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

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Abbreviations: **A**IM2 – absence in melanoma 2, CARD18 – caspase recruitment domain family member 18, COP – CARD-only protein, DAPI – 4,6-diamidino-2-phenylindole, DNase – deoxyribonucleases, GM-CSF – granulocyte macrophage colony-stimulating factor, IL – interleukin, IFN-ɣ - interferon-ɣ, TNF – tumor necrosis factor, IHC – immunohistochemistry, NHEK – normal human epidermal keratinocytes, PBS – phosphate-buffered saline, poly(dA:dT) – polydeoxyadenylic acid–polydeoxythymidylic acid double-stranded homopolymer, TS – tape stripping

Abstract

Caspase recruitment domain family member 18 (CARD18, Iceberg) is known as a negative regulatory molecule that inhibits inflammatory events by terminating inflammasome activation due to a direct interaction with pro-caspase-1.
During the investigation of molecular mechanisms in keratinocytes that contribute to the pathogenesis of psoriasis, we found that CARD18 expression differs in healthy and psoriatic skin; moreover, CARD18 demonstrated altered response under inflammatory conditions in healthy and psoriatic skin. In healthy skin, low basal CARD18 expression was detected, which showed significant elevation in response to inflammatory stimuli (lymphokine treatment or mechanical injury). In contrast, higher basal expression was observed in psoriatic non-involved skin, but no further induction could be detected.
We demonstrated that keratinocytes express CARD18 both at mRNA and protein levels and the expression increased in parallel with differentiation. The investigation of cellular inflammatory processes revealed that psoriasis-associated danger signals triggered the expression of inflammasome components (AIM2, Caspase-1) and CARD18 as well as IL-1β production of keratinocytes. Furthermore, gene-specific silencing of CARD18 in cells treated with cytosolic DNA (poly(dA:dT)) resulted in increased IL-1β secretion, suggesting a negative regulatory role for CARD18 in keratinocyte inflammatory signaling.
The differential regulation of CARD18 in healthy and psoriatic uninvolved epidermis may contribute to the susceptibility of psoriasis. Furthermore, our *in vitro* results indicate that CARD18 may contribute to the fine tuning of keratinocyte innate immune processes.

Keywords: psoriasis, inflammation, caspase-1, AIM2, IL-1β, CARD18

1. Introduction

The innate immune system constitutes the first line of defense that detects pathogen- and damage-associated molecular patterns. Inflammation is a protective physiological response; however, impaired activation and/or down-regulation of inflammatory signaling may result in inflammatory diseases, some of which involve multiple organs. Inflammasomes, located in the cytosol, are part of the innate immune system. These multi-molecular complexes are responsible for the recognition of various cytoplasmic danger signals and provoke inflammatory responses by recruiting and activating pro-caspase-1 through autocatalytic cleavage (Schroder and Tschopp, 2010). The activation of caspase-1 ultimately leads to the processing and, thus, secretion of pro-inflammatory cytokines, most importantly interleukin (IL) 1β and IL-18, and also induces pyroptotic cell death (Bauernfeind et al., 2011). IL-1β affects nearly all cell types and is fundamental for innate as well as adaptive immunity (Dinarello, 1996). Increased IL-1β abundance plays an important role in a number of chronic and acute inflammatory diseases (Li et al., 2008), such as psoriasis.

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

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

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

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

2. Materials and methods

2.1. Patients

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

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

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

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

2.2. Cell cultures

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

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

Saint Louis, MO, USA) transfection. The supernatants were collected and cells were harvested at the indicated time points after treatments.

2.3. Real-time RT-PCR

Total RNA was isolated from cells using TRIzol® Reagent (Invitrogen Corp., Carlsbad, CA, USA), following the manufacturer's instructions. cDNA was synthesized from 1 μg total RNA with the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). Real-time RT-PCR experiments were carried out with the Universal Probe Library system (F. Hoffmann-La Roche AG, Basel, Switzerland). The following primers were used: CARD18 forward AAGACCCCTCAACTTGCCTCA and reverse CTCCCTTGGAAGAAGCTCTG, (UPL probe number 50); AIM2 forward AGTCCTCTGCTAGTTAAGCTCTCTG and reverse TCTGACAACTTTGGGATCAGC (UPL probe number 87); and caspase-1 forward CCTTAATATGCAAGACTCTCAAGGA and reverse TAGCTGGGTTGTCCTGCACT (UPL probe number 17). PCR assays were performed with the C1000 Touch Thermal Cycler (Bio-Rad Laboratories). The expression of each gene was normalized to the 18S ribosomal RNA gene. Relative mRNA levels were calculated by the ΔΔCt method. Data from different treatments were compared using one-tailed t test. Differences were considered significant when $P \leq 0.05$.

2.4. Immunohistochemistry

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

dissolved in N,N-dimethylformamide, 0.01% H_2O_2, and 0.05 M acetate buffer). Sections were counterstained with hematoxylin for 10 seconds and subsequently analyzed with a Zeiss Axio Imager fluorescent light microscope (Carl Zeiss MicroImaging, Thornwood, NY, USA) fitted with a Carl Zeiss AxioCamMRc camera.

2.5. Immunofluorescence staining

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

2.6. ELISA

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

2.7. Gene specific silencing

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

3. Results

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

There is evidence that psoriatic non-involved epidermal keratinocytes carry molecular and cellular alterations that contribute to the occurrence of psoriatic symptoms. We have recently reported the results of a cDNA microarray experiments in which 61 annotated genes were differentially expressed in psoriatic non-involved and healthy epidermis upon T-cell lymphokine treatment (Szabó et al., 2014). In this experiment, organotypic skin cultures were treated with a cytokine mixture (GM-CSF, IL-3, IFN-ɣ) that has been shown to mimic the effect of psoriatic T-cell lymphokines (Bata-Csorgo et al., 1995). CARD18, identified as a differentially expressed gene in our experiment, encodes a protein that belongs to the COP family. CARD18 is a negative regulator of IL-1β maturation, inhibiting inflammasome activation directly by interacting with pro-caspase-1 (Bauernfeind et al., 2011). To validate the cDNA microarray results, real-time RT-PCR analysis was carried out to measure the abundance of CARD18 mRNA (Fig. 1.) using original RNA samples derived from organotypic skin cultures (healthy epidermis, n=5, and psoriatic non-involved epidermis, n=5). Our results confirmed the cDNA microarray results: we observed a 2-fold elevation of CARD18 mRNA level in psoriatic non-involved epidermis compared to healthy epidermis. Although the basal expression levels were relatively high in the psoriatic non-involved epidermis, it was not further induced in response to T-cell lymphokines. This was in contrast to the findings for healthy skin, where lower basal expression levels were induced with an average 1.65-fold elevation in response to the same treatment.

3.2. CARD18 expression differs in healthy and diseased skin

Using an organotypic skin model, we determined that CARD18 mRNA abundance was higher in psoriatic non-involved epidermis compared to healthy epidermis. To compare CARD18 protein levels in healthy and diseased skin, immunohistochemical (IHC) staining was performed on paraffin-embedded samples from healthy (Fig. 2A), psoriatic non-involved (Fig. 2B) and psoriatic involved skin samples (Fig. 2C) in an independent experiment. In all examined sections, CARD18 appeared exclusively in the epidermis, specifically in the cytoplasm of the epidermal keratinocytes. IHC revealed differential CARD18 expression in different skin samples: elevated levels were observed in the psoriatic-involved and non-involved epidermis compared to the healthy epidermis (Fig 2B and 2C vs 2A). These results

confirmed our real-time RT-PCR findings. No differences were detected in the different keratinocyte layers of the epidermis; however, the level of CARD18 staining was somewhat higher in the suprabasal layers of psoriatic non-involved and involved epidermis. Figure 2D-F provide the data from negative controls for Figure 2A-C.

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

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

3.4. CARD18 is abundantly expressed in differentiating keratinocytes

To examine the characteristics of CARD18 in NHEKs, which are non-professional immune cells, we examined mRNA and protein changes in spontaneously differentiating NHEKs. In spontaneously differentiating third-passage NHEKs, the differentiation process was detected as an increase in a differentiation marker, keratin 10, and the decrease in a proliferation-related marker, alpha5 integrin (data not shown). The 0-day samples were taken from subconfluent cultures. To determine the mRNA expression of CARD18, real-time RT-PCR was carried out using gene-specific primers. The *in vitro* gene-expression study revealed low-level CARD18 mRNA expression in the proliferative state of cells, and this expression

continuously increased during differentiation: up to 43.2-fold elevation in the 10-day samples (Fig. 4A).

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

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

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

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

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

These results encouraged us to further characterize the function of CARD18 in keratinocytes in which inflammatory processes had been induced. CARD18 expression was silenced by siRNA 24 hours before IFN-γ and TNF-α treatment and the subsequent poly(dA:dT) transfection. The applied siRNA effectively down-regulated CARD18 expression to 50% of the levels observed for the scrambled control cells (Fig. 6B).
The CARD18 silencing resulted in a significant decrease in AIM2 gene expression 12 and 24 hours after treatment (Fig. 6C) and significantly reduced caspase-1 mRNA expression (Fig. 6D) 24 hours after the treatment. For further confirmation, we determined whether the silencing of CARD18 had any effect on IL-1β secretion: our results indicated that downregulation of CARD18 expression affected an elevation of IL-1β production in keratinocytes (Fig. 6A). These results indicate that CARD18 might indeed contribute to the fine-tuning of inflammatory processes in keratinocytes and suggest that — similar to the role in professional immune cells (Humke et al., 2000) — functions as a negative regulator of inflammasome activation in keratinocytes.

4. Discussion

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

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

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

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

To gain some information about the function of CARD18 in keratinocytes under inflammatory conditions, we silenced CARD18 expression. CARD18 silencing resulted in a modest but significant elevation of IL-1β secretion in poly(dA:dT)-treated cells, suggesting that, similar to professional immune cells, CARD18 indeed has a negative regulatory role in keratinocyte innate immune functions. Interestingly, reduced inflammasome activation was detected as decreased AIM2 and caspase-1 gene expression in the CARD18 silenced cells. We hypothesize that IL-1β secretion is a highly regulated process both in professional and in non-professional immune cells, such as keratinocytes. Thus, the silencing of CARD18 might up- and down-regulate multiple process simultaneously, resulting in only a mild change in IL-1β secretion. The reduced inflammasome activation data suggest the present of a negative

feedback loop: elevated active caspase-1 down-regulates caspase-1 and AIM2 gene expression through a yet unknown control mechanism. Juruj et al has recently published results supporting this hypothesis: the authors demonstrated that caspase-1 negatively regulated the formation or stability of the AIM2 inflammasome complex, and that this regulation was specific to the AIM2 inflammasome pathway in macrophages (Juruj et al., 2013).

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

Conflict of interest statement

The authors declare no conflict of interest.

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Figures

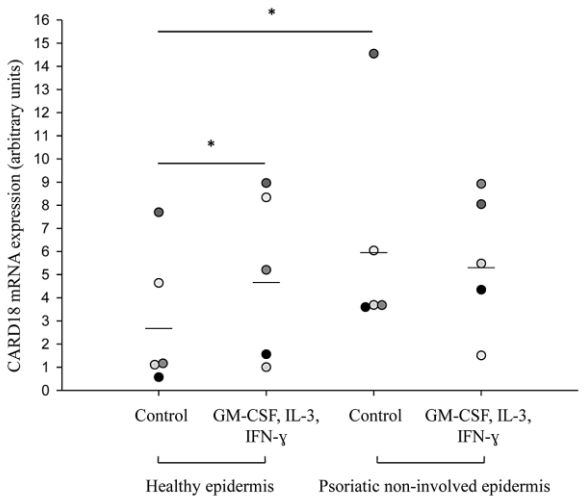

Figure 1. CARD18 gene expression in healthy and non-involved psoriatic epidermis. Total RNA was isolated from the epidermal compartment of organotypic skin cultures (n=5). CARD18 gene expression patterns in response to T-cell lymphokine (GM-CSF, IL-3, IFN-ɣ) treatment in healthy epidermis and in non-involved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

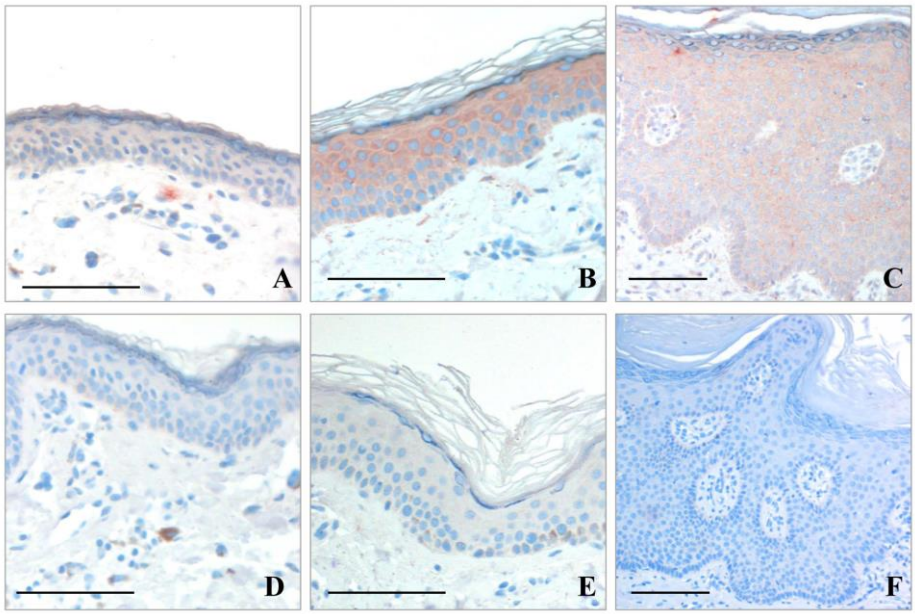

Figure 2. Immunohistochemical staining of CARD18 in healthy and psoriatic skin. CARD18 protein levels were analyzed in healthy (A), psoriatic non-involved (B), and psoriatic involved (C) skin. Negative controls were performed for healthy (D), psoriatic non-involved (E) and psoriatic involved skin (F).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

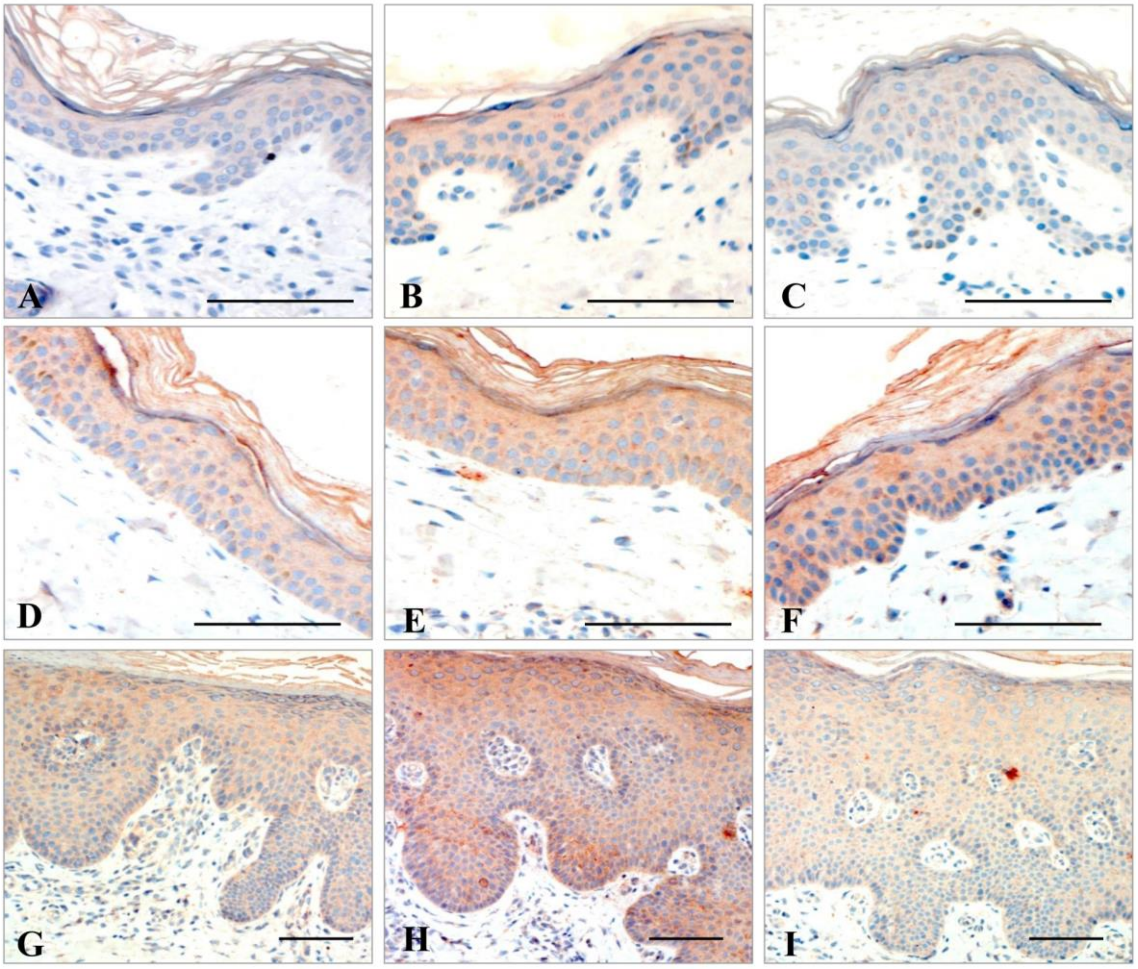

Figure 3. CARD18 expression upon acute barrier disruption. Tape stripping (TS) method was carried out on the skin of healthy (n=2, one representative picture is shown) and psoriatic volunteers (n=2, one representative picture is shown). Punch biopsies were taken at the indicated time points. Skin samples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

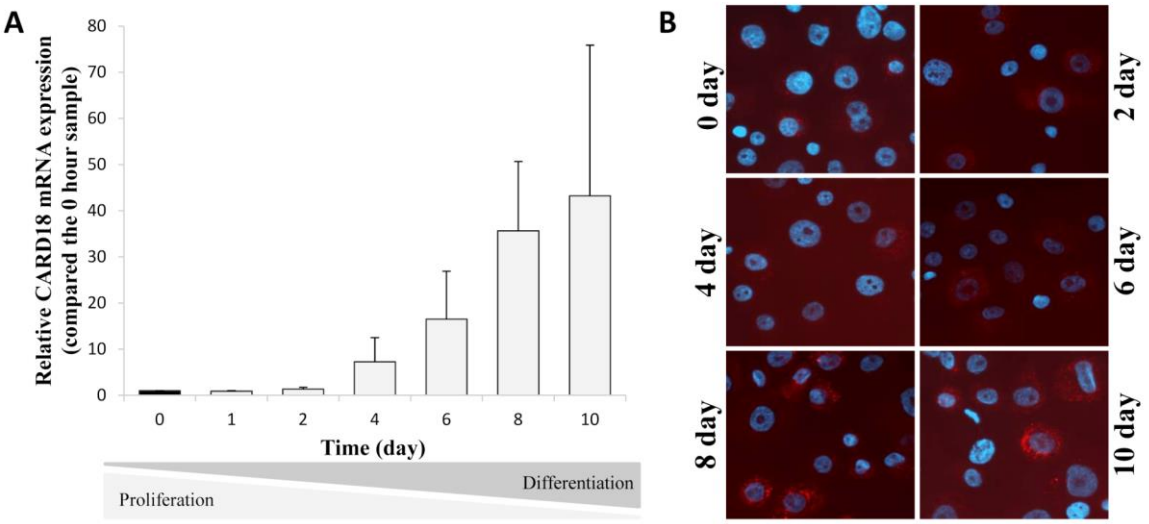

Figure 4. Expression of CARD18 mRNA and protein during keratinocyte proliferation and differentiation. Cultured NHEKs (n=3) were grown to subconfluency before samples were taken (0 day). (A) Changes in CARD18 mRNA expression were analyzed by real-time RT-PCR at the indicated time points. Relative expression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

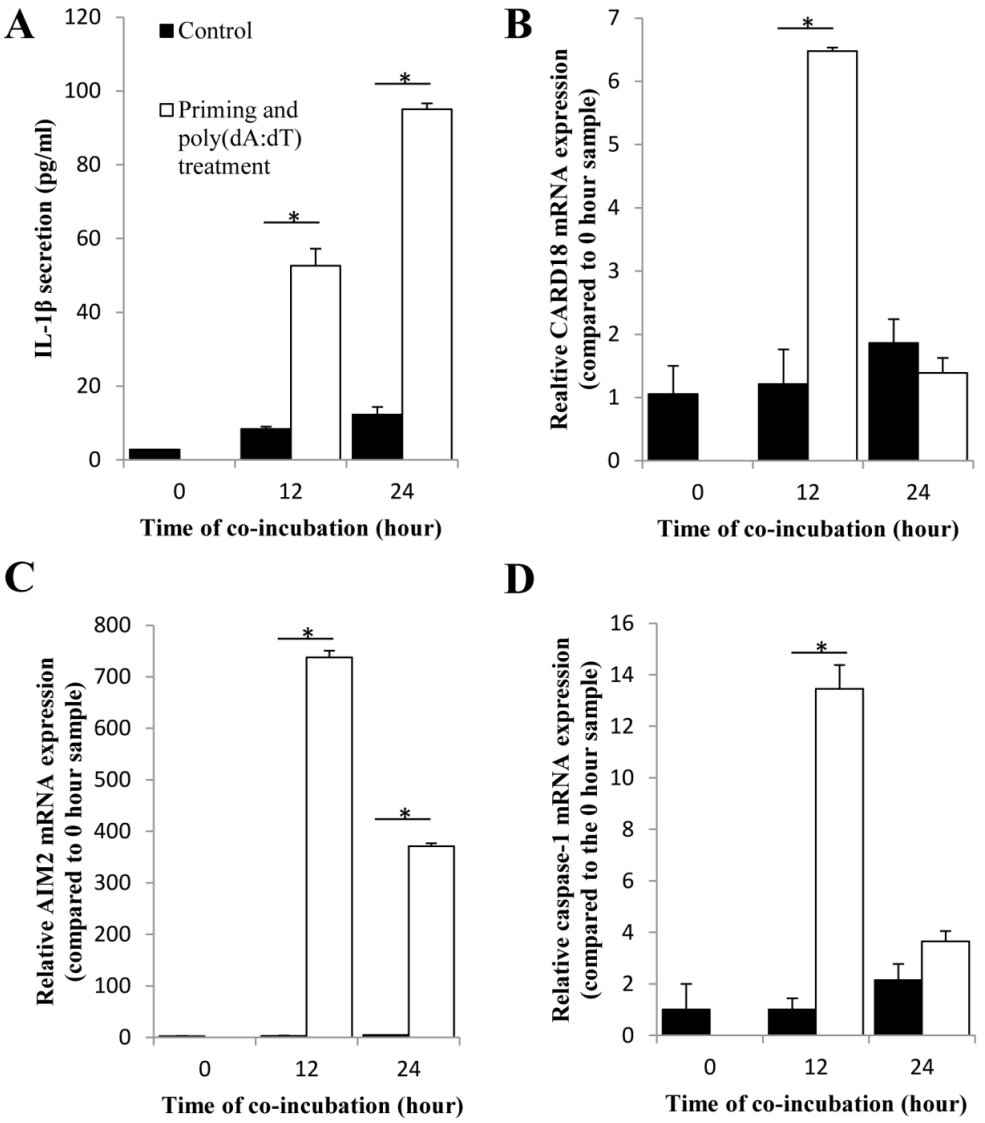

Figure 5. The response of cytokine-primed keratinocytes to synthetic DNA analogue (poly(dA:dT)) transfection. NHEK cultures (n=3) were incubated with IFN-ɣ and TNF-α for 24 hours, followed by poly(dA:dT) transfection. Samples were taken at the indicated time points. IL-1β secretion (A) was measured by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

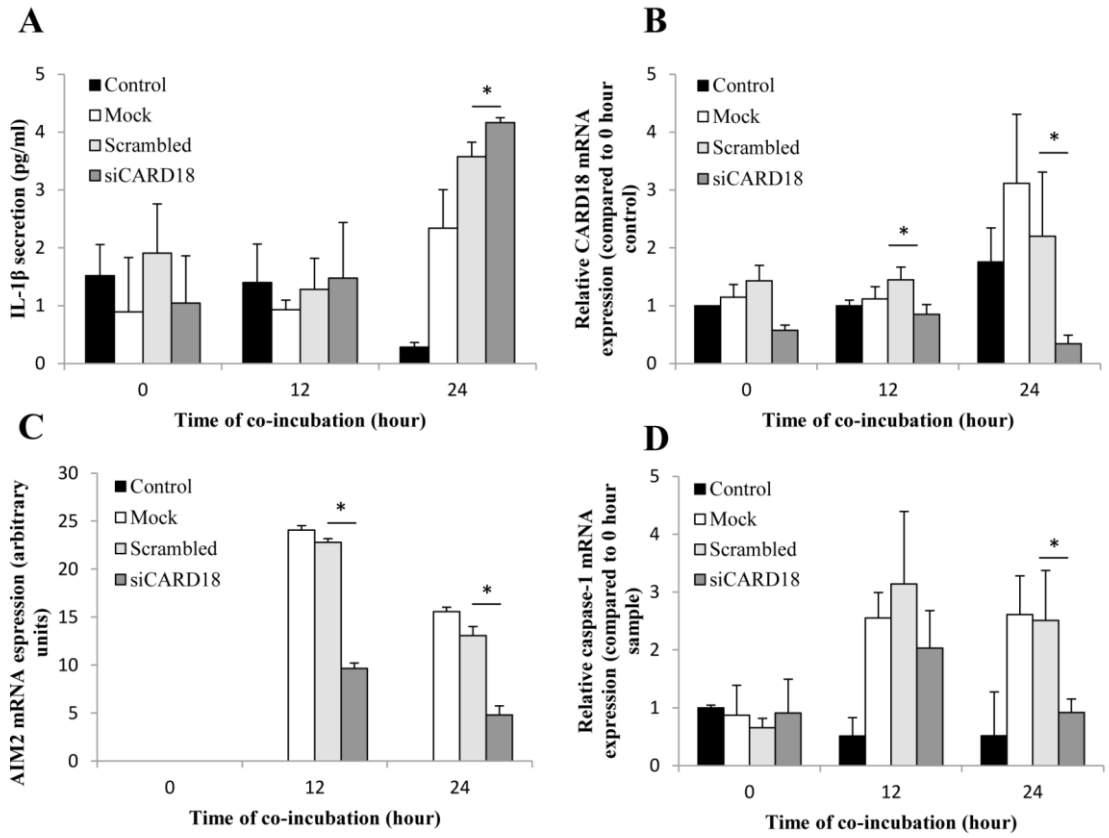

Figure 6. The effect of CARD18 gene-specific silencing in cytokine-primed poly(dA:dT) transfected NHEKs. CARD18 expression was silenced in NHEK cultures (n=3). CARD18 siRNA, scrambled control and mock-transfected cells were subsequently treated with a mixture of IFN-ɣ and TNF-α (12 hours) and transfected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