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CARD14sh-modulation of antiviral response in keratinocytes: Implications in Psoriasis

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II List of abbreviations

ACH	Psoriasis Acrodermatitis Continua of Hallpeau
AIP	Atrophin-interacting protein
AMPs	Antimicrobial peptides
ATG5	Autophagy-related protein 5
BCL10	B cell lymphoma/leukemia 10
CARD	Caspase activation and recruitment domain
CARMA	CARD-containing Membrane-Associated Guanylate Kinase (MAGUK) proteins
CBM	CARD-BCL10-MALT1 complex
CC	Coiled-coil
CCL	C-C motif chemokine
cIAP1	Cellular inhibitor of apoptosis 1
COX5B	Cytochrome c oxidase subunit 5B
CXCL1	C-X-C motif chemokine ligand
DAMP	Damage-associated molecular patterns
DC	Dendrictic cells
DD	Death domain
ER	Endoplasmic Reticulum
FAF1	Fas-associated factor
GPCRs	G protein-coupled receptors
GPP	Generalized Pustular Psoriasis
GWAS	Genome-wide association studies
HaCaT cells	Human Keratinocyte cells
HBD	Human beta-defensin
HEK293 cells	Human embryonic kidney cells
HR1	Hydrophobic heptad repeat region
IFN	Interferon
IKK	IκB kinase
IL	Interleukin
IRAK	IL1R-associated kinase

IRF3	IFN regulatory factor 3
KO	Knocked down
LGP2	Laboratory of genetics and physiology protein
LRRs	Leucine-rich repeats
LTA	Lipoteichoic acid
LUBAC	Linear ubiquitin chain assembly complex
MAGUK	Membrane-Associated Guanylate
MALT	Mucosa-associated lymphoid tissue
MAM	Mitochondrion-associated membrane
MAPK	Mitogen-activated protein kinase
MARCH5	Membrane-associated ring finger 5
MAVS	Mitochondrial antiviral signaling protein
MDA5	Melanoma differentiation-associated gene 5
NF-κB	Nuclear factor kappa B
MHC	Major histocompatibility complex
OPA1	Optic atrophy type 1
OTUD	Ovarian tumor family deubiquitinase
P-IRF3	Phosphorylated IRF3
PAMPs	Molecular patterns related to pathogens
PCBP	Poly(C)-binding protein
PGN	Bacterial peptidoglycan
PKC	Protein kinase C
POLY (I:C)	Polyinosinic-polycytidylic acid
PPP	Psoriasis Pustulosa Palmoplantaris
PRP	Pityriasis Rubra Pilaris
PRRs	Pattern recognition receptors
PsV	Plaque psoriasis or psoriasis vulgaris
SDD-AGE	Semi-denaturing detergent agarose gel electrophoresis
PSORS	Psoriasis-associated susceptibility loci
TANK	TRAF family member-associated NF-κB activator
TLR	Toll-like receptor
Th cell	T helper cell
WT	Wild type

III Resumo

A Psoríase, uma das doenças inflamatórias da pele mais prevalentes em seres humanos, resulta de uma complexa interação entre células imunes inatas e adaptativas que causa uma inflamação persistente. Esta condição tem origem em fatores genéticos e ambientais que desencadeiam um espectro de sintomas que vão desde desconforto leve até manifestações graves, afetando significativamente a qualidade de vida do indivíduo. Um elemento central na resposta imunoinflamatória da pele são os queratinócitos, um tipo de célula da pele. Estas células desempenham um papel fundamental na patogénese da psoríase, participando ativamente tanto na fase inicial como na fase de manutenção desta condição.

Esta tese tem por objetivo o estudo de aspetos moleculares da psoríase, com enfase específico na proteína CARD14 e, em particular, na variante de "splicing" CARD14sh. Esta variante é expressa de forma abundante na pele e está intimamente associada ao desenvolvimento da psoríase. Apesar de sua importância, os mecanismos de regulação celular envolvendo a CARD14sh têm permanecido pouco compreendidos. Além disso, têm sido descritas várias formas mutantes de CARD14sh em pacientes com psoríase, o que motivou este estudo sobre o impacto dessas mutações em características celulares relacionadas esta patologia.

Em particular, esta dissertação centra-se no impacto dos mutantes CARD14sh associados à psoríase em diversas características morfológicas dos queratinócitos evidentes na psoríase. Além disso, investigamos a modulação da via de sinalização TBK1/IRF3 pelo CARD14sh nos queratinócitos, tendo observado o seu efeito supressor nos níveis de P-IRF3 com possíveis implicações na patogénese da psoríase. Para além destes resultados, o nosso estudo revelou uma interação entre o CARD14sh e o MAVS nos queratinócitos, a qual poderá ser da maior importância para a compreensão dos mecanismos de regulação responsáveis pelo desenvolvimento da psoríase.

No conjunto, os nossos resultados contribuem de forma significativa para a compreensão do papel da CARD14sh nos mecanismos moleculares subjacentes a distúrbios inflamatórios da pele, como a psoríase.

Palavras-chave: Queratinócitos, mutantes de CARD14sh, TBK1/IRF3, MAVS, psoríase

IV Abstract

Psoriasis, one of the most prevalent inflammatory skin diseases in humans, arises from a complex interplay between innate and adaptive immune cells, causing persistent inflammation. This condition has its origins in both genetic and environmental factors, causing a spectrum of symptoms ranging from mild discomfort to severe manifestations, significantly affecting the individual's quality of life.

A central element in the skin's immunoinflammatory response are the keratinocytes, a type of skin cell. These cells are key players in the pathogenesis of psoriasis, actively participating in both the initiation and maintenance phases of the condition.

This thesis aims to study the molecular aspects of psoriasis, with a specific emphasis on the protein CARD14 and, more particularly, its splicing variant CARD14sh. This variant is abundantly expressed in the skin and is closely associated with psoriasis development. Despite its importance, the cellular regulatory mechanisms involving CARD14sh have remained poorly understood. Moreover, various mutated forms of CARD14sh have been described in psoriasis patients, motivating our investigation into the impact of these mutations on psoriasis-related cellular characteristics.

In particular, this dissertation centres on the impact of psoriasis-associated CARD14sh mutants on diverse keratinocyte morphological characteristics evident in psoriasis. Furthermore, we investigate the modulation of the TBK1/IRF3 signalling pathway by CARD14sh in keratinocytes, having observed its suppressive effect on the levels of P-IRF3 with potential implications for psoriasis pathogenesis. In addition to these results, our study reveals an interaction between CARD14sh and MAVS in keratinocytes; this may be of utmost importance for comprehending the regulatory mechanisms responsible for the development of psoriasis.

Overall, our data significantly contributes to the understanding of the role of CARD14sh in the molecular mechanisms underlying inflammatory skin disorders such as psoriasis.

Keywords: Keratinocytes, CARD14sh mutants, TBK1/IRF3, MAVS, psoriasis

V Introduction

Psoriasis is one of the most common chronic inflammatory skin diseases in humans [1], [2] characterized by an intricate interplay between the innate and adaptive immune cells [3]. The regulation of all dermal and epidermal elements that maintain barrier integrity in normal physiological conditions is disrupted, resulting in the establishment of a chronic inflammatory skin condition [4], [5].

The development of psoriasis is influenced by a combination of genetic and environmental factors, including smoking, alcohol consumption, UV exposure, diet, injury and infections [6], [7]. The interplay between these factors is considered critical for both the onset and progression of this condition. Psoriasis is characterized by a compromised epithelial barrier function and disrupted tissue homeostasis, stemming from stress or trauma within the epidermis, as well as a dysregulated immune response [8].

This condition involves the activation of the innate immune system driven by endogenous danger signals and cytokines, coexisting with an autoinflammatory perpetuation in some patients and T cell-driven autoimmune reactions in others [9]. Thus, psoriasis shows traits of autoimmune disease on an autoinflammatory background [10], with both mechanisms overlapping and even potentiating one another. The disease is characterized by abnormal T cell activation, particularly in focal skin regions [1], [11]. Keratinocytes, a type of skin cell, play a central role in regulating the immunoinflammatory response by rapidly inducing host defence molecules such as antimicrobial peptides (AMPs) and proinflammatory cytokines.

Psoriasis symptoms can vary in intensity, ranging from minor inconveniences to severe manifestations that significantly affect an individual's overall quality of life [12]. Moreover, this disorder is frequently associated with other health conditions such as diabetes, cardiovascular disease, and arthritis [13].

V.1 Comprehensive overview of psoriasis: clinical, histological, epidemiological features and variants

V.1.1 Clinical features

Psoriasis is a chronic cutaneous inflammatory disorder that manifests with varying morphology, intensity, distribution, and progression. This condition is characterized by the presentation of elevated, clearly defined, oval-shaped red patches and scaly plaques with distinct borders, accompanied by silvery, dry scales on the skin. These features are a result of irregular keratinization and an overgrowth of superficial keratinocytes [14]. Furthermore, the persistence of nucleated cells in the cornified layer, dilation of blood vessels in the dermis, and an inflammatory infiltrate of leukocytes are also characteristics of this pathology [15].

Predicted sites of psoriatic lesions are the elbows, knees, the sacral region as well as the capillitium and nails [1]. However, psoriatic plaques may also develop at sites of mechanical trauma known as Koebner's phenomenon [16], where skin injury can trigger the development of psoriatic lesions in areas that were previously uninvolved.

V.1.2 Histological features

Psoriatic plaques are histologically characterized by the thickened epidermis, so-called acanthosis, in combination with parakeratosis, hyperkeratosis, and elongated red ridges, so called papillomatosis [1]. Acanthosis and papillomatosis are caused by an increased proliferation of basal keratinocytes and a rapid differentiation of suprabasal cells, resulting in abnormal replacement of annular squames with nucleated cells in the stratum corneum (parakeratosis) and a loss of the normal granular layer with thickening of the stratum corneum (hyperkeratosis) [17]. Furthermore, suprabasal psoriatic keratinocytes are senescent, which contributes to the resistance of plaques to apoptosis and transformation [18].

The dermal compartment also exhibits changes in the immune cell composition, with T lymphocytes, DCs and macrophages, as well as a small proportion of neutrophils, mainly found in the tips of dermal papillae [13], [19].

V.1.3 Forms of psoriasis

Psoriasis is a chronic skin condition that is known for its variability in terms of morphology, severity, distribution and anatomical localization [20] as described in Table 1. The major distinctions include plaque and pustular forms, which differ in appearance and the immune cell infiltrate involved.

One of the most common forms of psoriasis is called Plaque Psoriasis or Psoriasis Vulgaris (PsV) , which accounts for 90 % of all cases [9], [15]. This form of psoriasis is characterized by sharply demarcated plaques that are covered in silvery scales, and are erythematous and pruritic in nature. The plaques are most commonly found on the extensor aspects of the elbows and knees, as well as the scalp, though they rarely extend beyond the hairline [15].

Plaque psoriasis is also associated with dilation and an increased number of blood vessels, which facilitate immune cell infiltration and contribute to the chronic nature of the disease. Comorbidities such as cardiovascular disease, Crohn's disease, obesity, and metabolic syndrome have been linked to PsV [21]–[23].

Guttate Psoriasis, which mostly affects children and adolescents, is marked by the formation of small, acute erythematous plaques and around one-third of patients with Guttate Psoriasis eventually develop Plaque Psoriasis as adults [9].

Pustular Psoriasis is another type of psoriasis that is marked by multiple coalescing sterile pustules. This form of psoriasis can be either localized or generalized with neutrophil and monocyte infiltration in the skin tissue being a hallmark of this variation [24]. When it is localized, it can be further classified into two distinct phenotypes: Psoriasis Pustulosa Palmoplantaris (PPP) and Acrodermatitis Continua of Hallpeau (ACH). PPP is restricted to the palms and soles, while ACH affects the tips of the fingers and toes. Generalized Pustular Psoriasis (GPP), on the other hand, presents with an acute and rapidly progressive course, characterized by diffuse redness and subcorneal pustules, and is often accompanied by systemic symptoms such as high fever, fatigue and muscle and joint pain [9].

In addition to PsV, there are several other forms of psoriasis that differ in terms of their morphology, occurrence, or distribution. These forms include Inverse Psoriasis, Psoriatic Erythroderma, Psoriatic Arthritis, and Psoriatic Nail Disease [1], [13], [25]. Each of these forms of psoriasis presents with distinct characteristics and presentations, differentiating them from PsV. These additional forms of psoriasis are important to

consider in the diagnosis and management of patients with psoriasis, highlighting the need for a comprehensive and thorough evaluation of each individual case.

Table 1: Different forms of psoriasis and their features (depicted from [1], [9], [15], [25]).

Type of psoriasis	Occurence	Distribution	Morphology
Psoriasis vulgaris	80 to 90%	Elbows, knees, scalp, trunk	Red plaques covered by silvery scales
Psoriasis guttate	2% post infectious	Trunk	Small, scattered papules
Psoriasis pustuolosa palmoplantaris	Preferably effecting smoking women over 40 years	Palms and soles	Sterile pustules of yellow-brown color with scales
Generalized Pustular Psoriasis	-	The pustules can occur anywhere on the body but often start in localized areas before spreading	Diffuse redness and subcorneal pustules, and is often accompanied by systemic symptoms
Psoriasis inversa	-	Intertriginous sites (inframammary, perineal and axillary)	Red, shiny well- demarcated plaques; w/o scales
Psoriatic erythroderma	-	Entire body surface	Erythema, sterile pustules and scaling
Psoriatic arthritis	25%	Distal interphalangeal joints, dactylitis, calcaneal enthesitis	Seronegative inflammatory arthritis
Psoriatic nail disease	50%	Nails	Small pits in nail plate with oil spots beneath nail plate

V.1.4 Epidemiological features

The global prevalence of Psoriasis is estimated to affect around 2 to 3 % of the worldwide population [26]. However, these vary depending on factors such as region, ethnicity, and country, with rates ranging between 0.2 and 10 % [10], [27]. It's interesting to note that this illness is more common among Caucasians, less frequent in Asians, and rarely observed in Africans [10]. Notably, higher prevalence rates are found in high-income countries, including Australia, Western Europe, Central Europe, and North America. Additionally, although psoriasis affects both genders equally, PPP is an exception, as it predominantly affects women [15], [28].

Two distinct forms of psoriasis have been established: an early-onset form occurring between the ages of 16 and 22 years, and a late-onset form occurring between the ages of 57 and 60 years, with the early onset being more prevalent [29]. The two types of psoriasis vary in both their age of onset and inheritance patterns. Early-onset psoriasis demonstrates a genetic component and is closely linked to the HLA-Cw6 allele, a particular allele of the major histocompatibility complex (MHC). HLA-Cw6 susceptibility allele alone is known to be recurrent in psoriatic patients and is particularly prevalent in those with early-onset psoriasis [29]–[31]. In contrast to the hereditary nature of the early-onset form, the late-onset form of psoriasis is sporadic and lacks a clear familial pattern of inheritance and the presence of HLA-Cw6 is less prevalent in patients with late-onset psoriasis [29], [32].

Furthermore, studies have indicated that individuals with late-onset psoriasis may exhibit unique symptoms compared to those with early-onset psoriasis, including a higher prevalence of anxiety [33]. Although more research is warranted regarding this matter, these findings underscore the significance of recognizing these two forms of psoriasis as distinct subtypes, each demanding tailored treatment approaches.

V.2 The interplay of innate and adaptive immunity in psoriasis

Psoriasis is a complex autoimmune disease that results from the interplay between innate and adaptive immune cells [1], [3]. It is described as a dendritic cell (DC) and T cell mediated illness, with intricate feedback loops involving keratinocytes, vascular endothelial cells, neutrophilic granulocytes, and the cutaneous nervous system [1], [11].

Keratinocytes play a key role in regulating the immunoinflammatory response in this disorder by producing and secreting psoriasis-associated AMPs such as LL-37, S100A7 and β-defensins in response to a mix of environmental factors and potential genetic predispositions [1], [9]. These molecules not only provide immediate protection against pathogens and physical dangers, but also recruit and activate myeloid and lymphatic immune cells, including neutrophils, monocytes, DCs, macrophages, and T lymphocytes, for long-term protection [3], [17]. These diverse components work in concert to create a complex network of cellular and molecular interactions, contributing to the characteristic inflammation, hyperproliferation, and aberrant differentiation of skin cells seen in psoriasis patients.

Keratinocytes are not only responsible for the recruitment of immune cells, but also involved in their activation via the secretion of cytokines, including interleukins (ILs) such as IL-1α/β, IL-6, IL-18, and tumor necrosis factor-α (TNF-α) [1].

A distinct subset of DCs, known as plasmacytoid dendritic cells (pDCs), are recruited from the bloodstream to injured tissue via a process called chemoattraction [35]. Keratinocytes have been identified as key players in initiating the activation and maturation of these immune cells into myeloid dendritic cells (mDCs) by releasing self-nucleotides and AMPs. Moreover, the interaction between the LL37 AMP and these self-nucleotides triggers the activation of toll-like receptors (TLRs), thereby intensifying the inflammatory cascade [34], [35]. In collaboration with keratinocytes, mDCs then generate pro-inflammatory cytokines, including TNF-α, IL-12, IL-23, interferon alpha (IFN-α), and IFN-β [34]. Nevertheless, the activation and maturation of mDCs are not solely triggered by keratinocytes and pDCs. For example, macrophages release cytokines including TNF-α and IFN-γ, as well as chemokines like C-C motif chemokine ligand 19 (CCL19). Additionally, TNF-α produced by natural killer T cells possesses the ability to activate DCs [1]. Upon activation, mDCs travel to the lymph

nodes, where they induce the differentiation of naïve T cells into effector cells such as type 1 helper cells (Th1) and type 17 helper cells (Th17) [36]. These T cells then migrate from dermal blood vessels into the dermis and ultimately into the epidermal compartment. This migration is thought to be facilitated by capillary dilation [36]. In the affected area, T cells release cytokines such as IFN-γ, TNF-α, IL-17A, IL-17F, and IL-22 [37].

Initially, psoriasis was primarily attributed to a Th1-driven immune response. However, the identification of the Th17 cell subset led to a substantial shift in our comprehension of the disease's pathogenesis. The production of IL-17A and the less potent IL-17F cytokines [13] by Th17 cells, as well as IL-17 producer immune cells such as neutrophils, mast cells, and macrophages [1], contributes to the activation of the IL-23/IL-17 cytokine axis. This axis is now considered a key factor in the development of the psoriasis [1], [17], [38]–[40], as it has been shown to be a major activator of cell signalling pathways such as mitogen-activated protein kinase (MAPK) and nuclear factor kappa B (NF-κB) signalling pathways [3], [13].

These proinflammatory mediators stimulate keratinocytes to proliferate rapidly and produce an abundance of chemokines, including C-X-C motif chemokine ligand 1 (CXCL1), CXCL11, and CCL20. This, in turn, stimulates additional keratinocytes and augments the recruitment of immune cells, including neutrophils, T cells, DCs, and macrophages. Consequently, a positive feedback loop is established, where the innate and adaptive immune systems intensify their own response (Figure 1) [3], [17], [41].

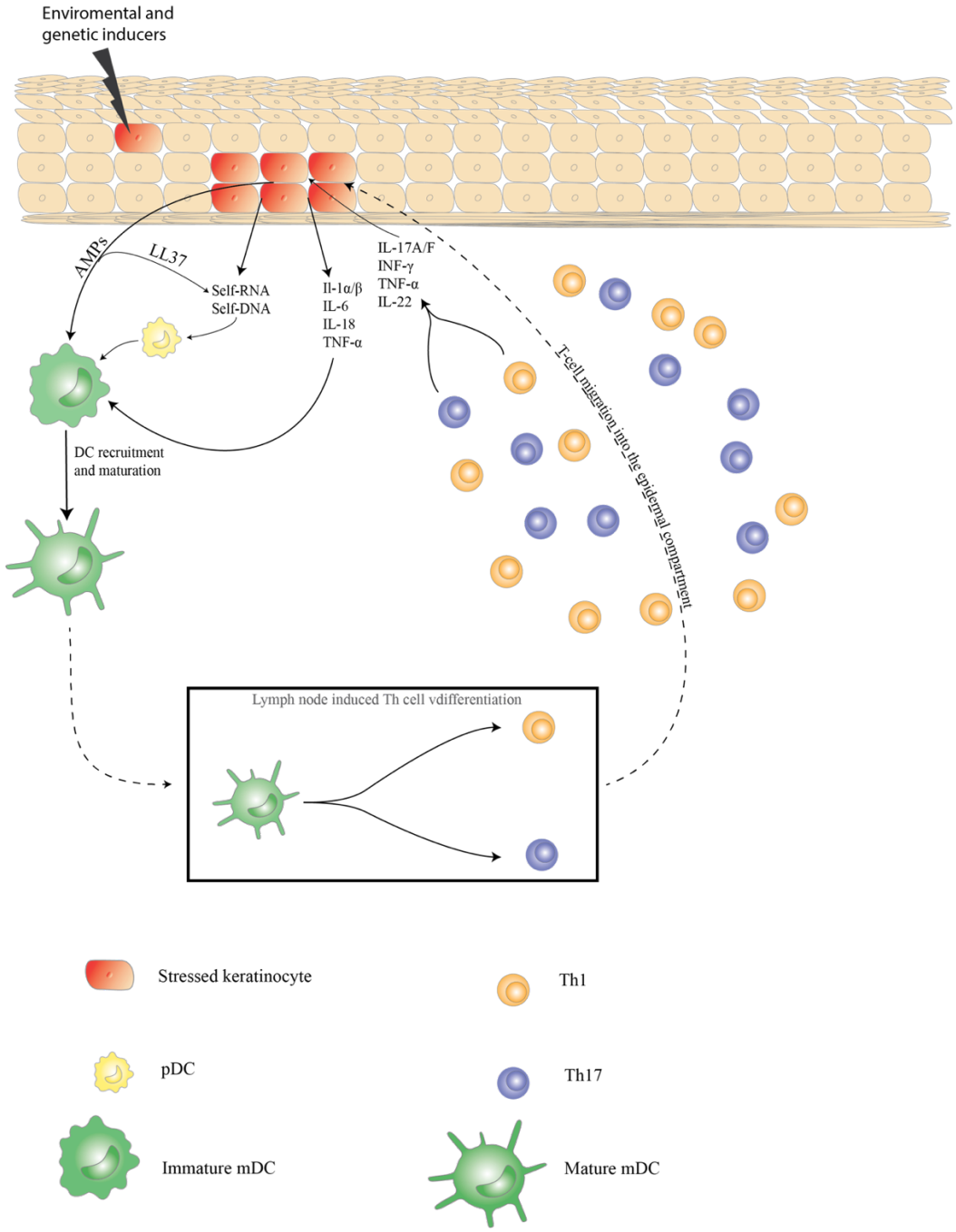

Figure 1: Molecular Mechanisms of Psoriasis Pathogenesis - Genetic predispositions, combined with environmental factors, initiate a positive feedback loop of autoimmune responses. Activated keratinocytes release AMPs such as LL-37, S100A7, and β-defensins, as well as cytokines like IL-1α/β, IL-6, IL-18, and TNF-α. This process leads to the activation of pDCs and the subsequent activation and maturation mDCs. pDCs contribute to this process by secreting IFN-α, which further induces mDC activation and maturation. mDCs then travel to lymph nodes and facilitate the differentiation of naïve T cells into Th1 and Th17 cells through antigen presentation. Th cells migrate to the epidermal compartment, where interactions among keratinocytes, immune cells, and proinflammatory mediators occur (adapted from [1]).

V.2.1 The CARD/BCL10/MALT1 complex

The CARD-BCL10-MALT1 (CBM) signalosomes are universally distributed across almost all tissues, and numerous caspase activation and recruitment domain (CARD) homologues have been identified as participants in these complexes [42]. These signalosomes are activated by a diverse array of stimuli and mediate context-dependent and cell type-specific inflammatory responses. They play a crucial role in preserving tissue homeostasis and defending the host against pathogens. Nevertheless, dysregulation of these signalling pathways and genetic alterations are commonly associated with the abnormal activation of NF-κB which has been associated as a crucial contributor to various pathologies including psoriasis, as the levels of active phosphorylated NF-κB appear to be lower in normal skin when compared with psoriatic skin [43], [44].

NF-κB can be triggered by a wide variety of stimuli, encompassing pro-inflammatory cytokines, environmental stressors, and antigenic stimulation in T and B lymphocytes. Bcl10 plays a pivotal role in promoting NF-κB activation through T and B cell receptors. Supporting this notion, T and B lymphocytes in Bcl10-deficient mice fail to activate NF-κB upon exposure to antigen-receptor stimulation [45]–[47]. Additionally, NF-κB can be triggered both indirectly, through MAPK signaling pathways, and directly, by the involvement of cytokines such as TNF-α and IL-17A, which are recognized as significant inducers of NF-κB. TNF-α enhances the inhibitor of kappa B (I-kB) degradation, while IL-17A triggers the formation of the CBM complex and amplifies the phosphorylation of I-κB subunits [48], [49].

B cell lymphoma/leukemia 10 (BCL10) is a regulatory molecule identified in mucosa-associated lymphoid tissue (MALT) lymphoma [50], BCL10 demonstrates multifunctionality by serving as a crucial initiator of apoptosis and a stimulator of NF-κB activation. Nonetheless, it is important to emphasize that BCL10 overexpression weakly activates pro-apoptotic pathways, yet robustly induces NF-κB activation [50], [51]. BCL10 is composed of two distinct domains: a serine/threonine-rich region located within the C-terminal domain, and an N-terminal CARD domain, known as the primary facilitator of its associated functions. Research suggests that BCL10 forms homotypic CARD-CARD interactions with CARD domain containing proteins such as CARD 9, 10, 11 and 14. These proteins can be found in the CARMA protein family, a

group of scaffold proteins thought to function as upstream regulators in NF-κB signalling [42], [51], [52].

The intramolecular interaction among the CARD, Coiled-Coil (CC) domain, and a flexible linker region maintains the inactivity of CARMA proteins. Their activation process is hypothesized to be consistent due to their similar structural features [42], [53]. Upon phosphorylation by kinases like protein kinase C (PKC), the autoinhibitory conformation of the linker region is disrupted, leading to CARMA protein activation [42]. This change in conformation facilitates oligomerization through the CC domain and enables the recruitment of BCL10 via the CARD domain.

CARD-CARD interactions between CARMA proteins and BCL10 initiate the activation of the CBM signalosome. This complex forms a helical filamentous structure in which CARMA proteins act as substoichiometric nucleation points for BCL10 filaments. The assembly of BCL10 filaments is highly cooperative, with the threshold for assembly becoming more sensitive due to CARMA oligomerization following receptor activation [42], [54].

Though BCL10 is crucial for NF-κB activation, it cannot accomplish this task on its own. Instead, the activation of NF-κB necessitates the formation of a BCL10-mucosa-associated lymphoid tissue 1 (MALT1) complex [45], [55]. MALT1 proteins, which consist of a death domain (DD) and two immunoglobulin (Ig) domains (Ig1/2) at the N-terminus, as well as a paracaspase domain and an Ig3 domain at the C-terminus [42], [51], engage in persistent interactions with BCL10. The presence of a proteolytically active paracaspase domain classifies this protein as a paracaspase. Effective binding between BCL10 and MALT1 relies on the Ig1/2 domains of MALT1. As mutants of MALT1 containing only the Death Domain (DD) or paracaspase domain seem incapable of binding [45], [55]. MALT1 proteins demonstrate two distinct molecular functions that facilitate NF-κB activation: their proteolytic activity and their capacity to act as scaffold proteins [53], [55]. In this discussion, the focus will be on MALT1's role as a scaffold protein.

The induced activation of MALT1 due to the CARD-CARD interactions of CARMA and BCL10 proteins promotes the oligomerization of MALT1 subsequently activating its caspase-like domain [53], [54]. Moreover, phosphorylation of the serine/threonine-rich region in the C-terminus of BCL10 can further mediate this interaction [42].

MALT1 will serve as a scaffold to recruit E3 ubiquitin ligases, such as TNF receptor-associated factor 6 (TRAF6), cellular inhibitor of apoptosis 1 (cIAP1), cIAP2 and linear

ubiquitin chain assembly complex (LUBAC) resulting in K63- and M1-linked ubiquitination of the CBM signalosome [42], [53], [54].
These polyubiquitin chains serve as binding sites for two distinct ubiquitin-binding complexes: the IκB kinase (IKK) complex and the transforming growth factor-β-activated kinase 1 (TAK1). The IKK complex, consisting of two catalytic subunits (IKKα and IKKβ) and a regulatory subunit IKKγ, also known as NEMO/FIP3/IKKAP1 [55]. IKK interacts with polyubiquitin chains via the ubiquitin-binding domain of its IKKγ subunit. Conversely, TAK1 forms connections with polyubiquitin chains by employing adapter proteins known as TAK-binding proteins 2 and 3 (TAB2/TAB3). The ubiquitination of NEMO and the TAK1-mediated phosphorylation of the IKKβ subunit activate the IKK complex. Upon activation, the IKK complex phosphorylates NF-κB inhibitory protein IκBα, prompting its proteasomal degradation. As a result, the NF-κB dimers, which were previously bound to IκBα, are released. These freed NF-κB transcription factors then transiently translocate to the nucleus and initiate the transcription of several genes, including psoriasis-associated genes (Figure 2) [42], [53].

V.2.2 Regulation of the CBM by CARD14/CARMA2 in Keratinocytes

Lymphocytes display the CARD-CC member CARD11/CARMA1 which transmits signals from the B cell T cell or activating Natural Killer cell receptors to MALT1. Meanwhile, CARD14/CARMA2 and CARD10/CARMA3 are expressed in non-hematopoietic tissues. The CARD14/CARMA2 protein is primarily found in keratinocytes and seems to have a significant role in the development of psoriasis. This is due to its capacity to activate the NF-κB pathway in cells, which is triggered by the stimulation of downstream receptors like IL-17R, as well as pattern recognition receptors (PRRs) such as TLRs [42], [53], [56].
CARD14/CARMA2sh is the most predominantly expressed isoform, which positively regulates NF-κB activity and has been linked to human inflammatory diseases including psoriasis [57]. Although the molecular signalling events downstream of the CARMA2sh/CARD14-BCL10-MALT1 signalling complex have not been delineated in keratinocytes, it is likely that they share similarities with those downstream of other well-studied CARMA proteins, such as CARD11/CARMA1 [54] (Figure 2).
Notably, CARD14/CARMA2sh exhibits unique properties compared to the other CARMA isoforms, as it depends on a functional TRAF2 adaptor protein for NF-κB

activation [68]. Moreover, the CBM complex activity of CARD14/CARMA2sh can be modulated through various mechanisms, including phosphorylation by the serine/threonine kinase Unc-51-like autophagy activating kinase 2 (ULK2). This phosphorylation event initiates the degradation of BCL-10 and modifies the ubiquitination states of MALT1 and NEMO via the action of the E3 ubiquitin ligase Ring Finger protein 7 (RNF7) [58].

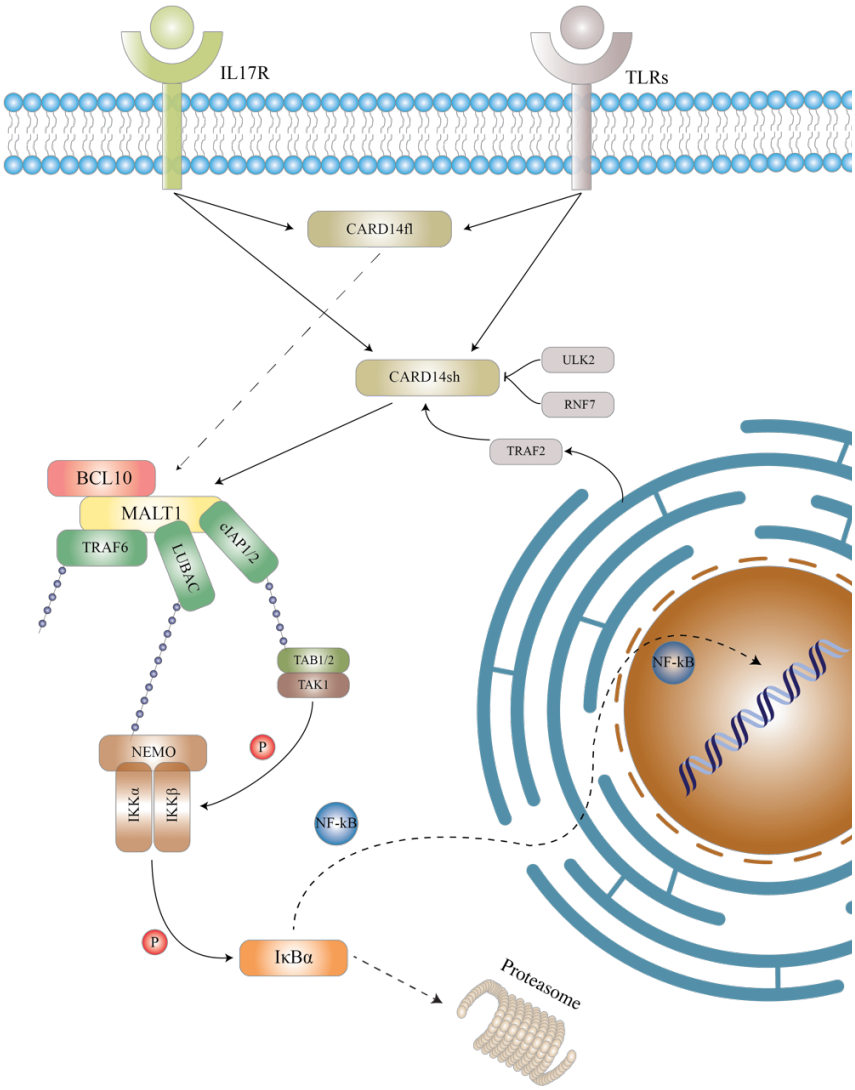

Figure 2: Molecular mechanisms of CBM complex activation by CARMA proteins - Upon activation of receptors, such as TLRs or IL-17R, kinase-mediated phosphorylation triggers the oligomerization of CARD14/CARMA2 and CARD14/CARMA2sh proteins. This conformational change enables the active CARD14 domain to recruit BCL10. BCL10 and MALT1 then translocate to the CARD14/CARMA2 protein site, forming the CBM signalosome. MALT1 serves as a scaffold to recruit E3 ubiquitin ligases, including TRAF6, cIAP1, cIAP2, and LUBAC, resulting in K63- and M1-linked ubiquitination of the CBM signalosome. These polyubiquitin chains provide binding sites for the NEMO subunit and the TAB2/3 adaptor proteins of TAK1. Ubiquitination of NEMO and phosphorylation of IKKβ by TAK1 activate the IKK complex. Upon activation, IKK phosphorylates IκBα, marking it for proteasomal degradation. This, in turn, triggers the liberation of NF-κB transcription factors, allowing them to translocate into the cell nucleus. In the nucleus, NF-κB initiates its crucial transcriptional functions. (adapted from [42], [58]).

V.3 The CARMA family of scaffold proteins

V.3.1 Scaffold proteins

Scaffold proteins are a diverse group of molecules that are central to cellular signalling pathways. These proteins serve as molecular scaffolds, enabling the assembly of large, multi-protein complexes that are required for the integration and transmission of signals from the extracellular environment to the intracellular space. The fundamental function of these proteins is to bind to at least two other signalling proteins and bring them into proximity, facilitating their interaction and promoting the formation of signalling complexes. Although scaffold proteins do not typically possess enzymatic transcriptional activity, they are essential for the efficient and selective activation of downstream signalling pathways [59].

One extensively studied role of scaffold proteins is their involvement in the activation of the NF-κB transcription factor, which exhibits ubiquitous expression in various cell types, including keratinocytes [42]. Among these scaffold proteins, the CARMA family has been shown to play a pivotal role in NF-κB-mediated activity, particularly during psoriasis [52], [58].

V.3.2 CARMA family of scaffold proteins

The CARD-containing Membrane-Associated Guanylate Kinase (MAGUK) proteins, also known as CARMA proteins, form a family of scaffold proteins that exhibit a high degree of homology in their amino acid sequences. This family is composed of three members: CARD11/CARMA1 (CARD11), CARD14/CARMA2 (CARD14), and CARD10/CARMA3 (CARD10), encoded by three genes on chromosomes 7, 17, and 22 in humans. Members of this protein family are scaffold proteins that are involved in a diverse array of cellular processes including cellular adhesion, signal transduction and cell polarity control [54].

These proteins have a similar structure, including a CARD domain, a CC region, and a MAGUK domain, consisting of PDZ, SH3, and GUK modules [58] (Figure 3).

However, despite its structural homology, each CARMA protein has distinct expression patterns.

CARD11 is essential for T and B cell activation, as shown by mouse studies where its genetic elimination caused severe disruption of antigen receptor-driven proliferation and cytokine production [60]. Additionally, CARD11 expresses primarily in lymphoid cells and hematopoietic tissues where it manages NF-κB via JNK signalling activated by antigen/Bcl10 stimulation of lymphocytes, setting it apart from CARD14 and CARD10 [61].

CARD14 remains the least characterized of the CARMA protein family and has a broad expression profile. In healthy skin, CARD14 is primarily abundant in placenta and keratinocytes of the basal layer of the epidermis, compared to psoriatic skin lesions that have increased levels of CARD14 in the upper layers of the epidermis and reduced CARD14 levels in the basal layer [47], [62]. CARD14 is unique among the CARMA protein family members as it is the only one known to have alternatively spliced isoforms. These isoforms are obtained through CARD14 mRNA splicing processes, each with different biological functions and distributions [62], [63]. Three known CARD14 isoforms have been described in humans so far: CARD14fl (full-length, 1,004 amino acids) with all typical CARMA domains and modules, CARD14sh (short, 740 amino acids) lacking SH3 and GUK modules, and CARD14cl (cardless, 434 amino acids) containing only a portion of the CC domain and the linker region and a PDZ module [58], [64].

Functionally, the distinctions between CARD14fl and CARD14sh remain largely undetermined, as both can engage in CARD-CARD interactions with BCL-10 and contribute to the CBM complex formation. In contrast, CARD14cl, which is minimally expressed, is unable to positively regulate NF-κB due to the absence of the CARD domain. This deficiency obstructs the formation of the CBM complex [58], [62].

CARD10 displays a broader non-hematopoietic expression pattern compared to CARD14. Its activation of NF-κB occurs through regulation following stimulation of G protein-coupled receptors (GPCRs) by various ligands, including angiotensin II, endothelin I, and lysophosphatidic acid [57], [63].

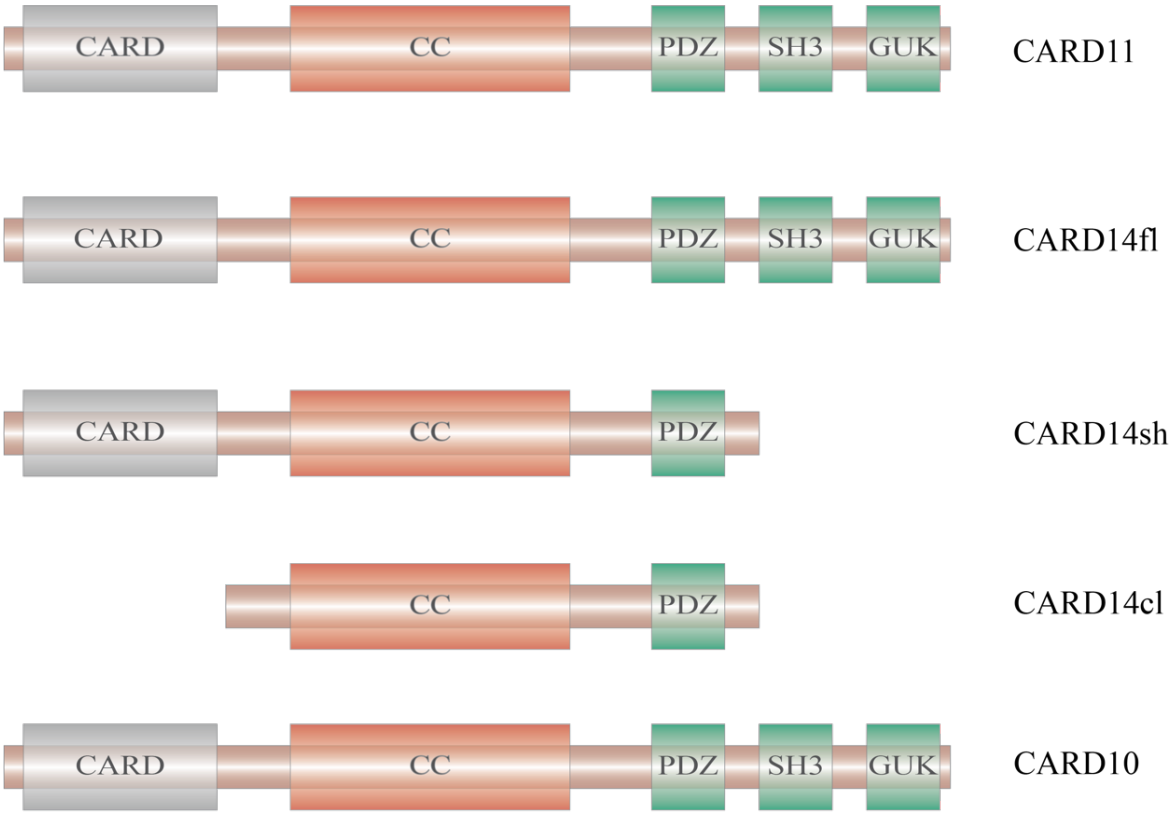

Figure 3: The CARMA Scaffold protein family and their respective domain organization - CARD11 features a CARD11 protein in its N-terminal domain, followed by a CC region and a complete MAGUK domain comprising three subunits: PDZ, SH3, and GUK. CARD14fl shares a structural resemblance with CARD11/CARD10; however, it contains a CARD14 protein in its CARD region. On the other hand, the CARD14 isoforms, CARD14sh and CARD14cl, both lack the SH3 and GUK subdomains. Additionally, CARD14cl is devoid of the CARD region. CARD10 presents a structure homologous to both CARD11 and CARD14fl, differing only in its CARD region, which houses a CARD10 domain (adapted from [65], [66]).

V.4 Mutations in psoriasis

The mode of inheritance of psoriasis is complex. Genome-wide association studies (GWAS) of the human genome have identified various susceptibility loci collectively referred to as psoriasis-associated susceptibility loci (PSORS), ranging from PSORS1 to PSORS16, which have been linked to the hereditary transmission of psoriasis [14]. To date, over 80 such loci have been identified, but the complete gene mapping of the genes responsible for these susceptibilities has not yet been uncovered [3], [62], [67]. In recent years, researchers have identified at least 100 genes associated with psoriasis susceptibility, many of which play a role in adaptive immunity, innate immunity, and skin barrier function [14], [68], [69].

Among the identified loci, PSORS1 stands out as the primary determinant of psoriasis susceptibility, accounting for approximately 35 to 50 % of the disease's heritability. It is located within the MHC region which includes the HLA-Cw6 susceptibility allele [70]. Despite the frequent occurrence of HLA-Cw6 among individuals with psoriasis, current immunogenetic and epidemiological evidence suggests that merely possessing the HLA allele is often insufficient for developing the condition [71]. This highlights the importance of additional genetic and environmental factors in the manifestation of the disease.

V.4.1 CARD14 mutations in psoriasis

Numerous non-MHC susceptibility loci also have been identified by linkage/association approaches. PSORS2 on chromosome 17q25 was the first identified as non-MHC locus that confers susceptibility to psoriasis [70]. Mutations within the CARD14 gene have been recognized as a significant factor in the development of psoriasis. These CARD14 gene mutations are associated with the PSORS2 [72], [73]. It is should be point out that, the presence of the HLA allele is not consistently observed in patients who carry these mutations [74], [75].

Numerous potential pathogenic mutations have been discovered in CARD14. Initially, 23 CARD14 gene variants were identified, predominantly in patients with PsV [76]. Several studies identified these CARD14 variants and others [77]–[80]. These variants have been identified in exons 2, 3, 4, 6, 7, 9, 13, 15, 18, and 21, with exons 3 and 4,

which partially encode the CARD and CC domains, being identified as a prominent hotspot, accounting for 63 % of documented variants [75].
Research has demonstrated that distinct mutations in the CARD14 gene can lead to a diverse range of CARD14 protein variants and diverse dermatological manifestations in individuals with psoriasis [73], [74], [81] such as PsV, Pityriasis rubra pilaris (PRP), and Psoriatic arthritis [58]. In addition to the association between various CARD14 variants and distinct disease entities, it is important to emphasize that certain CARD14 mutations have been linked to multiple forms of psoriasis skin disorder. For example, two CC domain-related variants (exon 4) exhibit this phenomenon: the p.D176H variant has been reported in patients with PsV, PPP, GPP, and PRP type V. Similarly, the E138A variant has been identified in individuals with both PsVand GPP [75].

V.4.1.1 CARD14 mutations and NF-κB regulation

Mutations in CARD14 often lead to its aggregation and hyperactivation, leading to increased formation of the CARD14-BCL10-MALT1 complex in keratinocytes [3], [82], [83]. This, in turn, triggers constant activation of the NF-κB pathway and elevated expression of inflammatory cytokines, chemokines, and AMPs. The regulation of NF-κB activity seems to exhibit significant variation among CARD14 mutants. For instance, mutants such as p.E138A, p.E138del, p.E142k/G, p.G117S, and p.D176H, are found to enhance its activity. In contrast, a set of mutants, namely p.R69W, p.R151Q, p.S200N, p.L209P, p.H171N, and p.T420A, appear to inhibit its activity. Interestingly, there are mutants, including p.R38C, p.R68Q, p.V191L, p.D285G, p.M338V, p.I593N, p.S602L, p.R682W, and p.G714S, that seem to either have no discernible effect or only minimally impact NF-κB activity [75] (Table 2).

Table 2: CARD14 variants and their ability to regulate NF-κB [75].

Protein Change	CARD14 domain	NF-κB reporter	Pathogenic signature upon O/E	Other functional testing
R38C	CARD	0.11	no	
R62Q	CARD	1.06	no	
R69W	CARD	0.144	nd	
G117S	Between CARD-CC	3.71	yes	- over expressed in EC in psoriatic skin - no spontaneous oligomerization - increased interaction with MALT1 and BCL10, increased ERK/p38 activation
M119R	Between CARD-CC	nd	nd	
M119T	Between	nd	nd	
	CARD-CC			
M119V	Between	nd	nd	
	CARD-CC			
L124P	Between CARD-CC	nd	nd	
C127S	Between CARD-CC	nd	nd	
Q136L	CC	nd	nd	
E138K	CC	nd	nd	
E138A	CC	8.95	yes	- increased CARD14 staining and proinfl. gene expression in lesional skin - over expressed in EC in psoriatic skin - spontaneous CARD14 oligomerization - increased interaction with MALT1 and BCL10, increased ERK/p38 activation
E138del	CC	≈2.5	yes (mouse)	- spontaneous CARD14 oligomerization - increased interaction with BCL10
E142G	CC	5	yes	
E142K	CC	4.03	yes	- increased interaction with MALT1 and BCL10, increased ERK/p38 activation
L150R	CC	1.79	no	
R151Q	CC	0.576	nd	
R151W	CC	1.766	nd	
L156P	CC	≈1.2	nd	- spontaneous CARD14 oligomerization
Q157P	CC	nd	nd	
R166H	CC	nd	nd	
H171N	CC	0.68	no	- increased interaction with MALT1 and BCL10, increased ERK/p38 activation
D176H	CC	2.78	no	- spontaneous CARD14 oligomerization
R179H	CC	1.38	no	
V191L	CC	1.02	no	
E197K	CC	1.667	nd	
S200N	CC	0.67	no	
L209P	CC	0.575	nd	
A216T	CC	nd	nd	
L228R	CC	1.5	nd	
D285G	CC	1.14	no	
M338V	CC	0.914	nd	
T420A	linker	0.663	nd	
E422K	linker	nd	nd	
R430W	linker	nd	nd	
R547S	linker	1	no	
V585I	linker	nd	nd	
T591M	PDZ	nd	nd	
I593N	PDZ	1.3	no	
S602L	PDZ	1.1	nd	
R682W	SH3	0.95	no	
G714S	SH3	1.02	no	
S802R	none	nd	nd	
R820W	GUK	nd	nd	
D973E	none	nd	nd	

Concerning the CARD14sh isoform, two notably penetrant point mutations, Gly117Ser and Glu138Ala [76], [78], seem to disrupt the auto-inhibitory conformation, subsequently stimulating NF-κB activity. Notably, the Glu138Ala and Glu142Gly mutations possess the ability to circumvent the negative regulation imposed by ULK2 and RNF7, emphasizing their distinctive attributes in connection with psoriasis [58]. Moreover, psoriasis patients have been found to harbour mutations, such as CARD14sh Arg38Cys, Arg69Trp, Arg151Trp, His171Asn, Ser200Asn, Ala216Thr, Thr420Ala, which do not seem to influence NF-κB activation [58].

This regulatory diversity emphasizes the complexity of NF-κB activity regulation among different CARD14 mutants and their subsequent influence on the psoriasis phenotype. It further implies that these mutations may employ regulatory mechanisms beyond NF-κB signalling, thus necessitating additional research to fully comprehend these intricate processes.

V.5 The significance of TLRs in the pathogenesis of psoriasis

The skin protects the body both by working as a physical barrier and as an immunologically active site. Consistent interaction with the environment results in ongoing exposure to pathogens and environmental stressors, therefore demanding a prompt and adaptable innate immune response.

In the event of infection or injury, pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) are released by either the invading pathogen or the damaged host cells. These molecular patterns may consist of pathogenic or host-derived nucleic acids, proteins, lipids, or lipoproteins, which are specifically identified by cytosolic or membrane-bound pattern recognition receptors (PRRs). This recognition process initiates a customized downstream signalling cascade, leading to the production of AMPs and pro-inflammatory cytokines [7], [84], [85]. As a result, various immune cells, including neutrophils, monocytes, DCs, macrophages, and T lymphocytes, are drawn to the affected site, contributing to an effective immune response [7].

In psoriasis, an overexpression of PRRs has been observed including the TLR family. TLRs are the most extensively studied class of innate immune receptors, serving a crucial role in skin-based defense mechanisms against bacterial, fungal, and viral pathogens [85]. These receptors are located in a variety of skin cells. In the epidermis, they can be found in keratinocytes and Langerhans cells. While in the dermis, they appear in macrophages, DCs, T, B cells and mast cells. Furthermore, they are present in skin blood vessel cells and supportive cells such as fibroblasts and adipocytes [86]. Each of these cells exhibits a distinct expression pattern of these receptors, resulting in a unique immune response and specialized recognition of microorganism components and serve as key molecules to recognize PAMPs and DAMPs and to initiate downstream innate immune host response [7]. This complex interplay of TLRs highlights their importance in orchestrating cutaneous immune responses.

V.5.1 The structure of TLRs

All TLRs are produced in the endoplasmic reticulum (ER), transported to the Golgi apparatus, and then directed to either the cell surface or intracellular compartments such as endosomes [87]. The human TLR family comprises 10 members (TLR1-TLR10) [87], [88], each with an identified chromosomal location. TLR1, TLR6, and TLR10 reside in the 4p14 region, while TLR2 and TLR3 are located at 4q32 and 4q35, respectively. TLR4 is found in the 9q32-33 region, and TLR5 at 1q33.3. TLR7 and TLR8 are positioned on the X chromosome at Xp22, and TLR9 is located at 3p21.3 [88], [89].

TLRs comprise single-pass type I transmembrane proteins that consist of three domains: an extracellular domain, a single transmembrane domain, and an intracellular domain [7], [90]. The extracellular domain of TLRs comprises multiple leucine-rich repeats (LRRs) that play a crucial role in recognizing specific PAMPs and DAMPs. This recognition occurs as either a homo- or heterodimer, in conjunction with a coreceptor molecule [7]. Meanwhile, the intracellular domain shares similarities with the IL-1 receptor, and is thus referred to as the Toll/IL-1 receptor (TIR) domain [90]. This domain is responsible for transmitting signals from the extracellular region to downstream signalling components [87]. Additionally, the transmembrane domain of TLRs not only anchors these receptors to the membrane but also plays a crucial role in the oligomerization of membrane proteins [91].

V.5.2 TLRs specific ligands and their interactions

Each TLR can specifically interact with various PAMPs or DAMPs, including lipopeptides, lipopolysaccharide, bacterial flagellin, double-stranded RNA (dsRNA), single-stranded RNA (ssRNA), and DNA. These interactions initiate a series of intracellular signalling events, ultimately triggering innate immune responses. Based on their cellular location and PAMP recognition abilities, TLRs can be divided into two subgroups. The first subgroup of TLRs is situated on the cell plasma membrane and includes TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10. These TLRs are responsible for identifying various components from gram-positive bacteria or mycoplasma cell walls, as well as protein derivatives from damaged host cells. The TLR2-TLR1 complex is able to recognize bacterial lipopeptides such as Pam3CSK4 (tripalmitoyl-S-

glyceroCys-Lys4), while the TLR2-TLR6 complex detects bacterial peptidoglycan (PGN), lipoteichoic acid (LTA), and diacylated lipopeptides like macrophage-activating lipopeptide-2 (Malp2). This selective binding facilitates the recognition of a variety of Gram-positive bacterial molecules. TLR4, in collaboration with its extracellular partner CD14, recognizes lipopolysaccharide, a cell wall component of gram-negative bacteria. TLR5 is capable of detecting flagellins from both gram-positive and gram-negative bacteria. The ligands for TLR10 have yet not been identified. The second subgroup includes TLR3, TLR7, TLR8, and TLR9, expressed in intracellular compartments like endosomes. These TLRs detect nucleic acids from viruses, bacteria, or damaged cells. TLR3 recognizes viral dsRNA, TLR7 and TLR8 identify ssRNA which is typically found during viral replication, and TLR9 detects unmethylated unmethylated deoxycytidylphosphate-deoxyguanosine (CpG) DNA motifs in bacterial and viral genomes [7], [88], [90].

V.5.3 TLRs signalling pathway

Upon recognizing PAMPs, the TLRs initiate the dimerization of their intracellular TIR domains. Each TLR selectively recruits specific TIR domain-containing adapter proteins, such as myeloid differentiation factor-88 (MyD88), TIR domain-containing adaptor protein (TIRAP), TIR domain-containing adaptor inducing interferon-beta (TRIF), and TRIF-related adaptor molecule (TRAM). These proteins assist in recruiting MyD88, which is essential for this process. TIRAP is specifically recruited during TLR2 and TLR4 activation, while TRIF and TRAM are involved in TLR3 and TLR4 activation.
TLR signalling can be classified into two separate pathways based on the specific adapter usage: those that rely on MyD88 and those that depend on TRIF.

V.5.3.1 MyD88-dependent pathway

Upon activation by their corresponding ligands, the TLR's TIR domain recruits MyD88, which in turn attracts various signalling molecules, including the IL1R-associated kinase (IRAK) family, such as IRAK1, IRAK2, IRAK4, and IRAK-M. These molecules form a complex known as the Myddosome [70]. This complex facilitates the induction of TRAF6 through the recruitment of E3 ubiquitin ligase ubiquitin-conjugating enzyme UBC13, and UEV1A. Working in tandem with TAB1/2, TRAF6 promotes K63-linked

polyubiquitination of both itself and the TAK1 protein complex. Additionally, the Myddosome enables the activation and autophosphorylation of IRAK1/4. Subsequently, the IRAK kinases dissociate from the Myddosome, and IRAK1 associates with TRAF6. The E3 ubiquitin ligase, UBC13, and UEV1A work together to facilitate K63-linked polyubiquitination of both TRAF6 and the TAK1 protein kinase complex. TAK1 can then interact with the IKK and the MAPK family, leading to the activation of NF-κB and AP-1 transcription factors, respectively. Moreover, the activation of endosomal TLRs7/8/9 not only instigates the activation of TAK1 through MyD88 recruitment but also stimulates the activation of TRAF6/TRAF3. This sequence of events ultimately facilitates the activation of IFN regulatory factor 7 (IRF7), mediated by IKKα. The subsequent nuclear translocation of this transcription factor fosters the transcription of IFNα and IFNβ type I IFNs [7], [86], [87], [92].

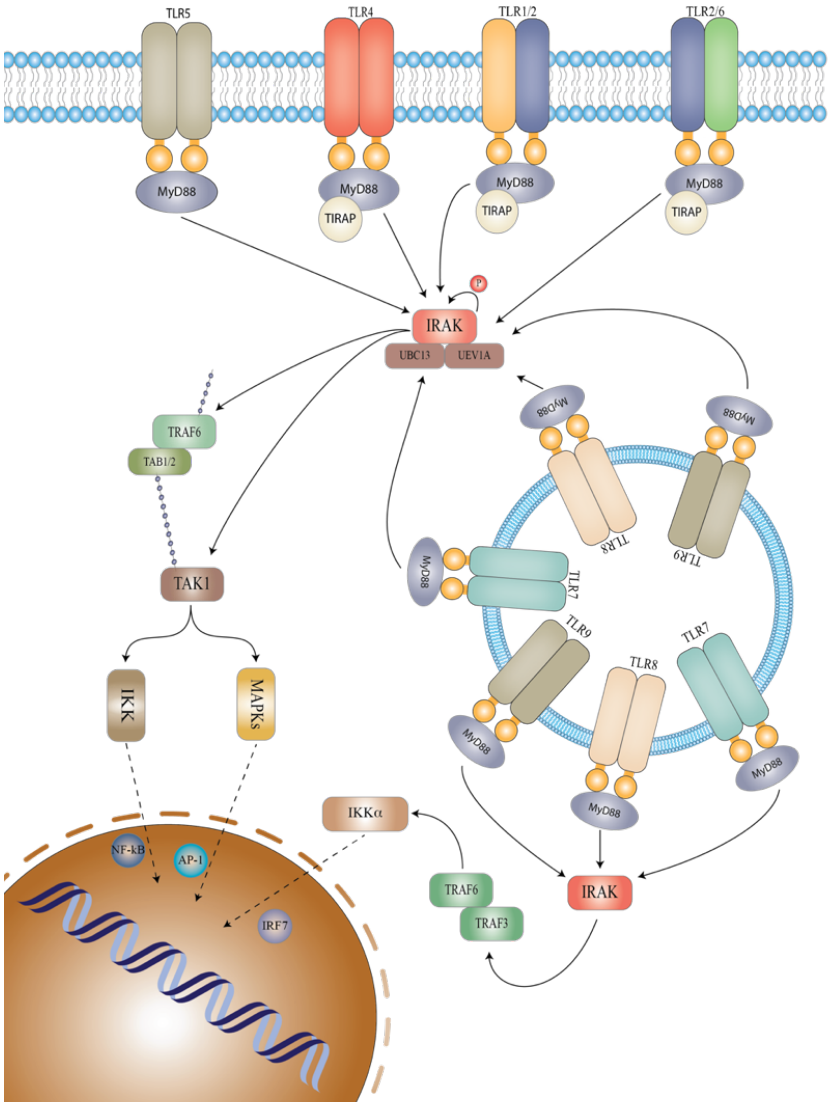

Figure 4: MyD88-dependent TLR signal transduction pathway - Upon encountering PAMPs, membrane-bound TLRs are activated: TLR2-1 responds to lipopeptides, TLR2-6 to PNGs, LTAs, and Malp2, TLR4 to lipopolysaccharides, and TLR5 to flagellins. Similarly, endosomal TLRs, TLR7/8 and TLR9, are triggered by DAMPs, recognizing ssRNA and CpG motifs respectively. Following the activation by their corresponding ligands, the TIR domain of the TLRs engages with the MyD88 adapter protein. MyD88 then recruits the IRAK family of kinases, including IRAK1, IRAK2, IRAK4, and IRAK-M, to form the Myddosome. This complex further enlists the E3 ubiquitin ligases, UBC13 and UEV1A, which facilitate the recruitment of TRAF6 and TAB1/2. Subsequently, TRAF6 promotes K63-linked polyubiquitination of the TAK1 complex. This sequence of events enables the activation of TAK1, which then interacts with the IKK complex and the MAPK family. This interaction triggers the activation of NF-κB and AP-1 transcription factors, respectively. Additionally, TLRs7/8/9 are also able to recruit TRAF6/7 which will induce IKKα-mediated activation of IRF7 transcription factor (adapted from [7], [86]).

V.5.3.2 TRIF-dependent pathway

Upon activation by their respective ligands, TLR3 or TLR4 are able to modulate the activity of NF-κB and AP-1 transcription factors. However, it is particularly crucial for the activation of IFN regulatory factor 3 (IRF3), which leads to the production of IFN-α/β.

Upon TIR activation, TRIF is recruited by TRAM, followed by the recruitment of TRAF6 and/or TRAF3. TRAF6 recruits the receptor-interacting protein 1 (RIP1) kinase, which interacts with and activates the TAK1 complex, leading to the activation of NF-κB and MAPK pathways. Conversely, TRAF3 recruits IKK-related kinases TANK-binding kinase 1 (TBK1), inhibitor of κB kinase ε (IKKε) and TRAF family member-associated NF-κB activator (TANK) for IRF3 phosphorylation. Subsequently, P-IRF3 forms a dimer and translocates from the cytoplasms to the nucleus where it induces the expression of IFN-α/β [7], [86], [87], [92]–[94]. Additionally, ligand binding of TLR3 also activates the AKT in a TBK1-dependent manner, and AKT contributes to IRF3 phosphorylation by interacting with TBK1 [95, p. 1].

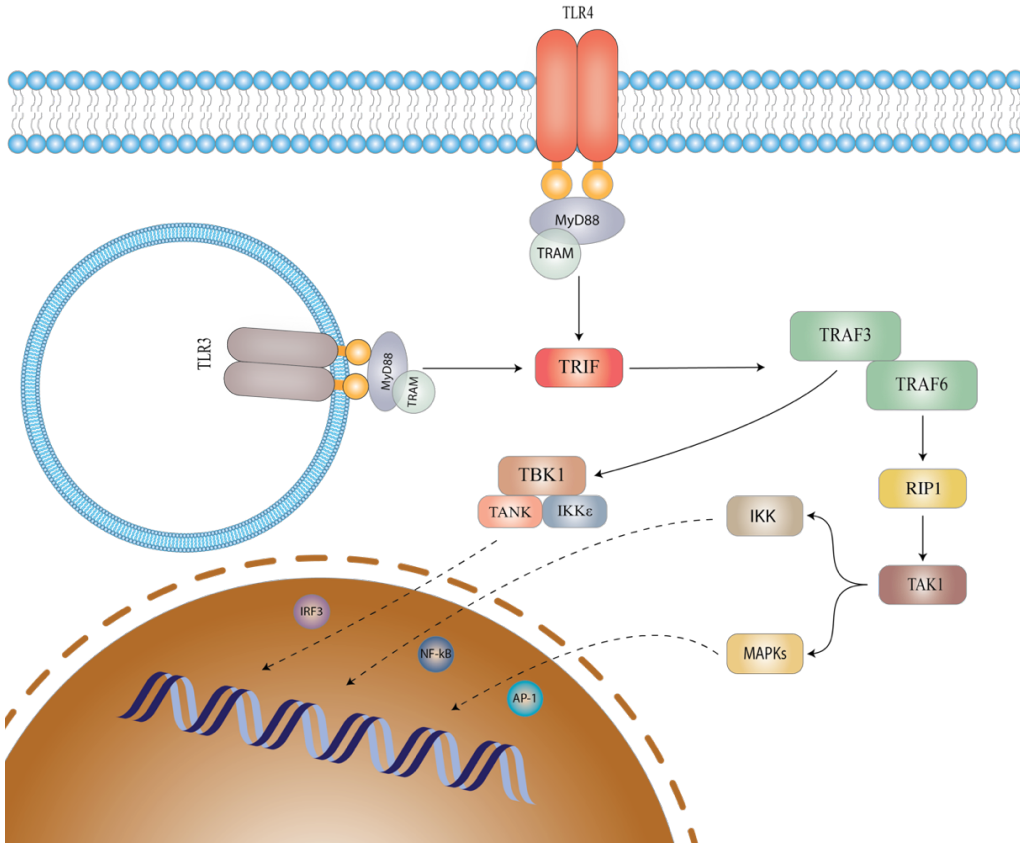

Figure 5: TRIF -dependent TLR signal transduction pathway - Upon activation through binding with dsRNA and lipopolysaccharides respectively, TLR3 or TLR4 set off a series of events. TRAF6 recruits the RIP1 kinase, which interacts with the TAK1 complex, leading to the activation of both the IKK complex and the MAPK family. Conversely, TRAF3 recruits TBK1 and IKKε, which in turn activate the IRF3 transcription factor (adapted from [7], [86]).

V.5.4 Expression and function of TLRs in keratinocytes

Keratinocytes, representing the primary cell population in the epidermis, effectively function as the initial line of defence against environmental stressors. When activated, these cells release a variety of pro-inflammatory cytokines and chemokines, including IL-6, IL-8, IL-36, IFN-β, TNF-α, CXCL10, and CCL20. Additionally, they produce AMPs such as LL37, human beta-defensin 2 (HBD2), and HBD3 [86]. Moreover, injured cells discharge DAMPs like dsRNA, ssRNA, and DNA [7], [96], which further amplify the inflammatory process.

The excessive production of LL37 by keratinocytes plays a pivotal role in the development of psoriasis and has been recognized as a key component in detecting DAMPs through its interaction with nucleic acids (DNA and RNA) [13], [96]. It has been demonstrated that LL37 when bound to self-RNA, activates pDCs through TLR7 and mDCs via TLR8 [97]. Moreover, the binding of LL37 to ssRNA has been shown to trigger the activation of TLR8 [7]. Interestingly, when LL37 binds specifically to dsRNA, it allows for the recognition of these molecules by TLR3 and the mitochondrial antiviral signalling protein (MAVS) pathways. This signalling pathway is highly expressed in keratinocytes compared to other skin cells [98]. Furthermore, LL37 converts inactive self-DNA into a potent activator of TLR9 on pDCs, thus stimulating these cells [99].

Keratinocyte activation through PRR-mediated signalling has been demonstrated to play a pivotal role in the innate immune response. These cells are known to express TLRs 1-6 and 9, with an increased expression of TLRs 1, 2, 5, and 9 observed in psoriatic skin compared to healthy skin [7], [100, p. 9]. The activation of each specific TLR elicits a unique immune response.

Research has revealed that activating Toll-like receptors (TLRs) 3, 4, 5, and 9 on keratinocytes leads to the release of various cytokines and chemokines, such as TNF-α, IL-8, CCL2, CCL20, and CCL27. Notably, when TLRs 3 and 5 are selectively activated, the secretion of CCL27 is enhanced, whereas specific stimulation of TLRs 3 and 9 induces the production of CXCL9 and CXCL10, resulting in the subsequent release of type I interferons (IFNα/β) [101, p. 9], [102], [102]. This cascade of cytokines and chemokines plays a pivotal role in recruiting and activating DCs and T cells. Furthermore, the activation of TLR5 and TLR9 has been demonstrated to be dependent on the transforming growth factor-alpha (TGF-α) [103]. This epidermal growth factor

(EGF) is of interest, as it activates MAPK signalling and is involved in the formation Koebner phenomenon [104], [105]. The Koebner phenomenon is characterized by an abnormal and exaggerated wound healing response following minor skin traumas, such as scratches or incisions. This phenomenon has been linked to psoriasis, as the cellular and molecular mediators involved in this response closely resemble those observed in psoriatic plaque sites [104].

To further support the importance of EGFs, a study revealed that treatment with the EGF receptor inhibitor PD153035 resulted in a decrease in both MAPKs-induced production of CCL20 and IL-17-induced production of CCL20 following induced scratch injury [106]. These observations suggest a synergistic effect which may be associated with the development of the psoriasis phenotype.

V.6 Mitochondrial antiviral signalling (MAVS) adaptor Protein

V.6.1 RIG-I-like receptor (RLR) family and MAVS

The RIG-I-like receptor (RLR) family represents another type of PRRs and consists of three members: retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated gene 5 (MDA5), and laboratory of genetics and physiology protein 2 (LGP2). Structurally, RIG-I comprises two tandem CARD domains at its N-terminal region, which are essential for CARD-CARD interactions and downstream signalling activation. It also contains a central DExD/H ATPase helicase domain, an ATP-binding motif, and a C-terminal repressor domain (RD) responsible for RNA binding. MDA5 shares similar functional CARD and ATPase domains with RIG-I. However, the role of its RD region remains unclear, as it is uncertain whether the C-terminal region of this RLR truly functions as an RD. LGP2 possesses a DExD/H domain and an RD but lacks the CARD domains, resulting in no signalling capacity. Moreover, LGP2 appears to inhibit RIG-I's antiviral function by interacting with RIG-I's RD domain through its own RD domain. Consequently, in contrast to the other two receptors, LGP2 is considered a negative regulator of antiviral responses [96].
These receptors are situated in the cellular cytoplasm and initiate antiviral responses upon interacting with viral dsRNA and RIG-I and MDA5 by interacting with mitochondrial antiviral signalling (MAVS) adaptor protein [107]. This protein is essential for the downstream activation of NF- κB and IRF3 transcription factors by RIG-I and MDA5 [108].

V.6.2 MAVS structure

The MAVS protein, composed of 540 amino acids and with a predicted molecular mass of 56 kD, consists of three domains: an N-terminal CARD domain, a middle proline-rich region (PRR), and a C-terminal transmembrane (TM) anchor domain [109]. The CARD domain enables CARD-CARD interactions between MAVS and RIG-1 or MDA-5, while the PRR facilitates the binding of MAVS to TRAF2, TRAF3, TRAF5, and TRAF6 family members. The TM domain enables MAVS to localize to various membranes, which is essential for effectively driving antiviral signalling [96], [109].

Primarily found in the mitochondrial outer membrane, MAVS can also be located in peroxisomes and the endoplasmic reticulum (ER) subdomain known as the mitochondrion-associated membrane (MAM) [109].

V.6.3 Regulation of MAVS

The expression and function of MAVS are subject to regulation by both post-transcriptional and post-translational mechanisms. This control is vital for ensuring a swift antiviral response that is also promptly restrained upon viral clearance, which helps prevent potential tissue damage that could arise from an extended immune response.

V.6.3.1 Mitochondrial regulation of MAVS

Regulation at the mitochondrial level is highly strategic, as signals generated by distinct cytosolic sensors converge on MAVS at the mitochondrial membrane. Consequently, regulators of MAVS can exert a more substantial level of control than they would if targeting upstream components of RLR signalling, such as RIG-I or MDA5 individually.

V.6.3.1.1 Mitochondrial dynamics

Mitochondrial dynamics, which involve the ongoing processes of mitochondrial fission and fusion, are crucial for cell survival and adaptation to ever-changing conditions necessary for cell growth, division, and the distribution of mitochondria during differentiation [110]. These dynamics have also been shown to play a significant role in the regulation of MAVS.
One study conducted on Human embryonic kidney (HEK293) cells demonstrated that the elongation and fusion of the mitochondrial network potentiate MAVS-mediated signalling, while mitochondrial fission attenuates it. This observation was made by knocking down (KO) mitochondrial dynamics regulators such as dynamin-related 1 (Drp1), Mitochondrial fission 1 (Fis1), optic atrophy type 1 (OPA1), and mitofusin 1 (Mfn1) proteins using short-hairpin RNA. Drp1 and Fis1 promote mitochondrial fission, while OPA1 and Mfn1 facilitate mitochondrial fusion. Upon KO of Drp1 or Fis1,

enhanced activation of IFNβ and NF-κB was observed, whereas cells with KO of OPA1 or Mfn1 exhibited inhibited activation of IFNβ and NF-κB [111].
In another study, also conducted in HEK293 cells, it was discovered that Mfn2 acts as a negative regulator of MAVS, in contrast to Mfn1. The study revealed that Mfn2 directly modulates MAVS through the interaction of its central hydrophobic heptad repeat (HR1) region with the C-terminal region of MAVS. This finding suggests that Mfn1 and Mfn2 are not functionally redundant [112].

V.6.3.1.2 ROS

Mitochondria are a significant source of physiological reactive oxygen species (ROS) production. Approximately 2 % of the oxygen consumed by the mitochondrial respiratory chain generates the superoxide radical, which subsequently leads to the formation of other ROS species, such as hydrogen peroxide and hydroxyl radical. Although the molecular mechanisms underlying the regulation of antiviral activity by ROS remain largely elusive, a connection has been identified between the MAVS-mediated antiviral pathway and the Cytochrome c Oxidase (CcO) subunit 5B (COX5B). Oligomerization of MAVS, induced by RIG-I CARD binding, leads to an increase in ROS production. This heightened ROS generation subsequently promotes the upregulation of COX5B, a known repressor of ROS production, and Autophagy-related protein 5 (ATG5), an essential component of the autophagic pathway. Both COX5B and ATG5 work together to downregulate MAVS activity, effectively modulating the antiviral response [113], [114]. This intriguing interaction establishes a connection between the mitochondrial electron transfer machinery and the autophagy pathway in controlling ROS production and host innate immunity.

V.6.3.2 Tripartite-containing motif (TRIM) protein family

Currently, TRIM31, an E3 ubiquitin ligase and a member of the TRIM protein family, is the sole known initiator of MAVS aggregation and activation [109]. Encoded within the locus associated with MHC class I proteins, TRIM31 is primarily localized in the cytoplasm, with a fraction also detected in the mitochondria [115]. It interacts with MAVS and catalyzes K63-linked polyubiquitination at Lys10, Lys311, and Lys461 on MAVS. Notably, TRIM31-mediated MAVS aggregation only occurs during viral infection and requires RIG-I engagement [116].

Another member of the TRIM superfamily, TRIM21, has been identified as a positive regulator of MAVS. Upon induction, TRIM21 interacts with MAVS and promotes K27-linked polyubiquitination at LYS325, resulting in the recruitment of TBK1 [117]. TRIM25, an E3 ubiquitin ligase, plays a crucial dual role in activating antiviral signalling. Firstly, TRIM25 mediates K63-linked polyubiquitination of RIG-I's 2CARD domains, stabilizing the tetramer and enhancing RIG-I binding to MAVS [118]. Secondly, TRIM25 catalyzes K48-linked ubiquitination of MAVS, which, despite typically being a negative regulator of RLR signalling, actually enhances P-IRF3 levels in this case [119]. The mechanism by which MAVS is removed from the mitochondrial outer membrane before proteasome degradation remains unknown.

V.6.3.3 K48-linked ubiquitination inhibitors

Proteins that inhibit K48-linked ubiquitination of MAVS, which promotes its proteasomal degradation, can be considered positive regulators of the antiviral response. For example, the endogenous protein cyclophilin A, overexpressed during viral infection, competes with TRIM25 for MAVS binding, thereby inhibiting K48-linked ubiquitination by TRIM25 [109]. Additionally, a recent study highlighted the role of ER-associated inactive rhomboid protein 2 (iRhom2) in augmenting MAVS activity by engaging with different E3 ubiquitin ligases, depending on the infection stage within the cell. In non-infected cells or during the early stages of infection, iRhom2 promotes auto-ubiquitination and degradation of the E3 ubiquitin ligase Ring Finger Protein 5 (RNF5), which hinders the formation of VISA-RNF5-GP78 complexes and counteracts ER-associated degradation (ERAD) of VISA. In the later stages of viral infection, iRhom2 is involved in proteasome-dependent degradation of the E3 ubiquitin ligase Membrane-Associated Ring Finger 5 (MARCH5), which obstructs mitochondria-associated degradation of VISA [120].

Additionally, the Ovarian tumor family deubiquitinase 4 (OTUD4) protein interacts with MAVS to cleave K48-linked polyubiquitin chains, inhibiting the proteasome-dependent degradation of MAVS [121].

V.6.3.4 E3 ubiquitin ligases

Several E3 ubiquitin ligases have been shown to mediate MAVS K48-linked ubiquitination, including atrophin-interacting protein 4 (AIP4), SMAD ubiquitin regulatory factor 1 (Smurf1), Smurf2, MARCH5, MARCH8 and RNF5. These proteins can be further regulated by various adapter proteins [109], [121]. For example, AIP4 can be recruited to ubiquitinate MAVS by poly(C)-binding protein 1 (PCBP1), PCBP2 and Tax1-binding protein 1 (TAX1BP1). TAX1BP1 bind to the MAVS CARD while PCBP1 and PCBP2 bind to the TM domain. PCBP1/2 promote the degradation of MAVS through the same mechanisms, however, PCBP1 is continuously expressed while PCBP2 increases expression after infection. Therefore indicating that PCBP1 serves as a maintainer of low MAVS activity in non-infected cells while PCBP2 serves as a controller for cellular overexposure to immune response [122]. Regarding Smurf1, its activity can be enhanced by either OTUD1 or Nedd4 family-interacting protein 1 (Ndfip1). OTUD1 interacts with Smurf1 and functions as a deubiquitinase to remove the K48-linked ubiquitination of Smurf1, prevent its self-ubiquitination degradation, and upregulate its protein level; whereas the binding of Ndfip1 to MAVS recruits Smurf1 to MAVS, and the interaction between Ndfip1 and Smurf1 enhances the self-ubiquitination [109].

V.6.3.5 Negative regulators of TRIM31

Two proteins were observed to limit TRIM31 interaction with MAVS: Fas-associated factor (FAF1) proteins which forms aggregates in unaffected cells maintaining these proteins inactive, but upon viral infection due to acetylation and lysosomal degradation triggered by IKKε-mediated phosphorylation it becomes active [123], and Rac1 which is activation by post-translational modifications upon viral infection which will induce its translocation to the MAMs [124]. These two proteins engage directly with MAVS and limit the interaction of Trim31 and, hence inhibiting it.

V.6.3.6 MAVS truncated isoforms

Currently, only a few studies have explored the regulation of full-length MAVS (FL MAVS) through its interaction with its truncated isoforms. Although the specific mechanisms governing the regulation of FL MAVS aggregation by truncated isoforms

remains unclear, it appears that their binding to FL MAVS impairs its function. The polycistronic MAVS mRNA contains six methionine residues: M1, M142, M303, M358, M367, and M449, potentially generating six distinct truncated isoforms: MAVS-M1/2/3/4/5/6. However, only MAVS-M1 and MAVS-M2 isoforms have been reported so far. These truncated isoforms lack the N-terminal CARD domain, making them unable to activate due to impaired aggregation. Instead, these N-terminal truncated isoforms interact with full-length MAVS through TM-mediated homotypic associations, ultimately inhibiting FL MAVS activation, likely by preventing the spatial proximity of neighboring CARDs and subsequent spontaneous aggregation of full-length MAVS [125].

V.6.3.7 CARD10/CARMA3

Interestingly, a study conducted on the CARD10 protein revealed an interaction between this scaffold and the MAVS protein. This research suggested that CARD10, acting as a scaffold, recruits BCL-10 and attaches to MAVS via its N-terminal GUK domain. This interaction effectively impedes the formation of MAVS molecular aggregates, thereby disrupting its crucial function. Furthermore, the study proposed an additional regulatory step in this process. It was suggested that CARD10 undergoes K-48 ubiquitination, marking it for proteasomal degradation. This degradation of CARD10 creates an environment conducive to MAVS aggregation, potentially enhancing its signalling activity in the context of antiviral responses [126].

V.6.4 MAVS signalling pathway

Upon activation of RIG-1 and MDA5 through viral RNA binding, these proteins undergo a conformational change, exposing their N-terminal tandem CARD domains. They subsequently form a tetramer using these CARD domains. This complex is then ubiquitinated by K63-polyubiquitin chains, facilitated by the E3 ubiquitin ligases TRIM25, RNF125, and RIPLET. This process allows for the binding of RIG-1 and MDA-5 to MAVS through CARD-CARD interactions with the MAVS CARD domain, ultimately activating MAVS. Consequently, MAVS binds to TRAF2, TRAF3, TRAF4, TRAF5, or TRAF6 via its PRR domain, which triggers the activation of the TBK1 complex by TRAF2/3/5/6 and the activation of the IKK complex by TRAF2/5/6. This promotes the activation of IRF3 and NF-κB transcription factors [107], [109], [127], [128].

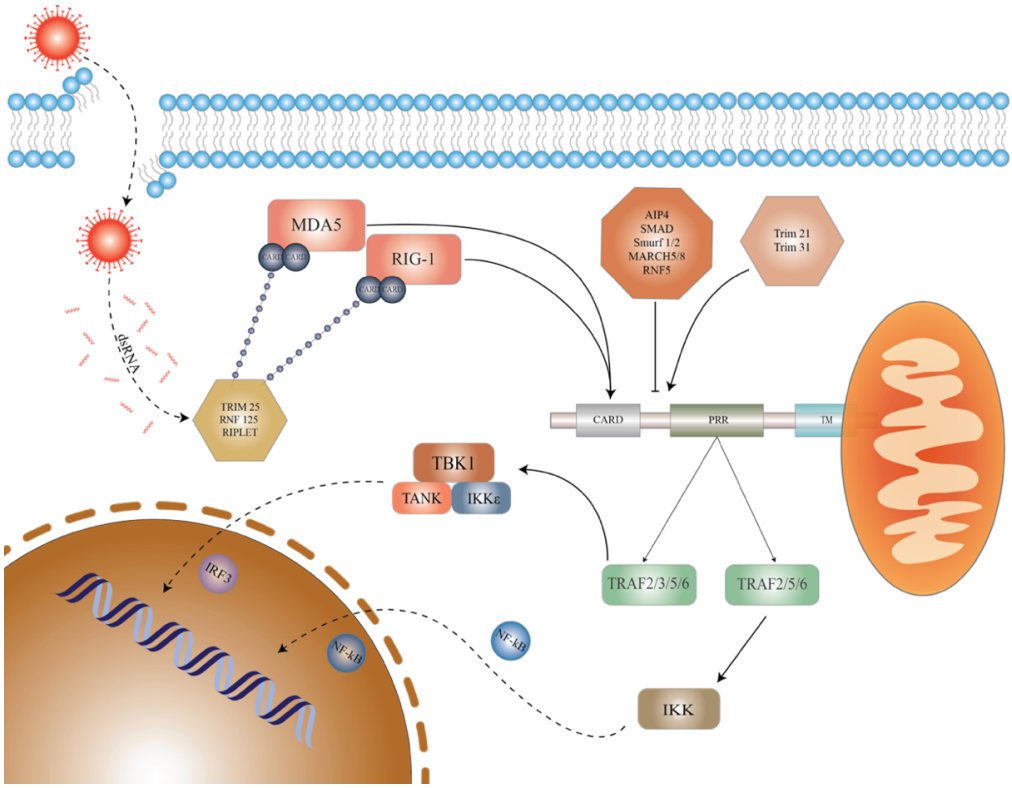

Figure 6: Molecular mechanisms of MAVS-dependent antiviral response - Upon recognition of viral dsRNA, RIG-1 and MDA5 activate and oligomerize in a manner that exposes their heterodimer CARD region for tetramer CARD formation. Subsequent K63-linked polyubiquitination by TRIM25, RNF125, and RIPLET induces the CARD-CARD binding of RIG-1 or MDA5 to MAVS. The interaction of these CARD domains activates MAVS, leading to the recruitment of TRAF2/3/5/6 for TBK1 complex activation, and the recruitment of TRAF2/5/6 for IKK complex activation. This process can be augmented through the K63-linked polyubiquitination or the K27-linked polyubiquitination of MAVS by TRIM31 and TRIM21 proteins respectively. Conversely, the process can also be attenuated by K48-linked polyubiquitination of MAVS by AIP4, Smurf1/2, MARCH5/8, and RNF5 proteins (adapted from [109]).

V.7 CARD14sh modulates the TBK1/IRF3 signalling

Interactions between the IKK complex and the TBK1/IRF3 response have been extensively investigated due to their crucial role in modulating the cell's innate response. IKKα/ß phosphorylates and enhances the activity of TBK1 and IKKε, thereby facilitating their functions. Conversely, TBK1 and IKKε can inhibit the activity of the IKK complex [129], [130]. However, the understanding of how other signalling mediators within the IKK axis modulate TBK1/IRF3 remains limited and requires further exploration.

In a recent study, the interaction between the CC domain of CARD14sh and TANK, a scaffold protein, was elucidated. TANK is known for its involvement in facilitating the interaction among TRAF3, TBK1, and IKKε during antiviral responses [131], [132]. Notably, the study also revealed that the psoriasis-associated CARD14sh E138A and E142G mutants displayed reduced efficacy in inhibiting P-IRF3 levels [133]. Moreover, the same research group conducted another investigation, identifying UBAC1 as a novel interactor of CARD14sh. UBAC1, the non-catalytic component of the E3 ubiquitin-protein ligase KPC complex, was found to associate with TANK and promote its K63- and K29-linked ubiquitination [134].

These recent findings underscore the regulatory role of the CARMA protein family in innate immunity through the modulation of the TBK1/IRF3 response. Additionally, it is important to note that the psoriasis-associated CARD14sh E138A and E142G mutants exhibited diminished effectiveness in inhibiting P-IRF3 levels when compared to CARD14sh wild-type, suggesting additional functions for these mutant forms beyond the well-documented NF-κB hyperactivation [43], [44].

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VI Objectives of the work

Psoriasis, an chronic autoimmune skin disorder, affects approximately 2 to 3 % of the global population [26]. Despite its widespread incidence, the precise molecular pro-inflammatory autoimmune mechanisms that instigate and perpetuate this condition remain largely undefined. CARD14 and its related mutations play a significant role in the initiation of psoriasis, with these mutations being often linked to the hyperactivation of the CBM complex and the subsequent hyperactivation of the NF-κB transcription factor. However, it is important to note that the influence of these CARD14 mutations on NF-κB activity is mutation-specific. While some mutations amplify NF-κB activity, others have no discernible effect [75], [83]. This variation highlights the crucial need for a deeper understanding of the complete scope of signalling transduction pathways in which they may be intricately involved.

In this study, our first aim is to investigate the impact of CARD14 mutations on the characteristic morphological features observed in psoriasis, including increased keratinocyte cell proliferation, viability, and migration. We will primarily focus on the CARD14sh isoform, given its recognition as the most prevalently expressed CARD14 protein in keratinocytes. We will closely examine three psoriasis-associated mutations E138A, E142G and R38C. Two of these mutations, E138A and E142G, display a pathogenic signature and are situated within the coiled-coil (CC) region of CARD14 and are known for amplifying NF-κB activity. Conversely, the impact of the third mutation, R38C, on psoriasis pathogenesis remains unclear, despite its prevalence among psoriasis patients. Located within the CARD region, R38C appears to inhibit CARD14's ability to activate NF-κB [75], [83], [135], [136].

Our secondary objective is to assess whether CARD14sh and its mutant counterparts could be implicated in a signal pathway distinct from the CBM complex-mediated NF-κB pathway. We focused on the TBK1/IRF3 response, given that CARD14sh has been demonstrated to interact with TANK - a protein essential for TBK1 activity.

VII Materials and methods

VII.1 Cell cultures

VII.1.1 HaCaT cells

The immortalized human keratinocyte (HaCaT) cell line was obtained from CLS Cell Lines Service GmbH (Eppelheim, Germany). These cells were previously stably transfected with CARD14sh wild type (WT), CARD14sh mutants (CARD14sh R38C, E142G, or E138A), or vector (control group) and maintained in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, 100 g/ml streptomycin, and G418. The transfected HaCaT cells were then cultured in six-well plates using RPMI medium (Biowest, Stockumer Kirchstraße, Düsseldorf, Germany) enriched with 10% FBS (Biowest, Stockumer Kirchstraße), 1x glutamine-penicillin-streptomycin solution (Biowest, Stockumer Kirchstraße), and G418. The cells were incubated at 37°C under 95% humidity and 5% CO2 conditions. The medium was refreshed approximately every 2-3 days, depending on its appearance and condition.

VII.1.2 HEK293 cells

The human embryonic kidney (HEK293) cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS (Fetal Bovine Serum) and antibiotics (penicillin-streptomycin). For transfection, a calcium phosphate precipitation method, a chemical-based transfection approach, was utilized. Prior to transfection, the cells were grown in a monolayer until reaching 30-40% confluence. In the transfection process, the specific amount of DNA for a single sample in a 6-well plate was dissolved in 220 µl of sterile distilled water. Then, 250 µl of 2x 35 Hepes-Buffered Saline (HBS) was added to the DNA mixture at room temperature. Subsequently, 30 µl of Calcium Chloride 2M was added dropwise with gentle swirling to ensure thorough mixing. After incubating the precipitates at room temperature for 20 minutes, they were added to the cells, which were then further incubated for 18-24 hours at 37 °C in a humidified incubator with 5% CO2. For 10 mm plates, the procedure remained similar, except for doubling the volume of the precipitation reaction mixture.

VII.2 Cell count

HaCaT cells were accurately seeded in a 12 multi-well plate, with a consistent density of 200,000 cells per well. This standardized seeding procedure was independently performed three times to ensure robustness and reliability in our results. Cell counting was performed at specific time points following cell seeding: 24, 48, 72, and 92 hours. The cells were imaged and visualized using the Burker camera, a reliable instrument for cell imaging and data acquisition. Utilizing the Burker camera, images were captured, and subsequently, the cell populations were quantified at each designated time point. The acquired images underwent rigorous analysis and quantification using ImageJ software, a widely recognized tool for processing biological image data.

VII.3 Cell viability assay

The viability assay was conducted using the MTT assay (Sigma-Aldrich, Saint Louis, MO, USA) following the manufacturer's instructions. In each well of a 96-well plate, 10 X 10^3 cells were seeded. At the specified time points, cells were incubated with MTT for 3 hours at 37°C in the dark. Subsequently, an equal volume of isopropanol was added to each well, and the plates were further incubated for 10 minutes at room temperature in the dark. Cell viability was determined by measuring the absorbance at 505 nm using a microplate reader (Seac Sirio S), with background absorbance at 630 nm subtracted from the signal absorbance. The experiments were conducted in triplicate, and the data are expressed as mean ± standard deviation (SD). For statistical analysis, the T-test was applied to evaluate significance, with a P value < 0.05 considered as statistically significant.

VII.4 Reagents

TRIzol reagent (15596-026) was obtained from Invitrogen. Poly (I:C) was purchased from SIGMA.

VII.5 Immunoblot analysis

In this study, the following primary antibodies were used: antibodies against HA (#7392), CARD14 (#99052), IRF3, and Cyclin D, which were purchased from Santa Cruz Biotechnology. For normalization, vinculin (#MCA4656A) was obtained from Bio-Rad Laboratories and β-actin (#A3854) from SIGMA. Secondary antibodies, anti-rabbit (7074P2) (1:3000) and anti-mouse (7076P2) (1:3000), were purchased from Cell Signalling Technology. Cell lysates were prepared in lysis buffer (150 mM NaCl, 50 mM Tris, pH 7.2, 1% NP40, 2 mM EDTA, and a mixture of protease inhibitors). Proteins were separated using SDS-PAGE, with samples loaded onto the gel following reduction in a solution containing 50 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.03% bromophenol blue, and subsequent boiling at 100°C for 10 minutes. Samples were subjected to a constant current of 100 V for 2 hours and 30 minutes in running buffer consisting of 25 mM Tris-HCl pH 8.3, 192 mM glycine, and 0.03% SDS. After electrophoretic separation, proteins were transferred onto a 0.2 µm pore-size nitrocellulose membrane pre-hydrated in Trans-Blot Turbo 5x Transfer Buffer (BIO-RAD LABORATORIES). The transfer apparatus was assembled and inserted into the Trans-Blot Turbo Transfer System. The membrane was then incubated with EveryBlot Blocking Buffer (BIO-RAD LABORATORIES) to saturate non-specific sites. A 1:1000 primary antibody solution was added and incubated overnight. Following incubation, the primary antibody solution was removed, and the membrane was washed three times with TBST (Tris HCl pH 7.5, NaCl, 0.1% Tween). The membrane was then incubated for 1 hour with a 1:3000 secondary antibody solution. After incubation, the antibody solution was removed, and the membrane was washed three times with TBST.

VII.5.1 Immunoprecipitation

For immunoprecipitation experiments, cells were lysed in lysis buffer and immunocomplexes were bound to protein A/G-Agarose beads (Roche, Basel, Switzerland), resolved by SDS-PAGE and analyzed by immunoblot assay. All immunoblots were done at least three times using different biological materials as sources. Phosphatase Inhibitor Cocktail was purchased from Sigma and used according to the instructions provided.

VII.6 SDD AGE

SDD-AGE gels (1.8% agarose gels) were prepared by melting 1.8 g of agarose in 100 mL of TAE buffer (10 mM Tris-HCl, 5 mM acetic acid, 5 mM EDTA). To prevent localized solidifying, 0.1% SDS was slowly added. Protein samples were mixed with 4X SDD-AGE loading buffer (2X TAE, 20% glycerol, 8% SDS, 1% Bromophenol blue) and incubated at room temperature for 5 minutes. Then, 20 µL of the sample was loaded onto the gel. Electrophoresis was performed for 1 hour at 12 V/cm and 4 °C in pre-chilled TAE running buffer containing 0.1% SDS. Protein transfer from the gel to a nitrocellulose membrane was achieved using a constant voltage of 250 milliAmperes for 5 hours.

VII.7 Wound-healing assay

HaCaT cells, previously seeded in 6-well plates, were incubated for 24 hours to reach confluency. On day 2, a uniform scratch was made across the entire well using a sterile tip. The cells were then washed twice with PBS buffer solution and incubated under the same conditions as the ones previously mentioned. The scratched region was photographed at 18, 44, and 67 hours post-wounding at 10x magnification. Scratch area analysis was performed using ImageJ software (U.S. National Institutes of Health, Bethesda, MD, USA), and the %age of wound closure was calculated using the following equation:

$$\%\, Wound\ closure = \left[\frac{(Surface\ area\ at\ time\ point\ 0 - Surface\ area\ at\ time\ point\ X)}{Surface\ area\ time\ point\ 0}\right] X\ 100$$

VII.8 Gene expression analysis

RNA was extracted from HaCaT cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). A reverse transcriptase reaction was carried out on 1 µg of RNA with the iScript™ Advanced cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA), followed by an amplification step using 30 ng of cDNA, along with 300 nM of each primer in a total volume of 15 µl. Real-time PCR reactions were performed in duplicate, employing SsoAdvanced Universal SYBR Green Supermix (Bio-Rad

Laboratories, Inc.) on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.). The cycling conditions were as follows: 25 seconds at 95°C; 37 cycles of 10 seconds at 95°C and 25 seconds at 60°C (with Plate Read); and a melting curve analysis to confirm single product amplification. Subsequently, gene expression analysis was conducted using Bio-Rad CFX Manager 3.1 (Bio-Rad Laboratories, Inc.), with β-actin serving as the reference for normalization. Target genes were amplified using the designated primers:

GENE	Primer Sequence (5'-3')	
	FORWARD	REVERSE
CARD14	TCCTAGACACGGCAGACCTT	CCGAGACATCAAGCCTTCCA
ACTIN	GAGCACAGAGCCTCGCCTTT	TCATCATCCATGGTGAGCTGG
CCL20	TTGTCTGTGTGCGCAAATCC	CCAACCCCAGCAAGGTTCTT

VIII Results

VIII.1 Exploring the role of CARD14sh and mutants in the manifestation of psoriasis-related keratinocyte morphological features

Psoriasis is a chronic inflammatory disorder characterized by dysregulated autoimmune and pro-inflammatory responses, leading to various abnormal morphological features [1]. In our experimental approach, our objective was to evaluate and compare cell proliferation, viability, and migration between CARD14sh and its mutant forms (R38C, E142G, and E138A), as well as the control group, in order to observe their potential contributions to the psoriasis-associated keratinocyte phenotype. Through this investigation, we sought to uncover distinctive regulatory effects that may exist between CARD14sh and its mutant counterparts.

Furthermore, we aimed to assess whether the CARD14sh R38C mutant exerts any significant effects on these cellular processes. This specific mutation is prevalent among psoriasis patients, despite not showing any apparent impact on NF-κB activity [75]. By examining the effects of this mutation on cell proliferation, viability, and migration, we sought to gain a better understanding of its potential role in the pathogenesis of psoriasis.

VIII.1.1 Ectopic expression of CARD14sh R38C, E142G, or E138A enhances proliferation in HaCaT cells

The initial objective of this study was to evaluate the impact of CARD14sh and its mutants on keratinocyte proliferation. To accomplish this, we used previously stably transfected HaCaT cells with different variants, including CARD14sh wild type (WT), CARD14sh mutants (CARD14sh R38C, E142G, or E138A), or vector (control). Cell proliferation was assessed through cell counts, and Cyclin D1 expression was evaluated using immunoblot analyses (Figure 1 and Figure 2).

Figure 1 illustrates the results of the initial cell count, revealing a noticeable increase in the proliferation rate of cells expressing the CARD14sh R38C, E142G, or E138A mutants compared to CARD14sh WT and the control group. However, it is important to note that the R38C variant only displayed statistically significant proliferation during the initial phases, specifically between day 1 and day 2. These findings indicate that the CARD14sh mutants have the potential to promote cellular proliferation, with the R38C variant showing a more pronounced effect during the early stages.

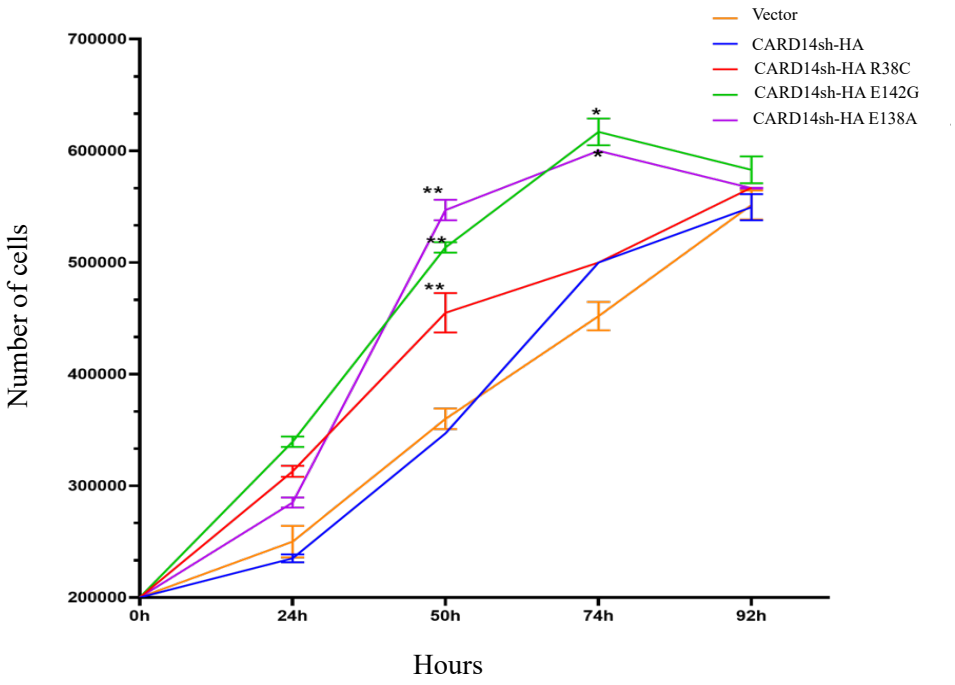

Figure 7:Impact of CARD14sh mutants on cell proliferation in stably transfected HaCaT cells - For statistical analysis, a t-test was conducted based on cell counts measured on Days 1, 2, 3, 4, and 5 (*P < 0.05; **P < 0.01). This experiment was performed three times independently.

Additionally, Cyclin D1 expression was assessed through immunoblot analyses (Figure 2). The results of this analysis provide further support for the increased keratinocyte proliferation observed in cells expressing the CARD14sh mutants. Elevated Cyclin D1 expression is indicative of cell cycle progression and is consistent with the enhanced proliferation observed in the mutant groups.

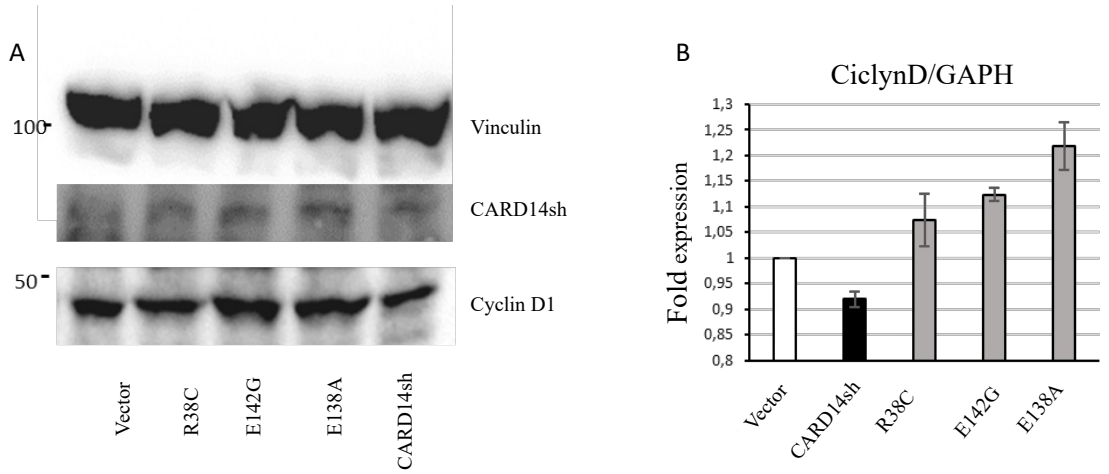

Figure 8:Evaluating the influence of CARD14sh on Cyclin D1 expression levels - Cyclin D1 expression levels were scrutinized through Western blotting. A Vinculin Western blot was used for normalization. An illustrative Western blot image representative of three independent experiments is provided (A), while the fold change in Cyclin D1 expression across different cell cultures is graphically represented (B). This experiment was performed three times independently.

Taken together, the results of the experiments provide compelling evidence that the CARD14sh mutants (CARD14sh R38C, E142G, and E138A) induce keratinocyte proliferation.

VIII.1.2 Ectopic expression of CARD14sh R38C, E142G, or E138A increases cell survival in HaCaT cells

Since CARMA proteins are known to be involved in signal transduction pathways related to cell proliferation and survival, we conducted additional experiments to investigate the effects of CARD14sh mutants on keratinocyte viability. To assess cellular viability, we employed the MTT assay on stably transfected HaCaT cells, a widely used method for quantifying cell viability.

As depicted in Figure 3, our results unequivocally demonstrate that both CARD14sh and its mutants (CARD14sh R38C, E142G, and E138A) significantly augmented cellular viability when compared to the control group. Notably, the observed increase in viability in cells expressing CARD14sh mutants became apparent after 24 hours, suggesting that these specific mutants actively promote cell survival and play a crucial role in maintaining overall cell viability.

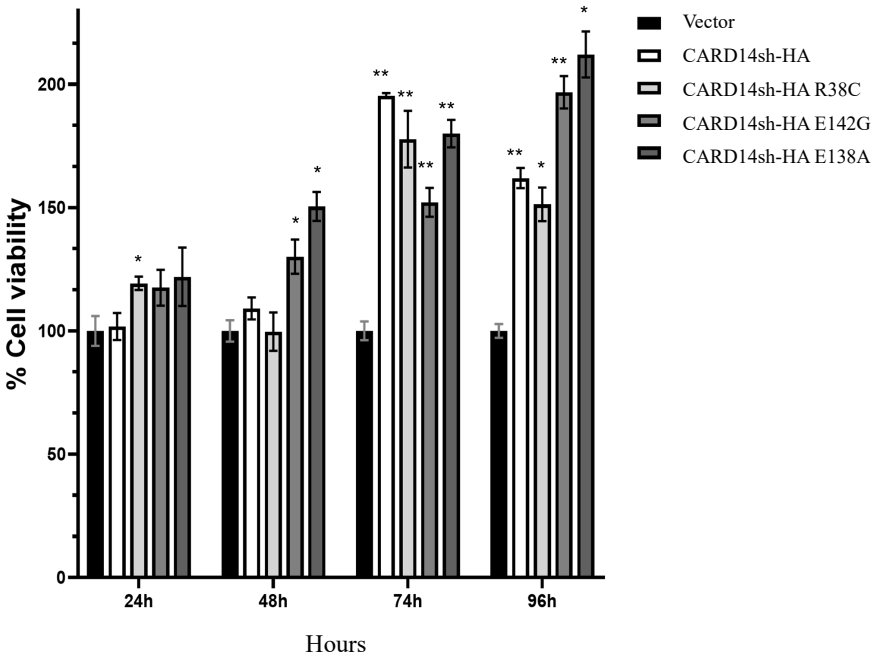

Figure 9: Effects of CARD14sh and CARD14sh mutants ectopic expression on cell viability - The number of viable cells was assessed on Days 2, 3, 4, and 5 using the MTT viability assay (*P < 0.05; **P < 0.01). This experiment was performed three times independently.

These findings provide strong evidence for the positive effects of CARD14sh mutants on keratinocyte viability.

VIII.1.3 Ectopic expression of CARD14sh R38C; E142G or E138A promotes cell migration in HaCat cells

In addition to assessing cell proliferation and viability, we also investigated the effect of CARD14sh mutants on cell migration using a wound-healing assay on stably transfected HaCaT cells. This investigation was motivated by two observations: the Koebner phenomenon and the intriguing finding that individuals with psoriasis tend to experience faster healing of small wounds compared to unaffected individuals [104], [137].

To specifically focus on cell migration and eliminate the influence of cell proliferation, we performed the assay without serum by creating a scratch in confluent keratinocyte cells using a sterile tip and monitored the closure of the scratch over time. The area of the scratch was measured precisely at 0 hours, 18 hours, 44 hours, and 67 hours, and the changes in the area were analyzed (Figure 4). Figure 4A clearly demonstrates that the expression of all three CARD14sh mutants resulted in faster closure of the scratch compared to cells expressing CARD14sh WT and control cells. Moreover, statistical analysis supports these findings (Figure 4B).

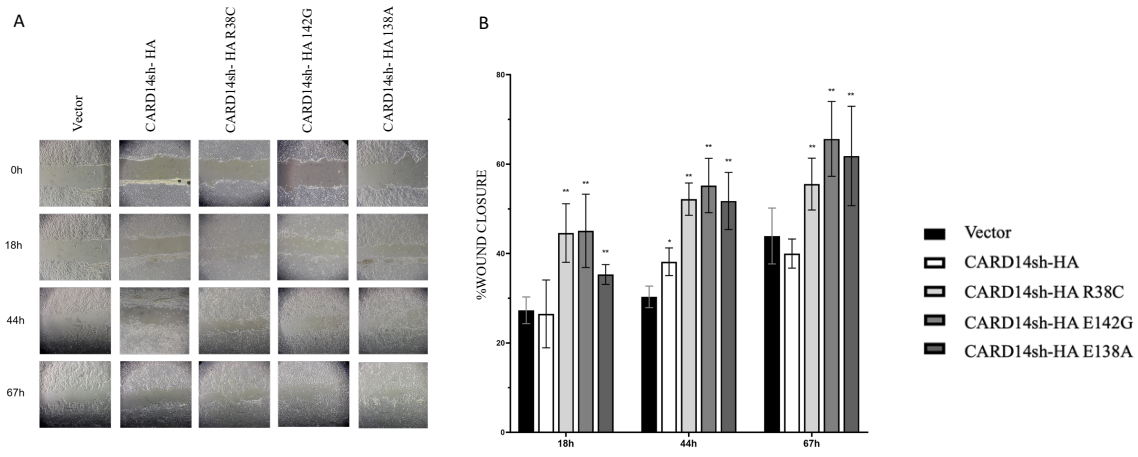

Figure 10: CARD14sh mutants stably transfected HaCaT cells exhibit accelerated wound closure following scratch injury induction - Images captured at 10X magnification of HaCaT cells at time points 0, 18, 44, and 67 hours are representative of three independent experiments is provided (A). Wound closure in the various HaCaT cell cultures (B) is shown, with a t-test employed for statistical analysis (*P < 0.05; **P < 0.01). This experiment was performed three times independently.

These results provide compelling evidence for the role of CARD14sh mutants in promoting keratinocyte migration. The faster closure of the scratch in cells expressing CARD14sh mutants suggests that these mutants enhance the migratory capacity of cells.

VIII.2 Expanding the role of CARD14sh: investigating its functions beyond the NF-κB hyperactivation

Driven by the limited impact observed on NF-κB activity modulation in psoriasis-associated mutations, such as R38C, and considering the previously observed effects of CARD14sh in TBK1/IRF3 activity [133], our focus shifted towards exploring an alternative pathway. In our experiments, we aimed to investigate the potential influence of CARD14sh and its mutants on the TBK1/IRF3 response. To induce TBK1/IRF3 activity, we utilized polyinosinic-polycytidylic acid (POLY (I:C)), a synthetic analogue of dsRNA that mimics a characteristic feature found in certain viruses [138]. POLY (I:C) acts as a ligand for TLR3 and RLRs, triggering antiviral responses and leading to increased expression of NF-κB and IRF3 transcription factors [139]. By utilizing POLY (I:C) as a stimulus, we aimed to investigate the potential crosstalk between CARD14sh and the TBK1/IRF3 signalling pathway.

Moreover, building upon the previously observed interaction between CARD10 and MAVS, we explored the possibility of a similar occurrence with CARD14sh. Given the striking similarities between CARD14sh and CARD10, it is plausible that a comparable mechanism could come into play when CARD14sh interacts with MAVS, potentially leading to abnormal functioning, which, in turn, might be closely associated with the onset and progression of Psoriasis.

VIII.2.1 The effects of POLY (I:C) on CARD14 expression levels

In this experiment, our primary objective was to observe the effects of induced antiviral response on the levels of CARD14 in keratinocytes. To achieve this, we examined both mRNA expression and protein levels of CARD14 in response to POLY (I:C) treatment. In the first part of the experiment, we aimed to demonstrate the impact of POLY (I:C) exposure on the expression levels of CARD14sh and MAVS in HaCaT cells. Our results unequivocally showed a gradual increase in the levels of both CARD14sh and MAVS upon exposure to POLY (I:C), providing strong evidence of POLY (I:C)'s influence on their expression (Figure 5A). Furthermore, we analyzed the mRNA expression levels of CARD14 in response to POLY (I:C) exposure. Our findings revealed a significant increase in the mRNA expression levels of CARD14sh (Figure 5B), indicating its transcriptional upregulation upon POLY (I:C) stimulation. Moreover, we investigated the relative mRNA expression levels of IFN-β and CCL20 under conditions with or without POLY (I:C) treatment. Notably, the results demonstrated a significant increase in the levels of IFN-β and CCL20 mRNA compared to the control group (Figure 5C). This suggests the enhanced activity of P-IRF3 and NF-κB transcription factors, respectively, in response to POLY (I:C) stimulation, highlighting the activation of the antiviral signalling pathway in keratinocytes.

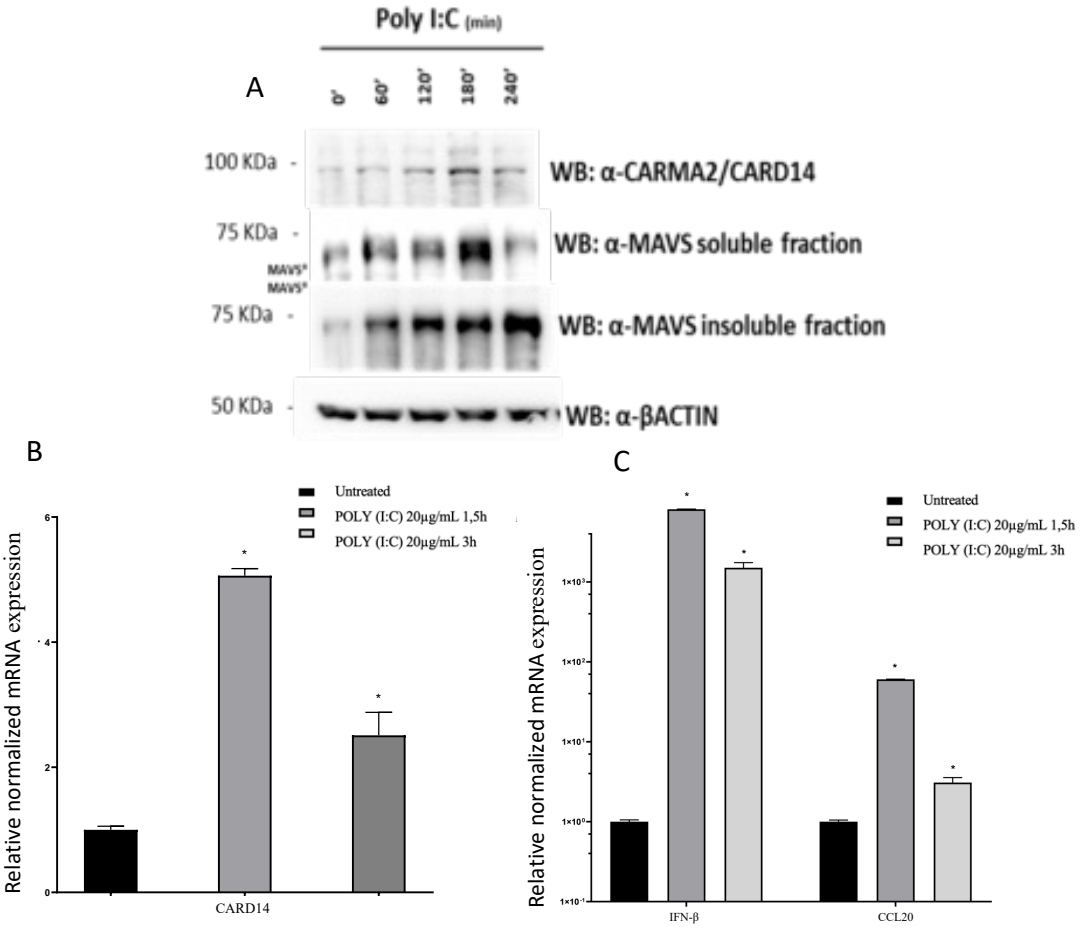

Figure 11: The effects of POLY (I:C) on CARD14 expression - The expression levels of CARD14 and MAVS were assessed through western blotting in HaCaT cells that were previously treated with POLY (I:C) - It should be noted that during the extraction procedure for western blotting, some MAVS proteins may or may not remain attached, leading to variations in the detection. Therefore, soluble, and insoluble forms of MAVS were accounted for. Here, an illustrative Western blot image representative of three independent experiments is provided for reference (A). The relative mRNA expression levels of CARD14 among distinct concentrations of POLY I:C and control are presented (B) and also the relative mRNA expression levels of IFN- β and CCL20 among distinct POLY I:C treatment times and control are presented (C). (*P < 0.05; **P < 0.01). These experiments were performed three times independently.

Collectively, the stimulation appears to produce an increase in protein and mRNA CARD14sh levels.

VIII.2.2 CARD14sh effects on TBK1/IRF3 response induced with POLY I:C

To investigate the impact of CARD14sh protein on IRF3 expression, the mRNA levels of IFN-β were analyzed in different cellular conditions, including vector (control), vector + POLY (I:C), CARD14sh, and CARD14sh + POLY (I:C) (Figure 6). Notably, upon exposure to POLY (I:C), control cells exhibited a significant increase in IFN-β mRNA expression compared to cells transfected with CARD14sh.

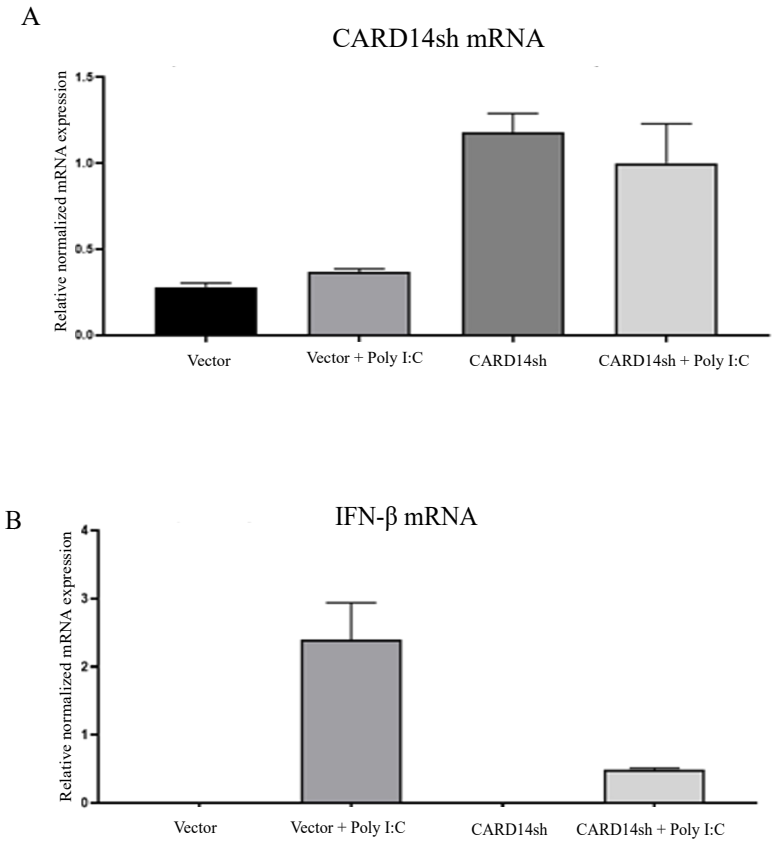

Figure 12:CARD14sh effects on IFN- β mRNA expression levels upon POLY (I:C) stimulation - The mRNA expression levels of CARD14 were utilized as a normalization approach (A). The relative expression levels of IFN-β mRNA among the different conditions (B). This experiment was performed three times independently.

Furthermore, a representative western blot analysis presented in Figure 7 demonstrated a noticeable reduction in P-IRF3 protein levels in the presence of CARD14sh.

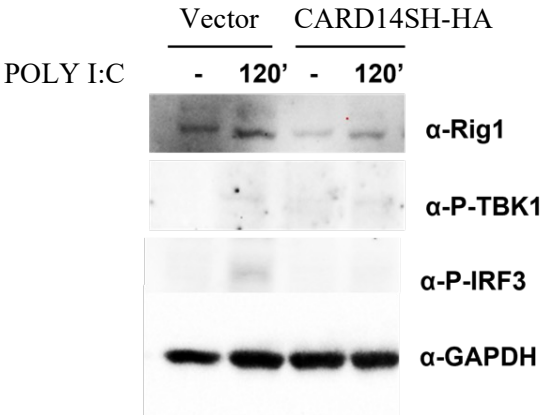

Figure 13:Evaluating the influence of CARD14sh on IRF3 signalling - Expression levels of TBK1, P-IRF3 and RIG1 were observed through a western blot. A GAPDH Western blot was used for normalization. An illustrative Western blot image representative of three independent experiments is provided. This experiment was performed three times independently.

The observed decrease in both IFN-β mRNA and P-IRF3 protein levels suggests that CARD14sh plays a role in modulating the activity of TBK1/IRF3response.

VIII.2.3 CARD14sh mutants are significantly less effective than CARD14sh WT in restraining the TBK1/IRF3 response induced with POLY I:C

Our experimental approach for this experiment involved subjecting HaCaT cells to POLY (I:C) stimulation for 180 minutes, followed by the examination of P-IRF3 levels using immunoblot analysis and expression of IFN-β through mRNA analysis.
The results presented revealed an interesting finding regarding the effectiveness of CARD14sh mutants compared to CARD14sh WT in restraining TBK1/IRF3 activity. As depicted in Figure 8, the expression levels of IFN-β were noticeably higher in cells expressing CARD14sh mutants compared to those expressing CARD14sh WT. This suggests that CARD14sh mutants have a diminished ability to inhibit the TBK/IRF3 response activity.

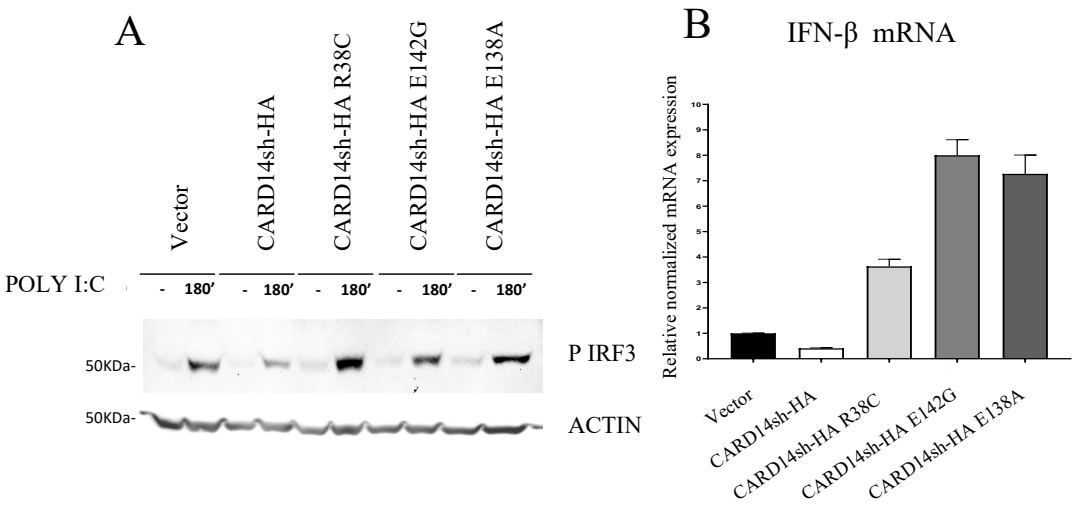

Figure 14:Effects of CARD14sh mutants ectopic expression on antiviral response - The expression levels of P-IRF3 were assessed by western blotting HaCaT cells pre-exposed to POLY (I:C). Actin western blot was used for normalization. An illustrative Western blot image representative of three independent experiments is provided for reference (A). The relative expression levels of IFN-β mRNA among the different conditions (B). This experiment was performed three times independently.

These findings highlight the functional differences between CARD14sh mutants and CARD14sh WT in regulating the TBK1/IRF3 pathway. The reduced effectiveness of CARD14sh mutants in restraining this pathway activity may have implications for the downstream signalling events involved in psoriasis.

VIII.2.4 The interaction between CARD14/CARMA2sh and MAVS

To confirm the interaction between CARD14sh and MAVS, we transfected HEK293 cells with MAVS and/or CARD14sh.

The transfection process involved introducing CARD14sh-HA, MAVS-FLAG, CARMA2sh-HA + MAVS-FLAG, or a vector control into HEK293 cells. Co-immunoprecipitation of MAVS revealed the migration of CARD14sh-HA+MAVS-FLAG bands on the Western blot probed with an HA antibody (Figure 10). This finding provides compelling evidence for the physical association of CARD14sh with MAVS, firmly establishing their interaction in HEK293 cells. To further validate the presence of MAVS in the transfected cells, an additional Western blot analysis was performed using an FLAG antibody specifically recognizing the FLAG tag.

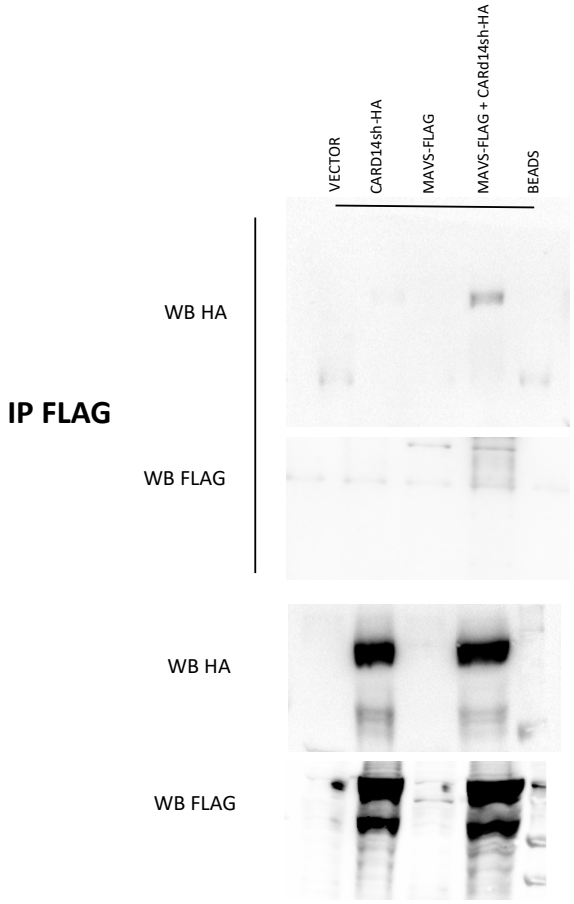

Figure 15:Interaction between CARD14sh and exogenous MAVS in HEK293 cells - Immunoprecipitation of MAVS-FLAG, followed by a Western blot using an HA antibody, was conducted to assess the interaction between CARD14sh and MAVS. An additional Western blot using a FLAG antibody was performed to validate the presence of MAVS within the precipitated complex. An illustrative Western blot image of the co-immunoprecipitated CARD14sh, representative of three independent experiments, is provided as a reference. This experiment was performed three times independently.

To further confirm the interaction between CARD14sh and MAVS, an additional experiment was performed. In this experiment, we investigated the potential interaction between endogenous MAVS and CARD14sh. To assess this interaction, HaCaT cells were transfected with CARD14sh and subjected to varying exposure times to POLY (I:C) treatment: 0 minutes (0'), 90 minutes (90'), or 180 minutes (180') (Figure 9). Following transfection, co-immunoprecipitation analysis revealed the presence of both MAVS and CARD14sh in the precipitated complex.

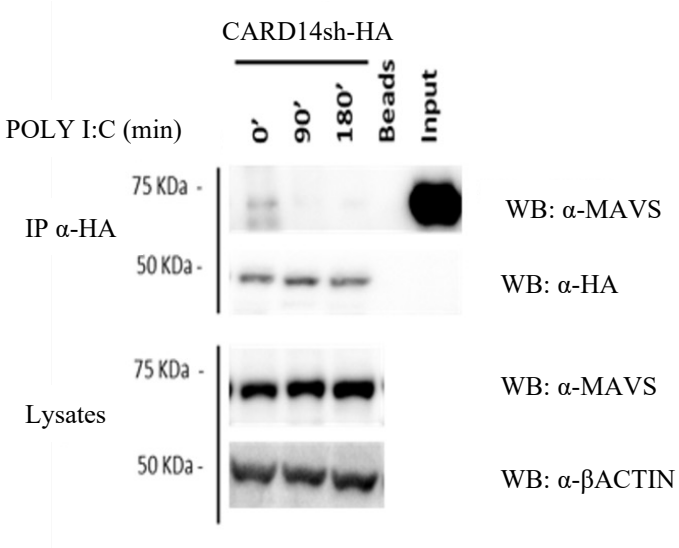

Figure 16:Interaction between CARD14sh and endogenous MAVS in HaCaT cells - Immunoprecipitation of CARD14sh was performed, followed by a Western blot using an HA antibody to assess the interaction between endogenous CARD14sh and MAVS. MAVS and β-actin lysate levels were utilized for normalization. An illustrative Western blot image of the co-immunoprecipitated CARD14sh, representative of three independent experiments, is provided as a reference. This experiment was performed three times independently.

Taken together, our co-immunoprecipitation experiments revealed a robust interaction between CARD14sh and MAVS in both HaCaT and HEK293 cells. Moreover, our observations suggest that CARD14sh and MAVS interact in unstimulated cells.

VIII.2.5 The interaction between CARD14/CARMA2sh mutants and MAVS

To determine if the observed CARD14sh-MAVS interaction extends to the CARD14sh mutants (E142G, E138A, and R38C), the cells were transfected with the following constructs: Vector, CARD14sh-HA, CARD14sh R38C-HA, CARD14sh 142G-HA, CARD14sh E138A-HA, MAVS-FLAG, CARD14sh-HA + MAVS-FLAG, CARD14sh R38C-HA + MAVS-FLAG, CARD14sh 142G-HA + MAVS-FLAG, or CARD14sh E138A-HA + MAVS-FLAG.
Co-immunoprecipitation analysis demonstrated the presence of MAVS in the precipitated complex of all CARD14sh mutant forms (Figure 11).

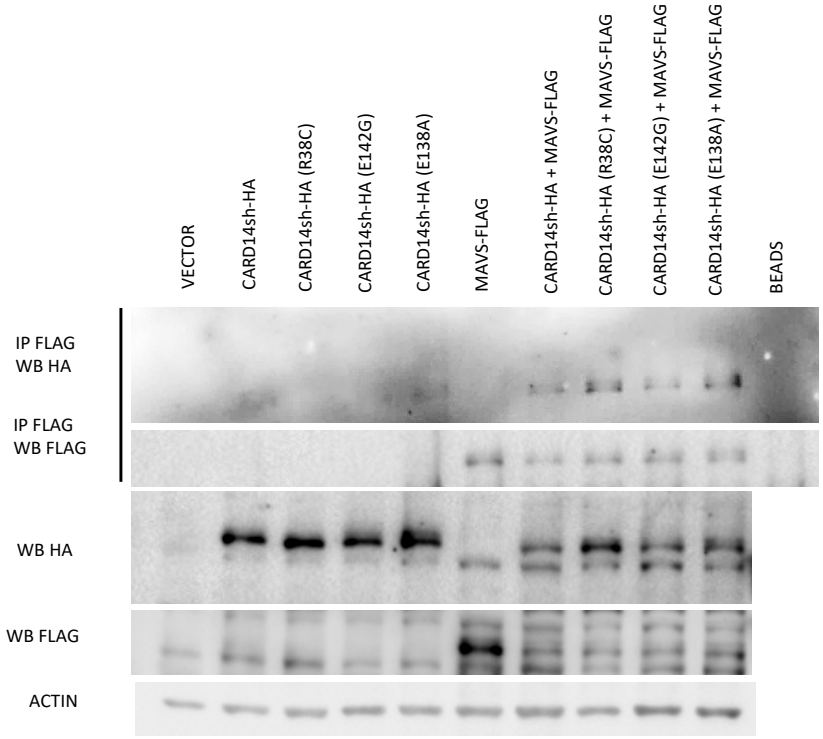

Figure 17:Interaction between CARD14sh mutants and MAVS - Immunoprecipitation of MAVS-FLAG, followed by a Western blot using an HA antibody, was performed to investigate the interactions of CARD14sh WT, CARD14sh R38-C, CARD14sh E142G, and CARD14SH E138a with MAVS. An illustrative Western blot image of the co-immunoprecipitated MAVS, representative of three independent experiments, is provided as a reference. This experiment was performed three times independently.

These findings reveal that not only the CARD14sh WT is able to interact with MAVS but also the psoriasis-associated mutants E142G, E138A and R38C.

VIII.2.6 CARD14sh role on MAVS aggregation

Upon the activation of the induced antiviral response, MAVS undergoes aggregation [140]. In this experiment, we sought to investigate whether the interaction of CARD14sh with MAVS affects its aggregation. To assess this, we performed a semi-denaturing detergent agarose gel electrophoresis (SDD-AGE) analysis. Surprisingly, the SDD-AGE analysis revealed no strong effects on MAVS aggregation levels on HEK293 when co-transfected with CARD14sh-HA and MAVS-HA, as depicted in Figure 12.

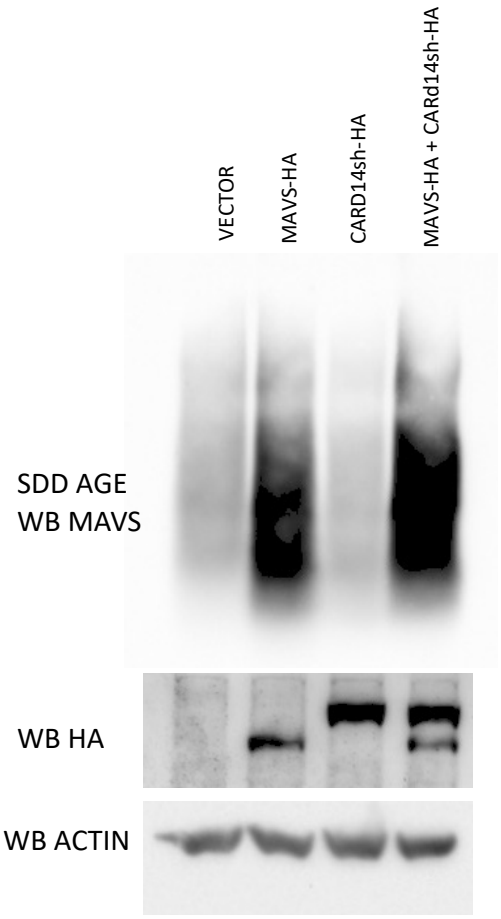

Figure 18:CARD14sh effects on MAVS aggregation levels in HEK293 cells - MAVS SDD-AGE and CARD14-HA Western blot were employed to investigate the role of CARD14 in regulating MAVS expression levels. An actin Western blot was used for normalization. An illustrative Western blot image of MAVS SDD-AGE, representative of three independent experiments, is included as a reference. This experiment was performed three times independently.

Altogether, the lack of significant changes in MAVS aggregation in the presence of CARD14sh suggests that this interaction may not have a substantial impact on MAVS aggregation.

IX Discussion

Among the various proteins linked to psoriasis, CARD14, more specifically, its splice variant CARD14sh is our focus of attention, as it exhibits the highest level of CARD14 expression in the skin [57]. This protein presents an intriguing subject for study and a potential target for psoriasis treatment, given its undeniable association with psoriasis development. However, the precise regulatory mechanisms involving CARD14 remain poorly understood. Multiple mutations in CARD14 have been identified in psoriasis patients, some of which are exclusive to particular forms of psoriasis [75], while others are observed across multiple forms. Certain mutations, such as CARD14sh E138A and CARD14sh E142G, are known to hyperactivate NF-κB, while others like CARD14sh R38C render CARD14 unable to activate this process NF-κB in a CBM-dependent manner [75], [83], [135], [136].

In this thesis, we first examined the regulatory variations in CARD14 mutants compared to CARD14 WT and investigated how these mutations might impact characteristic morphological features typically observed in keratinocytes during psoriasis, including increased cell proliferation, survival, and migration. Moreover, extensive documentation supports that the R38C mutant, in contrast to E138A and E142G, does not hyperactivate NF-κB, yet it is frequently observed in psoriasis patients. This intriguing observation prompted us to investigate the possibility of an alternative pathway through which these mutations may exert their effects. In this study, we directed our attention towards elucidating the impact of CARD14sh on MAVS-mediated TBK1/IRF3 modulation.

IX.1 Impact of CARD14sh mutations on the development of psoriasis-related keratinocyte features

Our results demonstrated that CARD14sh R38C, E142G, and E138A mutants induced cell proliferation to a significant extent compared to CARD14sh WT and the control group. With the exception of CARD14sh R38C mutation which only displayed statistically significant effects initially. This observation suggests that these CARD14sh mutants may contribute to the abnormal cell proliferation observed in psoriatic lesions. Additionally, the increased cell migration observed in cells expressing CARD14sh mutants suggests their involvement in the abnormal wound healing process observed in psoriasis. The faster scratch closure observed in cells expressing CARD14sh mutants compared to CARD14sh WT and the control group indicates their potential role in promoting excessive cell migration, which may contribute to the Koebner phenomenon and the faster healing of small wounds observed in individuals with psoriasis [104], [141].

The amplified expression of these morphological features in psoriasis-associated CARD14sh mutants compared to CARD14sh WT, suggests a role for these mutants in amplifying these phenomena during psoriasis. Furthermore, even though the R38C mutation only enhances cell proliferation during the initial phases of cell growth, its contribution to increased cell viability and migration suggests that despite not enhancing NF-κB expression, it could participate in distinct intracellular mechanisms regulating skin homeostasis.

IX.2 The modulatory role of CARD14sh in TBK1/IRF3 signalling

IX.2.1 Distinct roles of CARD14sh and its mutants in the TBK1/IRF3 response

Our experiments have provided compelling evidence supporting the modulation of the TBK1/IRF3 pathway by CARD14sh. It is important to point out the intriguing observation that in CARD14sh transfected HaCaT cells treated with POLY (I:C) the level of P-IRF3 was lower than in control. Thereby suggesting an inhibition of antiviral response by CARD14sh. We then sought to expand upon these findings by investigating the regulatory distinctions between the CARD14sh WT and the E138A, E142G and R38C mutants on the TBK1/IRF3 response. We successfully observed decreased levels of P-IRF3 in the CARD14sh WT condition compared to its mutant counterparts. These findings corroborate previous observations and expand upon them by introducing the R38C mutation to our experimental system. Consistent with prior studies, we observed that CARD14sh WT exhibits a higher efficacy in inhibiting the TBK1/IRF3 response compared to its mutant counterparts [133].

These discoveries are highly intriguing as they support the role of CARD14sh in modulating the TBK1/IRF3response, emphasizing that CARD14sh is capable of regulating additional mechanisms beyond the CBM-dependent NF-κB activation, which becomes dysregulated in the presence of mutations in the CARD14 gene, including E142G and E138A [75]. Understanding the full spectrum of mechanisms regulated by CARD14sh could significantly advance our knowledge of autoimmune skin diseases, such as psoriasis, and pave the way for potential future treatment approaches.

IX.2.2 CARD14sh and MAVS: unveiling a novel interaction

The results obtained from both HaCaT and HEK293 cells provide clear evidence of the interaction between CARD14sh and, both endogenous and exogenous MAVS. Moreover, the observed increase in MAVS levels upon prolonged treatment with POLY (I:C) may indicate an unknown regulatory mechanism that mediates this interaction. This observed interaction may imply a regulatory mechanism in which CARD14sh influences MAVS activity. The decrease in TBK1/IRF3 activity observed in the presence of CARD14sh suggests that this scaffold may downregulate MAVS activity. Upon MAVS activation, it is likely that some unidentified molecular signalling events occur, impairing CARD14sh modulation of MAVS. However, it is important to note that, according to our results, CARD14sh WT overexpression does not affect MAVS aggregation, therefore suggesting that CARD14sh interaction with MAVS modulates its activity in an unknown way. Furthermore, a supplementary study should be conducted to determine if the MAVS aggregation levels also remain untouched in the presence of CARD14sh mutants.

It is worth considering the study conducted in CARD10 [126] has mechanisms similar to those identified in that study may also be applicable to CARD14sh proteins. If this is indeed the case, it is possible that CARD14sh mutations could exhibit variations in MAVS modulation, similar to their effects on CBM complex modulation. This observation could help elucidate the prevalence of various CARD14 mutations among psoriasis patients, particularly those mutations that do not affect NF-κB activity. Nevertheless, it is crucial to acknowledge that these findings are preliminary, and further research is required to comprehensively elucidate the functional consequences of this novel interaction. Future studies should focus on exploring the downstream signalling pathways activated by the interaction between CARD14sh and MAVS. Taken these findings together, a thorough investigation is required to ascertain whether an interplay between CARD14sh proteins and the TLR3 and MAVS pathways exists during psoriasis, given that POLY (I:C) treatment results in elevated P-IRF3 levels in CARD14sh mutations compared to those in CARD14sh WT.

X Conclusions

Psoriasis initiation is often linked to a combination of genetic and environmental influences, yet the specific pro-inflammatory autoimmune mechanisms that incite and sustain this condition remain elusive. Central to this disorder are keratinocytes, which exert regulatory control through their release of pro-inflammatory mediators. These mediators induce stress in adjacent keratinocytes and attract immune cells to the epidermal layer, resulting in a complex interplay between adaptive and innate immunity [1], [142].

CARD14sh mutations are well documented as being connected to contribute to psoriasis, our research has provided insights into the role of CARD14sh mutations in psoriasis-related morphological characteristics, such as keratinocyte cell proliferation, viability, and migration. We observed an increase in these features when comparing psoriasis-associated CARD14sh mutants to its wild-type counterpart, suggesting a pivotal role of these mutants in exacerbating psoriasis phenomena.

While this condition was initially believed to be primarily regulated by Th1 cells, the recent discovery of Th17 cells has challenged this assumption [143]. Current evidence strongly supports the critical role of the IL-17/IL-23 axis in the development of this disease [1], [17], [38]–[40]. Nevertheless, it is becoming increasingly evident that the dysregulation of innate immune mechanisms also play a crucial role in both the onset and progression of this skin disorder [49], [56]. This may include a potential dysregulation of TLRs and MAVS pathways. It is well documented that CARD14sh and some of its identified mutants can promote CBM oligomerization upon activation by receptors such as IL-17R [38], subsequently resulting in the overexpression of NF-κB. However, the extent to which CARD14sh plays a role in the regulation of other transcription factors remains unclear. In our research, we observed that CARD14sh WT exhibits a greater ability to inhibit the phosphorylation of IRF3 when compared to its mutant counterparts. These findings suggest that CARD14sh may indeed inhibit P-IRF3 levels by interacting with the signal transduction pathways that mediate the activity of this transcription factor, including TLR3/4 and MAVS, therefore suggesting that CARD14sh mutants could be involved in the hyperactivation of both NF- κB and IRF3 transcription factors.

Moreover, our observations on the CARD14sh R38C mutation are of particular interest. This psoriasis-associated mutation revealed sustained elevated cell proliferation levels only initially when compared to the wild type variant. It's understood that the R38 residue, located within the CARD domain of CARD14sh, is crucial for CARD-CARD interactions between this protein and BCL10. Previous research has demonstrated that when an induced mutation on the R38 residue of CARD14 E138A occurs, this interaction ceases, leading to a decrease in NF-κB expression levels [135]. Therefore, this implicates a decrease in CBM-dependent NF-κB induction in CARD14sh R38C mutants. Surprisingly, despite this predicted decrease in NF-κB induction, our study revealed that the CARD14sh R38C mutation increases the levels of P-IRF3. Following nuclear translocation, P-IRF3 induces the transcription of IFN- α and IFN- β. These interferons activate MAPK pathways, which are implicated in cell proliferation and the emergence of the psoriasis phenotype [9], [18]. These findings underscore the existing knowledge gaps surrounding the signalling pathways that may be regulated by CARD14sh R38C during the progression of psoriasis, as well as of other CARD14sh mutations.

Taken together, our observations further highlight the importance of the CARD14sh protein mutations in the progression and onset of psoriasis. However, these findings emphasize the necessity of a comprehensive investigation to ascertain the potential interplay of CARD14sh mutants on additional signalling pathways. Such an inquiry could provide a more in-depth understanding of the psoriasis pathogenesis, therefore paving a way for more targeted therapeutic treatment solutions for this disorder.

Our research has yielded a noteworthy finding regarding the interaction between CARD14sh and MAVS. Notably, the CARD14sh mutants (E142G, E138A, and R38C) also demonstrate an interaction with MAVS, which prompts the need for further investigation to assess the impact of these interactions on MAVS activity when compared to the wild type CARD14sh.

Interestingly, we observed that the overexpression of CARD14sh did not affect MAVS aggregation. This observation raises intriguing questions about the potential role of CARD14sh in modulating MAVS activity. Considering the substantial homology between CARMA proteins, such as CARD10 and CARD14sh, it is plausible that they may share a similar function in MAVS regulation. However, it is important to note that the interaction between CARD14sh and MAVS observed in our study may not be equivalent to the CARD10-MAVS interaction. The CARD10 protein was found to bind

to MAVS via its GUK domain [126], a domain which is absent in the CARD14sh isoform [67], [68]. This distinction suggests that the mechanisms underlying the interactions of CARD10 and CARD14sh with MAVS could differ significantly. These findings open up new avenues of research into the intricate interplay between CARD14sh and MAVS in the context of psoriasis. Further exploration of these interactions will contribute to a deeper understanding of the signalling pathways involved in this complex autoimmune disease.

Overall, our discovery of the CARD14sh-MAVS interaction underscores the importance of investigating the intricate molecular mechanisms involved in psoriasis. As we continue to unravel the complexities of this skin disease, we move closer to the development of more effective and tailored treatment options for individuals suffering from psoriasis.

X.1 Future perspectives

One significant barrier to expanding our understanding of this pathology is the lack of relevant animal models. In this regard, the development of genetically modified animal models that target the locus encoding CARD14 could significantly enhance our understanding of the functions of the CARD14 protein and its corresponding mutations. For instance, murine studies have shown that Card14$^{138A/+}$ and Card14$^{\Delta Q136/+}$ heterozygous mice exhibit an overexpression of the CBM complex. Remarkably, these mice spontaneously manifested a phenotype akin to the one observed in psoriasis [72], [144]. Hence, such models would facilitate the identification of mutations that can independently manifest the psoriasis phenotype while also allowing for a more accurate delineation of the intrinsic pathways that are disrupted during this disorder by these specific mutations.

Moreover, the utilization of a 3D co-culture system incorporating keratinocytes and other resident skin cells could augment our capacity to examine the complex cellular interactions characteristic of this disease, representing an advantage over the traditional 2D cell culture models. Remarkably, a limited number of studies have constructed 3D skin models for psoriasis research, integrating keratinocytes, fibroblasts, and specific T cell subsets. These innovative models have already proven valuable in quantitatively assessing T cell activation and migration, including IL-17A dynamics [145], [146]. However, it's important to note that psoriasis involves a broader spectrum of immune cells, including neutrophils, monocytes, macrophages, and DCs [3], [17]. Considering this, the development of a comprehensive *in vitro* psoriatic skin model, which captures the full spectrum of cellular interactions in psoriasis, would be highly beneficial. Such a model would offer us the means to quantitatively assess the abnormal release of pro-inflammatory mediators, as well as the activation and recruitment of immune cells resulting from the complex dialogue between epithelial and immune cells. Moreover, it would present a more physiologically relevant platform to evaluate potential pharmacological interventions for this intricate skin disorder.

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