin and serve as protective factors by counteracting plaque formation and contributing to the maintenance of the nonlesional phenotype.

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Introduction

Psoriasis is a multifactorial, chronic inflammatory skin disease with a spectrum of clinical phenotypes affecting approximately 2% of the population worldwide. The most common form is plaque-type psoriasis, which is characterized by well-demarcated, erythematosus oval plaques and adherent silvery scales caused by the abnormal proliferation and differentiation of basal keratinocytes (KCs) and their dysregulated interplay with professional immune cells (Di Meglio et al., 2011; Nestle et al., 2009). These lesions and plaques are surrounded by clinically healthy-looking skin, which is referred to as nonlesional or uninvolved skin. Even though the nonlesional skin of patients with psoriasis appears macroscopically identical to the normal skin, it contains molecular, cellular, and extracellular alterations and, in several aspects, has a prepsoriatic phenotype (Bata-Csorgo et al., 1995; Jackson et al., 1999; Körver et al., 2006; McKenzie and Sabin, 2003; Sonkoly et al., 2005; Szabó et al., 2014; Széll et al., 2004).

Psoriatic lesional epidermis can be distinguished from the nonlesional epidermis by the presence of infiltrating leukocytes, inflammation, and hyperproliferation and abnormal differentiation of KCs. Although there is no clear infiltration of immune cells in nonlesional skin, compared with the healthy skin, an increase in T helper and suppressor cells, $CD11b^+$ cells, and some subsets of dendritic cells (DCs) can be observed (Baker et al., 1984; Placek et al., 1988; Prens et al., 1991). Whereas the psoriatic lesional epidermis has a strong type I cytokine profile, the nonlesional epidermis also exhibits significant mRNA expression of several cytokines (e.g., *IL1α*, *IL1β*, *IL17*, *TNF-α*, and *IFN-γ*) compared with the healthy epidermis (Uyemura et al., 1993), of which the TNF-α/IL-23/IL-17 axis has a crucial role in maintaining the psoriatic plaques (Griffiths et al., 2021); however, initiation of plaque formation might depend on other cytokines as well. These results suggest altered proinflammatory conditions in the nonlesional skin, which might be responsible for the initiation of psoriatic lesions (Chiricozzi et al., 2016; Johnson-Huang et al., 2012; Yoshinaga et al., 1995). The inflammatory mediators in psoriatic lesions promote the proliferation of KCs (Bata-Csorgo et al., 1998) and also modify the expression of their adhesion molecules (Uyemura et al., 1993). This state allows recruited leukocytes to infiltrate the epidermis, leading to the release of additional inflammatory mediators that further promote the hyperproliferative state of the epidermis, interconnecting inflammation and proliferative overresponse in psoriatic lesions. However, the initiating factors that drive psoriatic plaque development from nonlesional skin are still unknown.

Our research group has long been focused on the identification of factors that differentiate nonlesional psoriatic epidermis from the healthy epidermis to understand why nonlesional cells form plaques in response to the cytokines produced by skin-infiltrating lymphocytes. We have performed several large-scale gene- and protein-expression experiments comparing the expression profiles of healthy with those of

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Abbreviations: DC, dendritic cell; ECM, extracellular matrix; EDA^+FN, extra domain A–containing fibronectin; KC, keratinocyte; lncRNA, long noncoding RNA; miRNA, microRNA; MMP, matrix metalloproteinase; ncRNA, noncoding RNA; NET, neutrophil extracellular trap; PRINS, psoriasis-related noncoding RNA induced by stress

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nonlesional epidermis samples (Sonkoly et al., 2005; Szabó et al., 2014; Szél et al., 2019; Széll et al., 2004). The primary aim of these studies was to identify the susceptibility factors in the nonlesional epidermis, but unexpectedly, functional studies identified molecules that likely play protective roles in the nonlesional skin against plaque formation rather than being susceptibility factors. These studies highlighted altered expression of cellular adhesion and extracellular matrix (ECM) molecules that contribute to the maintenance of tissue structure and the upregulation of anti-inflammatory processes in psoriatic nonlesional epidermis.

In this paper, we summarize the existing knowledge of the factors specific to the nonlesional psoriatic epidermis that are likely to have protective functions against psoriatic plaque formation and thereby contribute to the maintenance of the clinically asymptomatic phenotype. Two aspects are discussed in detail: immune regulation and structural alterations in the uninvolved epidermis.

Anti-inflammatory potential of molecules highly expressed in nonlesional psoriatic skin

Although several inflammatory mediators are expressed in nonlesional psoriatic skin, it is not inflamed, suggesting that inhibitory factors in the nonlesional epidermis might counteract the inflammatory processes to maintain a noninflamed state.

In healthy skin, KCs are the main source of the proinflammatory cytokines of the IL-1 family. These cytokines are produced as inactive precursors (i.e., pro−IL-1β and pro−IL-18) and are activated by inflammatory stimuli by the multimolecular inflammasome complexes containing caspases (Beer et al., 2014; Feldmeyer et al., 2007). In psoriatic lesional skin, the AIM2 inflammasome is expressed and activated by cytosolic DNA fragments (Dombrowski et al., 2011; Kopfnagel et al., 2011) leading to the subsequent release of active IL-1β and IL-18, causing pronounced inflammation and hyperproliferation. Nucleic acids in the psoriatic skin associate with the antimicrobial protein LL37, and these complexes propagate plasmacytoid DC and KC activation (Chiliveru et al., 2014; Dombrowski et al., 2011; Ganguly et al., 2009; Herster et al., 2020; Lai et al., 2011; Lande et al., 2007; Morizane et al., 2012). The involvement of nucleic acid fragments in the disease is underlined by the elevated amounts of cell-free DNA found in the circulation of patients with psoriasis (Beranek et al., 2017), and that topical nanoparticles binding DNA fragments ameliorate disease phenotype (Liang et al., 2020). However, the source of nucleic acids that start these initial inflammatory events remains unknown. They could originate from KCs because in psoriatic skin, deoxyribonucleases, which remove extracellular DNA during terminal differentiation, have reduced activity, resulting in the presence of excess DNA fragments (Fischer et al., 2011). Moreover, mitochondrial dysregulation can lead to the secretion of mtDNA in psoriasis, leading to increased serum mtDNA levels (Therianou et al., 2019), which could act as danger-associated molecular patterns (Theoharides, 2016). Similarly, disturbed ribonuclease activities were described in psoriatic skin (Ruiz-Romeu et al., 2016), which might result in the accumulation of excess RNA fragments. Recently, activation of neutrophils and neutrophil extracellular trap (NET) formation were shown in psoriatic skin as the source of nucleic acids. Unexpectedly, the authors showed that not the DNA component of NETs but the associated RNA could be bound by LL37. It was also shown that these could activate inflammatory reactions and lead to a self-propagating vicious cycle contributing to chronic inflammation in psoriasis (Herster et al., 2020).

Interestingly, AIM2 is also highly expressed in psoriatic nonlesional epidermis but without activation (de Koning et al., 2012). Our results might reveal a possible explanation for this phenomenon: we found higher expression of CARD18 in psoriatic noninvolved epidermis (Göblös et al., 2016; Szabó et al., 2014). CARD18 negatively regulates inflammasome activation by binding to the amino-terminal caspase recruitment domain of inflammatory procaspases, inhibiting their interaction with the inflammasome (Druilhe et al., 2001; Humke et al., 2000). Both terminal differentiation (Qin et al., 2017) and barrier disruption (Göblös et al., 2016) induce the expression of CARD18 in the skin, and gene-specific silencing of CARD18 in KCs resulted in increased IL-1β secretion, in accordance with its negative regulatory role in inflammasome signaling (Göblös et al., 2016; Qin et al., 2017).

Although biological therapies against IL-1β are not effective in plaque-type psoriasis (Tsai and Tsai, 2017), patients with psoriasis were shown to present higher plasma levels of IL-1β and IL-18, however, without any correlation to skin lesion severity (Verma et al., 2021). Upregulation of inflammasome components is found in lesional psoriatic skin samples (Tervaniemi et al., 2016). Whether IL-1β and IL-18 in plasma originate from skin lesions or from other cells is not known because not only KCs can express inflammasome components, but a constitutive expression of inflammasome sensors NLRP1, NLRP3, and AIM2 and an activity of caspase-1 were found in peripheral blood cells of patients with psoriasis. Activation of the inflammasomes in psoriatic myeloid cells is mediated by TNF-α, and its blockade also reduces IL-1β levels. Although IL-1β blockade in most patients with psoriasis is ineffective, inflammasome activation is still contributing to the maintenance of the systemic inflammation (Verma et al., 2021). However, the major hallmarks of the disease are rather TNF-α, IL-17, and IL-23 even though their targeting only leads to disease clearance in about half of the patients, pointing out the need for further studies to better understand the right therapeutic choices for each individual patient (Griffiths et al., 2021; Kamata and Tada, 2020).

GATA3 transcription factor is also upregulated in nonlesional skin compared with that in lesional skin samples, and therapeutic healing of psoriatic lesions also leads to increased GATA3 expression (Rácz et al., 2011; Wang et al., 2020). GATA3 is a suppressor of IL-6 and IL-8 expression and an activator of the anti-inflammatory cytokine IL-37. The expression of IL-37 closely follows GATA3 expression (Wang et al., 2020) and is highly expressed in nonlesional skin but is undetectable in lesional skin (Li et al., 2014). IL-37 is an upstream inhibitor of NF-κB, making it an attractive therapeutic target (Boutet et al., 2019; Conti et al., 2019).

These observations suggest that the upregulation of anti-inflammatory molecules in nonlesional epidermis can contribute both to the downregulation of inflammatory processes and to the maintenance of the nonlesional phenotype. Deregulation of these processes might result in the initiation of plaque formation.

Besides the protein-coding transcripts differentially expressed in nonlesional epidermis, noncoding RNAs (ncRNAs) are also differentially expressed in psoriasis (Sonkoly et al., 2005). Both long ncRNAs (lncRNAs) and short ncRNAs are known to be dysregulated in psoriasis. lncRNAs are longer than 200 nucleotides and share similar structural features with mRNAs but do not code for proteins. Our group was one of the first to identify and characterize an lncRNA that is highly expressed in psoriatic nonlesional epidermis compared with that in psoriatic lesional or healthy epidermis. The identified lncRNA is referred to as Psoriasis susceptibility-related RNA Gene Induced by Stress (PRINS) (Sonkoly et al., 2005). Initially, PRINS was considered a susceptibility factor because T-cell lymphokine–induced (Bata-Csorgo et al., 1995) proliferation of nonlesional KCs was accompanied by decreased expression of PRINS (Sonkoly et al., 2005). However, recently, we also showed an inhibitory role for PRINS in psoriasis-related inflammatory responses of KCs. Overexpression of PRINS in KCs—to mimic the high PRINS expression of nonlesional epidermis—resulted in ameliorated expression of the inflammatory genes *IL6* and *CCL5*, also known as *RANTES*. Our in silico and in vitro results suggest that the PRINS lncRNA might directly bind to the mRNAs of *IL6* and *CCL5* through distinct binding sites (Danis et al., 2017). IL-6 is not considered a key cytokine in psoriasis because its blockade does not ameliorate symptoms (Blauvelt, 2017); however, its elevated levels contribute to the maintenance of systemic inflammation (Beranek et al., 2017), together with TNF-α and IL-1β (Verma et al., 2021). However, a complete lack of IL-6 can result in the upregulation of compensatory inflammatory reaction (Fritz et al., 2017), which could explain why we only see partial downregulation by PRINS in KCs (Figure 1).

MicroRNAs (miRNAs) are short (~22 nucleotides) ncRNAs that negatively regulate the expression of protein-coding genes at the post-transcriptional level (Sonkoly et al., 2008). Changes in the expression of miRNAs are known to play a role in several human diseases through their involvement in the regulation of immune system development (Cheng et al., 2007; Hansen et al., 2007; Sonkoly et al., 2008). Effective biological therapies in psoriasis also result in upregulation of circulating miRNAs targeting TNF-α or IL-17, the driving cytokines of psoriasis (Khan and Ansar Ahmed, 2015). In a study comparing the expression in psoriatic skin with that in healthy skin, 42 miRNAs were upregulated, and 5 miRNAs were downregulated in lesional skin, whereas only 9 miRNAs were upregulated and 1 downregulated in nonlesional skin. The upregulated miRNAs in the lesional epidermis are targeting anti-inflammatory genes, resulting in increased inflammation through the decrease of anti-inflammatory molecules (Gubán et al., 2016; Moldovan et al., 2019; Sonkoly et al., 2007; Zibert et al., 2010). In contrast, highly expressed miRNAs of the nonlesional epidermis are targeting inflammatory genes (e.g., *IL17*, *TNF-α*, and others) and members of proliferative signaling pathways (Mizuno et al., 2018; Sonkoly et al., 2008; Teng et al., 2013; Wei et al., 2017); thus, their expression in psoriatic nonlesional epidermis might counteract the inflammatory and hyperproliferative signals. These data indicate that both lncRNA and miRNA deregulation is involved in the pathogenesis of psoriasis, providing an additional layer of regulation.

Structural changes contributing to the symptomless state of nonlesional psoriatic skin

Alterations of ECM proteins in nonlesional epidermis could be susceptibility factors because ECM-derived signals can influence the T-cell response in psoriasis (Gliński et al., 1993) and also affect KC functions. Abnormalities directly below the basal cells of the epidermis concern the dermal–epidermal junction region and alteration of the basal membrane. A discontinuous pattern has been observed in the LAMA1 chain within the basement membrane (BM) laminin layer of nonlesional psoriatic skin (Mondello et al., 1996; Vaccaro et al., 2002). Moreover, on the sites of the disrupted laminin layers in nonlesional skin, the expression of other ECM molecules, such as the fibronectin splice variant extra domain A–containing fibronectin (EDA^+FN) and its ligand (the α5β1 integrin), is increased, indicating an abnormal anchoring mechanism (McFadden et al., 2012). In addition, α5β1 integrin is required for the development of the hyper-responsiveness of KCs to proliferative signals (Bata-Csorgo et al., 1998) because abnormal integrin-driven focal adhesion kinase activity and downstream signaling affect the proliferation capacity of nonlesional KCs (Chen et al., 2001). Furthermore, the key role of α5β1 integrin in the mediation of proliferation is also supported by the fact that mice expressing the β1 integrin subunit alone or in combination with the α5 integrin subunit exhibit epidermal hyperproliferation (Carroll et al., 1995).

In addition, KC GF—highly expressed in nonlesional skin (Gubán et al., 2016)—induces the expression of α5 integrin in human KCs and in transgenic mice (Andreadis et al., 2001), resulting in epidermal hyperplasia (Guo et al., 1993) as well as the production of EDA^+FN by fibroblasts (Gubán et al., 2016) and nonlesional psoriatic KCs (Széll et al., 2004). EDA^+FN is normally expressed in developing tissues, especially at BMs (Vartio et al., 1987), but a distinct accumulation of EDA^+FN in nonlesional psoriatic skin can be observed (Gubán et al., 2016). The accumulated EDA^+FN forms an autologous loop through the fibronectin receptor α5β1 integrin, predisposing psoriatic KCs to proliferate (Bata-Csorgo et al., 1998; Ting et al., 2000). These changes in the expression of integrins and EDA^+FN observed in nonlesional skin lead us to consider them as susceptibility factors in the development of psoriatic plaques.

However, there are molecules in the nonlesional skin that could counteract proliferation. One such counteracting molecule could be the COMP, which shows an increased expression and colocalization with basal KC β1 integrin and EDA^+FN and a decreased colocalization with LAMA1 in nonlesional skin. In healthy skin, COMP is expressed in the papillary dermis and contributes to the stabilization of the

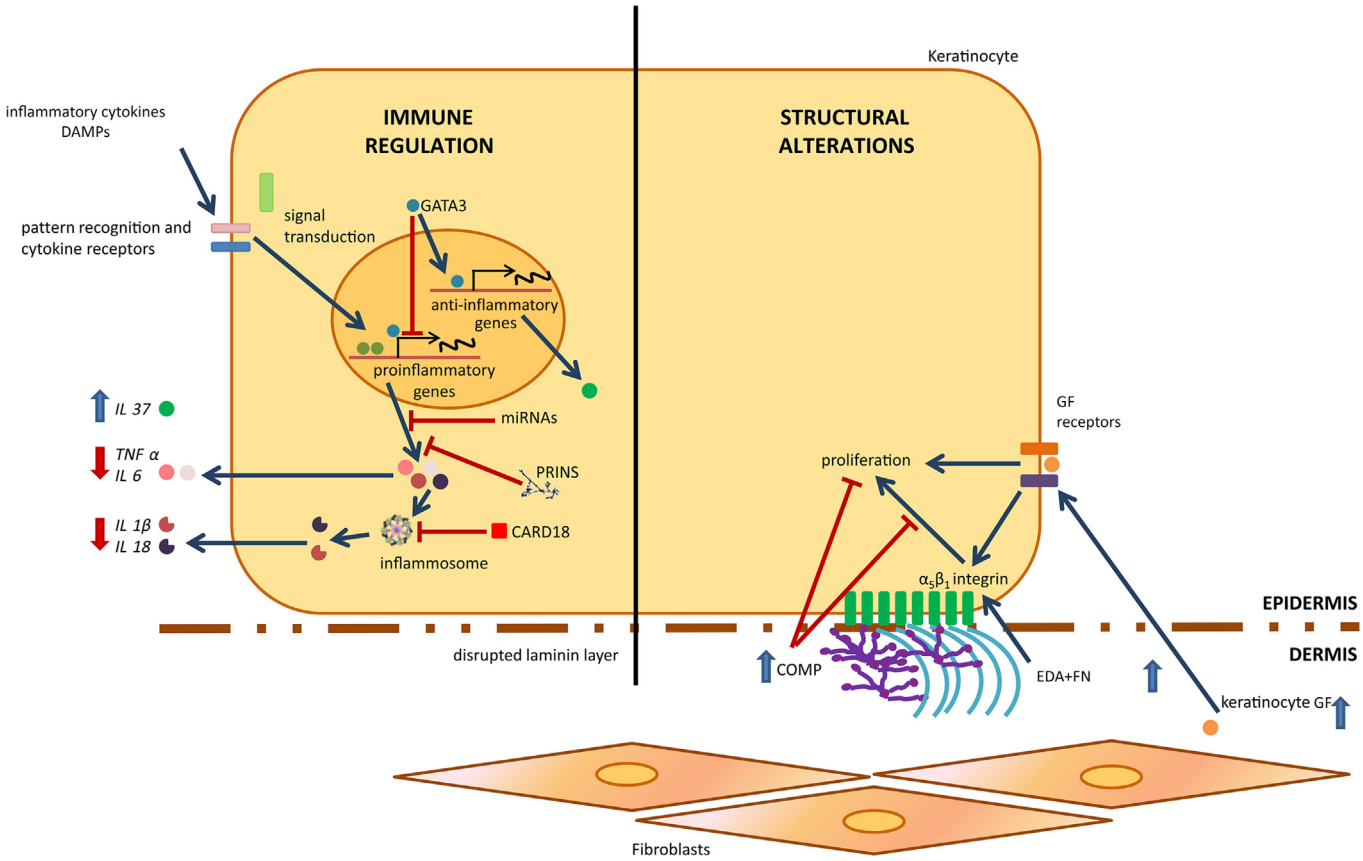

Figure 1. Potential protective factors in psoriatic nonlesional skin: immunological and structural alterations. DAMPs and inflammatory cytokine predispose nonlesional keratinocytes to respond with proinflammatory gene expression; however, protective factors counteract these effects. GATA3 transcription factor inhibits the expression of proinflammatory genes (e.g., *IL6*, *IL8*) and induces the expression of anti-inflammatory genes (e.g., *IL37*). The mRNAs of proinflammatory genes are regulated by miRNAs and the PRINS lncRNA. Inflammasome activation can be inhibited by highly expressed CARD18. These processes together lead to ameliorated inflammatory responses in psoriatic nonlesional keratinocytes. Structural alteration in the basal membrane also contributes to the maintenance of the nonlesional phenotype. The dermal fibroblast of the nonlesional skin produces a higher amount of keratinocyte GF and EDA$^+$FN, predisposing keratinocytes to proliferation through α5β1 integrin. Through the disrupted laminin layers, COMP can interact with keratinocytes through α5β1 integrin, leading to the inhibition of proliferation. DAMP, danger-associated molecular patterns; EDA$^+$FN, extra domain A–containing fibronectin; lncRNA, long noncoding RNA; miRNA, microRNA; PRINS, psoriasis-related noncoding RNA induced by stress.

dermal–epidermal junction (Agarwal et al., 2012), whereas in psoriatic nonlesional skin, it extends deeper into the dermis, forming a more continuous layer. Our in vitro results indicate that this increased COMP expression may restrain the proliferation rate of nonlesional basal KCs through α5β1 integrin. Although the elevated expression of EDA$^+$FN and α5β1 integrin could predispose nonlesional KCs to hyperproliferation, the elevated and continuous expression of COMP at the dermal–epidermal junction can control this process through interactions with both EDA$^+$FN and α5β1 integrin. Thus, COMP can aid in maintaining the nonhyperproliferative state of psoriatic nonlesional epidermis (Bozó et al., 2020) (Figure 1).

In psoriasis, matrix metalloproteinases (MMPs) and their inhibitors can contribute to structural changes, and MMPs can be potential targets during the therapy (Mezentsev et al., 2014). Several cytokines, including IL-17, are known to be responsible at least partially for the elevated MMP levels in the psoriatic lesional skin (Starodubtseva et al., 2011). The enhanced IL-17 signaling described in the nonlesional skin (Chiricozzi et al., 2016) could play a role in the formation of structural alterations in the nonlesional skin.

Conclusions

On the basis of these findings, alterations in nonlesional psoriatic skin, on the one hand, contribute to the initiation of lesions and represent prepsoriatic, preactivated skin. In contrast, it is interesting to note that some alterations in nonlesional skin can also contribute to the maintenance of the symptomless state of nonlesional skin, indicating the presence of special compensation mechanisms. Thus, these compensatory alterations allow the nonlesional skin to maintain the appearance of being asymptomatic when, in fact, this skin type is a special type of psoriatic skin without obvious hyperproliferation and immune-cell infiltration.

The large-scale studies comparing the expression profiles of healthy skin with those of psoriatic skin have predicted several susceptibility factors for psoriatic plaque formation. However, the functional validation of many of these molecules unexpectedly revealed a protective role against plaque formation in nonlesional skin. We believe that reanalysis of these large-scale gene-expression studies could differentiate between susceptibility and

protective factors in nonlesional skin. Thus, deep characterization of molecules arising through large-scale studies is indispensable for identifying additional factors, which could uncover novel layers of psoriasis pathogenesis and possibly lead to novel therapeutic strategies.

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CONFLICT OF INTEREST

The authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS

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