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RAGE as a regulatory molecule in cutaneous chronic inflammation

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Declarations according to § 8 (3) b) and c) of the doctoral degree regulations:

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Kathrin Tarnanidis

This thesis is dedicated to

my family

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III. Abstract

Psoriasis is a complex genetic, chronic inflammatory disease of the skin with a prevalence of 2 % among the population. It is characterized by a multi-faceted interplay of keratinocytes, dendritic and T cells, as well as downstream transcription factors and proinflammatory cytokines. The IL-23/T_H17 axis is thought to play an important role in the pathogenesis of psoriasis. However, the relative contributions of keratinocytes and immune cells to disease initiation and maintenance remain unclear. Recent studies implicate a role of the receptor for advanced glycation end-products (aptly known as RAGE) in the development of chronic disorders due to its expression on involved cell types and its constitutive signaling in inflammatory conditions.

The here presented thesis revealed a role for RAGE in psoriasis by demonstrating its upregulation in human and murine psoriatic specimens. In addition, RAGE drives and maintains chronic inflammation as the initial phase and the chronic stage of the inflammation were found to be diminished in Rage-deficient mice upon topical Imiquimod treatment. Moreover, RAGE was expressed on psoriatic effector cells such as keratinocytes and dermal inflammatory cells including $CD11c^+$ dendritic cells. While the inflammation-associated activation of keratinocytes resulting in a release of the alarmins S100B and HMGB1 was demonstrated to be independent of RAGE, the functionality of plasmacytoid dendritic cells, which are major activators of dermal dendritic cells, strongly relied on active RAGE signaling. Indeed, both HMGB1 and S100B in complex with self-DNA activated plasmacytoid dendritic cells *via* RAGE. The finding that RAGE additionally controls the transcription of inflammatory response genes involved in the differentiation of naïve T cells into the T helper cell lineage further supported the hypothesis that RAGE signaling is essential for the activation of the IL-23/T_H17 axis in psoriasis. This was underlined by the rescue of the defective inflammatory phenotype of Rage-deficient mice by intradermal injections of recombinant IL-23.

Taken together, these findings illustrate an epidermal-innate immune crosstalk mediated by RAGE signaling that in turn affects adaptive immune responses and points towards a central role of RAGE in the IL-23/T_H17 axis of psoriasis. Therefore, this thesis highlights RAGE signaling as a potential target for new therapeutic strategies intervening ‘upstream’ of IL-23.

IV. Zusammenfassung

Psoriasis ist eine komplexe genetische, chronisch entzündliche Erkrankung der Haut mit einer Prävalenz von 2 % in der Bevölkerung. Sie wird durch ein komplexes Wechselspiel von Keratinozyten, dendritischen und T-Zellen, sowie nachgeschalteten Transkriptionsfaktoren und Zytokinen charakterisiert. Eine wichtige Rolle in der Pathogenese der Psoriasis wird der IL-23/T_H17-Achse zugesprochen. Die relative Beteiligung von Keratinozyten und Immunzellen an der Initiierung und Aufrechterhaltung dieser Erkrankung bleibt jedoch unklar. Jüngste Studien implizierten eine Rolle für den Mustererkennungsrezeptor RAGE (*engl.* 'receptor for advanced glycation end-products') bei der Entwicklung von chronischen Erkrankungen basierend auf seiner Expression durch beteiligten Zelltypen und seiner kontinuierlichen Signaltransduktion während Entzündungsreaktionen.

Die hier präsentierte Arbeit enthüllte eine funktionelle Rolle von RAGE in der Psoriasis durch eine nachweisliche Hochregulierung der Rezeptorexpression in humanen und murinen psoriatischen Gewebeproben. Die Verminderung der initialen wie auch der chronischen Entzündungsphase in Imiquimod behandelten Rage-defizienten Mäusen zeigte, dass RAGE nicht nur an der Enstehung einer chronischen Entzündung beteiligt ist, sondern auch deren Aufrechterhaltung ermöglicht. Als RAGE exprimierend wurden psoriatische Effektorzellen wie Keratinozyten und kutane Entzündungszellen, $CD11c^+$ dendritischen Zellen inbegriffen, identifiziert. Während die entzündungsbedingte Aktivierung von Keratinozyten, die zur Freisetzung der Alarmine S100B und HMGB1 führt, unabhängig von RAGE vonstattengeht, stützt sich die Funktionalität von plasmazytoiden dendritischen Zellen, die als wichtigste Aktivatoren dermaler dendritischer Zellen gelten, stark auf einen aktiven RAGE Signalweg. Komplexe, bestehend aus körpereigener DNA und HMGB1 oder S100B, aktivieren plasmazytoide dendritische Zellen durch Bindung an RAGE. Die Erkenntnis, dass RAGE die Transkription von Genen der Entzündungsantwort, die unter anderem an der Differenzierung von naiven T-Zellen in die T-Helfer-Zelllinien beteiligt sind, kontrolliert, unterstützt die Hypothese, dass die RAGE Signalkaskade für die Aktivierung der IL-23/T_H17 Achse essentiell ist. Vorangetrieben wurde diese Vermutung durch die korrigierende Wirkung intradermaler rIL-23-Injektionen, die vergleichbare Phänotypen in Wildtypen- und Rage-defizienten Mäuse induzierten.

Die Studie veranschaulicht somit nicht nur ein Wechselspiel von epidermalen und angeborenen Immunzellen, das wiederum die erworbenen Immunreaktionen beeinflusst, sondern weist auch auf eine zentrale Rolle des RAGE-Signalweges in der IL-23/T_H17-Achse der Psoriasis hin; dies identifiziert RAGE als potentielles IL-23-vorangeschaltetes Angriffsziel neuer therapeutischer Strategien.

V. Introduction

V.1. Inflammation

V.1.1. Definition

Inflammation (*latin*, inflammatio, *engl.* 'to set on fire') is part of a complex response that occurs in reaction to chemical or physical irritation, injury or infection in living tissue. It involves a well-organized cascade of vascular and cellular changes and has both beneficial and detrimental, as well as local and systemic effects [1]. Closer examinations of inflammation-associated processes uncovered a close relationship between inflammation and the immune system [2].

V.1.2. Innate immune system

The innate immune system is a universal system and part of the host defense against invading pathogens. It consists of chemical and physical barriers, the complement system, as well as phagocytic granulocytes such as neutrophils, eosinophils, macrophages, and dendritic cells (DCs). These granulocytes are capable of sensing microorganisms as soon as they penetrate the epithelial surface, thereby contributing to the 'first line of defense' [3]. Pathogens are not only recognized by granulocytes but also by the complement system, which becomes activated and initiates proteolytic lysis of the infiltrating microorganisms [3]. Germline-encoded pattern recognition receptors (PRRs) expressed on granulocytes bind common structures of pathogens, thus inducing the secretion of proinflammatory cytokines and chemokines, which initiate the process of inflammation [3], [4]. During initiation, neutrophils and macrophages derived from monocytes engulf and destroy the infiltrating pathogen. Moreover, eosinophils contribute *via* the 12/15-lipoxygenase route to the resolution of the inflammation [5]. Simultaneously, antigen-presenting DCs migrate through afferent lymphatics to local lymph nodes and present the antigens *via* the major histocompatibility complex (MHC) to naïve T cells. This feature, as well as the ability of DCs to discriminate between host and non-self antigens highlights their importance as key players in the interface between the innate and adaptive immune system. Indeed, the activation of T lymphocytes is dependent on the interaction between receptors like the cluster of differentiation (CD) 28 and costimulatory molecules, such as CD80 and CD86, expressed by DCs [3].

V.1.3. Adaptive immune system

In contrast to the innate immune recognition the adaptive immune system consists of B and T lymphocytes that are activated through antigen presentation by antigen-presenting cells (APCs) like DCs, thereby representing the 'second line of defense'. Naïve T cells

continuously circulate in the blood and the lymphoid organs. In the latter, naïve T lymphocytes differentiate into T effector cells and clonally expand upon antigen presentation of APCs. After differentiation and proliferation, effector T cells are recruited to inflamed tissue by the expression of selectins, adhesion molecules and chemokines [6]. Effector T cells can be grouped into two main subtypes: $CD4^+$ helper T (T_H) cells that recognized MHC class II molecules on APCs, and $CD8^+$ cytotoxic (T_C) cells, which bind to MHC class I molecules [3]. T_C cells directly respond to infected cells with the release of cytotoxic proteins, such as granzyme and perforin [7]. This release is highly effective declaring the need of required costimulation by, for instance, binding of costimulatory molecules on APCs to CD28 or by the present of T_H cells [3]. The T helper cell subset consists of different subtypes. T_H1 cells are characterized by the clearance of infiltrating pathogens and the production of interferon (IFN)-γ, whereas T_H2 lymphocytes are involved in the clearance of specific parasitic infections and the production of interleukin (IL) -4, IL-5 and IL-13 [8]. The third subtype of T_H cells are the so-called T_H17 cells, which are characterized by the production of IL-17 and known to have an impact on autoimmune diseases as well as on tumorigenesis. T helper cell responses can be modulated with the help of other immune cells; for instance, IL-12 expression by DCs differentiates naïve T lymphocytes into type 1 T helper (T_H1) cells, while the expression of IL-23 leads to the proliferation of T_H17 cells [8]–[10]. Autoimmune diseases also involve the T_H1 subset, whereas T_H2 cells are thought to be associated with asthma [11]. Another main function of T helper cell subsets is the promotion of a B lymphocyte response [12]. These are crucial components of the immune system enable the removal of pathogens and toxins due to their ability to produce antibodies. B cells have membrane-bound immunoglobulins on their surface, so-called B cell receptors (BCRs), that bind and internalize foreign antigens leading to the production and secretion of antigen-specific antibodies [13], [14]. As essential mediators of adaptive host defense, the antibodies are able to directly neutralize pathogens or opsonize them for phagocytosis [3]. In addition, the appearance of a T cell-independent B cell activation has been described [12]. Furthermore, B cells differentiated into diverse effector B cells have additional functions such as antigen presentation and production of multiple cytokines [15]–[17].

V.1.4. Classification of inflammation

Inflammation can be classified by means of time and action as peracute, acute and subacute inflammatory response, as well as chronic inflammation. Peracute inflammation shows a rapid onset within minutes to a few hours and is usually induced by a potent stimulus such as bacterial or viral infection. The acute form of inflammation emerges due to bacterial or viral infections, but also upon mechanical trauma, physical and chemical agents or hypersensitivity reactions (immune reactions). Acute inflammation is mainly characterized by increased blood flow, increased vascular permeability for plasma proteins, and the

emigration of leukocytes, predominantly neutrophils [18]. The accumulation of leukocytes requires a series of successive processes. First, leukocytes adhere on endothelial surfaces due to the interactions between complementary adhesion molecules on leukocytes (*e.g.* C5a) and endothelial cells [19], [20]. Integrins, including members of the CD11/CD18 adhesion family modulate cell-matrix and cell-cell interactions [21]. Additionally, endothelial-leukocyte adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), ICAM-2 and vascular cell adhesion molecules (VCAMs) on endothelial cells contribute to the leukocyte adhesion to the endothelium [22], [23]. After adhesion, cells emigrate in a process controlled by chemotaxis. Leukocytes express specific chemotactic receptors on their cell surface allowing them to bind a variety of chemotactic agents, and in turn follow respective chemical gradients. Accumulated at the site of injury or infection, leukocytes, such as neutrophils and macrophages, recognize, engulf, degrade microorganisms and foreign materials, and secrete leukocyte-specific enzymes; a process, which is known as phagocytosis [18], [24]. Acute inflammation is limited by apoptosis and subsequent clearance of activated cells resulting in the inflammatory resolution [25].

Clinically, acute inflammation exhibits five cardinal signs first described by Aulus Celsus (30-38 B.C.) and Virchow (1821-1902), namely redness (*rubor*), swelling (*tumor*), heat (*calor*), pain (*dolor*), and loss of function (*function laesa*) [26]. Redness and heat result from the vasodilation of blood vessels, whereas the swelling arises from edema. The local release of prostaglandin and kinins can lead to pain.

The subacute inflammation differs to the aforementioned in its extended duration and displays the period between acute and chronic responses [27]. Under normal conditions the inflammatory response is self-limiting, and has beneficial aspects including elimination of injury causes, neutralization of offending agents, and restoration of tissue integrity. Under pathological conditions inflammation becomes continuous and leads to the development of chronic inflammatory diseases [1]. Clinically, chronic inflammation exhibits less redness and heat compared to acute inflammatory reactions, while showing a tendency for swelling and firmness. Chronic inflammation represents a prolonged deregulated immune response that, in contrast to acute inflammation, is not only exudative, but also productive or proliferative (angiogenesis, wound healing). Characteristics are proliferation of blood vessels and connective tissue, as well as the presence of macrophages, T and B lymphocytes [28]–[31]. Macrophages and lymphocytes employ comparable processes in order to migrate into injured or infected tissue as known for neutrophils. Upon activation, for example by IFN-γ, macrophages execute neutrophil-like tasks, however because of their durability this response can be sustained over a longer period [24]. T lymphocytes have been demonstrated to be an

important source of macrophage-inducing cytokines, thereby setting up an ongoing inflammation.

Chronic inflammation is linked to several human diseases such as neurological, metabolic, cardiovascular, pulmonary and Alzheimer´s disease as well as different types of autoimmune diseases and cancer [32].

V.1.5. Mouse models of inflammation

The understanding of complex biological and pathogenic processes requires *in vivo* systems that avoid the risk of harming a human being. The development of animal models that recapitulate the human system is crucial for the research field. Rodent disease models have expanded the knowledge of the pathogenesis of many human disorders [33]. However, findings from immunological animal studies cannot be directly translated to clinical intervention for human diseases due to differences between murine and human immune system [34], [35].

In the last decades, the mouse (*mus musculus*) has developed into the premier mammalian model system for research because of its close genetic and physiological similarities to humans. Several different mouse models exist, which can be used for the study of chronic inflammation, regardless of whether it is experimental, spontaneous, negative or orphane [36], [37]. Because of the amount of mouse models available today, the following section will focus on mouse models for psoriasis, a well-described chronic inflammatory disease of the skin.

Psoriasis is a disease solely observed in humans, with the notable exception of two monkey species [38], [39]. Given the many disparities between human and mouse skin, it cannot be expected that psoriasiform phenotypes in mice will mirror the human disease in every respect [40]–[42]. The lack of suitable animal models impeded the investigations regarding psoriatic pathogenesis; however, in the last twenty years several psoriatic mouse models have been identified or created that mirror at least some aspects of human psoriasis [43]. Notably, mouse models are usually based on single gene manipulations, which stands in contrast to psoriasis that arises from interaction of multiple susceptibility loci [44]. An ideal mouse model of psoriasis should reflect many histological features such as epidermal hyperproliferation, altered differentiation of the epidermis, and moreover it should reproduce immunological features of the disease such as inflammatory infiltration and altered vascularity [40], [45], [46]. A number of spontaneous mouse models have been described that display histological features of psoriasis but lack pathological symptoms [40], [47]–[49]. Another category are the genetically engineered mouse models comprising transgenic and knockout models [40]. Contrary to spontaneous models these include pathological features

of the human disease. In most of these models, either increased expression or knockout of specific genes in the basal or suprabasal layer of the epidermis has been achieved by using promoters for the keratin genes keratin 5 or keratin 14, or respectively keratin 1, keratin 10, or involucrin. Existing models comprise the overexpression of the endothelial-specific receptor tyrosine kinase in basal keratinocytes (K5-Tie2) [50], the overexpression of the latent form of transforming growth factor β (TGF-β) 1 in basal keratinocytes (K5-TGFb1) [51], and the basal keratinocyte-specific overexpression of a constitutively active mutant of signal transducer and activator of transcription 3 (STAT-3) (K5-Stat3C) [52]. Xenotransplantation, where skin biopsies of psoriatic patients are transplanted on immunocompromised mice from spontaneously mutated or genetically modified strains, can also be used to study the pathogenesis of psoriasis [48]. Furthermore, a psoriasiform phenotype can be induced upon external stimuli, such as the intradermal injections of recombinant IL-23 or the application of Imiquimod (IMQ) [53], [54]. IMQ-induced phenotype differs in a fundamental way to the aforementioned mouse models. This model´s phenotype arises from direct stimulation of the immune system resulting in skin inflammation, immune cell infiltration, followed by hyperproliferation of keratinocytes and enhanced dermal vascularity [54].

V.2. Psoriasis

V.2.1. Definition

Psoriasis (*greek*, ψωρίασις, *engl.* 'itching condition') is one of the most common chronic inflammatory skin diseases in humans, mediated by cells and molecules of both the innate and the adaptive immune system [55], [56]. All dermal and epidermal elements guaranteeing barrier integrity under physiological conditions are deregulated in psoriasis, thereby leading to the development of a chronic skin inflammation [57], [58]. Since its pathology fits the definition of "a clinical syndrome caused by activation of T cells and B cells, or both, in the absence of an ongoing infection or other discernable causes" psoriasis is ranked among autoimmune diseases [59]. A variety of studies suggested that intra-lesional activated T cells produce cytokines that trigger basal stem cell keratinocytes to proliferate and perpetuate the disease [60]–[62]. In contrast, several reports have emphasized the causative role of epidermal defects in the initiation and maintenance of the disease [55], [63]. It is still under investigation what exactly causes psoriasis, however, research suggests that it may be founded in a deregulation of T cell differentiation or epidermal defects.

The severity of psoriasis varies markedly between individuals, ranking from minor irritations to intense symptoms with serious impact on the patients quality of life [64]. Psoriasis is frequently associated with a certain form of arthritis, as well as cardiovascular diseases and

diabetes [65]–[68]. Psoriasis vulgaris is the most common clinical variant of the disorder affecting approximately 80 to 90 % of the patients [55]. Therefore, statements made in this thesis relate to psoriasis vulgaris except when otherwise noted.

V.2.2. Clinical, histological, and epidemiological characterization

V.2.2.1. *Clinical features*

Psoriasis is a cutaneous chronic inflammatory disease with variable morphology, severity, distribution, and course. It is characterized by raised, erythematous, scaling plaques with a well-defined boarder and silvery dry scales all over the body. Predicted sites of psoriatic lesions are the elbows, knees, the sacral region as well as the capillitium and nails. However, psoriatic plaques may also develop at sites of mechanical trauma known as Koebner´s phenomenon [55]. Since the amount of skin lesions covering the body as well as their severity can vary strongly, Fredriksson and Pattersson proposed the `Psoriasis Area and Severity Index' (PASI) to assess the occurrence of psoriasis [69]. This tool combines erythema, induration, desquamation of psoriatic lesions and the area affected into one single score. Nevertheless, the PASI is not a universal definition for the severity of psoriasis, because not all features of psoriasis are revealed and considered. For example, it is still unclear if the severity correlates with the presence and intensity of pruritus; however, it is known that pruritus is frequent symptom of psoriasis [70].

V.2.2.2. *Histological features*

Psoriatic plaques are histologically characterized by thickened epidermis, so-called acanthosis, in combination with parakeratosis, hyperkeratosis, and elongated rete ridges, so-called papillomatosis [55].

Acanthosis and papillomatosis are caused by accelerated proliferation of basal and precipitous differentiation of suprabasal keratinocytes with abnormal replacement of annular squames with nucleated cells in the stratum corneum (parakeratosis) and by a loss of normal granular layer with thickening of the stratum corneum (hyperkeratosis) [71]. Furthermore suprabasal psoriatic keratinocytes are senescent, which contributes to the resistance of plaques to apoptosis and transformation [72]. Instead, erythema can be explained as greater number of dilated dermal blood vessels within the dermal papillae [55]. During plaque development neutrophils infiltrate the inflamed tissue, build spongiform pustule, so-called Munro´s microabscesses by invading the epidermis or migrate into the stratum corneum. These microabscesses have been only observed in epidermal compartments with parakeratotic phenotype [73]. The number of infiltrating neutrophils correlates with the severity of psoriatic plaques and is only restricted to pustule and guttate psoriasis [74]. Besides neutrophils, also $CD4^+$ and $CD8^+$ T lymphocytes are found in psoriatic epidermis.

Not only the epidermis is infiltrated, also the dermal compartment exhibits changes in the immune cell composition. The dermal inflammatory infiltrate mainly consists of T lymphocytes, dendritic cells and macrophages, as well as a small proportion of neutrophils [55]. Accumulation of T cells consisting of approximately 75 % $CD4^+$ and only 25 % $CD8^+$ T lymphocytes are mainly found in the tips of dermal papillae [74]. In contrast, the destination of infiltrating $CD11c^+$ dendritic cells during inflammatory psoriatic responses is most often the upper part of the dermis [55].

V.2.2.3. *Epidemiological features*

Psoriasis is estimated to affect 2 % of the population in Germany; however, variance in its prevalence has been observed between different ethnicities and countries ranging from 0.2 to 10 % [75]. Both genders are equally affected by psoriasis, except for psoriasis pustulosa palmoplantaris, where women are preferentially concerned [44], [76].

In 1985, it was reported that the onset of psoriasis has two predicted age groups. Based on this finding a bimodal onset model of psoriasis was established comprising an early-onset at ages between 16 and 22 years (psoriasis type I) and a late-onset from 57 to 60 years, wherein early-onset cases are more frequent [77]. However, both types do not only differ in age but also in other clinical settings, such as inheritance. Consequently, there are two different types of psoriasis, on the one hand the hereditary early-onset form and on the other hand the sporadic late-onset form [77].

V.2.3. Forms of psoriasis

Psoriasis can be variable in morphology, severity, and distribution. The most common variant is psoriasis vulgaris, affecting around 80 to 90 % of psoriasis patients [76]. Psoriasis vulgaris is also referred to as ‘plaque psoriasis’ reasoned by its typical hallmark, which is the development of plaques all over the body (described in V.2.2.1.). Psoriasis guttate is another well-described form of psoriasis. It arises upon infection and is an acute and self-limiting form in contrast to psoriasis vulgaris [76]. The transitions between the individual forms of psoriasis can be smooth. For instance, a study in 1996 was able to demonstrate that 33 % of patients with psoriasis guttate are more prone to develop a chronic psoriasis vulgaris [78].

Additional forms of psoriasis are pustulosa palmoplantaris, psoriasis inversa, psoriatic erythroderma, psoriatic arthritis, and psoriatic nail disease that differ in morphology, occurrence, or distribution from psoriasis vulgaris, as described in Table 1.

Type of psoriasis	Occurrence	Distribution	Morphology	Special features
Psoriasis vulgaris	80 to 90 %	Elbows, knees, scalp, trunk	Red plaques covered by silvery scales	High prevalence of pruritus
Psoriasis guttate	2 %; post infectious	Trunk	Small, scattered papules	Self-limiting, potential co-occurrence with psoriasis vulgaris
Psoriasis pustulosa palmoplantaris	Preferably effecting smoking women over 40 years	Palms and soles	Sterile pustules of yellow-brown color with scales	Acute form, co-occurrence with psoriasis vulgaris
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	Psoriasis is not the only cause; life-threatening
Psoriatic arthritis	25 %	Distal interphalangeal joints, dactylitis, calcaneal enthesitis	Seronegative inflammatory arthritis	5 different kinds
Psoriatic nail disease	50 %	Nails	Small pits in nail plate with ´oil spots` beneath nail plate	Nail plate can detach and may become thickened and discolored

Table 1 Features of different forms of psoriasis. [76], [79]

V.2.4. Etiology

In psoriasis, the cause leading to the establishment of a chronic inflammation with contribution of both the innate and adaptive immune system remains unknown. When associated with an abnormal skin barrier, either as a primary or secondary event, the activity of the immune system manifests the disease [55].

In the 1960s, studies investigating the family history of psoriasis patients suggested that relatives have a higher incidence for the development of the disease [80], [81]. Evidence for such a genetic predisposition was provided by twin studies several years later; indeed, the concordance rate for monozygotic twins with approximately 72 % for northern European individuals and 35 % for Australian individuals was higher when compared to dizygotic twins (15 to 13 % for European individuals, 12 % for Australian individuals) [82]–[84]. Furthermore, in both studies the character of psoriasis regarding age of onset, distribution, severity, and course was similar for monozygotic twins; a fact not observed in dizygotic twins [85].

Interestingly, the absence of 100 % concordance in twin studies and a clear inherence pattern suggested a multifactorial etiology [86], [87].

V.2.4.1. *Genetic factors*

The mode of inheritance of psoriasis is complex. During the past twenty years classical genome-wide association studies (GWAS) have identified at least nine chromosomal loci with statistically significant linkage to psoriasis. These loci are known as psoriasis susceptibility loci (PSORS) 1 to 9 [88], whereas several others are still under discussion, *e.g.* PSORS 10 to 13 [89].

The MHC class I molecule HLA-C is identified as a major susceptibility factor associated with the development of psoriasis and thereby defined as PSORS1 [90]–[92]. PSORS1, located on chromosome 6p21, accounts for 35 to 50 % of the psoriasis heritability [55]. Fine linkage mapping sequence and haplotype analysis suggested HLA-C and its allele HLA-Cw*0602 as the most likely PSORS1 gene [93], [94]. In up to 60 % of psoriasis patients the allelic variant HLA-Cw*0602 has been identified which confers a 20-fold-increased susceptibility [95]. In line with this finding, individuals who are homozygous for this allele have a fivefold-increased risk compared to heterozygous individuals [96]. To date, the precise role of HLA-C in the pathogenesis of psoriasis remains unknown. The high homology between MHC class I genes and the polymorphic nature of HLA-C impede functional studies. However, HLA-C is involved in innate and adaptive immune responses: APCs, such as DCs expressing HLA-C, are well-described triggers of adaptive immunity by presenting processed antigens to epidermal $CD8^+$ T lymphocytes. In addition, HLA-C expressing keratinocytes contribute to the immune response by interacting with killer immunoglobulin-like receptors (KIRs) on natural killer T lymphocytes, a cell subset that is involved in psoriatic disease [97], [98]. The HLA-C gene is not the only gene associated with PSORS1. For instance, CCHCR1 that encodes the coiled-coil α-helical rod protein 1 is overexpressed in psoriatic epidermis [99]. Upregulation of corneodesmosin (CDSN), uniquely expressed in the granular and cornified layers of the epidermis, has also been reported in psoriasis [100].

Notably, diverse psoriasis-related genes belong to the IL-23/T_H17 axis, the NF-κB signaling pathway, and the epidermal differentiation complex (EDC) [97], [98]. IL-23 is a heterodimeric cytokine composed of a unique IL-23p19 subunit and a common IL-12p40 subunit, and signals upon binding *via* the heterodimeric IL-23 receptor (IL-23R). Perera and others have identified single nucleotide polymorphisms (SNPs) in the *IL-12B* gene as well as in the *IL-23R* gene, both associated with psoriasis [101], [102]; the latter is located in PSORS7 on chromosome 1p. The presence of SNPs leading to an arginine to glutamine (381R/Q) substitution at position 381 within the cytoplasmic domain of the IL-23R lends protection against psoriasis by impairing T_H17 effector function [101]–[104]. This raises the option that

the protective *IL-23R* gene variant is involved in the contribution of a different phenotype in psoriasis like seen in Crohn´s disease patients [105]. Associated gene variants of regulatory components of NF-κB signaling pathway in psoriasis have been identified in multitude GWAS [104], [106]–[108]. Observed variants comprise genes such as *TNFAIP3,* which encodes for TNF-inducible protein 3, and *TNIP1* encoding for ABIN-1, both regulating NF-κB activation [104], [107]. Moreover, associations with *TRAF3IP2* encoding the signaling adaptor molecule ACT1 as well as *NFKBIA* that codes for the inhibitory protein IκBα give further hints for an impaired negative regulation of the NF-κB signaling pathway [106]–[109].

In addition to the psoriasis susceptibility genes aforementioned, various other gene sets were reported to be involved in psoriasis. Genes from the EDC, including genes encoding for the S100 calcium-binding proteins have been identified as PSORS4 [89], [110], [111]. PSORS4 is a cluster of more than 80 genes on chromosome 1q21, of which at least 45 genes are involved in skin cornification [112], [113]. As psoriasis displays abnormal epidermal differentiation, the EDC genes are candidates for psoriasis susceptibility, as members of all three families belonging to the EDC are deregulated in psoriatic inflammation [114]–[117]. In addition, genes for STAT-3 associated with PSORS2 on chromosome 17q25.3, and genes for Jun-B identified as PSORS6 on chromosome 19p13 were listed among various others [89]. Beside these factors, epigenetic mechanisms might also largely influence the pathogenesis of psoriasis.

V.2.4.2. *Epigenetic and environmental factors*

Epigenetic network might be a causative element in psoriasis. The deregulation of keratinocyte differentiation is closely linked to the epigenetic state of basal keratinocytes [118], [119]. The discordance in occurrence among twin studies as well as plaque formation have indicated that in addition to genetic predisposition, environmental and epigenetic factors might play a central role in the progression of psoriasis [120]. One epigenetic process postulated to play a role in psoriasis pathogenesis is DNA methylation, a silencing factor responsible for genome stability, cellular differentiation, imprinting, and X-linked inactivation [121]. Global DNA methylation profiling showed an increased DNA methylation in PBMCs from psoriasis patients when compared to healthy controls [122]. A second epigenetic regulatory process claimed to be from importance for the pathogenesis of psoriasis is histone modification [89]. It was shown that abnormal expression of a nicotinamide adenine dinucleotide (NAD^+) dependent deacetylase sirtuin-1 leads to hyperproliferation of keratinocytes or promotion of keratinocyte differentiation [123], [124]. Beside DNA methylation and histone modification, another important mechanism is the posttranscriptional regulation of expression *via* microRNAs (miRNAs). However, the influence of epigenetic processes in psoriasis needs further investigations in order to elucidate their importance in

the development of psoriasis. In addition to genetic predisposition and epigenetic regulation, environmental factors may contribute to the formation of psoriasis. Especially streptococcal infections, but also other kinds of infections are listed among important factors inducing the development of psoriasis [125]. Psoriatic lesions formed at site of minor injury in previously unaffected skin areas occur with an incidence of 5 to 50 %. Such plaque formations upon physical trauma, the so-called Koebner's phenomenon, illustrate another environmental factor leading to psoriasis [126]. The list of environmental factors is supplemented by certain medication such as antidepressants, antihypertensives and anticytokine therapies [127], [128], as well as smoking and alcohol [129], [130]. In addition, emotional factors, such as stress can contribute to the development or (re)activation of the disease [130].

V.2.5. Pathogenesis

The complex pathogenesis of psoriasis is driven by various cells, regulatory modulators, and pathways of the immune system – both, innate and adaptive – as well as by the skin epithelium and connective tissue [131]. In a psoriatic plaque two cellular responses are excluded: the balance between activation of immune cell types and the production of factors secreted by keratinocytes directly affecting T lymphocytes as well as dendritic cells and *vice versa* [56].

Non-lesional skin of psoriatic patients appears normal until progression into a psoriatic lesion is induced by skin injury, inflammation or infections [132]. The combination of environmental and potential genetic predisposition causes cell death and leads to the production and secretion of antimicrobial peptides. Among these peptides are molecules such as the cathelicidin LL-37, S100A7 (psoriasin), and β-defensins, which are produced and secreted by keratinocytes, and which are known to have also chemotactic impacts on DCs besides their antimicrobial function [133]. A small subpopulation of DCs, known as plasmacytoid dendritic cells (pDCs), is chemoattracted from the blood into injured tissue [134]. Indeed, keratinocytes are not only responsible for the recruitment of DCs, but also involved in their activation *via* the secretion of cytokines such as IL-1α/β, IL-6, IL-18, and tumor necrosis factor-α (TNF-α) [55]. These cytokines also function as activators of other keratinocytes and epidermal Langerhans cells. Sustained activation of keratinocytes resulting in the release of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) molecules to the extracellular matrix [135]. LL-37, secreted by keratinocytes, has the ability to build complexes with DNA and RNA, thereby activating pDCs and myeloid dendritic cells in a toll-like receptor (TLR)-dependent manner. This illustrates how host DNA is turned into a proinflammatory stimulus in psoriasis [136]. Activated pDCs represent the main source of INF-α, a cytokine essential for the maturation and activation of dermal dendritic into inflammatory dendritic cells [137], [138].

Activation and maturation of myeloid dendritic cells is not only induced by keratinocytes and pDCs. For instance, macrophages secrete TNF-α, IFN-γ, and chemokines (*e.g.* CCL19), and also TNF-α derived from natural killer T cells has the capacity to activate DCs [55]. Activated myeloid dendritic cells recognize and capture antigens, migrate to local draining lymph nodes and present antigens to T cells [139]. Presentation of antigens induces the differentiation of naïve T cells into effector cells such as type 1 helper cells (T_H1 cells), and type 17 helper cells (T_H17 cells), as well as type 1 cytotoxic T cells (T_C1 cells) and type 17 cytotoxic T cells (T_C17 cells) [55]. Chemokine profiling performed in the dermal compartment of inflamed tissue uncovered the recruitment of T_H1 cells *via* binding to its chemokine receptor CXCR3. Additionally, T_H17 cells follow the same chemokine gradients due to the expression of the chemokine receptors CCR6 and CCR4. In fact, T helper cell migration into the psoriatic lesion is the beginning of disease maintenance [55] (Figure 1).

Key processes during the maintenance phase are the presentation of putative auto-antigens to T cells and the release of IFN-γ, TNF-α, IL-17A, IL-17F, and IL-22 by T helper cell subsets (T_H1, T_H17, and T_H22 cells). Furthermore, dermal dendritic cells produce IL-23 and a special subgroup of myeloid dermal DCs (TIP-DCs) releases high levels of TNF-α and iNOS [140]. IL-23, elevated in psoriatic plaques and produced by dendritic cells, neutrophils, macrophages, and keratinocytes, is one of the key cytokine in the survival and proliferation of T_H17 lymphocytes and signals *via* binding to the IL-23R on their cell surface [141]–[144]. In turn, all these proinflammatory mediators act on keratinocytes, leading to the activation, proliferation and sustained secretion of antimicrobial peptides, chemokines (*e.g.* CXCL1, CXCL9 through CXCL11, and CCL20), and S100 proteins [145], [146]. CCL19, a chemokine secreted by macrophages, initiates the formation of perivascular clusters and lymphoid-like structures consisting of dendritic cells and T lymphocytes [55]. Migration of T cells from the dermis into the epidermal compartment is an important event in psoriasis, which is controlled by the interaction of α1β1 integrin on T cells with collagen IV in the basal membrane of psoriatic epidermis [147].

Feedback loops involving keratinocytes, fibroblasts, and endothelial cells contribute to tissue reorganization with endothelial-cell activation, proliferation, and deposition of extracellular matrix [55] (Figure 1).

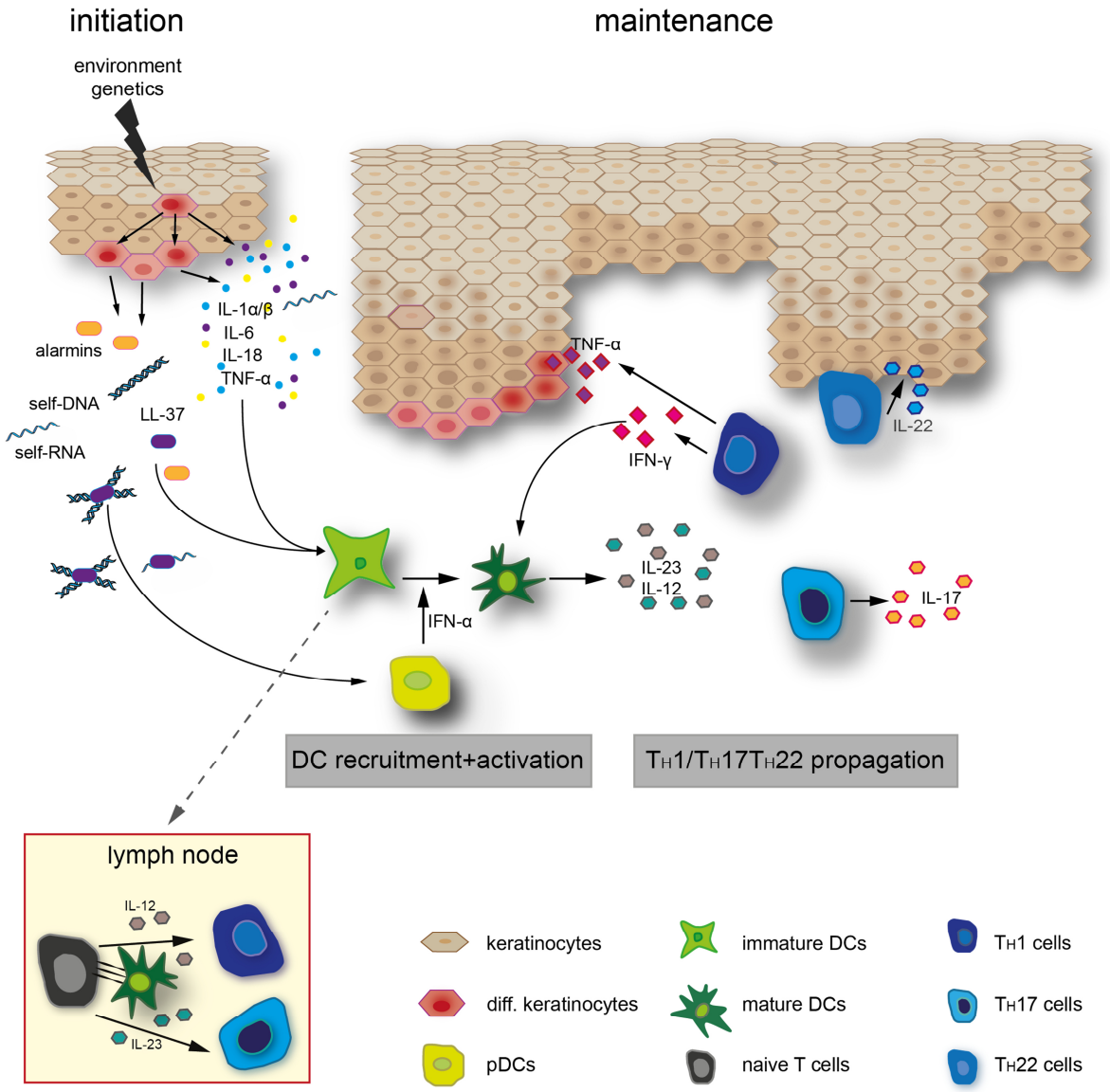

Figure 1 Summary of psoriasis pathogenesis.

Genetic deposition and/or environmental stress lead to immune responses from both the innate and adaptive immune system. Stressed keratinocytes activate dermal DCs and lead to differentiation of other keratinocytes. Differentiated keratinocytes release DNA and RNA molecules, which build complexes with LL-37 thereby leading to the activation of pDCs and the secretion of IFN-α. IFN-α activates and matures dermal DCs, which circulate to the lymph nodes, where antigens are presented to naïve T cells that differentiate into different T helper subsets (T_H1, T_H17, T_H22). Differentiated T_H cells migrate into the dermis, where an interaction between keratinocytes, innate immune cells, and T_H cells as well as cytokines such as TNF-α, IFN-γ, IL-17, IL-22, and IL-23 takes place *(adapted from* [97]*)*.

V.2.5.1. *Role of keratinocytes in psoriasis*

The epidermis is mainly formed by slowly differentiating keratinocytes that build the mechanical barrier of the body. Keratinocytes have a variety of functions including important roles in the initiation, maintenance and regulation of cutaneous immune responses [148].

In general, the function of keratinocytes is based on their state of activation and differentiation. In psoriatic lesions the terminal differentiation potential of keratinocytes is incomplete leading to the activation and proliferation of early-differentiated keratinocytes displayed by an upregulation of early differentiation markers like involucrin and a downregulation of filaggrin and loricrin [97]. Activated keratinocytes display a hyperproliferative and migratory phenotype that differs from differentiated keratinocytes, *e.g.* regarding cytokine secretion. The vascular endothelial growth factor (VEGF) is secreted by activated keratinocytes and is important for the recruitment of circulating lymphocytes [149] as well as for the induction of migration, survival, and proliferation of epithelial cells [150]. The complex situation in psoriasis is based on an incomplete terminal differentiation and deactivation of keratinocytes as well as their deregulated cytokine expression [151]. For instance, keratinocyte-derived IL-1 is one of the most important deregulated cytokines in psoriasis with multiple roles in orchestrating immune responses [152], [153]. Both IL-1 forms, IL-1α and IL-1β, are stored in the cytosol as precursor proteins [154], [155]. Activated caspase-1 cleaves the precursor pro-IL-1α, pro-IL-1β and pro-IL-18, hereby inducing the release of the active forms of these proinflammatory cytokines [156]–[158]. The IL-1 family exhibits diverse function during an inflammatory response. While IL-1 act as direct chemoattractant for lymphocytes [159], and as activator for fibroblast proliferation [160], IL-18 and IL-1β are involved in the differentiation of naïve T cells into T_H1 and T_H17 cells [161]–[163]. Furthermore, keratinocytes are entangled in leukocyte recruitment by producing immune-related proteins such as the MHC class II cell surface receptor HLA-DR, CD40, ICAM-1 and S100 proteins [164]. Through the expression of ICAM as well as MHC II and programmed death-ligand 1 (PD-L1) keratinocytes are directly involved in T cell activation [165]. Moreover, Li and colleagues have shown that differentiated keratinocytes signal through TLR-7 leading to the activation of the NF-κB pathway. This results in the secretion of proinflammatory cytokines such as IL-8 and TNF-α, contributing to an inflammatory phenotype [166]. As mentioned previously, clusters formed by keratinocyte-derived antimicrobial peptides (LL-37, S100A7, and β-defensins) and DNA as well as RNA molecules are able to activate different DC subsets and to induce infiltration of inflamed tissue by neutrophils and macrophages, thereby sustaining inflammation [97]. Production of AMPs by keratinocytes can be increased by T cell-derived cytokines like IL-17A and IL-22 leading to the generation of a positive feed-forward loop [167].

V.2.5.2. *Role of plasmacytoid dendritic cells*

Dendritic cells are innate immune sentinel cells linking the innate and adaptive immunity by acting as APCs and inducing T cell-mediated adaptive immune responses [167]. Normal skin is comprised of epidermal Langerhans cells and dermal dendritic cells, whereas in psoriatic skin both lesional and non-lesional skin, additional DC subsets are present: TNF-α and

iNOS-producing inflammatory DCs (Tip-DCs), slan-DCs and dermal pDCs [168]–[170]. This altered immune cell composition contributes to the specificity of the inflammatory response [171].

Human $CD123^+$ $BDCA\text{-}2^+$ $BDCA\text{-}4^+$ pDCs are a rare subset of dermal dendritic cells (0.2 to 0.8 %) characterized by a plasma cell-like morphology and a unique surface phenotype [138], [172], [173]. These cells are key effector cells in the innate immune system in response to viruses due to their unique ability to produce large amounts of IFN-α [174], [175]. pDC-derived IFN-α promotes the maturation and activation of dermal myeloid DCs that force the activation and expansion of pathogenic T cells [170].

Under homeostatic conditions, pDCs are exclusively found in secondary lymph nodes and the peripheral blood, but upon viral infection they are recruited from the blood to peripheral infected tissue [176]. Nestle and colleagues have shown increased number of pDCs in 'normal-appearing pre-psoriatic skin' of patients in comparison to the healthy control group; thereby demonstrating that the elevated amounts of pDCs in the skin represents a conditioning factor for future plaques of psoriasis [170]. Since skin injury is a well-known initiator of psoriasis [177], pDC activation might be explained by the release of skin-derived products upon mechanical stress or infection [170]. For instance, fibroblast-derived chemerin has been reported to promote the recruitment of pDCs in dermal compartment [134]. TLR-7 and TLR-9, selectively expressed on pDCs have the ability to bind nucleic acids at non-methylated CpG-rich motifs or phosphodiester backbones [178], [179], which consequently activates pDCs and initiates inflammatory responses. Under homeostatic conditions pDCs are tolerant to nucleic acids releases from stressed or dying host cells. Tolerance to self-nucleic acid molecules occurs when self-RNA or self-DNA form complexes with LL-37 [135], an AMP released from keratinocytes, as mentioned before. Normally, host nucleic acids are rapidly degraded in the extracellular environment and are not able to enter the endosomes spontaneously [180]. LL-37 protects host nucleic acid from cellular degradation, and transports it into endosomal compartments of pDCs, where the complex of DNA-LL-37 activates TLR-9 [136], and respectively the aggregation of RNA and LL-37 leads to the activation of TLR-7 [135].

More specifically, self-DNA-LL-37 binding to TLR-9 leads to the association with the adaptor molecule MyD88 (myeloid differentiation factor 88), which then recruits IL-receptor associated kinase (IRAK) 1 and 4. After association of IRAK-1 with MyD88, IRAK-1 is phosphorylated by activated IRAK-4, and directly associates with TNFR-associated factor (TRAF) 6 [181]. Recruitment of TRAF-6 to the cytoplasmic transductional-transcriptional processor (CTTP) complex leads to the activation of the interferon regulatory factor (IRF) 7, a transcription factor for early IFN genes [182]–[185]. Furthermore, the CTTP complex is also

activating IRF-5 that induces together with NF-κB the secretion of proinflammatory cytokines, including IL-6 and TNF-α in pDCs [186].

pDCs are key effector cells in inflammatory conditions linking innate and adaptive immunity. The production of large amounts of IFN-α induces several inflammatory responses: survival and differentiation of T lymphocytes, activation of NK cells, maturation of monocytes to fully-differentiated, and activated dendritic cells as well as involvement in the differentiation of B lymphocytes [187]–[192].

V.2.6. Therapies

Although a range of diverse therapies is available for the treatment of psoriasis, there is no definite cure.

The selection of therapy and potential combination of therapies is dependent on various factors. First, patient-related factors such as age, gender and physical and mental health. Second, psoriasis-related factors including type, severity disease duration, as well as comorbidities and previous therapies [56]. Existing therapies aim to induce remission and to mitigate the severity of psoriasis (PASI 50, i.e. 50% reduction of PASI), thereby improving quality of life [193]. Four therapy modalities are available for the treatment of psoriasis, which can be used either alone or as a combination: topical monotherapy, phototherapy, systemic treatments and biological agents.

V.2.6.1. *Topical treatment*

Despite their disadvantages, topical monotherapies remain the first-line treatment for most patients with mild psoriasis. Among those, topical corticosteroids, vitamin D_3 derivatives, dithranol as well as retinoids are listed. They exist in different bases such as creams, lotions, foams and many more [194]. Keystones in psoriasis therapy are glucocorticoids, a member of corticosteroids that exhibit antiinflammatory and immunosuppressive features. Local side effects such as skin atrophy, purpura, as well as systemic effects including the iatrogenic Cushing´s syndrome limit the application period [195], [196]. Hyperproliferation of keratinocytes is hampered by the usage of dithranol as well as tazarotene; the latter represents the only topical retinoid available for treatment and is often combined with corticosteroids or vitamin D_3 analogues. Monotherapy of tazarotene is moderately effective leading to local skin irritations [197]. Also vitamin D_3 derivatives exhibit a beneficial effect on psoriatic plaques by modulating proliferation and differentiation, as well as infiltration and activation of neutrophils and other immune cells in psoriatic skin lesions [198].

V.2.6.2. *Phototherapy*

One of the most common treatments for managing psoriasis is phototherapy [199]. Narrow ultraviolet-B (nbUVB), highlighted at 311 nm, and psoralen combined with ultraviolet-A

between 315 and 400 nm (PUVA) are second-line therapies applied in between topical and systemic therapy [200], [201]. The dose of both therapeutic approaches is based on the minimal erythermogenic dose (MED). Their benefits are mainly based on suppressed T_H1/T_H17 inflammatory axis through altered cytokine expression, immune suppression of Langerhans cells and other immune cells, as well as induction of apoptosis [202]. Nevertheless frequent phototherapeutic treatments increase the risk of non-melanoma skin cancer [203]; both phototherapies are further associated with squamous cell carcinoma (SCC), whereas the PUVA method indicates a significantly higher risk [204]. Based on its decreased association with carcinogenesis and due to a comparable efficiency, nbUVB therapy is therefore the preferred phototherapy in psoriasis [201].

V.2.6.3. *Systemic treatment*

In moderate to severe psoriasis, affecting approximately 20 % of psoriatic patients, a systemic therapeutic approach is the most common choice of therapy [205]. Approved drugs are methotrexate (MTX), fumaric acid esters, acitretin, and cyclosporine. MTX, a folic acid antagonist, is a specific chemotherapeutic inhibiting DNA synthesis and cell cycle, but also acting on T cell and keratinocyte proliferation [194]. Nevertheless, other side effects are liver fibrosis, hepatotoxicity and gastrointestinal diseases. The latter is also described for fumeric acid esters, which in turn positively impacts clinical outcome by inhibiting the NF-κB pathway and increasing apoptotic effects on T lymphocytes [194], [206], [207]. A systemic drug that has no immunosuppressive effects is the retinoid acitretin. Its treatment leads to a normalization of keratinocyte proliferation and differentiation and is considered excellent for use in combination with phototherapies [194], [208]. Cyclosporines possess immunosuppressive effects by inhibiting T lymphocyte activation and production of cytokines as well as by decreasing the amounts of dendritic cells, and additionally effecting epidermal keratinocytes [201], [209]. However, side effects such as nephrotoxicity and increased risk for malignant melanoma are associated with cyclosporine treatment [194].

V.2.6.4. *Biologica*

Recent advances in psoriasis research led to the development of new therapeutical agents, the so-called biologica; a group of recombinant proteins, fusion proteins and/or monoclonal antibodies. Two of the best-known representatives of biologica are T cell agents and TNF-α inhibitors [194]. Alefacept is a human leukocyte function associated antigen-3/IgG1 fusion protein that interferes with T cell antigen presentation and is involved in apoptosis of memory effector T cells in the skin [210]. Another example is Efalizumab, also referred to as T cell agent, which targets a crucial step in the pathogenesis of psoriasis, and resembles the adhesion between T lymphocytes and endothelial cells. After a 12-week cycle of T cell agent injections 20 to 25 % of the patients achieve a PASI 75 (75 % reduction of PASI) [194]. TNF-

α inhibitors that have been approved by the 'Food and Drug Administration' (FDA) are Etanercept, Infliximab, and Adalimumab. Blockade of TNF-α results in many cases in disease improvement, but a clinical resolution is not achieved [211]. Novel biologicals such as the recently approved Ustekinumab or Secukinumab target interleukin-17A or p40, a subunit of interleukin 12/23, respectively [212], [213].

The psoriatic cascade starts with the activation and/or maturation of keratinocytes and DCs leading to the differentiation of T lymphocytes. Lymphocytes, DCs, neutrophils and keratinocytes produce cytokines, growth factors, as well as chemokines and activate diverse signaling pathways, including the NF-κB and the STAT-3 pathway, thereby driving inflammation and the formation of psoriatic plaques. However, many questions regarding the observed imbalanced regulation mechanisms supporting progression of psoriasis remain. Among others, the identity of factors guaranteeing the sustained activation of these pathways remains unresolved. Recent data provided evidence for a potential contribution of pattern recognition receptors (PRR). Among those, a role for the receptor for advanced glycation end-products (RAGE) in various inflammatory setting has been implicated, pointing towards a potential function also in the pathogenesis of psoriasis.

V.3. The receptor for advanced glycation end-products (RAGE)

V.3.1. Characterization

The receptor for advanced glycation end-products, short RAGE, is a multiligand pattern recognition receptor and belongs to the super-immunoglobulin gene family [214], [215]. RAGE was first identified from bovine lung endothelium as a transmembrane receptor for advanced glycation end-products (AGEs) [215].

The gene *AGER*, which is encoding for RAGE, is located within the MHC class III region on human chromosome 6p21.3 [216], [217] and murine chromosome 17 [218]. Human *AGER* consists of 11 exons and 10 introns [216], [217] and is further characterized by a multiple number of functional elements within the regulatory 5'-untranslated region (UTR). Common elements regulating *AGER* transcription comprise binding sites for transcription factors such as NF-κB, activator protein (AP) -1 and AP-2, or specificity protein 1 (SP-1) [219], [220], as well as methylation sites, thereby additionally allowing an epigenetic regulation of *AGER* [221].

The expression of RAGE and thereby its function is also affected by several single nucleotide polymorphisms (SNPs) in the *AGER* promoter. GWAS described a threonine to alanine

substitution at position -374 (-374T/A) and a threonine to cysteine substitution (-429T/C) [222], [223]. At position +82 a glycine to serine substitution is enhancing expression of *AGER* gene and has been associated with chronic inflammation, which is predicted by increase expression of proinflammatory cytokines [224]–[227]. Additionally, alternative promoters for *AGER* are described [228]. RAGE mRNA is 1.4 kilo base pairs (kb) in size and undergoes further regulations *via* polyadenylation at the 3'UTR as well as binding of miRNAs to target sites, leading to its transcriptional degradation or translational inhibition [229], [230].

The unspliced mRNA is translated into a protein of 404 amino acids (aa) with a molecular mass of 55 kDa, known as full-length RAGE (fl-RAGE) [214]. RAGE is a highly conserved protein, showing 78 % amino acid homology between human and mice [231], and composed of a single hydrophobic transmembrane domain, a highly charged C-terminal intracellular tail and an extracellular region. This N-terminal extracellular region comprises a signal sequence for secretion (1-22 aa), one viable (V-type) immunoglobulin domain (23-116 aa), and two constant (C-type) immunoglobulin regions (124-221 aa, 227-317 aa) [232], [233]. The V-type domain together with the C1 domain builds an integrated structural unit that is principally required for RAGE binding to extracellular ligands [234], [235]. Only two of the wide variety of RAGE ligands shows a low binding affinity to the C2 domain [236], [237]. The separation of the V-C1 complex to the C2 region is provided by a 5 aa flexible linker sequence, thereby allowing the latter to function in a fully independent manner to the V-C1 unit [234]. The cytoplasmic tail (364-404 aa) is of high importance for RAGE-mediated intracellular signaling, despite the absence of known signaling motifs [232], [233], [238].

Alternative splicing of the *AGER* transcript creates 19 natural splice variants beside fl-RAGE, mainly characterized by different extends and localizations of truncations [239], resulting in distinct amino acid sequences. For instance, these alterations affect the ligand binding domain or lead to the removal of the transmembrane helix [240]–[243]. The metalloproteases ADAM 10 and matrix metallopeptidase (MMP) 9 have been shown to restrict the presence of RAGE on the cell surface by receptor shedding. More specifically, cleavage of RAGE close to the transmembrane region leads to a soluble RAGE (sRAGE) isoform that shares the same V-C1-C2 region with fl-RAGE but lacks the transmembrane and intracellular domains [244], [245]. Extracellular sRAGE molecules can function as "decoy" receptors by competing with fl-RAGE for ligand binding, and thereby attenuate the activation of the RAGE signaling pathway [228], [246]. Besides that, the cleaved ectodomains can also accumulate in intracellular vesicles, while the C-terminal region has been found to localize in the cytoplasm or the nucleus [244], [245], [247].

Moreover, besides posttranscriptional modification by proteolysis RAGE function can be regulated by phosphorylation and glycosylation, both known to promote ligand binding and

signaling, as well as by disulfide bond formation between conserved cysteine residues within the immunoglobulin domains [214].

Not only intrastructural alterations within the RAGE molecule can lead to different properties, but also the preassembly into dimers or oligomers affects its function. Fluorescence resonance energy transfer (FRET) analyses showed that RAGE molecules can assemble and form homodimer and homooligomers, as well as heterodimer with heparin sulfate proteoglycans (HSPGs) and TLRs. Ligand binding enhances the heterodimerization; for instance, S100B stimulus leads to the stabilization of RAGE-TLR-2 heterodimers [235]. However, dimerization or oligomerization is not only a consequence of ligand binding, Xie and colleagues could show that the formation of homodimers and homooligomers of RAGE on the cell surface appears in a ligand-independent manner [248].

During embryonic development, RAGE is highly expressed in a constitutive manner especially in the brain [249]. In comparison to embryonic cells, RAGE expression is predominantly reduced to basal levels in many differentiated adult cells like neutrophils, macrophages, lymphocytes, DCs, and vascular endothelial cells [249], [250]. An exception is the adult lung where RAGE is constitutively expressed at high level in the alveolar epithelium [249], [251]. It is believed that basal levels of RAGE but also the high expression in the lung guarantee homeostasis of cells and tissue [252], [253]. Under pathological conditions RAGE expression is drastically induced on the basis of accumulated RAGE ligands and/or inflammatory mediators in most of the tissues. [232], [252], [253]. Human disorders with observed high expression of RAGE are *inter alia* diabetes, Alzheimer´s disease, and acute and chronic inflammatory conditions [254].

RAGE represents a key molecule in the interplay between the innate and adaptive immune system highlighted by its expression on lymphocytes [255]–[258] and DCs [259]–[261]. Additionally, its sustained signaling leads to the establishment of chronic inflammatory diseases [262], [263].

V.3.2. RAGE ligands

RAGE is a pattern recognition receptor mediating physiological and pathological effects through interaction with a variety of ligands characterized by diverse molecular properties [264]. Most ligands bind to the positive charged V-C1 domain of RAGE, implying an electrostatic and not a pattern-dependent interaction between RAGE and its ligands [265]–[267].

V.3.2.1. *Advanced glycation end-products*

Non-enzymatic glycation and oxidation of proteins and lipids during maillard reaction generate negatively charged advanced glycation end-products (AGEs) with high affinity for

RAGE [214], [268]. AGE accumulation is associated with diverse biological setting such as diabetes, aging, Alzheimer´s disease, and inflammation [269]–[273].

V.3.2.2. *Damage-associated molecular pattern molecules*

Damage-associated molecular pattern (DAMP) molecules, also known as alarmins, are either released actively or passively by stressed cells. These endogenous danger signals promote systemic inflammatory responses in the absence of an infection, which distinguishes them from exogenous pathogen-associated molecular pattern (PAMPs). Increased serum levels of DAMPs are associated with many diseases including sepsis, arthritis, cancer, and inflammation [63], [274]–[276]. DAMPs are characterized by their ability to recruit and activate receptor-expressing immune cells and to promote reconstruction of destroyed tissues [277]. They include proteins such as the high-mobility group box 1 (HMGB1), S100 family members, heat shock proteins, uric acid, adenosine triphosphate (ATP), fibrils of amyloid-β, serum amyloid A, and DNA [277]–[284].

The next sections will focus on HMGB1 and the S100 protein family, since they represent well-known ligands of RAGE with high importance for chronic inflammatory diseases including psoriasis [285]–[287].

V.3.2.2.1. S100 proteins

The S100 family consists of more than 20 family members that are 9 to 13 kDA acidic proteins, characterized by the presence of two calcium binding EF-hand motifs [288]. Most of these proteins are encoded within the EDC on chromosome 1q21 (PSORS4). S100 proteins are able to form dimers or oligomers spontaneously [289]–[291]. Despite the fact that S100 family members show a high similarity regarding their sequence and structure, they are not functionally redundant and involved in diverse biological processes such as proliferation, migration, differentiation, and inflammation [292]–[294]. In the intracellular compartment S100 proteins modulate many processes, including calcium homeostasis, cytoskeletal organization, cell cycle progression as well as cell growth and differentiation [290], [295]. However, S100 proteins can also be released into the extracellular space, through a mechanism that remains elusive. They are not secreted *via* the classical Golgi pathways due to their lack of a leader sequence. Furthermore, it is still under discussion if S100 proteins are actively secreted or passively released by necrotic cells. [294]. Nevertheless, their extracellular functions are manifold and not been fully clarified for all family members. Most extracellular functions with impact on cancer and inflammatory responses are mediated *via* binding to RAGE. In general, S100-RAGE interaction activates multiple signaling pathways, including NF-κB, AP-1, and STAT-3 pathways, thereby resulting in enhanced expression of proinflammatory cytokines and cellular adhesion [287].

Many members of the S100 family are connected to chronic inflammatory disorders. For example antimicrobial peptides as S100A8, S100A9, and S100A12 were shown to be involved in the attraction of immune cells to inflamed tissue, in the communication between keratinocytes and immune cells, as well as in enhanced production of proinflammatory cytokines by keratinocytes [238], [296], [297]. S100A8/A9, common antiparallel heterodimers of the S100 family, signal *via* RAGE with subsequent activation of the NF-κB pathway and induction of proinflammatory mediators, including themselves. This leads to the generation of a self-sustained proinflammatory feed-forward signaling loop [263], [298]. Accordingly, S100A8/A9 as well as S100A12 serum levels are often used as inflammatory biomarkers [299]–[301]. Moreover, cytokines released by T_H1, T_H17, and T_H22 cells induce S100A7 and S100A15; both act as chemoattractants for different leukocytes, thereby linking innate and adaptive immune reactions [302]–[304].

In most inflammatory lesions and blood of patients with different inflammatory and autoimmune disorders increased S100 protein levels have been found [278], [305]. Moreover, for several S100 proteins antimicrobial functions are described [133]. Nevertheless, their exact contribution to inflammation-associated process remains to be examined in detail.

V.3.2.2.2. High-mobility group box 1

The high-mobility group box 1 (HMGB1) is a small protein consisting of two tandem HMG box domains and an acidic C-terminal tail. Depending on its location HMGB1 is involved in DNA replication and repair, or in inflammatory immune responses. Within the nucleus HMGB1 acts as a non-histone DNA binding protein, binding DNA in a conformation-dependent but sequence-independent manner, and as a transcriptional regulator [306], [307]. In order to function as proinflammatory cytokine, HMGB1 has to be released which requires protein shuttling from the nucleus to the cytoplasm or/and prevention of nuclear transport of newly synthesized proteins. Furthermore, posttranslational modification such as phosphorylation, methylation, and acetylation leads to the inhibition of the nuclear import and thereby to the accumulation of HMGB1 in the cytoplasm [308]–[310]. The non-classical secretion of HMGB1 can be triggered by several stimulatory cytokines such as lipopolysaccharide (LPS), IFN-γ, TNF-α, and IL-1β [311]–[313]. As the localization of HMGB1 is crucial for its function, inhibitory mechanisms for the HMGB1 secretion also exist. For instance, the presence of ethyl pyruvate and cholinergic agonist prevent the secretion of HMGB1 [314], [315]. Furthermore, the function of HMGB1 is not only controlled by its location, but also regulated by its ability to interact with different molecules. Amongst molecules with inhibitory effects on HMGB1 function proinflammatory cytokines such as CD24 and thrombospondin are listed [316], [317]. In terms of thrombospondin, the inhibitory molecule directly binds to HMGB1,

thereby blocking its interaction with RAGE [318]. However, there are binding partners of HMGB1 that show a positive influence on the accessibility and signaling of HMGB1 through different receptors. Positive modulators include nucleosomes, PAMPs (in particular LPS), cytokines, and chemokines such as IL-1β and CXCL12, as well as DNA molecules [319]–[323].

A role of HMGB1 has been associated with a diversity of human diseases such as sepsis and cancer, where HMGB1-RAGE interactions regulate the migration and invasion of tumor cells [276], [324], as well as with inflammation and autoimmune diseases [285], [325]. At specific inflammatory sites high amounts of HMGB1 as well as a multitude of HMGB1-sensing cells were detected [326], [327]. HMGB1-RAGE interaction on dendritic cells promotes their maturation and activation, and simultaneously induces upregulation of HMGB1 [328], [329]. In other words, HMGB1 is activating DCs in an autocrine as well as in a paracrine loop [330]. Moreover, binding of HMGB1 to RAGE influences T cell differentiation, invasion of monocytes to inflammatory regions, and the transmigration of neutrophils [256], [260], [331]–[333]; major processes during inflammatory response.

V.3.3. RAGE signaling pathway

RAGE ligation is capable of inducing an array of cell signaling pathways, including the activation of mitogen-activated protein (MAP) kinases [334]–[336], PI3K/Akt (phosphoinositide 3-kinase) pathways [337], and the induction of Rho GTPases [338]. All these pathways are involved in different important cell regulatory mechanisms. Downstream transcription factors such as NF-κB, AP-1, or STAT-3 induce the expression of chemokines, cytokines, and other regulatory molecules. For example in DCs, binding of HMGB1 to RAGE results in the activation of MAP kinases ERK1/2 and p38, and consequently in the induction of NF-κB. In turn, NF-κB leads to the expression of cytokines like IL-6 and TNF-α, which promotes DC maturation [328], [339].

The outcome of RAGE signaling depends on a variety of conditions *inter alia* the identity of the ligand, ligand concentration, presence of co-receptors, co-ligands or adaptor molecules, as well as the surface concentration of RAGE and its oligomerization status [339]. Moreover, RAGE signaling is also dependent on cell types involved. For instance, RAGE engagement in macrophages induces signaling cascades including ERK1/2 and p38 MAP kinases, whereas neutrophils respond with an activation of PI3K-dependent signaling pathways instead of ERK1/2 or p38 MAP kinases activation [337], [340], [341] (Figure 2).

While the ectodomain of RAGE is important for converting an extracellular into an intracellular signal, the C-terminal tail of RAGE is essential for translating the signal into a cellular response [342]. Indeed, studies showed that the cytoplasmic deletion mutant of

RAGE impaired intracellular signaling upon ligand engagement in a dominant-negative manner [276], [343], [344]. Recent studies using immunoprecipitation assays and yeast two-hybrid system described the MAP kinases ERK1/ERK2 and the diaphanous 1 (DIA-1) as direct interaction partners of the cytoplasmic tail of RAGE [338], [345]. Due to ligand binding, the C-terminal tail of RAGE gets phosphorylated allowing the binding of adaptor molecules like MyD88 (Figure 2). Noteworthy, also the TLR-9/MyD88 pathway plays a critical role in the initiation of adaptive immune responses and thereby in the establishment of inflammatory conditions [346], linking RAGE to TLR signaling in these contexts.

A characteristic feature of the RAGE signaling is the ability to induce, despite a diverse set of signaling molecules, also its own expression *via* a NF-κB-dependent positive feed-forward loop [342], [347]. The existence of two NF-κB response elements at position -1518/-1510 and -671/-663 in the promoter region of *AGER* enables the binding of NF-κB, which results in enhanced RAGE expression and signaling [220]. This self-induced mechanism allows the cells to immediately respond to external stimuli.

However, deregulated RAGE signaling can cause hyperactivation of important signaling pathways leading to pathological features. Nevertheless these pathways are highly complex and have not been fully elucidated.

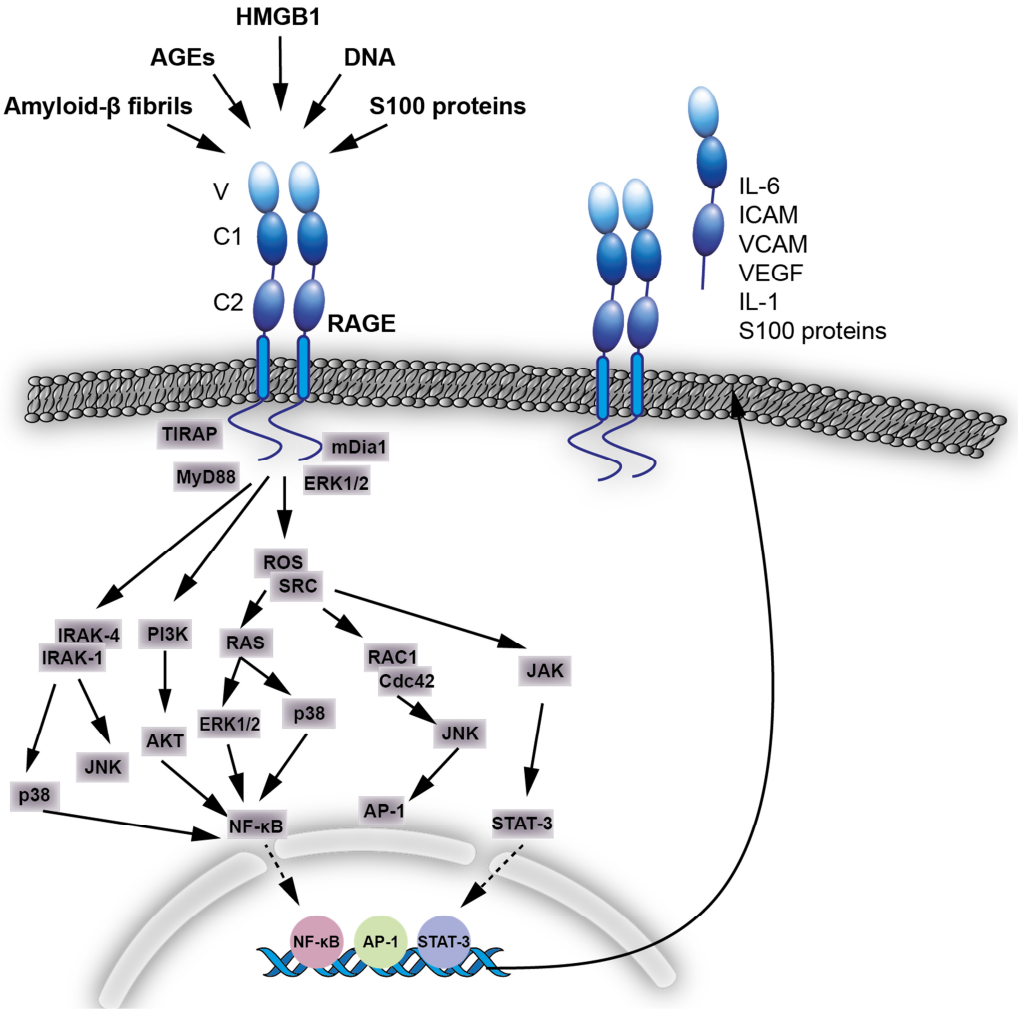

Figure 2 Signaling pathway downstream of RAGE.

RAGE serves as a receptor for various groups of ligands. Engagement of RAGE with one of its ligands induces a cytosolic signaling cascade resulting in the activation of NF-κB, AP-1 or STAT-3. Activation of transcription factors increases *inter alia* the secretion of proinflammatory cytokines, RAGE, and its ligands (*adapted from* [285], [348]).

V.3.3.1. *RAGE signaling in homeostasis*

While most studies focus on RAGE and its function in pathological conditions such as inflammation and cancer, little is known about the physiological function of RAGE, yet.

Genetically engineered Rage-deficient mice (*Rage*$^{-/-}$) appear normal and show no obvious alteration under physiological conditions. Furthermore, these mice exhibit any obvious developmental defects and functional neural development [349]. However, potential compensatory mechanisms in *Rage*$^{-/-}$ mice have not been studied in detail; therefore RAGE effects in homeostasis cannot be ruled out.

In 2008, Reynolds and colleagues have proposed that RAGE is crucial for the embryonic lung development and postnatal pulmonary morphogenesis [350]. However, expression of

RAGE during lung development as well as in adult lung can lead to lung hyperplasia and alveolar destructions [351], [352].

The physiological role of RAGE in the lung as well as in other tissues remains exclusive and needs further investigation.

V.3.3.2. *RAGE signaling under pathological conditions*

Since RAGE signaling mediates the recruitment of inflammatory immune cells as well the secretion of proinflammatory cytokines and chemokines [238], [333], [353], a role in pathological conditions has been hypothesized. Indeed, RAGE has been implicated in many different diseases including Alzheimer's disease [354], [355], atherosclerosis [356], arthritis [224], diabetes mellitus [357], cancer [263], [276], and inflammatory diseases [262], [358].

In terms of Alzheimer´s disease, RAGE has been described as a receptor for amyloid-β, a major component of amyloid plaques [359]. More specifically, RAGE engagement generates oxidative stress and activates NF-κB in neurons, while microglia respond with enhanced cell proliferation and migration. This identifies RAGE as a mediator of neurotoxicity [215], [354], [355], [360]. Moreover, Amyloid-β/RAGE interaction is suggested to be involved in impaired memory in Alzheimer´s disease by decreasing on long-term potentials [361]. Several studies have demonstrated that also the RAGE-S100 interaction is associated with neurodegenerative diseases. For example, high doses of S100B induce death of neurons [362], [363] as well as activation of microglia [364], [365] and astrocytes [366].

RAGE has been detected in macrophages, T lymphocytes, as well as in some B lymphocytes in synovial tissue. As these cells are implicated in the development of arthritis, a role of RAGE in the pathogenesis of this disease has been suggested [367]. Enhanced activation of RAGE due to the accumulation of AGEs, HMGB1, and S100 proteins alters the mechanical properties and metabolism of the flexible connective tissue, cartilage, contributing to its degradation, as seen in arthritis [368], [369].

Furthermore, elevated RAGE expression has been detected in a variety of tumors, including liver [370], gastric [371], colon [372], breast tumors [370], as well as malignant melanoma [373]–[375]. The role of RAGE in carcinogenesis depends on the tissue the tumor is located in and the involved coherent signaling pathways. A study from 2013 suggested that a possible explanation for this observation could be the presence of distinct isoforms of RAGE that effect tumor migration, invasion, and adhesion differential [376]. In contrast to most types of cancers, lung carcinomas exhibit decreased expression of RAGE, while, as mentioned before, RAGE is highly expressed in healthy lung tissues [377]. However, in general it is known that RAGE-dependent chronic inflammatory conditions predispose to carcinogenesis and also promote tumor progression [378].

V.3.3.2.1. RAGE signaling in cutaneous chronic inflammation

RAGE expression is low in adult tissue, including innate and adaptive immune cells such as neutrophils, T and B lymphocytes, monocytes, macrophages, dendritic cells, and endothelial cells but increases upon activation [253], [258], [379]. However, despite the expression of RAGE, these cells also secrete its ligands. Therefore, they are most likely not only contributing to RAGE-dependent inflammatory responses but also crucial for their initiation and propagation [380]. For instance, the autocrine/paracrine RAGE signaling of DCs facilitates the maturation of these cells and is necessary for their migration into draining lymph nodes allowing their interaction with naïve T lymphocytes [261]. Moreover, Chen and colleagues demonstrated increased levels of T_H2-related cytokines in RAGE-deficient T cells, and *vice versa* a decreased expression of IFN-γ, a T_H1 proinflammatory cytokine, thereby illustrating the importance of RAGE on T lymphocytes not only for the induction of an immune response but also for its modulation [256].

Furthermore, RAGE engagement and consequent activation of proinflammatory transcription factors such as NF-κB leads to the expression of target genes, encoding for some well-described regulators of the innate and adaptive immune system [347]. Another contribution of RAGE to the inflammatory response is given by its expression on endothelial cells and its ability to interact with leukocyte β2 integrin Mac1, thereby acting as an adhesion receptor for leukocytes [278], [381], [382]. Herold and coworkers demonstrated that oligomeric ligands have a higher affinity to RAGE thereby inducing a persistent signaling which leads to a chronic inflammation; whereas monomeric ligands are only able to induce an acute inflammation [383]. Upon signal transduction, RAGE-dependent activation of intracellular pathways leads to the subsequent induction of NF-κB and downstream target genes. Through the utilization of the aforementioned positive feedback loop, RAGE guarantees sustained signaling, thereby contributing to the maintenance of a chronic inflammation such as psoriasis.

Besides immune cells also other cell types within the skin, such as keratinocytes, contribute to an inflammatory phenotype of the skin. Using *Rage*$^{-/-}$ mice, it was shown that RAGE signaling in keratinocytes is influencing epidermal structure and is involved in the dermal immune response, at least under acute inflammatory conditions [384].

V.3.3.2.2. Impact of RAGE in the pathogenesis of psoriasis

The expression of RAGE in immune and cutaneous cells, the high stability of RAGE-ligand complexes, as well as the existence of a positive feed-forward loop sustained RAGE signaling in inflammatory conditions strongly recommend a role of RAGE in the initiation and maintenance of a cutaneous chronic inflammation such as psoriasis.

A first strong connection between RAGE and psoriasis is given by a genetic link; indeed, *AGER* encoded within the psoriasis susceptibility locus 1 (PSORS1) on chromosome 6p21.3 [216], [217]. Furthermore, genes for the members of the S100 family, S100A7 and S100A15, are positioned within the PSORS4 on chromosome 1q21 in the EDC [385]. That psoriasis is genetically linked both, receptor and ligands, is not the only evidence for a potential role of the RAGE axis in psoriasis. During skin inflammation keratinocytes are characterized by increased production and secretion of S100A7 and S100A15 [386]–[388]. In 2008, Wolf and colleagues further demonstrated that the S100A7-RAGE interaction chemoattracts leukocytes into inflamed skin, displaying an important step in the induction and maintenance of an inflammation [302]. Two years later the same group exhibited that the overexpression of S100A7/A15 increases a RAGE-dependent secretion of T_H1- and T_H17-related proinflammatory cytokines by applying a double-transgenic mouse model [303].

Another early event in the initial phase of psoriasis is the induction of S100A8/A9 and their consequent binding to RAGE [63]. This interaction is connected to hyperplasia, a hallmark of psoriasis, by the stimulatory effects of the S100/RAGE axis on the growth of normal human keratinocytes *in vitro* [389].

Moreover, the elevation of other RAGE ligands such as AGEs has been described as a characteristic of an inflammatory psoriatic response [390]. AGE-RAGE interaction stabilizes the active state of the receptor, thereby leading to enhanced production of proinflammatory cytokines [245], [391]. An additional characteristic of the inflammatory response found in psoriasis is the induced migration of T lymphocytes to the sites of skin inflammation that is at least partially established by RAGE-mediated signaling [331], [338].

Considering RAGE as a potential regulator of several processes involved in the development of a psoriatic disease, a better understanding of its activation in psoriasis, comprising the contribution of distinct ligands, and its functional relevance in different cell types associated with psoriasis can be beneficial for the development of new therapeutic targets.

VI. Material and Methods

VI.1. Materials

Reagents and kits	Company	Catalog #
2-(4-Aminophenyl)-1H-indole-6-carboxamidine (DAPI)	Roche Diagnostics	10236276001
β-Mercaptoethanol	Gibco® Life Technologies	31350-010
7-AAD Viability Staining Solution	affymetrix	00-6993-50
20 % Sodium dodecyl sulfate (SDS)	G-Biosciences	786016
100bp DNA ladder	Thermo Scientific	SM1143
Acetone	Sigma-Aldrich	32201
Acid alcohol	Sigma-Aldrich	56694
Agarose NEEO ultra-quality	Carl Roth	2267.4
Albumin Fraction V	Carl Roth	8076
Antibody Diluent	Dako	S0809
Arcturus PicoPure RNA isolation Kit	Applied Biosystems	KIT0204
autoMACS® Columns	Miltenyi	130-021-101
autoMACS™ Rinsing Solution	Miltenyi	130-091-222
autoMACS™ Running Buffer	Miltenyi	130-091-221
Braunol®	Braun	3864154
Calcium (Ca^{2+})	Sigma-Aldrich	215147
Citric acid	Sigma-Aldrich	C-6445
Cloroform ($CHCl_3$)	Sigma-Aldrich	32211
Color Reagent A + B	R&D	DY999
Dako Fluorescent Mounting Medium	Dako	S302380-2
Delimiting Pan	Dako	S2002
Diamond pDC isolation Kit	Miltenyi	130-092-402
Diamond pDC isolation Kit II	Miltenyi	130-097-240
Eosin	Sigma-Aldrich	HT110132

Ethanol (EtOH)	Sigma-Aldrich	459844
Ethidiumbromide	Carl Roth	7870
Ethylene diamine tetraacetic acid (EDTA)	Gerbu	1034
Hematoxylin	Sigma-Aldrich	GHS132
HIER citrate buffer	Zytomed systems	ZUC028
HMGB1 ELISA Kit	IBL	ST51011
IL1β/IL1F2 DuoSet ELISA Kit	R&D	DY201
MACS BSA Stock Solution	Miltenyi	130-091-376
Methanol (MeOH)	Sigma-Aldrich	32213
Paraformaldehyde	Sigma-Aldrich	P6148
Phosphate Buffered Saline (PBS)	Biochrom	L182-50
Phusion Taq	Thermo Scientific	F-530
peqGOLD RNAPure	peqlab	30-1020
Peroxidase block solution	Dako	S2023
Potassium chloride (KCl)	Carl Roth	6781
ProcartaPlex mouse $T_H9/T_H22/T_H17$/Treg cytokine panel (6plex)	affymetrix	EPX-060-20822-901
Proteinase K	New England Biolab	P8102S
RevertAid First Strand cDNA Synthesis Kit	Thermo Scientific	K1622
RT^2 Profiler™ Mouse Innate & Adaptive Immune Response PCR Assay	QIAGEN	PAMM-052Z
RNase-Free DNase Set	QIAGEN	79254
RNeasy Mini Kit	QIAGEN	74106
S100B ELISA Kit	Abnova	KA0037
Sodium azide	AppliChem	A1430
Sodium chloride (NaCl)	Sigma-Aldrich	31434
Sucrose	Sigma-Aldrich	S79003
Sulfuric acid (H_2SO_4)	Carl Roth	4623
SYBR Green PCR Master Mix	Applied Biosystems	4309155

Tissue Tek® Paraffin Wax	Sakura	4502
Tissue Tek® Mold Release	Sakura	4141
Tween® 20	Applichem	A1389
Tris-hydrochloride	Carl Roth	9090
Tri-sodium citrate	Merck	1110371000
Verikine™ IFN-α ELISA Kit	Pbl assay science	41100-1
Xylene	Sigma-Aldrich	247642

Table 2 Reagents and Kits

Cell culture reagents	Company	Catalog #
Corning™ cell scraper	Sigma-Aldrich	CLS3010
Dimethylsulfoxide (DMSO)	Carl Roth	A994
Dulbecco's Phosphate Buffered Saline (PBS)	Sigma-Aldrich	D8537
EDTA pH 8.0	Ambion	AM9260G
EpiLife® Medium with 60µM Calcium	Gibco® Life Technologies	MEPI-500CA
Fetal Calf Serum (FCS)	Biochrom	S0415
Ficoll/Pancoll, human	Pan-Biotech	P04-60500
Human Keratinocyte Growth Supplement (HKGS) 100x	Gibco® Life Technologies	S0015
Lab Tek chamber slide	Thermo Scientific	177402
Normocin	Invivogen	ant-nr
Nylon cell strainer (40-µm)	BD falcon	352340
Nylon cell strainer (100-µm)	BD falcon	352360
Penicillin-Streptomycin	Sigma-Aldrich	P4333
RPMI-1640+GlutaMAX™-I	Gibco® Life Technologies	61870-044
Trypan blue solution	Fluca	93595
Trypsin-EDTA solution	Sigma-Aldrich	T3924

Table 3 Cell culture reagents

Stimulatory molecules	Company	Catalog #
Disulfide HMGB1, LPS-free	HMGBiotech	HM-120
Mouse IL-23 recombinant protein, carrier-free	affymetrix	34-823185
S100-b bovine brain	Merck	559290
Class A CpG oligonucleotide (ODN-2216)	Invivogen	tlrl-2216
Recombinant human IL-3	PeproTech	200.03
Imiquimod	Invivogen	tlrl-imq
Aldara cream (5 % Imiquimod)	MEDA	

Table 4 Stimulatory reagents

Antibodies	Company	Catalog #
Primary Antibodies		
Goat-anti-Calgranulin A	SantaCruz	sc-8113
Goat-anti-Calgranulin B	SantaCruz	sc-8114
Hamster-anti-CD11c	BD	550283
Hamster-anti-CD11c-APC	Biolegend	117309
Rat-anti-CD45	BD	550539
Mouse-anti-CD123-FITC	Miltenyi	130-090-897
Mouse-anti-CD303-APC	Miltenyi	130-090-905
Rabbit-anti-Filaggrin	Abcam	ab24584
Rat-anti-F4/80	Abd Serotec	MCA497GA
Rabbit-anti-IL17	Abcam	ab79056
Rabbit-anti-IL-22	Abcam	ab18499
Rabbit-anti-IL-23	LSBio	LS-B1366
Mouse-anti-Involucrin	Sigma	I9018
Mouse IgG2B isotype ctrl	R&D	MAB004
Mouse-anti-Keratin-14	Covance	PRB-155P
Rabbit-anti-Ki67	Abcam	ab16667

Rat-anti-Ly-6B.2	Abd Serotec	MCA771GT
Rabbit-anti-RAGE	Abcam	ab37647
Mouse-anti-RAGE	R&D	MAB11451
Secondary Antibodies		
Donkey-anti-goat Cy3	Dianova	705-485-147
Donkey-anti-rabbit Cy2	Dianova	711-225-152
Donkey-anti-rabbit Cy3	Dianova	711-166-152
Goat-anti-hamster Cy3	Dianova	127-165-160
Goat-anti-mouse Cy3	Dianova	115-165-062
Goat-anti-rabbit Cy3	Dianova	111-165-045
Goat-anti-rabbit HRP AEC+	Dako	K400911-2
Goat-anti-rat Cy 3	Dianova	112-165-062

Table 5 Antibodies

VI.2. Buffer solutions

Lysis buffer
100 mM Tris-HCl (pH 8.8)
5 mM EDTA (pH 8.0)
0.2 % SDS
200 mM NaCl
prior use: 100 µg/ml Proteinase K

Salt buffer
4.21 M NaCl
0.62 M KCl
10 mM Tris (pH 8.0)

Citrate buffer pH 6.0
10 mM Citric acid
0.05 % Tween® 20

Sodium citrate buffer pH 6.0
10 mM Tri-sodium citrate
0.05 % Tween® 20

Demasking buffer
1.8 % Citrate buffer pH 6.0
8.2 % Sodium citrate buffer pH 6.0

Washing buffer (IF) pH7.6
PBS
0.05 % Tween®20

FACS buffer
PBS
10 % FCS
1 % Sodium azide

Analysis software	Source
7500 Software v2.0.5	Applied Bioscience
BD FACSDiva™	Biolegend
FlowJo 7.2.2.	FlowJo
GraphPad Prism 5	GraphPad Prism
iControl 1.10©2012	TECAN
ImageJ	National Institute of Health (NIH)
NIS-Elements Viewer	Nikon

Table 6 Analysis software

Devices	Company
AB 7500 Real Time PCR machine	Applied Biosciences
AutoMACS Pro separator	Miltenyi
Biovision + 1000/26MX	Peqlab
Cryostat CM3050S	Leica
Digital Caliper	ETS Product Service AG
DM LS light microscope	Leica
FACS Canto 4.0	BD biosciences
Micro Dismembrator S	Satorius
NanoDrop ND-1000 Spectrophotometer	Peqlab Biotechnologie GmbH
Nikon Eclipse Ti Fluorescence microscope	Nikon
TECAN infinite F200 pro microplate reader	Tecan
Tissue Tek® TEC 5 Embedding System	Sakura

Table 7 Devices

VI.3. Methods

VI.3.1. *In vivo* mouse experiments

Rage-deficient (*Rage*$^{-/-}$) mice were described previously [349], [392] and wildtype (*wt*) controls were obtained from Charles River Laboratories, France. Both, *wt* and *Rage*$^{-/-}$ mice were on a C57BL/5 background [349] and were housed in groups of 3 animals per cage with food and water *ad libitum* under specific pathogen-free and 12- hour light/dark cycle conditions. All animal experiments were performed with 11 to 13 week old female *Rage*$^{-/-}$ and *wt* mice.

The procedures for performing animal experiments were in accordance with the principles and guidelines of the 'Arbeitsgemeinschaft der Tierschutzbeauftragten in Baden-Württemberg', Germany, and were approved by the 'Regierungspräsidium Karlsruhe', Germany (G-13/12).

VI.3.1.1. *In vivo Imiquimod mouse experiments*

Chronic skin inflammation of mice back skin was generated according to the protocol of Imiquimod-induced psoriasis [54]. Mice received a daily topical dose of 62.5 mg Aldara cream (5 % Imiquimod) on the shaved back skins for six consecutive days. Progression of inflammatory response was measured daily by the adapted PASI score. Animals were euthanized 24 h after the last Imiquimod administration by cervical dislocation. Skin samples for RNA isolation were immediately frozen in liquid nitrogen. For histological analysis, tissue was fixed with 4 % (w/v) paraformaldehyde in PBS (pH 7.4) (PBS/PFA), paraffin-embedded, and subsequently cut into 1- µm sections. Blood samples were taken, prepared and frozen in liquid nitrogen (VI.3.8.).

VI.3.1.2. *In vivo rIL-23 mouse experiments*

Chronic skin inflammation of the ear was generated according to the protocol of rIL-23-induced psoriasis [53]. 20 µl PBS containing 500 ng recombinant mouse IL-23 (ebioscience) was intradermally injected into the ears of anesthetized mice. Injections were repeated every other day for 16 days. Ear thickness and adapted PASI score were measured directly before injections. Ear measurements were made in the center of the ear using a digital slide gauge (Digital Caliper). 24 h after the last treatment mice were euthanized by cervical dislocation, and tissues were collected. Skin samples for RNA isolation, histological analysis, and blood samples were prepared as described above. Lymph nodes were processed for FACS analysis (VI.3.9.).

VI.3.2. Cell culture

All cells were grown in a humidified atmosphere at 37 °C and 5 % CO_2 and have been routinely tested for mycoplasma. Individual culture conditions are listed below.

VI.3.2.1. *Cell culture of primary human keratinocytes*

Human keratinocytes were cultivated in EpiLife® Medium with 60 µM calcium supplemented with 1 % (v/v) human keratinocyte growth supplement (HKGS), 100 units/ml penicillin and 100 µg/ml streptomycin, from here on referred to as keratinocyte medium. Every 5 to 6 days when 90 % confluence was reached the cells were subcultured using a 0.25 % to 0.5 % trypsin solution.

VI.3.2.2. *Isolation of primary human keratinocytes*

Human keratinocytes were obtained from foreskins, kindly provided by Dr. Uysal, Mannheim. Donor age ranged from newborns to three years old patients. Excised foreskins were incubated for 15 min at room temperature (RT) in 10 % Braunol solution, were washed with PBS, freed from subcutaneous adipose and loose connective tissues, and cut into strips about 2-3 mm width. Samples were incubated with the dermal side down in 0.25 % trypsin with 100 units/ml penicillin and 100 µg/ml streptomycin for 16 to 24 h at 4 °C. The epidermis of overnight digested tissue was peeled off and incubated in a 0.05 % trypsin/EDTA solution for 15 min at 37 °C. Primary keratinocytes were washed and transferred to 35- mm dishes containing keratinocyte medium supplemented with 0.05 % normocin. Approximately six days after isolation cells were attached and fibroblasts were removed by aspiration with 1x phosphate buffered saline (PBS) with 0.2 % EDTA.

VI.3.2.3. *Stimulation of primary human keratinocytes*

Keratinocytes from passage 1 to 3 were used for all stimulation experiments.

VI.3.2.3.1. Stimulation of primary human keratinocytes with RAGE ligands

Keratinocytes were plated in 48- well plates at a density of 5 x 10^4 cells per well. Cells were stimulated with indicated concentrations of ligands (HMGB1 and S100B) alone, or pre-complexed with CpG-A (ODN 2216) (Table 8).

Stimulatory factors	Concentration
Disulfide HMGB1	5 µg/ml
S100-b bovine brain	5 µg/ml
CpG-A (ODN 2216)	300 nM

Table 8 Stimulatory factors of primary human keratinocytes

IL-1β protein concentration in cell culture supernatant was measured by ELISA. IL-1β mRNA levels were determined *via* RT-qPCR.

VI.3.2.3.2. Stimulation of primary human keratinocytes with Imiquimod

Keratinocytes were plated in 6-well plates at a density of 5 x 10^5 cells per well. Cells were differentiated for 7 days in keratinocyte medium containing 1.2 mM calcium and control cells

were cultivated in normal keratinocyte medium. After differentiation period, keratinocytes were stimulated with 100 µM Imiquimod for one to six hours. HMGB1 and S100B protein concentrations in cell culture supernatant were measured by ELISA.

VI.3.2.4. *Cell culture of primary plasmacytoid dendritic cells*

Human plasmacytoid dendritic cells (pDCs) were cultivated in RPMI-1640 medium supplemented with 10 % (v/v) heat inactivated fetal bovine serum (FCS), 100 units/ml penicillin, 100 µg/ml streptomycin, 0.05 % (v/v) normocin and 10 ng/ml interleukin-3, from here on referred to as pDC medium.

VI.3.2.5. *Isolation of primary human plasmacytoid dendritic cells*

pDCs were isolated from primary human peripheral blood mononuclear cells (PBMCs). For the isolation of PBMCs buffy coats, concentrated leukocyte suspension, from IKTZ Heidelberg were used. The buffy coat sample was diluted with an equal volume of 2 mM EDTA in PBS (PBS/EDTA) and overlayed on 15 ml ficoll (human density 1.077 g/l). The following ficoll gradient centrifugation was performed at 970 rcf for 15 min without brake. The PBMC interphase was transferred to 30 to 35 ml PBS/EDTA and centrifuged for 7 min at 580 rcf with brake. Cell pellet was resuspended in 50 ml PBS and filtered through a 40- µm nylon mesh to provide a single cell suspension. Cells were counted with trypan blue and centrifuged for 10 min at 300 rcf at 4 °C. 4 x 10^6 cells were stored at 4 °C for FACS analysis. The remaining cells were used to isolate pDCs using the 'Plasmacytoid dendritic cell Isolation Kit' (Miltenyi) according to manufacturer's instructions. Briefly, PBMCs were magnetically labeled with a biotin-antibody cocktail and anti-biotin microbeads. Non-plasmacytoid dendritic cells were depleted with an autoMACS separator. The unlabeled fraction was collected, magnetically labeled with CD304 diamond microbeads and positively separated. The positive fraction contains pDCs, of which 5 x 10^5 were used for purity analysis *via* FACS.

VI.3.2.6. *Stimulation of primary human plasmacytoid dendritic cells*

pDCs were plated in 48- well plates at a density of 7.5 x 10^4 cells per well and either incubated with RAGE blocking antibody (MAB11451) or the corresponding isotype control (MAB004) at a concentration of 10 µg/ml 3 h prior as well as during stimulation. Cells were stimulated with indicated concentrations of ligands (HMGB1 and S100B) alone, or pre-complexed with CpG-A (ODN 2216) for 24 h at 37 °C (Table 9).

Stimulatory factors	Concentration
Disulfide HMGB1	5 µg/ml
S100-b bovine brain	5 µg/ml
CpG-A (ODN 2216)	37.5 nM

Table 9 Stimulatory factors of primary plasmacytoid dendritic cells

After 24 h of stimulation supernatants were collected and stored at -20 °C until IFN-α ELISA analysis was performed. Cell lysates were resuspended in 70 µl extraction buffer and RNA was isolated (Arcturus PicoPure RNA isolation Kit). IFN-α mRNA levels were determined by RT-qPCR.

In addition, pDCs were plated in a 4- well Lab Tek chamber slide at a density of 2.5 x 10^4 cells per well, stimulated with Rage ligands alone or in complex with CpG and were prepared for immunofluorescence staining.

VI.3.3. RNA isolation from cultivated cells

RNA extraction was performed using the RNeasy Mini Kit (QIAGEN) or PicoPure RNA isolation kit (Life Technologies) according to manufacturer's instructions. Briefly, pelleted cells were dissolved using RLT lysis buffer containing 1 % β-Mercaptoethanol followed by RNA extraction using a column-based purification. Every sample was DNase I-treated for 15 min at RT followed by two washing steps and the elution in RNase-free H_2O. RNA concentration and quality was measured using a NanoDrop ND-1000 spectrophotometer. Samples fulfilling the quality recommendations were further processed.

VI.3.4. RNA isolation from murine skin

For RNA isolation of murine skin, a teflon capsule was cooled down in liquid nitrogen, filled with 500 µl peqGOLD RNAPure and put in liquid nitrogen for another 20 to 30 sec. A precooled metal ball and tissue sample were added into the capsule. Covered capsule was inserted in a dismembrator and tissue was fragmented at 2500 rcf for 30 sec. Dismembered tissue was transferred into a corex tube, filled with 2 ml peqGOLD RNAPure and was incubated at RT until tissue powder was solved. Afterwards, 400 µl chloroform was added to the samples, followed by 15 sec shaking period and incubation on ice for 10 min. The following chloroform gradient centrifugation was performed at 12700 rcf for 20 min at 4 °C. The upper aqueous phase, containing RNA, was transferred into RNase-free tube and incubated on ice until RNA extraction was performed using the RNeasy Mini Kit (QIAGEN). RNA concentration and quality was measured using a NanoDrop ND-1000 spectrophotometer. Samples fulfilling the quality recommendations were further processed.

VI.3.5. cDNA transcription

500 ng RNA of each sample were then used for cDNA generation. In brief, RNA and H_2O, as non-template control, were incubated with oligo $(dT)_{18}$ primers in a volume of 12 µl for 5 min at 65 °C. Reverse transcription was performed using the RevertAid First Strand cDNA Synthesis Kit according to manufacturer's advice. As control for successful removal of genomic DNA also reactions without reverse transcriptase were performed. Before use cDNA was diluted at a 1 : 5 ratio in nuclease-free H_2O.

VI.3.6. Quantitative real-time polymerase chain reaction (qPCR)

VI.3.6.1. *Standard qPCR analysis*

QPCR was performed using 2x SYBR Green PCR Master Mix and an Applied Biosystems 7500 Real-Time PCR System. In each qPCR experiment 12.5 ng cDNA per sample were applied and amended by a non-template control as well as minus-reverse-transcriptase controls to monitor the quality of the assay. All primers used in this study show an efficiency within the range of 85 to 110 % as analyzed by cDNA dilution curves. After testing all samples for low variances of housekeeping gene expression (< 2 cycles) the results of human targets were normalized to hs_18S and for murine targets to mm_βActin. All samples were analyzed in triplicates and gene quantification was calculated using the Pfaffl method [393], calculating the delta-delta Ct (ΔΔCt). Statistical analysis was carried out in Excel and visualization of graphs in GraphPad Prism 5. Primers used in the study are listed in Table 10.

	Forward primer	**reverse primer**
human primer		
Hs_18S	*GAGGATGAGGTGGAAACGTGT*	*TCTTCAGTCGCTCCAGGTCT*
Hs_HMGB1	*CTAAGAAGTGCTCAGAGAGGTG*	*GGAAGAAGGCCGAAGGAG*
Hs_IL-1β	*TGTGAAATGCCACCTTTTGA*	*GGTCAAAGGTTTGGAAGCAG*
Hs_IFN-α	*AGTCTCTTCCACCCCAACCT*	*TGGGAACAGAGCCTCCTAGA*
Hs_Involucrin	*TGTGAGTCTGGTTGACAGTAGC*	*ATTCTTGCTCAGGCAGTCCC*
Hs_S100B	*CCACCAATATTCTGGAAGGG*	*TCCACAACCTCCTGCTCTTT*
murine primer		
Mm_βActin	*TCACCCACACTGTGCCGATCTACGA*	*GGATGCCACAGGATTCCATACCCA*
Mm_Il-17	*CAGCAGCGATCATCCCTCAAAG*	*TGAGGTTGACCTTCACATTCTGGA*
Mm_Il-22	*ATACATCGTCAACCGCACCTTT*	*AGCCGGACATCTGTGTTGTTAT*

Mm_Il-23	*TATCCAGTGTGAAGATGGTGGTG*	*CACTAAGGGCTCAGTCAGAGTTG*
Mm_Ager	*TGGGCAGAGATGGCACAGGT*	*AGCTGGCACTTAGATGGGAAACTT*

Table 10 QPCR primer

VI.3.6.2. *RT² Profiler™ PCR Array Mouse Innate & Adaptive Immune Responses*

The applied RT² Profiler™ Mouse Innate & Adaptive Immune Responses PCR Array profiles the expression of 84 immune response related genes, 5 house-keeping genes, as well as reverse transcriptase- and positive controls. Relevant genes out of following signaling pathways were analyzed and normalized to two house-keeping genes (*βActin* and *Gapdh*): Innate immunity, adaptive immunity, humoral immunity, inflammatory response, defense response to bacteria and defense response to viruses (Table 11). The average correlation coefficients of these arrays is higher than 0.99, ensuring reliable detection of differences in the expression between biological samples. cDNA from Imiquimod treated wildtype and Rage-deficient mice were used as biological samples in this assay. The arrays were performed using 2x SYBR Green PCR Master Mix and an Applied Biosystems 7500 Real-Time PCR System according manufacturer's protocol.

RT² Profiler™ PCR Array Mouse Innate & Adaptive Immune Responses	
Pathway	Genes
Innate Immunity	
PRRs	*Ddx58 (RIG-I), Nlrp3, Nod1 (Card4), Nod2, Tlr1, Tlr2, Tlr3, Tlr4, Tlr5, Tlr6, Tlr7, Tlr8, Tlr9.*
Innate cytokines	*Ccl12 (MCP-5, Scya12), Ccl5 (RANTES), Csf2 (GMCSF), Cxcl10 (INP10), Ifna2, Ifnb1, Il18, Il1a, Il1b, Il2, Tnf.*
Other innate immunity genes	*Apcs, C3, C5ar1 (Gpr77), Casp1 (ICE), Cd14, Cd4, Cd40 (Tnfrsf5), Cd40lg (Tnfsf5), Cd8a, Crp, H2-Q10, H2-T23, Il1r1, Irak1, Irf3, Irf7, Itgam, Ly96 (MD2), Lyz2, Mapk1 (Erk2), Mapk8 (JNK1), Mbl2, Mpo, Mx1, Myd88, Nfkb1, Nfkbia (Iκbα, Mad3), Stat1, Ticam1 (TRIF), Traf6.*
Adaptive Immunity	
Th1 marker	*Ccr5, Cd80, Cxcr3, Ifng, Il18, Il23a, Slc11a1, Stat4, Tbx21, Tlr4, Tlr6.*
Th2 marker	*Ccr4, Ccr8, Cd86, Gata3, Ifnb1, Il10, Il13, Il18, Il4, Il5, Il6, Nod2, Stat6.*
Th17 marker	*Ccr6, Il17a, Rorc, Stat3.*
Treg marker	*Ccr4, Ccr8, Foxp3, Il10.*

T cell activation	*Cd80, Cd86, Icam1, Ifng, Il23a, Il6, Slc11a1.*
Adaptive cytokines	*Ccl12 (MCP-5, Scya12), Ccl5 (RANTES), Csf2 (GMCSF), Cxcl10 (INP10), Ifna2, Ifng, Il10, Il13, Il17a, Il18, Il2, Il23a, Il4, Il5, Il6, Tnf.*
Other adaptive immunity genes	*Cd4, Cd40 (Tnfrsf5), Cd40lg (Tnfsf5), Cd8a, Crp, Fasl (Tnfsf6), H2-Q10, Ifnar1, Ifngr1, Il1b, Il1r1, Irf3, Irf7, Itgam, Jak2, Mapk8 (JNK1), Mbl2, Mx1, Nfkb1, Rag1, Stat1.*
Humoral immunity	*C3, C5ar1 (Gpr77), Ccl12 (MCP-5, Scya12), Ccr6, Crp, Ifnb1, Ifng, Il6, Mbl2, Nod2, Tnf.*
Inflammatory response	*Apcs, C3, Ccl5 (RANTES), Crp, Foxp3, Il1a, Il1b, Il4, Il6, Mbl2, Stat3, Tnf.*
Defense response to bacteria	*C5ar1 (Gpr77), Ifnb1, Ifng, Il23a, Il6, Lyz2, Mbl2, Myd88, Nod1 (Card4), Nod2, Slc11a1, Tlr1, Tlr3, Tlr4, Tlr6, Tlr9, Tnf.*
Defense response to viruses	*Cd4, Cd40 (Tnfrsf5), Cd86, Cd8a, Cxcl10 (INP10), Ddx58 (RIG-I), H2-Q10, Ifnar1, Ifnb1, Il23a, Il6, Irf3, Nlrp3, Ticam1 (TRIF), Tlr3, Tlr7, Tlr8, Tyk2.*

Table 11 Genes involved in immune response pathways covered by RT² Profiler™

VI.3.7. PASI score

The Psoriasis Area Severity Index (PASI) is a quantitative rating score for measuring severity of psoriasis. It combines plaque characteristics like erythema, induration and desquamation and the percentage of affected area [69]. Shaved back skin area was set up as 100 % body area for calculating the adapted PASI score.

VI.3.8. Blood withdrawal and serum extraction

Blood was withdrawal by cardiac puncture from the right ventricle or by puncture of vena saphena. Blood was transferred into a 1.5 ml tube, incubated 1 h at RT, and centrifuged for 15 min at 400 rcf and 4 °C to separate the serum. The serum was transferred to fresh tubes, frozen in liquid nitrogen and kept at -80 °C.

VI.3.9. Preparation of murine lymphoid organs

Cervical lymph nodes were isolated from recently sacrificed mice. Lymph nodes were removed and transferred to a 100- µm cell strainer. Back of a syringe plunger was used to macerate the cells through the filter, and the filter was washed with PBS/EDTA. Isolated cells were centrifuged for 5 min at 300 rcf at 4 °C, before supernatant was discarded. Red cell

lysis was performed by incubating the cells 20 to 30 sec in 2.3 ml 1/6x PBS on ice and by adding 310 µl 10x PBS to the cells. Afterwards cell were centrifuged and counted with trypan blue.

VI.3.10. Genotyping of mice

Tail biopsies were lysed overnight at 55 °C in 500 µl lysis buffer. Lysates were vortexed for 15 sec and afterwards centrifuged for 5 min at 3000 rcf at RT. Supernatant was then mixed with 139 µl salt buffer and centrifuged again. Addition of 640 µl 95 % EtOH led to the precipitation of DNA, which was pelleted by a centrifugation step (5 min; 3000 rcf; RT). DNA pellet was washed with 500 µl 70 % EtOH, centrifuged, air-dried for 15 min at 37 °C and resuspended in 100 µl ddH_2O.

PCR reactions were set up in 20 µl including 50 ng DNA, 5 µl 5x buffer, 10 mM primer mix (Table 12), 10 mM dNTPs and 0.2 µl Phusion Taq.

	Forward primer	**reverse primer**
Mm_Rage	*AGCTGGCACTTAGATGGGAAACTT*	*TGGGCAGAGATGGCACAGGT*
Mm_eGFP	*CCTACGGCGTGCAGTGCTTCAGC*	*CGGCGAGCTGCACGCTGCGTCCTC*

Table 12 PCR primer

Program was designed as followed (Table 13).

PCR step	**Temperature**	**Time**
1. step: initial denaturation	94 °C	60 sec
2. step: annealing	60.5 °C	30 sec
3. step: elongation	72 °C	20 sec
4. step: denaturation	94 °C	30 sec
Repeat step 2 to 4 for a total of 35 cycles		
5. step: final elongation	72 °C	5 min
6. step: storage	4 °C	∞

Table 13 Cycling condition

VI.3.11. Agarose gel electrophoresis

PCR reactions were run on 1.5 to 2 % agarose gels. 4 µl of 6x DNA loading dye were added to the samples and 10 µl of each samples were loaded. A 100 bp DNA ladder was used as marker. Gels were run at 110 V until the bands were separated and afterwards incubated in

an Ethidiumbromide bath for 20 min before bands were visualized by using the gel documentation system 'Biovision'.

VI.3.12. Embedding of murine skin

VI.3.12.1. *Paraffin-embedding of murine skin*

For histological analysis, tissue was fixed with PBS/PFA for 20 to 28 h at 4 °C. Once fixed, tissue was processed as follows using a gentle agitation (Table14).

Solution	Time	Temperature
1x PBS	1 h	4 °C
0.85 % NaCl	30 min	4 °C
1:1 0.85 % NaCl: EtOH	15 min	RT
1:1 0.85 % NaCl: EtOH	15 min	RT
70 % EtOH	15 min	4 °C
70 % EtOH	30 min	RT
85 % EtOH	30-60 min	RT
95 % EtOH	1 h	RT
100 % EtOH	3 x 1 h	RT
100 % Xylene	2 x 1 h	RT
100 % Xylene	10-16 h	RT
1:1 Paraffin:Xylene	50 min	56 °C
Paraffin	3 x 1 h	56°C

Table 14 Paraffin-embedding condition

Afterwards a small amount of molten paraffin was put in a mold and tissue was transferred into mold in upended position. Mold was then completely filled with molten paraffin. When the wax was cooled and hardened, the tissue within the paraffin block was sectioned in 1- µm sections using a microtome. The generation of tissue sections was kindly performed by Sayran Arif-Said, Clinical Cooperation Unit Dermato-Oncology, Mannheim.

VI.3.12.2. *Cryo-embedding of murine skin*

Tissue was fixed with PBS/PFA for 20 to 28 h at 4 °C, wash with 1x PBS at 4 °C for 1 h and subsequently incubated in 25 % sucrose for 20 to 24 h. Tissue samples were placed in a cryomold and were overlayed with Tissue Tek. Embedded samples were stored at -80 °C and cut in 2 to 3- µm slides by using the cryostat from Leica.

VI.3.13. Hematoxylin and eosin (H&E) staining

Paraffin-embedded tissue was deparaffinized three times in 100 % xylene and rehydrated in descending alcohol series (twice for 10 min in 100 % EtOH, for 4 min in 95 % EtOH, 80 % EtOH and 70 % EtOH). Followed by a washing step with ddH_2O for 5 min and stained with filtered hematoxylin for 6 min. Tissue was then washed with running tap water for 20 min before it was decolorized in acid alcohol, washed and counterstained in eosin for 15 sec. After counterstaining tissue slides were dehydrated in ascending alcohol series (twice for 3 min in 95 % EtOH and twice for 3 min in 100 % EtOH), cleaned in 100 % Xylene two times for 5 min and subsequently mounted in Dako Fluorescent Mounting Medium (Dako).

All HE stained tissue samples were analyzed under a Leica DM LS light microscope.

VI.3.14. Immunofluorescence

For immunofluorescence staining following antibodies were used (Table 15).

Primary antibodies	Concentrations	Secondary antibodies	Concentrations
Rabbit-anti-Ki67	1 : 50	Goat-anti-rabbit Cy3	1 : 500
Hamster-anti-CD11c	1 : 50	Goat-anti-hamster Cy3	1 : 500
Rat-anti-F4/80	1 : 200	Goat-anti-rat Cy3	1 : 500
Rat-anti-Ly-6B.2	1 : 100	Donkey-anti-goat Cy3	1 : 500
Rat-anti-CD45	1 : 50	Goat-anti-mouse Cy3	1 : 500
Goat-anti-Calgranulin A	1 : 150	Donkey-anti-rabbit Cy3	1 : 500
Goat-anti-Calgranulin B	1 : 150	Donkey-anti-rabbit Cy2	1 : 500
Rabbit-anti-IL-17	1 : 100	Goat-anti-rabbit Cy3	1 : 500
Rabbit-anti-IL-22	1 : 500		
Rabbit-anti-IL-23	1 : 250		
Rabbit-anti-RAGE	1 : 100		
Rabbit-anti-Keratin-14	1 : 250		
Mouse-anti-Involucrin	1 : 500		
Rabbit-anti-Filaggrin	1 : 100		
DAPI	1 : 5000/ 10000		

Table 15 Primary and secondary antibodies used in immunofluorescence

VI.3.14.1. *Immunofluorescence on paraffin-embedding tissues*

Paraffin-embedded murine tissue samples were deparaffinized and rehydrated in descending alcohol series (as described in H&E staining VI.3.13.). Rehydration was followed by an antigen unmasking step by incubating the slides for 40 min in demasking buffer pH 6.0 in a streamer and a washing step for 5 min in 1x PBS. To block unspecific bindings of the antibodies slides were incubated in 1 % (w/v) BSA in 1x PBS (PBS/BSA) for 1 h at RT in humid chamber. Afterwards tissue slides were incubated in primary antibody, diluted in PBS/BSA over night at 4 °C. On the next day slides were washed three times in 1x PBS for 6 min and then incubated with secondary antibody and 4',6-diamidin-2-phenylindol (DAPI) diluted in 1 % BSA/PBS for 2 h in the dark at RT. Staining procedure was finished by three washing steps with 1x PBS for 6 min and mounting of the samples with Dako Fluorescent Mounting Medium (Dako). Stainings were analyzed by fluorescence microscopy using a Nikon ECLIPSE Ti fluorescent microscope. Exposure time was set with isotype control or secondary antibody control stained samples to achieve minimal background and further applied for the analysis of all stainings. Images were analyzed using Nikon NIS-Elements AR 4.00 image analysis software.

VI.3.14.2. *Immunofluorescence on cryo-embedding tissues*

Cryo-embedded tissue slides were fixed with 100 % acetone for 10 min at RT, washed for 5 min with 1x PBS and blocked with PBS/BSA for 1 h at RT to limit unspecific binding of the antibodies. Slides were then stained following the protocol for paraffin-embedded tissue samples (IV.3.14.1.).

VI.3.14.3. *Immunofluorescence on seeded cells*

In Lab Tek chamber slides seeded cells were rinsed briefly in 1x PBS, and fixed with 4 % PFA/PBS for 5 min on ice and for 10 min at RT, followed by a 1 min-wash step in 1x PBS. Cell were permeabilized with methanol for 1 min at -20 °C and washed with 1x PBS for 1 min. To avoid unspecific bindings of the antibodies cells were incubated in PBS/BSA with 0.05 % Tween-20 for 30 min at RT in a humid chamber. Removal of the blocking buffer was followed by addition of primary antibody diluted in PBS/BSA over night at 4 °C. Cells were washed three times with 1x PBS for 1 min and incubated with secondary antibody and DAPI diluted in PBS/BSA for 60 min in a light-protecting humid chamber. After the second staining, cells were washed three times for with 1x PBS for 1 min each and were mounted with Dako Fluorescent Mounting Medium (Dako). The next day samples were analyzed with a Nikon ECLIPSE Ti fluorescent microscope. Exposure time was set with isotype control or secondary antibody control stained samples to achieve minimal background and further applied for the analysis of all stainings. Images were analyzed using Nikon NIS-Elements AR 4.00 image analysis software.

VI.3.15. Immunohistochemistry

Paraffin-embedded human tissue samples were deparaffinized and rehydrated in three washes of 5 min with xylene and a descending alcohol series (100, 90, 80 and 70 % EtOH) for 3 min each, and rinsed distilled water (dH_2O) and Tris-buffered saline (TBS). Antigen retrieval was performed by heating the sample for 60 min at 100 °C in zytomed HIER citrate buffer (pH 6.0). After additional washing steps with TBS/TBST (TBST containing 1 % Tween-20), blocking of the endogenous peroxide containing sodium azide for 10 min, as well as washing steps with dH_2O and TBS/TBST followed. Then, tissue slides were stained with a specific anti-RAGE antibody (ab37647) in Dako antibody diluents overnight at 4 °C. Next, slides were washed twice for 2 min with PBS and stained with Dako EnVision™ anti-rabbit secondary antibody conjugated with horse-radish peroxidase (HRP) for 60 min. After two washing steps with TBS/TBST for 5 min each samples were developed with AEC (Dako EnVisionTM) for 15 min, followed by an additional washing step and counterstaining with hematoxylin. Afterwards, the samples were mounted with Dako Fluorescent Mounting Medium (Dako). All tissue samples were analyzed under a Leica DM LS light microscope.

VI.3.16. FACS analysis

1 x 10^5 to 1 x 10^6 cells per well were transferred in 10 % FCS in PBS (PBS/FCS) into a round bottomed 96-well plate and centrifuged for 5 min at 300 rcf at 4 °C. Removal of the supernatant was followed by incubation of primary antibodies (Table 16) diluted in PBS/FCS for 15 min on ice. Next, cells were resuspended in 200 µl PBS/FCS and centrifuged for 5 min at 300 rcf at 4 °C. Plate was turned down to remove the supernatant and cells were incubated in secondary antibodies (Table 16) including DAPI diluted in PBS/FCS for 15 min on ice. If instead of DAPI 7-aminoactinomycin D (7-AAD) was used, it was added for 3 min. If direct-labeled primary antibodies were used, DAPI staining was performed during primary antibody incubation. Cells were washed, centrifuged, resuspended in 200 µl PBS/FCS.

Antibodies	Concentrations
Rabbit-anti-RAGE	1 : 100
Hamster-anti-CD11c-APC	1 : 50
Mouse-anti-CD123-FITC	1 : 2
Mouse-anti-CD303-APC	1 : 2
DAPI	1 : 10000
7-AAD	1 : 150
Donkey-anti-rabbit Cy3	1 : 1000

Table 16 Primary and secondary antibodies used in FACS analyses

FACS analyses were performed using FACS Canto 4.0 and FACSDiva provided by the DKFZ Flow Cytometry Core Facility and the FlowJo Single Cell Analysis software 7.2.2

VI.3.17. Enzyme-linked immunosorbent assay (ELISA)

For the quantification of different factor concentrations in the supernatant of cultivated cells we performed different sandwich ELISA kits. All ELISA protocols contain the same procedure consisting of coating, blocking, addition of standard probes and samples in duplicates, detection, enzyme reaction and development. However, the ELISA kits vary according to the desired factor particularly in the antibodies and buffers used as well as in the incubation times. Variances of the different ELISA kits were adapted to the specific protocols of the manufacturer. Between the individual working steps, unbound molecules were removed by multiple washing steps using a multichannel pipette. ELISAs were analyzed by absorbance measurement using the TECAN infinite F200 pro microplate reader and the TECAN iControl 1.10.©2012 software.

VI.3.17.1. *Interferon-α ELISA*

To quantify the concentration of the cytokine interferon (IFN)-α in the supernatant of stimulated pDCs the VeriKine™ human IFN-α ELISA kit was used. Supernatant samples were used undiluted or up to a dilution of 1:20. The ELISA procedure was performed to manufacturer's instructions.

VI.3.17.2. *Interleukin-1β ELISA*

To quantify the concentration of the cytokine interleukin (IL)-1β in the supernatant of stimulated keratinocytes the human IL-1β/IL-1F2 DuoSet ELISA kit was used. Supernatant samples were used undiluted or 1:2-diluted. The ELISA procedure was performed to manufacturer's instructions.

VI.3.17.3. *HMGB1 ELISA*

To quantify the concentration of the Rage ligand HMGB1 in the supernatant of stimulated keratinocytes the human HMGB1 ELISA kit from IBL was used. Supernatant samples were used undiluted or up to a dilution of 1:10. The ELISA procedure was performed to manufacturer's instructions.

VI.3.17.4. *S100B ELISA*

To quantify the concentration of the Rage ligand S100B in the supernatant of stimulated keratinocytes the human S100B ELISA kit from Abnova was used. Supernatant samples were used undiluted or up to a dilution of 1:10. The ELISA procedure was performed to manufacturer's instructions.

VI.3.18. Multiplex immunoassay (Luminex)

ProcartaPlex™ Immunoassays use the xMAP® technology (multi-analyte profiling beads) to enable the detection and quantitation of multiple protein targets (IL-9, IL-10, IL-17A, IL-22, IL-23, IL-27) simultaneously in diverse matrices. The xMAP system combines a flow cytometer, fluorescent-dyed microspheres (beads), dual laser design and digital signal processing to effectively allow multiplexing within a single sample. ProcartaPlex™ Immunoassay was kindly performed by Ludmila Umansky, T cell tumor immunity, DKFZ, Heidelberg, according to manufacturer's instructions.

VI.3.19. Image analysis

Quantification of immunofluorescence and H&E images was performed with skin of *wt* and *Rage*$^{-/-}$ mice. Acanthosis was measured by counting epidermal layers and cellular infiltration by counting the dermal infiltrates on H&E stained tissue sections from six to nine different mice of each genotype. Image analysis of immunofluorescence stainings was performed by counting positive cells in the dermal or the epidermal compartment and by setting these cells in relation to the DAPI positive cells (selected unit [%]). Immunofluorescence stained cells were counted on tissue sections from six to nine different mice per genotype.

VI.3.20. Statistical analysis

Tests for all data were performed using GraphPad Prism version 5.00 (2007) with the appropriate tests. Significance in two-tailed t-tests was assumed for p-values < 0.05 (*), < 0.01 (**) or < 0.005 (***).

VII. Aims of the thesis

The identification of a complex interplay of epidermal and immune cells, inflammatory cytokines, and downstream transcription factors contributing to psoriasis resulted in advances that elucidate the pathomechanism of the disease. However, the exact qualitative and quantitative influence of keratinocytes, dendritic cells, T lymphocytes, and signaling pathways orchestrating the pathogenesis of psoriasis remains largely elusive and limits our understanding of the disease.

Recent studies exhibit an evidence for the involvement of RAGE during initiation and maintenance of psoriasis. The expression of RAGE on immune and epidermal cells, a high stability of RAGE-ligand complexes, as well as the existence of a positive feed-forward loop sustaining RAGE signaling in inflammatory conditions, strongly recommend for a role of RAGE in the initiation and maintenance of a cutaneous chronic inflammation such as psoriasis. Although these findings suggested a central role of RAGE in psoriasis, cellular sources of aberrant RAGE signaling as well as the exact mechanisms of action remain.

Taken together, this thesis aims to address the following questions:

a) Is RAGE involved in chronic inflammatory skin diseases such as psoriasis?
   If yes, which consequences result from a lack of Rage during the establishment of a chronic inflammation *in vivo*?

b) Is RAGE contributing to the activation of epidermal and/or immune cells in the inflammatory environment of a psoriatic disease?
   If yes, what are the soluble factors/ligands that activate RAGE signaling in these cells?

By applying two different mouse models of psoriasis as well as suitable *in vitro* assays investigating functional impacts of RAGE on disease-associated effector cells, such as keratinocytes and dendritic cells, this thesis examines the importance of RAGE as a control element in the pathogenesis of psoriasis linking innate and adaptive immunity.

VIII. Results

VIII.1. RAGE is overexpressed in psoriatic skin

RAGE expression was evaluated between non-lesional skin and psoriatic plaques using human biopsy specimens. Immunohistochemical staining using an anti-RAGE antibody showed that RAGE is expressed in both, psoriatic plaques and non-lesional skin. Furthermore, RAGE expression in lesional skin sections of psoriatic patients was higher compared to corresponding non-lesional skin. Representative images for psoriatic plaque (right panel) and non-lesional skin samples (left panel) are illustrated in Figure 3A. Reasoned by the multitude of cells involved in the pathogenesis of a chronic inflammation, such as psoriasis, it is nearly impossible to create an appropriate controlled environment *in vitro*. Mouse models have often demonstrated remarkable similarity to human diseases since many of the genes responsible for complex diseases are shared between mice and men. Therefore, two previously established mouse models, comprising IMQ treatment and rIL-23 injections, that display many features of human psoriasis were applied to C57/BL6 wildtype (*wt*) and Rage-deficient ($Rage^{-/-}$) mice. Detailed information about both mouse models will be provided in section VIII.2. and VIII.7., respectively. *Ager* transcript level expression was analyzed in untreated murine skin samples of *wt* mice and samples exhibiting a IMQ-induced psoriasiform phenotype *via* qPCR analysis with *βActin* as endogenous control. Mice with psoriasiform phenotype showed an approximately 5-fold increased Rage expression, when normalized to the corresponding untreated mouse sample (Figure 3B).

In addition, immunofluorescence analysis of Rage expression revealed a remarkable difference in control mice (Figure 3C, upper panel) compared to the skin of rIL23-treated mice (Figure 3C, bottom panel). Indeed, Rage expression was upregulated in the epidermis as well as in dermal compartment of mice exhibiting a psoriasiform phenotype. This observation might be due to an enhanced expression of Rage by resident stroma cells, or due to infiltration of Rage-expressing immune cells. Evidence for the latter will be provided in chapter VIII.2.2.

The demonstration of an upregulation of Rage upon induction of a psoriasiform phenotype in mice as well as the finding of elevated RAGE protein in human psoriatic plaques strongly supports a role of RAGE in the development and/or progression of psoriasis in both mice and men.

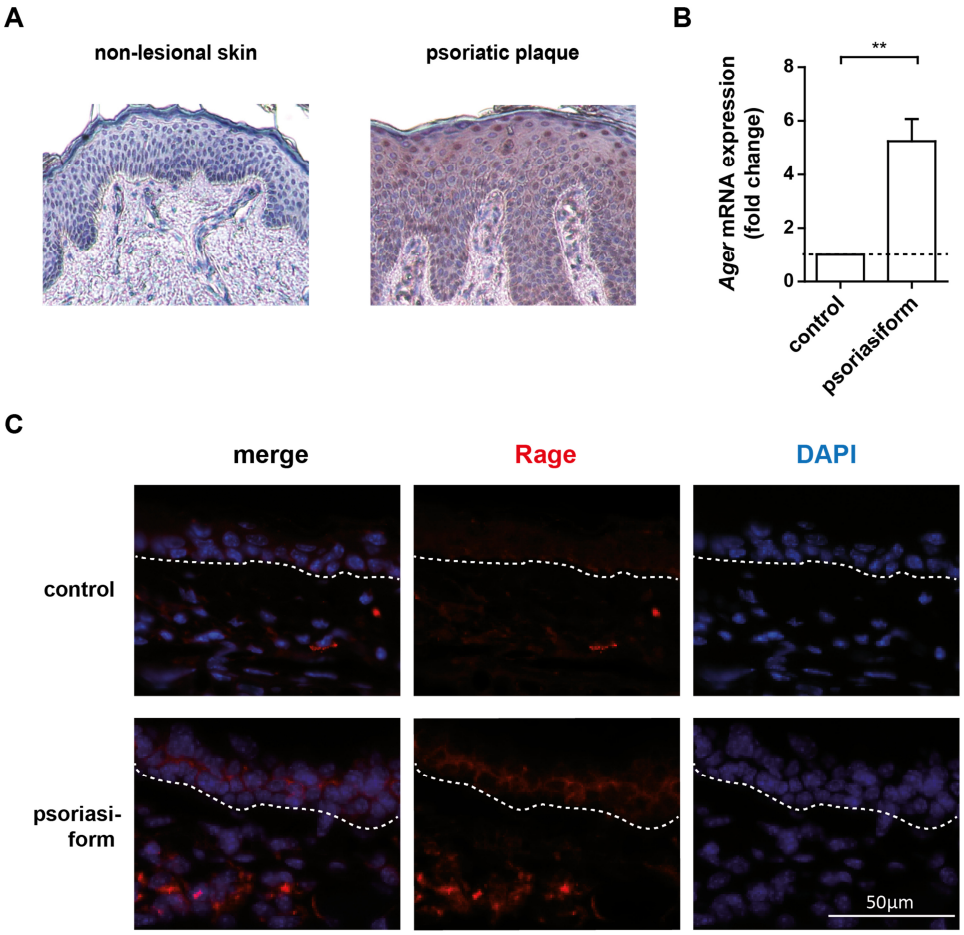

Figure 3 RAGE is overexpressed in human and murine psoriatic skin.

Representative microscopic pictures of the immunohistochemical expression of RAGE using an anti-RAGE antibody on human lesional (psoriatic plaque) and non-lesional skin specimens of psoriasis patients (**A**). *Ager* transcript levels in psoriasiform *wt* mice topically treated with repetitive IMQ doses (n=3) were verified using qPCR analysis with *βActin* as endogenous control and untreated (control) *wt* as reference sample. Error bars indicate 95 % confidence intervals (**B**). Rage expressing cells in the epidermis and the dermis were detected by immunofluorescence staining using anti-Rage antibody (red staining; nuclei were stained with DAPI, blue signal) on skin sections derived from repeatedly PBS-treated (control) (n=3) or rIL-23 treated (psoriasiform) (n=3) ears of *wt* mice; the dotted lines represent the dermal-epidermal junction (**C**). P values were calculated by two-tailed, unpaired sample t-test. Two asterisks indicate t-test p value of ≤ 0.01 in comparison to the respective reference.

VIII.2. Imiquimod-induced inflammation *in vivo* is dependent on Rage

In order to investigate the functional role of Rage in psoriasis *in vivo*, two previously established mouse models that share many features of human psoriasis were applied on *wt* and *Rage*$^{-/-}$ mice. Genotypic analysis was performed with primer targeting fl-Rage and eGFP confirming Rage deletion by eGFP detection (Figure 4A).

The first mouse model used in this thesis, referred to as Imiquimod (IMQ) model, induces a psoriasiform chronic inflammatory phenotype of the skin represented by epidermal proliferation and dermal immune cell infiltration [54].

VIII.2.1. Histological hallmarks of psoriasis are reduced in *Rage*$^{-/-}$ skin

The shaved back skin of *wt* and *Rage*$^{-/-}$ mice was topically treated with Aldara cream (5 % IMQ). According to the protocol of van der Fits and colleagues, induction of a psoriasiform phenotype requires a six-time IMQ treatment every 24 h. In order to describe the severity of inflammatory reactions occurring during the experiment the 'Psoriasis Area and Severity Index' (PASI), originally designed for classifying human psoriatic severity, was adapted to mice and measured immediately before the application of IMQ. The timeline and structure of the applied mouse model are illustrated in Figure 4B. To examine the IMQ-inducibility of both mice strains, gene expression of involved receptors was measured. Normalizing gene expression of *Tlrs* to two housekeeping genes (*βActin* and *Gapdh*) and comparison of *Rage*$^{-/-}$ to *wt* data sets revealed that all investigated toll-like receptors were deregulated. However, the deregulation of both receptors (Tlr7 and Tlr8) engaged by IMQ was of an opposite character, so that no difference in the entirety of IMQ-induced receptors was investigated (Figure 4C).

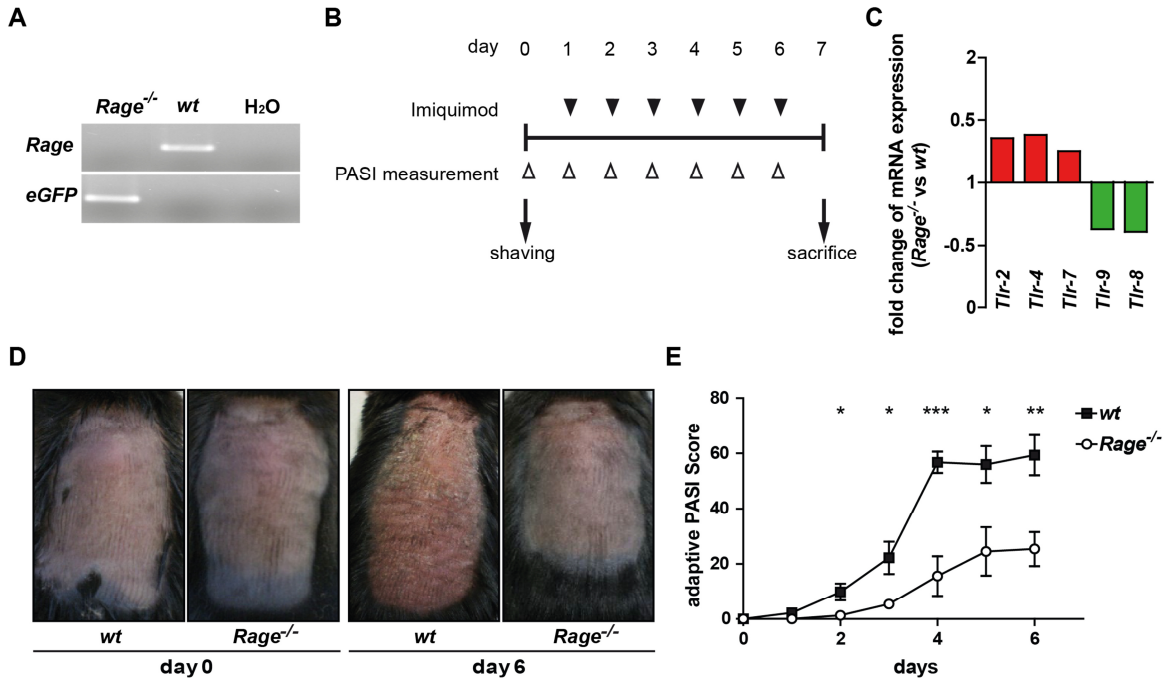

Figure 4 Delayed inflammatory response to IMQ treatment in *Rage*$^{-/-}$ mice.

Confirmation of receptor for advanced glycation end-products (Rage) deletion; genomic DNA was prepared from the tail of *Rage*$^{-/-}$ and *wt* mice. Equal amounts of DNA (50 ng) were used for PCR analysis with Rage- and eGFP- specific primers (**A**). Shaved back skins of *wt* (n=9) and *Rage*$^{-/-}$ mice (n=9) were topically treated with Aldara cream (5 % IMQ) daily for six days (**B**). IMQ-treated *Rage*$^{-/-}$ and *wt* skin was analyzed for differentially regulated *Tlr* genes applying the 'RT^2 Profiler^{TM} PCR Array'. Transcript levels of *Rage*$^{-/-}$ skin were illustrated by their fold change to *wt* skin samples. As endogenous controls *βActin* and *Gapdh* were used and mRNA expression changes of *Tlr-2*, *-4*, *-7*, *-8* and *Tlr-9* were displayed (**C**). Representative macroscopic pictures of inflamed murine back skins before start of the treatment and after induction of the chronic inflammation at day 6 (**D**). Prior the daily IMQ administrations, cutaneous inflammatory response was measured using an adapted PASI score (**E**). P values were calculated by two-tailed, unpaired sample t-test. Asterisk indicates t-test p value of ≤ 0.05 in comparison to the respective reference (** p value ≤ 0.01; *** p value ≤ 0.005).

Murine skin of *wt* and *Rage*$^{-/-}$ mice did not show any morphological differences after shaving the back skins and before the first IMQ administration (Figure 4D, left panel). The skin was hairless and without any shaving injuries. Two days after the start of the IMQ application the *wt* back skin started to display signs of erythema, scaling and thickening, whereas the *Rage*$^{-/-}$ mice displayed a delayed onset of these symptoms on day three to four. Scoring the severity and scope of these lesions by applying the adapted PASI score verified a delayed clinical response of *Rage*$^{-/-}$ mice. In the period from day 0 to day 4 for *wt* mice and up to day 5 for *Rage*$^{-/-}$ mice the psoriasiform inflammation continuously increased. From these dates on the inflammatory response exhibited a plateau phase (Figure 4E). Phenotypical presentation of both back skins at the end of the experiment revealed that the inflammatory response to IMQ is dampened in mice lacking Rage (Figure 4D, right panel). The PASI score confirms the reduction of the inflammatory reaction to IMQ, showing that PASI levels were significantly decreased at day 6 in *Rage*$^{-/-}$ mice in comparison to control *wt* mice (Figure 4E). These data

revealed that the development of psoriasiform skin inflammation upon IMQ treatment is diminished in Rage-deficient mice in comparison to *wt* controls.

The typical skin lesion associated with psoriasis vulgaris is the psoriatic plaque. These plaques are histologically characterized by epidermal hyperplasia (acanthosis) with a downward extension of the epidermal rete ridges (papillomatosis), parakeratosis, as well as an inflammatory infiltrate of leukocytes, predominantly in the dermis [55], [97]. Hematoxylin-eosin staining (H&E staining) of IMQ-treated murine back skins exhibited a multiplicity of these histological hallmarks of psoriasis (Figure 5A). Rudimentary formation of rete ridges could be seen in brightfield microscopic pictures of *wt* back skins, but were absent in Rage-deficient skin. Furthermore, differences in the severity of acanthosis were noted. More specifically, the epidermis of IMQ-treated *wt* mice showed severe acanthosis, while *Rage*$^{-/-}$ mice displayed a mild form of this psoriatic feature. Additionally, hyperplasia of the epidermis was found to be significantly reduced in *Rage*$^{-/-}$ mice in comparison to the levels in *wt* mice (*** $p \leq 0.005$) (Figure 5B). The statistical analysis of the H&E staining showed significantly decreased dermal infiltration of immune cells in *Rage*$^{-/-}$ compared to *wt* mice (*** $p \leq 0.005$) (Figure 5C).

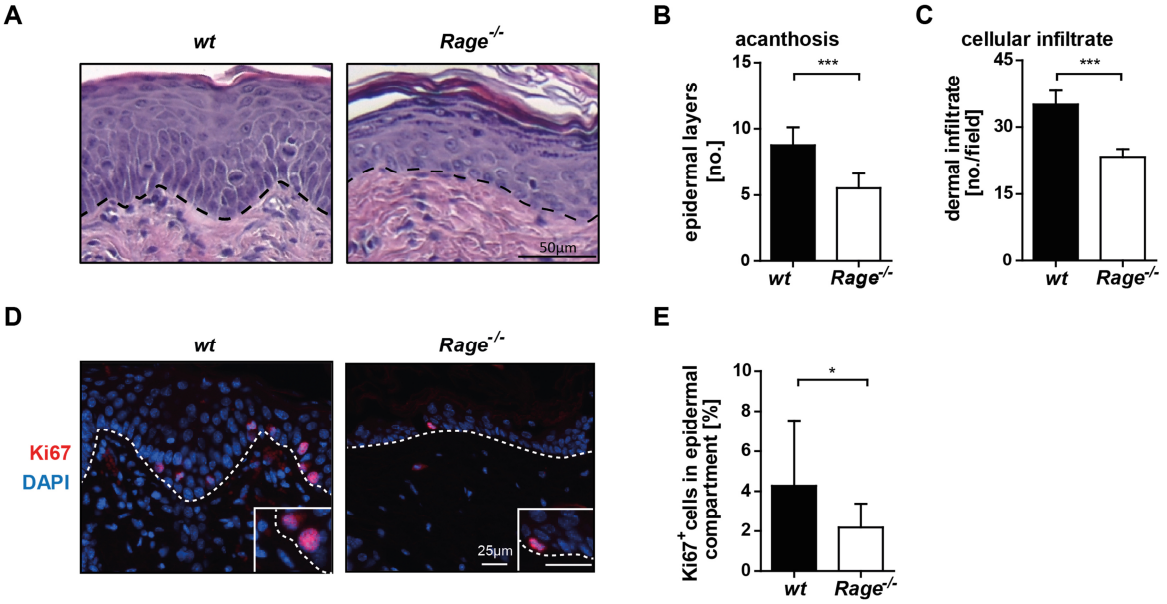

Figure 5 Impairment of typical hallmarks of psoriasis in IMQ-treated *Rage*$^{-/-}$ mice.

Brightfield microscopic pictures of hematoxylin-eosin stained (H&E staining) sections of *Rage*$^{-/-}$ (n=9) and *wt* (n=9) back skins 24 h after the last IMQ administration (**A**). Acanthosis was measured by counting epidermal layers (**B**) and cellular infiltration by counting dermal infiltrating cells (**C**). Proliferative cells in the epidermis of IMQ-treated skins were detected by immunofluorescence staining using anti-Ki67 (red staining; nuclei were stained with DAPI, blue signal) (**D**) and were statistically analyzed by counting Ki-67^{+}cells within the epidermal compartment (**E**). The dotted lines represent the dermal-epidermal junction. P values were calculated by two-tailed, unpaired samples t-test. Asterisk indicates t-test p value of ≤ 0.05 in comparison to the respective reference (** p value ≤ 0.01; *** p value ≤ 0.005).

The number of proliferative keratinocytes is highly upregulated in psoriatic plaques and is known to correlate with the clinical severity of psoriasis [394]. In more detail, acanthosis as well as papillomatosis are caused by accelerated proliferation of basal and suprabasal keratinocytes [71]. Here, Ki67 was used as a marker of proliferation in the epidermis. Representative immunofluorescence images of Ki67 staining for *wt* and *Rage*$^{-/-}$ skin sections are displayed in Figure 5D, showing a positive nuclear staining of proliferative cells. Figure 5E shows that approximately 4 % of all epidermal cells in *wt* mice were positive for Ki67, while only 2 % of *Rage*$^{-/-}$ epidermal cells were characterized as proliferative. This diminished proliferation marked by lower expression of the nuclear Ki67 protein in the epidermal compartment of *Rage*$^{-/-}$ back skin in contrast to the *wt* epidermis was found to be statistically significant (* $p \leq 0.05$).

VIII.2.2. Dermal infiltration is diminished in *Rage*$^{-/-}$ mice

Since the infiltration of leukocytes into the dermal compartment plays an important role in the initiation and maintenance of a chronic disease such as psoriasis, and furthermore the Rage-deficient dermis exhibited less infiltrating cells (Figure 5A, C), these cells were next examined in more detail.

Therefore, immunofluorescence stainings using antibodies specific against Cd11c for dendritic cells (DCs), F4/80 for macrophages, and Ly-6B.2 for neutrophils were performed. The enumeration of infiltrating cells, for instance, of dendritic cells was realized by expressing the number of Cd11c-positive ($Cd11c^+$) as percentage of $DAPI^+$ cells. All immunofluorescence analyses were performed according to this method.

Dermal dendritic cells function as antigen-presenting cells during inflammation and are upregulated in psoriatic skin [97], [395]. Immunofluorescence staining and accompanied statistical analysis of dermal DCs revealed significantly reduced amounts of $Cd11c^+$ DCs in IMQ-treated *Rage*$^{-/-}$ (** $p \leq 0.01$) (Figure 6A upper row, B). Not only DC amounts were impaired in *Rage*$^{-/-}$ skins, also diminished numbers of macrophages, known to secrete pro-psoriatic cytokines and to activate DCs [55], were found in *Rage*$^{-/-}$ mice in comparison to the *wt* situation (*** $p \leq 0.005$) (Figure 6A middle row, C). In line with the finding that numbers of infiltrating neutrophils correlate with the severity of psoriatic plaques [74], these cells were less abundant in the skin of *Rage*$^{-/-}$ mice compared to control mice (* $p \leq 0.05$) (Figure 6A bottom row, D).

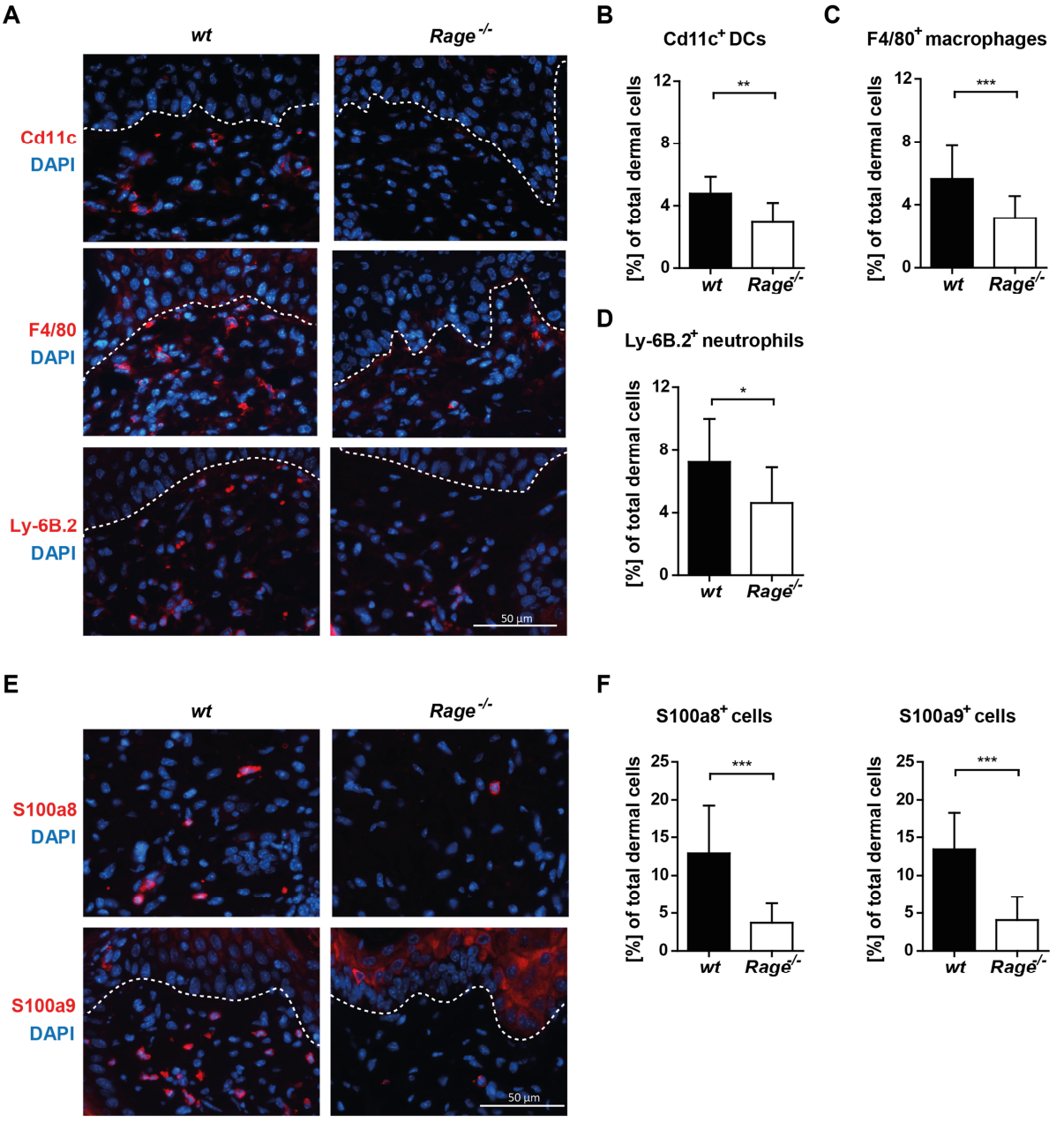

Figure 6 Impaired infiltration of immune cells in the dermis and decreased dermal expression of Rage ligands upon IMQ treatment in *Rage*$^{-/-}$ mice.

Infiltrating immune cells in the dermis of IMQ-treated *wt* and *Rage*$^{-/-}$ skin (n=9) were analyzed by immunofluorescence stainings using anti-Cd11c for dendritic cells, anti-F4/80 for macrophages, and anti-Ly-6B.2 for neutrophils (red stainings; nuclei are stained with DAPI, blue staining). The dotted lines represent the dermal-epidermal junction (**A**). S100a8^{+} and S100a9^{+} cells of IMQ-treated back skins (n=9) were detected using immunofluorescence with anti-S100a8 and anti-S100a9 antibodies (red staining; nuclei were stained with DAPI, blue staining) (**E**). Statistical analysis of immunofluorescence pictures was done by expressing the number of staining-positive as percentage of DAPI^{+} cells (**B**-**D**, **F**). P values were calculated by two-tailed, unpaired sample t-test. Asterisk indicates t-test p value of ≤ 0.05 in comparison to the respective reference (** p value ≤ 0.01; *** p value ≤ 0.005).

Moreover, IMQ treatment led to an upregulation of Rage ligands, here S100a8 and S100a9 expression in the dermal compartment of *wt* mice. The statistical analyses of the respective immunofluorescence stainings revealed approximately 12 % $S100a8^+$ and $S100a9^+$ dermal cells in *wt* mice but only 4 to 5 % in Rage-deficient mice. Indeed, the decreased amount of $S100a8^+$ and $S100a9^+$ cells of $Rage^{-/-}$ mice compared to *wt* mice was provided to be statistically significant (*** $p \leq 0.005$) (Figure 6E, F). As described before, these proteins are important for the maintenance of a chronic cutaneous inflammation due to their ability to sustain activation of Rage signaling *via* the aforementioned positive signaling feed-forward loop [263], [298].

Taken together, the results presented in this section demonstrated a dependence of Rage signaling in a chronic cutaneous inflammation induced by topical IMQ treatment. Lack of Rage led to diminished hallmarks of psoriasis and, furthermore, to decreased abundance of infiltrating cells of the innate immune system in the dermis.

In the next chapters, the impaired inflammation will be investigated in more detail. More specifically, cells involved in the so-called 'psoriatic cascade', as well as signal transduction mediated by RAGE/ligand interaction and the effects of Rage signaling onto these cells during psoriasis as chronic inflammation model will be described.

VIII.3. Rage controls the transcription of inflammatory response genes *in vivo*

As described in V.2. psoriasis is a chronic inflammatory disease mediated by cells and molecules of both the innate and the adaptive immune system [55], [56]. In order to investigate a potential impact of RAGE onto the activation state or differentiation of immune cells involved in the pathogenesis of a chronic inflammation, effects of the loss of RAGE onto genes encoding for transcription factors of innate and adaptive immune cells or for effector molecules were investigated. More specifically, the applied 'Mouse Innate and Adaptive Immune Responses RT^2 Profiler™ Array' enabled the simultaneous monitoring of 79 immune responses-related genes as well as a potential differential expression upon IMQ treatment of *wt* or $Rage^{-/-}$ mice. The monitored genes were further allocated to distinct subgroups, namely '$T_H1/T_H2/T_H17$ differentiation', 'Treg markers', 'T cell activation', 'inflammatory response', 'innate immunity', 'adaptive immunity', as well as 'humoral immunity', and 'viral/bacterial defense'.

Normalizing gene expression of molecules involved in inflammatory innate and adaptive immune responses to two housekeeping genes (*βActin* and *Gapdh*) and comparison of the

Rage$^{-/-}$ to *wt* data sets revealed a tremendous downregulation of *Cd4,* a marker of T helper cells. While some genes associated with the differentiation of $CD4^+$ T cells into T_H1, T_H2 or T_H17 cell were found to be deregulated, no clear pattern emerged (Figure 7A). T_H22 cells that are also known to be important for the pathogenesis of psoriasis, could not be investigated using this assay. Nevertheless, 71.43 % of the genes affecting T cell activation were found to be downregulated in *Rage*$^{-/-}$ psoriatic skin in comparison to matched *wt* samples (Figure 7B). However, as most affected subgroup the 'inflammatory response' was identified. In terms of numbers, 90 % of genes involved in inflammatory response were downregulated upon Rage loss under chronic inflammatory conditions. More specifically, *Apcs*, *C3*, *Crp*, *Foxp3*, *Il-1a*, *Il-1b*, *Il-4*, *Il-6*, *Stat-3* and *Tnf-α* were found to be downregulated, solely an upregulation for *Ccl5* transcript level was detectable (data not shown).

Next, the data sets were clustered in the context of psoriasis. Transcript levels of already described molecule with an involvement in psoriasis were investigated for differences between *wt* and *Rage*$^{-/-}$ mice. More than 70 % of the investigated psoriasis-associated transcript levels were downregulated in inflamed Rage-deficient mice compared to their inflamed controls, including proinflammatory cytokines like *Il-1β, Il-6, Il-17a, Il-23a Ifn-α,* and *Tnf-α*, co-stimulatory factors like *Cd40* and *Cd80,* the adaptor protein *Myd88*, or transcription factors such as *Nf-κb* and *Stat-3*. In contrast, *Nfkbia*, *Il-1r,* and *Cd86* transcript levels were found to be upregulated upon the loss of Rage (Figure 7A).

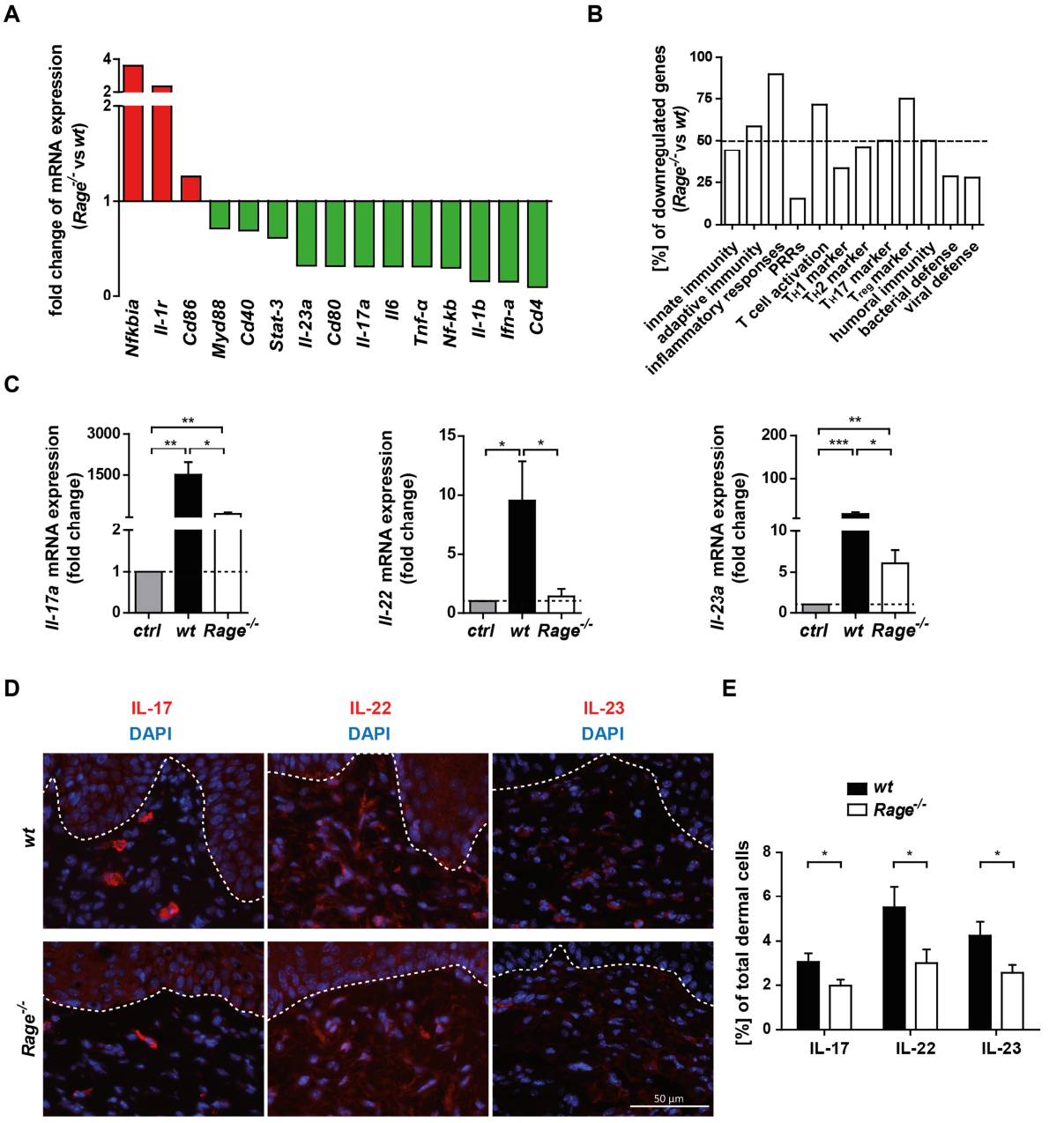

Figure 7 Psoriasis-related inflammatory genes are downregulated upon IMQ treatment in *Rage*$^{-/-}$ mice.

IMQ-treated *Rage*$^{-/-}$ and *wt* skin was analyzed for differentially regulated innate and adaptive immune system-associated genes applying the 'RT^2 Profiler^{TM} PCR Array'. Transcript levels of *Rage*$^{-/-}$ skin were illustrated by their fold change to *wt* skin samples. As endogenous controls *βActin* and *Gapdh* were used. Representative graphs for mRNA expression of psoriasis-related genes (**A**) and percentage of downregulated genes per cluster (**B**) were indicated. *Il-17a*, *Il-22*, and *Il-23a* transcript levels in IMQ-treated *wt* (n=9) and *Rage*$^{-/-}$ (n=9) mice were verified in more detail using qPCR analysis. Untreated (ctrl) *wt* served as reference sample and *βActin* as endogenous control. Error bars indicate 95 % confidence intervals (**C**). Cytokine-secreting cells within the dermis of inflamed back skins were detected by immunofluorescence staining with anti-IL-17, anti-IL-22, and anti-IL-23 antibodies (red staining; nuclei were stained with DAPI, blue staining) (**D**). Statistical analysis of immunofluorescence pictures was done by expressing the number of staining-positive as percentage of DAPI$^+$ cells. The dotted lines represent the dermal-epidermal junction (**E**). P values were calculated by two-tailed, unpaired sample t-test. Asterisk indicates t-test p value of ≤ 0.05 in comparison to the respective reference (** p value ≤ 0.01; *** p value ≤ 0.005).

Analysis of the cellular infiltrate during IMQ-induced inflammation showed reduced accumulation of DCs in the dermal compartment of *Rage*$^{-/-}$ mice (Figure 6A, B). Under pathological conditions, these cells secrete high amounts of IL-23, a cytokine that is described to propagate a $T_H1/T_H17/T_H22$ polarized immune response [9], [10]. The differentiation into these T helper subsets leads to the secretion of proinflammatory cytokines like IL-17 and IL-22 [55]. In order to confirm the observed downregulation of *Il-17a* and *Il-23a* by RT-PCR profiling, *interleukin* transcript levels comprising *Il-17a*, *Il-22*, and *Il-23a* were measured in IMQ-treated *wt* and *Rage*$^{-/-}$ mice; with untreated (ctrl) *wt* skin samples as reference and *βActin* as endogenous control. The analysis revealed a significant upregulation of all three interleukins in IMQ-treated *wt* mice compared to the healthy counterpart. IMQ-treated *Rage*$^{-/-}$ skin samples also displayed an upregulation of *Il-17* and *Il-23*, while *Il-22* transcripts remained at their basal level (Figure 7C). Nevertheless, transcript levels of all three interleukins were significantly decreased in IMQ-treated *Rage*$^{-/-}$ skin samples in comparison to the inflamed *wt* counterpart (Figure 7C). This was confirmed *via* immunofluorescence staining and subsequent statistical analysis, exhibiting a significant reduction of interleukin-positive cells in *Rage*$^{-/-}$ mice in comparison to the *wt* mice (* $p \leq 0.05$) (Figure 7D, E).

The *in vivo* data obtained from the IMQ model indicates that IMQ-induced inflammation is dependent on Rage and that Rage has an impact on $CD4^+$ T cell differentiation, T cell activation, as well as on psoriasis-related molecules. Furthermore, the investigation of IL-17 and IL-23 revealed a consistent downregulation of these cytokines upon Rage-deficiency in chronic inflammation, and therefore a possible mechanism of action. In order to further verify if RAGE signaling contributes to the development of chronic inflammation by upregulating proinflammatory cytokines, cells involved in the pathogenesis of psoriasis were investigated in more detail.

VIII.4. Activation of keratinocytes is independent of RAGE

Keratinocytes are known to be involved in the pathogenesis of psoriasis. Most of the clinical hallmarks of psoriasis feature keratinocyte deregulation; for instance, acanthosis and papillomatosis are caused by accelerated proliferation of keratinocytes [71]. Keratinocytes modulate immune reactions in the skin, but their role as initiators or amplifiers of the inflammatory reaction in psoriasis is still elusive [396].

For this reason, the next chapter deals with the activation potential of keratinocytes by investigating potential stimulators as well as associated effects on their functional properties including the release of proinflammatory mediators, which contribute to the activation of RAGE signaling.

VIII.4.1. Keratinocytes are not activated by alarmins

Normal human epidermal keratinocytes (NHEK) were isolated from epidermis of juvenile foreskin as described in VI.3.2.2. and validated *via* immunofluorescence using the early keratinocyte marker K14. Representative images of the immunofluorescence staining are displayed in Figure 8A and verified the keratinocytic character of the cells. Moreover, immunofluorescence staining revealed expression of RAGE by keratinocytes (Figure 8B). Further experiments investigating the functional impact of RAGE axis were performed with these cells.

Recent studies provide evidence that cell-free DNA levels can be directly linked to psoriasis severity [397]. Indeed, extracellular DNA as well as unmethylated cytosine-phosphate-guanine (CpG) dinucleotides are known to stimulate immune effects either alone or in complex with proteins, such as HMGB1, a RAGE ligand described to be upregulated in psoriasis [398]–[400]. Therefore, primary human keratinocytes were stimulated with CpG, HMGB1 or another RAGE ligand S100B alone, or with a complex of CpG-HMGB1 or CpG-S100B. The activation state of keratinocytes was monitored by levels of the proinflammatory cytokine IL-1β. The administration of RAGE ligands alone or in complex with CpG as well as the dinucleotide alone failed to upregulate *IL-1β* transcripts, when compared to untreated control keratinocytes serving as reference sample (Figure 8C). Furthermore, released IL-1β by keratinocytes upon stimulation was measured using enzyme-linked immunosorbent assays (ELISA). In line with the qPCR data, stimulation of keratinocytes with CpG, HMGB1, and HMGB1-CpG complexes could not lead to increased release of IL-1β. Moreover, increasing concentrations of HMGB1 could not change IL-1β release (Figure 8D). Similar results were obtained for keratinocytes stimulated with S100B instead of HMGB1 (Figure 8E). However, increasing concentration of S100B alone seemed to enhance the release of IL-1β to a small extent, but the differences were not significant and the complex showed no ability to activate keratinocytes.

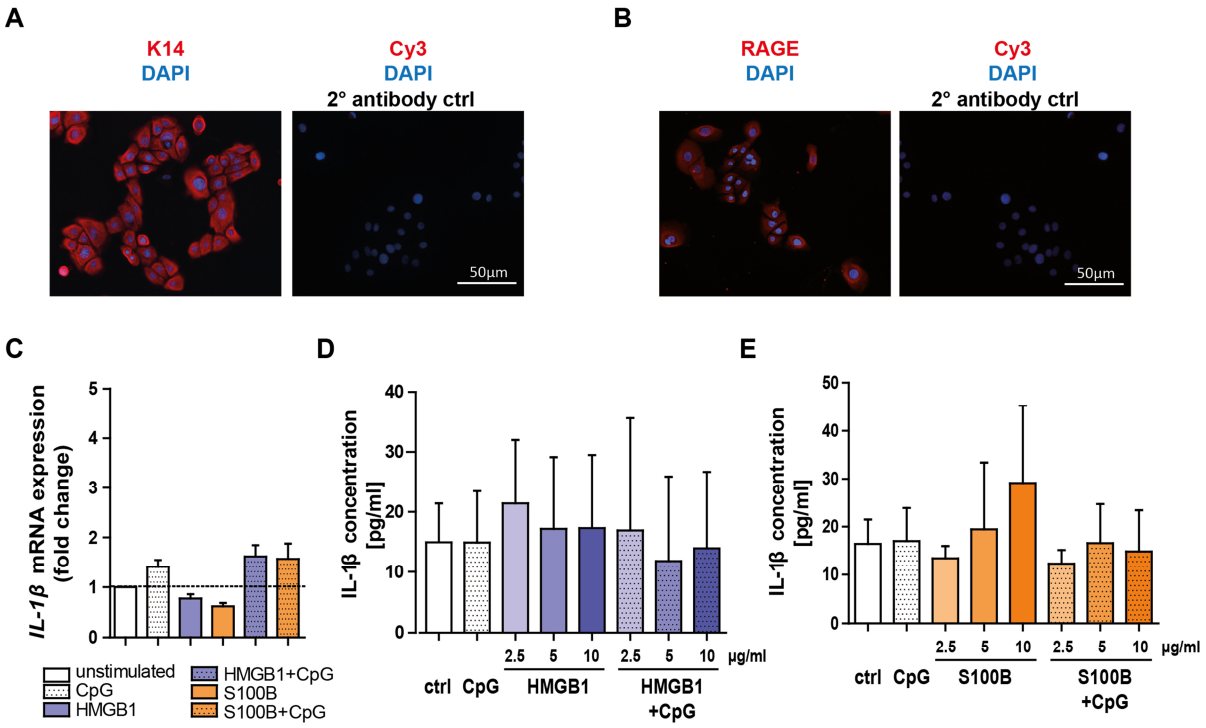

Figure 8 Alarmin-CpG-complexes fail to activate keratinocytes.

NHEKs were isolated from epidermis of juvenile foreskin. Verification of keratinocytes was performed by immunofluorescence staining using anti-K14 (red staining; nuclei are stained with DAPI, blue staining) (**A**), and RAGE expression was measured using anti-RAGE antibody (ab37647) (**B**). Sections stained with secondary antibody alone served as a control for antibody specificity (**A**, **B**). Keratinocytes were stimulated with 5 µg/ml HMGB1, 5 µg/ml S100B, 300 nM CpG, HMGB1-CpG, or S100B-CpG (5 µg/ml, 300 nM CpG). Total RNA from stimulated keratinocytes was isolated, reverse transcribed and analyzed by qPCR for relative transcript levels of *IL-1β*. Expression levels were normalized to unstimulated (ctrl) with *18S* as endogenous control. Error bars indicate 95 % confidence intervals (**C**). 24 h after stimulation, cell culture supernatants were harvested to determine IL-1β protein levels by ELISA (**D**, **E**). Data are shown as mean ± SD of two (qPCR) and three (ELISA) independent experiments.

Taken together, these results demonstrate that RAGE ligands alone or in complex with CpG fail to stimulate keratinocytes towards IL-1β secretion. However, there has to be an activator for keratinocytes, since these cell were found to be hyperactivated in psoriasis. Li and colleagues could show that upon differentiation keratinocytes upregulate *TLR-7* transcripts. Treatment with the TLR-7 agonist IMQ and keratinocyte differentiation cooperatively led to the secretion of proinflammatory cytokines such as IL-8 and TNF-α [166].

That raised the question if differentiated keratinocytes could be encouraged to additionally secrete proinflammatory RAGE ligands such as HMGB1 and S100B.

VIII.4.2. IMQ-induced activation of keratinocytes

Primary human keratinocytes were differentiated for 10 days with medium containing high Calcium (Ca^{2+}) concentrations (Figure 9A). Differentiation was validated by morphological changes and mRNA expression of keratinocyte markers. Keratinocytes cultured in 60 µM Ca^{2+} conditions, defined as non-differentiated keratinocytes, were small polygonal cells growing in relatively homogenous monolayers (Figure 9B, left panel). Upon Ca^{2+} administration keratinocytes became more heterogeneous, displaying irregular sizes and shapes (Figure 9B, right panel). Additionally, these cells, from here on referred to as differentiated keratinocytes, were validated for their *involucrin* transcript levels using qPCR. When normalized to the corresponding non-differentiated cells, high Ca^{2+} concentration resulted in a marked upregulation of *involucrin*, thereby verifying the differentiated phenotype of the cells (** $p \leq 0.01$) (Figure 9C).

Differentiated and non-differentiated keratinocytes were then stimulated with 100 µM IMQ and the resulting upregulation of RAGE ligands S100B and HMGB1 was measured. Protein levels of HMGB1 and S100B measured by ELISAs revealed equal secretion of these proteins in untreated keratinocytes independent of their differentiation status. Moreover, also IMQ stimulated non-differentiated keratinocytes were unable to increase the secretion of proinflammatory RAGE ligands. In contrast, differentiated keratinocytes stimulated with IMQ showed a significantly increased amount of secreted HMGB1 and S100B in comparison to unstimulated differentiated as well as IMQ-stimulated non-differentiated cells (*** $p \leq 0.005$) (Figure 9D, E). Along with that, qPCR analyses using untreated non-differentiated keratinocytes as reference sample and *18S* as endogenous control showed that mRNA expression of *HMGB1* as well as *S100B* was independent of the differentiation state of untreated keratinocytes (** $p \leq 0.01$) (Figure 9F). In contrast, stimulation of differentiated keratinocytes with IMQ led to a significant upregulation of *HMGB1* and *S100B* transcripts in comparison to untreated cells (** $p \leq 0.01$).

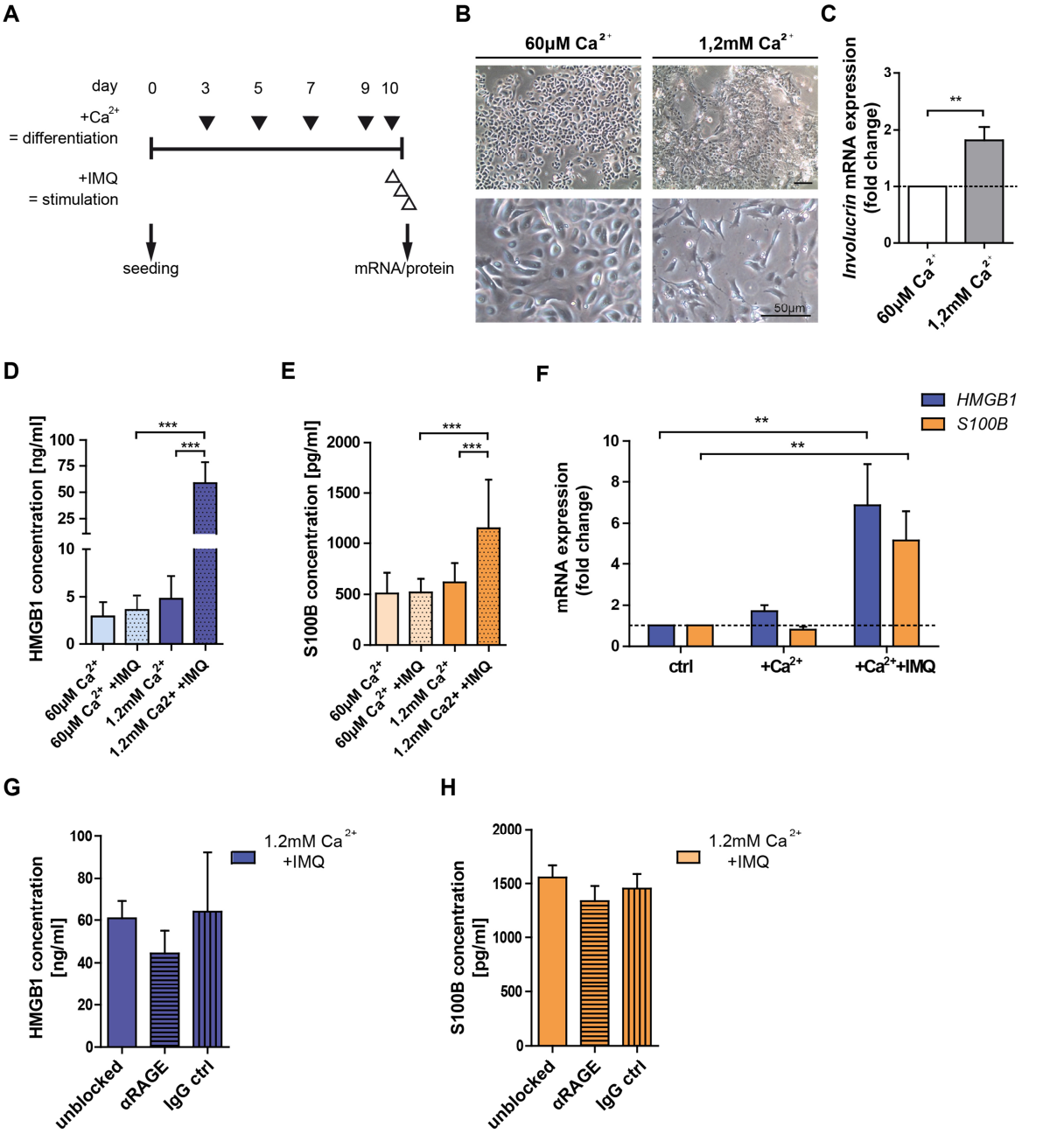

Figure 9 Differentiated keratinocytes secrete RAGE ligands upon IMQ stimulus.

NHEKs were cultured under low calcium conditions (60 µM; non-differentiated) or in the present of 1.2 mM calcium (differentiated) for 10 days (**A**). Representative macroscopic pictures of non-differentiated and differentiated keratinocytes before IMQ treatment (**B**). Total RNA from cells was isolated, reverse transcribed and analyzed by qPCR for relative transcript levels of *involucrin*. Expression levels were verified with *18S* as endogenous control (**C**). Keratinocytes were treated with IMQ in a concentration of 100 µM. At 1 to 6 h after stimulation, cell culture supernatants were harvested to determine HMGB1 (**D**) and S100B (**E**) protein levels by ELISA and total RNA was collected for measurement of *HMGB1* and *S100B* transcript levels *via* qPCR (**F**). Expression levels were normalized to untreated non-differentiated keratinocytes and *18S* was used as endogenous control. Error bars indicate 95 % confidence intervals. In IMQ-stimulated keratinocytes RAGE activation was blocked by the administration of an anti-RAGE antibody (MAB11451) while IgG2B (MAB004) served as isotype control. HMGB1 (**G**) and S100B (**H**) protein levels were determined by ELISA. Data are shown as mean ± SD of two (qPCR) and three (ELISA) independent experiments. P values were calculated by two-tailed, unpaired sample t-test. Asterisk indicates t-test p value of ≤ 0.05 in comparison to the respective reference (** p value ≤ 0.01; *** p value ≤ 0.005).

Next, a possible involvement of RAGE in the activation of keratinocytes was investigated. Therefore, RAGE signaling was blocked using an anti-RAGE antibody and the corresponding IgG isotype control to exclude artefact distortions by the blocking antibody. RAGE blockade was initiated 3 h before IMQ was added to the differentiated keratinocytes. No alteration in HMGB1 protein levels were detected in IMQ-stimulated cells treated with the anti-RAGE antibody compared to either corresponding IgG control cells or unblocked stimulated keratinocytes (Figure 9G); similar results were obtained for S100B protein levels (Figure 9H). Enzyme-linked immunosorbent assays revealed that RAGE neither plays a role for the secretion of HMGB1 nor for S100B under the here described conditions.

In summary, *in vitro* experiments with keratinocytes clearly showed that RAGE ligands, at least HMGB1 and S100B, are no potential stimulators of keratinocytes, but were consequently secreted by those cells upon IMQ-induced activation. Moreover, these findings implicate that RAGE is not involved in the keratinocyte contribution to the development of a chronic inflammation.

While results from the IMQ model suggest an involvement of Rage in psoriasis, consequently, an investigation of the potential role of RAGE on DCs was performed in the following two chapters.

VIII.5. Expression of RAGE on DCs during inflammation

As shown in VIII.1. RAGE expression is upregulated under inflammatory conditions in human and mouse (Figure 3). Moreover, with the *in vivo* model the involvement of Rage in the psoriatic context was locally limited to keratinocytes or/and DCs. Although keratinocytes do express RAGE (Figure 8 B), the activation of keratinocytes under inflammatory conditions was independent of RAGE as described before (Figure 9).

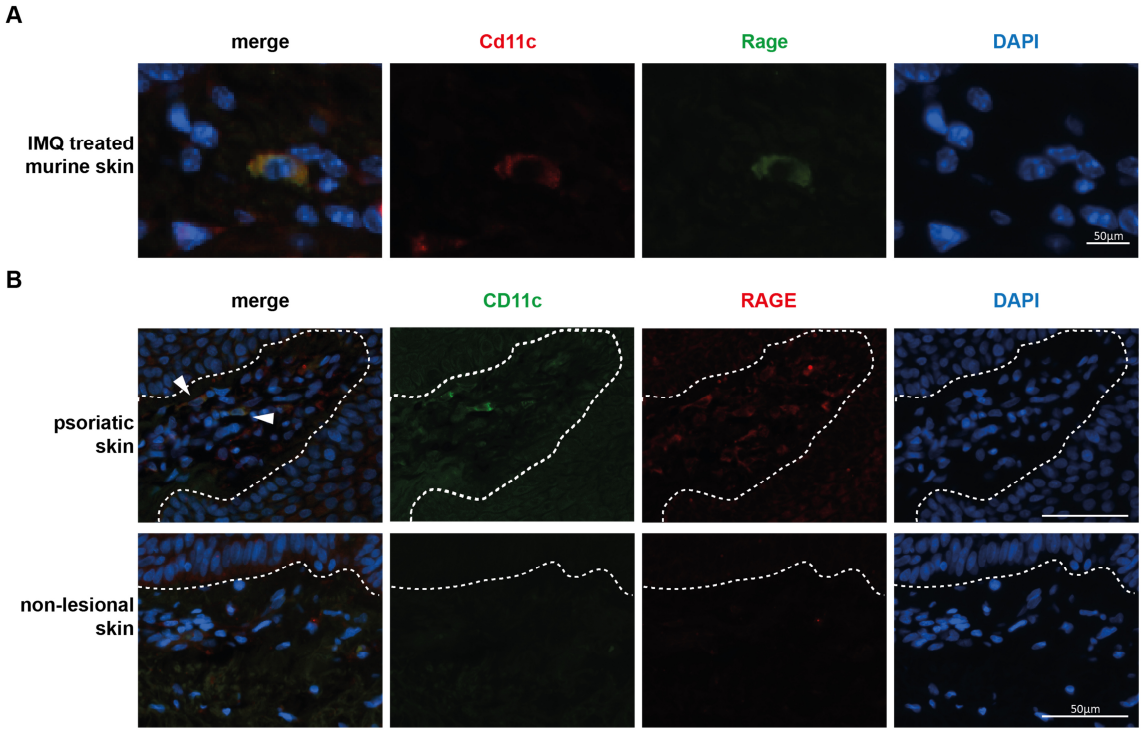

Figure 10 RAGE is expressed on dendritic cells within psoriasiform inflammation.
Representative images of immunofluorescence stainings using an anti-Cd11c (red stainings) and anti-Rage antibody (green staining; nuclei are stained with DAPI, blue staining) performed on IMQ-treated murine back skin (**A**). Immunofluorescence stainings of human lesional (psoriatic plaque) and non-lesional skin specimens of psoriasis patients were conducted using an anti-CD11c (green staining) and an anti-RAGE antibody (red staining; nuclei are stained with DAPI, blue staining). The dotted lines represent the dermal-epidermal junction (**B**).

For all of these reasons, RAGE expression on DCs was investigated under pathological conditions seen in psoriasis. Immunofluorescence co-staining analysis was performed with murine skin sections from IMQ-induced inflamed *wt* mice using an anti-Rage and an anti-Cd11c antibody. Representative images revealed that upon psoriasiform conditions Rage was predominantly expressed on DCs (Figure 10A). However, not only $Cd11c^+$ DCs in murine psoriasis-like skin displayed Rage expression but a similar expression pattern was observed for human samples from patients exhibiting a high PASI score (Figure 10B, upper panel). Here, almost all DCs in the dermal compartment were expressing RAGE. Nevertheless, RAGE was also expressed on $CD11c^-$ cells within the dermis, showing that DCs are not the only cell type expressing RAGE in the context of chronic inflammation. In contrast, the investigation of human non-lesional skin of psoriatic patients barely showed $RAGE^+$ DCs (Figure 14, bottom panel), indicating that RAGE is upregulated on DCs during psoriasis progression. As plasmacytoid dendritic cells are known to be involved in psoriasis, the next chapter will focus on the role of RAGE on this DC subtype.

VIII.6. Alarmin-CpG-complex-induced activation of plasmacytoid dendritic cells is mediated by RAGE

The DC population can be divided into diverse subsets, one of these subpopulation are the so-called plasmacytoid dendritic cells (pDCs). Under pathological conditions, these cells are key effector cells due to their unique ability to produce large amounts of IFN-α, a cytokine of high importance in the pathogenesis of psoriasis [170], [174], [175]. Understanding all mechanisms of pDC activation is of high importance elucidating the pathogenesis of psoriasis. Recently, it was shown that self-DNA triggers TLR-9 signaling in pDCs by forming a complex with LL-37 [136]. Moreover, Tian and colleagues revealed that pDCs in systemic lupus erythematosus (SLE) are activated by a self-DNA-HMGB1-complex [257]. And another study linking cell-free DNA and psoriasis severity further highlighted the importance of self-DNA in the pathogenesis of psoriasis [397].

Therefore, this chapter concentrates on the ability of RAGE ligand-self-DNA-complexes to activate pDCs in the context of psoriasis.

pDCs were obtained from peripheral blood mononuclear cells (PBMCs), purified by magnetic beads, and flow cytometric-analyzed to determine purity of the population using the CD123 and CD303 antibody as pDCs-specific marker combination. Figure 11A illustrates the enrichment of the pDCs population upon magnetic isolation. pDCs represent less than 1 % of all cells in the blood (Figure 11A, left panel). Representative image of the flow cytometry analysis shows the efficiency of the magnetic pDC isolation, demonstrating a pDC population with a purity of more than 80 % (Figure 11A, right panel). Populations used for the experiments contained at least 75 % pDCs.

IFN-α is secreted by activated pDCs and is used in the here-displayed experiments as a read-out system for the activation status of these cells. By stimulating pDCs with 300 nM CpG a strong stimulatory effect on pDCs was observed (Figure 11B). However, under physiological conditions such effects are minimized by consequent degradation of DNA molecules by specific enzyme. Therefore, the minimal concentration of CpG sufficient to stimulate pDCs at low extent was detected by dilution series. Figure 11B demonstrates that CpG in concentration of 300 nM to 75 nM is able to activate the cells solely by its presence. In contrast, concentration below 75 nM of CpG showed only a slight induction of IFN-α secretion (Figure 11B).

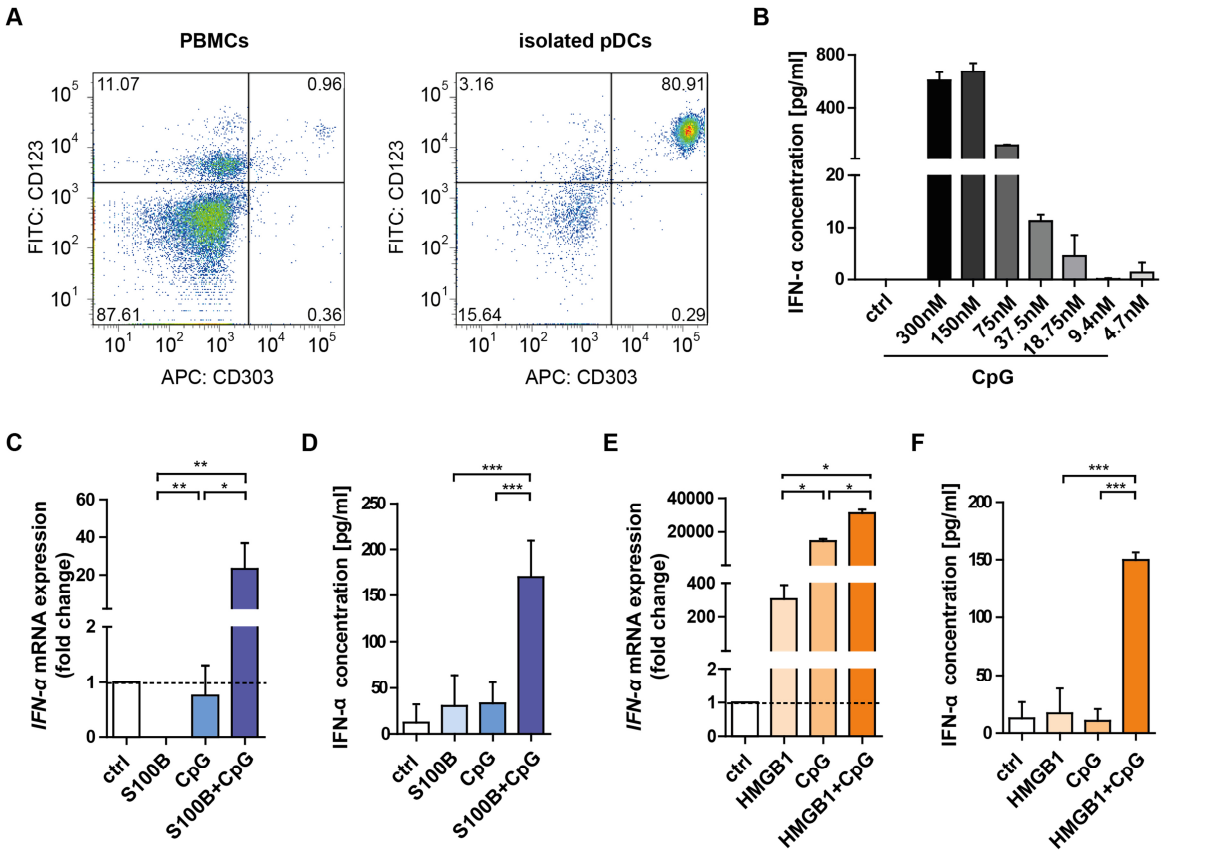

Figure 11 Plasmacytoid dendritic cells are activated by S100B-CpG- and HMGB1-CpG-complexes.

Isolated PBMCs and primary human pDCs were stained with anti-CD123-FITC, anti-CD303-APC, and 7-AAD to identify the purity of pDC population. An illustration of a flow cytometric analysis of $CD123^{+}CD303^{+}$ subpopulation is shown (**A**). pDCs were stimulated with different concentrations of CpG for 24 h. Stimulatory effects of indicated CpG concentrations in pDCs were determined by measuring the IFN-α protein levels in the supernatant of cultivated pDCs *via* ELISA (**B**). pDCs were stimulated with 5 µg/ml S100B, 37.5 nM CpG or CpG-S100B (37.5nM, 5 µg/ml) (**C**, **D**), or with 5 µg/ml HMGB1, 37.5 nM CpG or CpG-HMGB1 (37.5nM, 5 µg/ml) (**E**, **F**). Total RNA from stimulated and untreated pDC was isolated, reverse transcribed and analyzed by qPCR for relative transcript levels of *IFN-α*. Expression levels were normalized to untreated (ctrl) pDCs with *18S* as endogenous control. Error bars indicate 95 % confidence intervals (**C**, **E**). At 24 h after the stimulation, cell culture supernatants were harvested to determine IFN-α protein levels by ELISA (**D**, **F**). Data are shown as mean ± SD of two (qPCR) and three (ELISA) independent experiments. P values were calculated by two-tailed, unpaired sample t-test. Asterisk indicates t-test p value of ≤ 0.05 in comparison to the respective reference (** p value ≤ 0.01; *** p value ≤ 0.005).

For the following experiments a CpG concentration of 37.5 nM was used. Cultivated primary human pDCs were stimulated with CpG, 5 µg/ml S100B alone or with a complex of CpG and S100B (CpG-S100B). In brief, after a stimulus period of 24 h, total RNA was isolated, reverse transcribed and analyzed *via* qPCR using *18S* as an endogenous control. When normalized to the corresponding unstimulated cells (ctrl) S100B as well as CpG stimulated cells displayed a consistent upregulation of *IFN-α* transcript levels. A detailed examination of the qPCR analysis revealed that the stimulatory effects of CpG-S100B were highly significant in

comparison to all other cells either stimulated or unstimulated (** $p \leq 0.01$) (Figure 11C). To consider this observation an IFN-α ELISA using cell culture supernatants of stimulated and unstimulated cells was performed. Expression levels of IFN-α demonstrated that only the complex of CpG and S100B was able to induce high amounts of IFN-α secretion by pDCs (*** $p \leq 0.005$) (Figure 11D). Moreover, HMGB1 was used to examine the activation potential of alarmin-CpG complexes. *IFN-α* transcripts (* $p \leq 0.05$) (Figure 15E) as well as levels of secreted IFN-α (*** $p \leq 0.005$) (Figure 11F) revealed that the CpG-HMGB1 complex activated pDCs in a significantly higher manner than single stimuli.

These results showed that both RAGE ligands, S100B and HMGB1, in complex with CpG nucleotides are able to stimulate pDCs towards IFN-α secretion and raises the question if RAGE is involved in the investigated activation of pDCs.

To address this, freshly isolated human pDCs were cultured for 16 h in chamber slides and were afterwards stimulated with the CpG-HMGB1 complex or left untreated. Cells were permeabilized and stained with an anti-RAGE antibody and DAPI. Immunofluorescence stainings demonstrated that less than one third of unstimulated cells expressed RAGE (Figure 12A, left panel). In contrast, almost all cells, stimulated with CpG-HMGB1, showed high expression levels of RAGE in the immunofluorescence analysis (Figure 12A, right panel). These observations were independent of the fact whether cells grew as single cell suspensions or in confluent cell layers.

This immunofluorescence staining indicated a role for RAGE for the here-investigated mechanism of pDCs activation and consequent secretion of proinflammatory cytokines associated with chronic inflammations such as psoriasis.

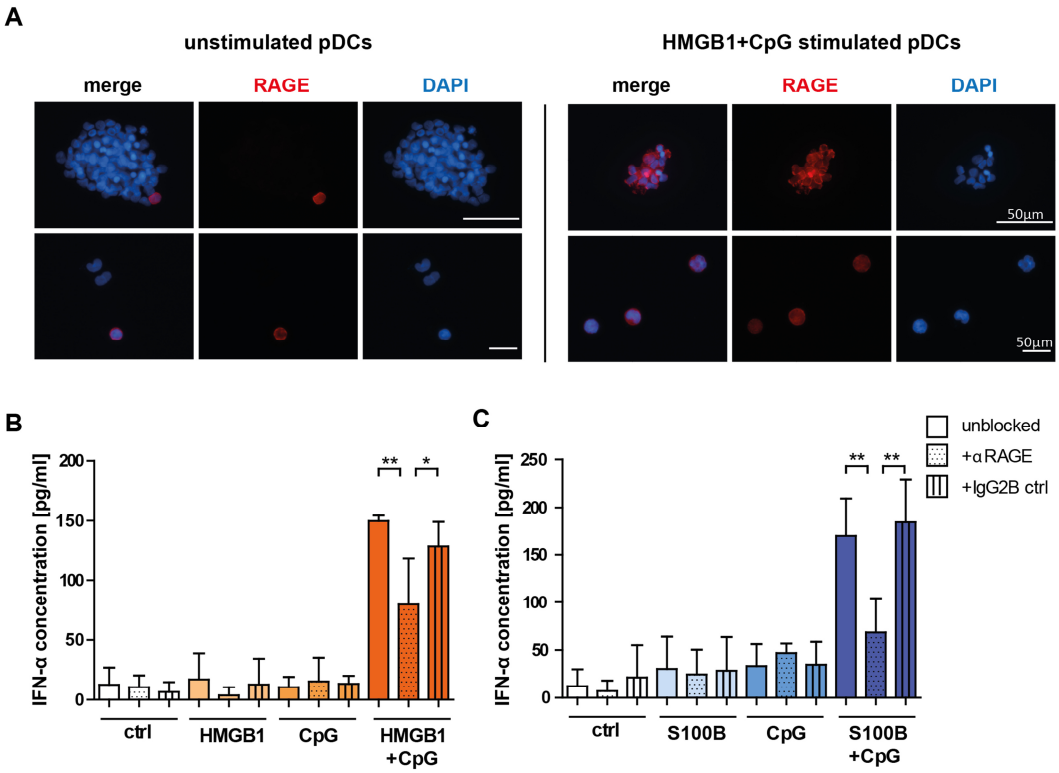

Figure 12 Alarmin-CpG-complexes activate pDCs *via* RAGE.

Expression of RAGE in human primary unstimulated and HMGB1-CpG stimulated pDCs was detected by immunofluorescence staining using anti-RAGE antibody (ab37647, red staining; nuclei were stained with DAPI, blue signal) (**A**). pDCs were treated for 24 h with 5 µg/ml HMGB1, 5 µg/ml S100B, 37.5 nM CpG, HMGB1-CpG, or S100B-CpG (5 µg/ml, 37.5 nM CpG) and RAGE activation was blocked by the administration of an anti-RAGE antibody (MAB11451; αRAGE) 3 h prior treatment. The IgG2B isotype control (MAB004) was used for the exclusion of artefact distortions by the blocking antibody. At 24 h after the stimulation, cell culture supernatants were harvested to determine IFN-α protein levels by ELISA (**B**, **C**) Data are shown as mean ± SD of three independent experiments. P values were calculated by two-tailed, unpaired sample t-test. Asterisk indicates t-test p value of ≤ 0.05 in comparison to the respective reference (** p value ≤ 0.01).

To further investigate the participation of RAGE in pDC activation blocking experiments were performed. Therefore, prior stimulation with the respective alarmin-CpG-complex, pDCs were treated with a specific RAGE blocking antibody (αRAGE). The αRAGE antibody was applied three hours before the stimulation to the cells, and was maintained without interruption. To exclude side effects of the blocking antibody a third group of pDCs was treated with the corresponding IgG isotype control (IgG2B).

Investigation of the secreted IFN-α amount *via* ELISA revealed that the unstimulated or CpG-stimulated cells showed no effect upon administration of α-RAGE or IgG isotype control (Figure 12B, C). However, cells stimulated with HMGB1 alone showed a slide reduction upon treatment with αRAGE, whereas the IgG isotype control had no effect on the behavior of pDCs stimulated with HMGB1. Focusing now on the decisive part of the experiment, ELISA

analyses revealed that activated pDCs (CpG-HMGB1 stimulated cells) displayed a significantly reduced secretion of IFN-α upon blockade of RAGE signaling, whereas the IgG isotype control exhibited the same secretion capacity as the unblocked stimulated pDCs (** $p \leq 0.01$) (Figure 12B). Similar results were obtained using S100B instead of HMGB1 (** $p \leq 0.01$) (Figure 12C).

Taken together, these pDC experiments demonstrated that HMGB1 as well as S100B in complex with the dinucleotide CpG were able to activate the specialized DC population pDCs and furthermore that the activation of these cells is dependent on RAGE signaling.

To confirm the role of RAGE on dendritic cells under chronic cutaneous conditions a second mouse model was applied to *Rage*$^{-/-}$ and *wt* mice. Intradermal injections of recombinant murine IL-23 (rIL-23) mimic constitutive DC activation resulting in the development of a psoriasiform inflammation in mice [53].

VIII.7. Normal inflammatory manifestation in *Rage*$^{-/-}$ mice upon rIL-23 injection

VIII.7.1. Clinical observations are unaltered in *Rage*$^{-/-}$ mice

DC-secreted IL-23 is known to differentiate naïve T cells into T_H17 lineage, a cell type found to be elevated in psoriasis. IL-23 is described to induce a psoriasiform inflammation in mice [53]. Intradermal rIL-23 injections into the ear of mice over a period of 14 days induce a chronic inflammation, which phenotypically resembles human psoriasis. Every 48 h 500 ng rIL-23 were injected into the left ear of *Rage*$^{-/-}$ and *wt* mice (Figure 13A), whereas the right ear was left untreated and served as a control for ongoing inflammation (Figure 13B). During the treatment, the developing inflammatory response of *Rage*$^{-/-}$ and *wt* mice was graded using the adaptive PASI score and *via* measurement of ear swelling.

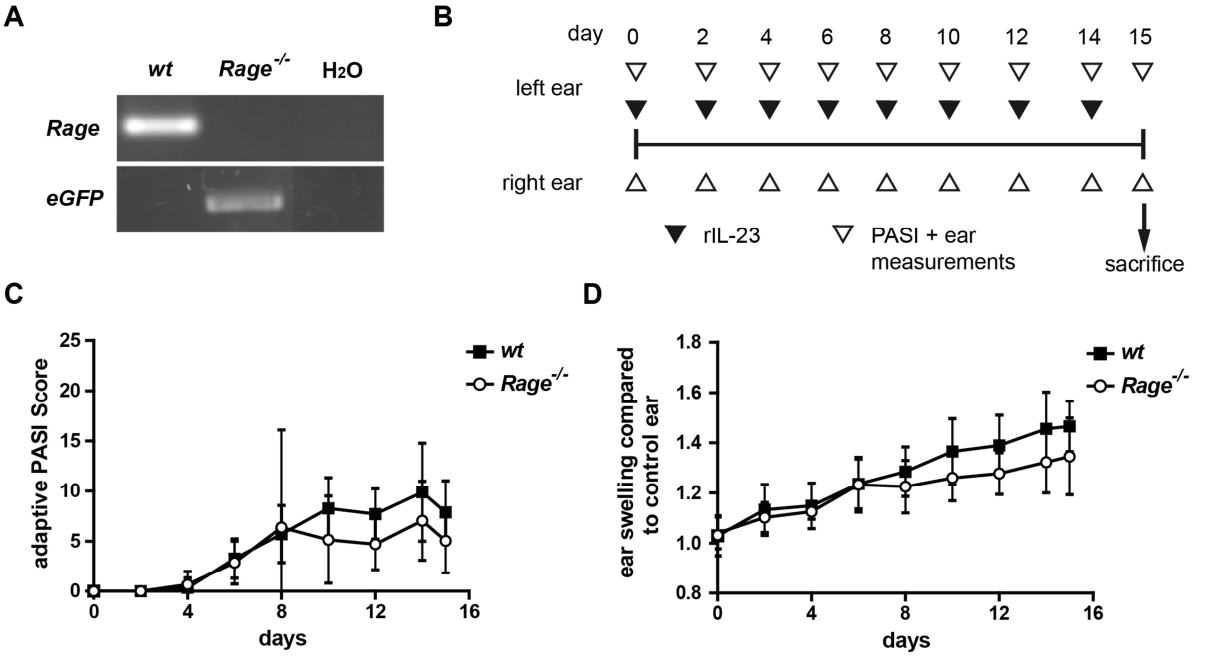

Figure 13 *Rage*$^{-/-}$ and *wt* mice show similar inflammatory responses to rIL-23 treatment.
Confirmation of Rage deletion; genomic DNA was prepared from the tail of *Rage*$^{-/-}$ and *wt* mice. Equal amounts of DNA (50 ng) were used for PCR analysis with Rage- and eGFP-specific primers (**A**). Experimental design of the intradermal rIL-23 ear injections (**B**). Plaque formation was measured by applying the adaptive PASI score onto *wt* (n=9) and *Rage*$^{-/-}$ (n=9) mice (**C**) and by analysis of the ear swelling (**D**). P values were calculated by two-tailed, unpaired sample t-test.

The ears treated with rIL-23 showed erythema as well as scales (data not shown). Analyzing the PASI score revealed two things: First, from day 1 to 8 under Rage-deficient conditions, and from day 1 to 10 in the wildtype situation the inflammation continuously increased. However, this slight delay was not significant. The inflammation became chronic at day 8 in *Rage*$^{-/-}$ mice and at day 10 in *wt* mice. Second, the statistical analysis of the PASI score revealed that the intensity of the inflammation caused by rIL-23 injections was not significantly altered in *Rage*$^{-/-}$ mice (Figure 13C). Another feature besides erythema and scale development, which displays the inflammatory progression, was the ear swelling. Figure 13D demonstrated that ear thickening started at the first day after initiation, and continuously increased until day 15. Again, no significant difference between *Rage*$^{-/-}$ and *wt* ear swelling was observed during the experiment (Figure 13D). This provided first evidence for rIL-23 injections to bypass the action of Rage in chronic inflammation. In order to further address this topic, microscopic investigations of the inflamed tissue were performed, as described in the next section.

VIII.7.2. *Rage*$^{-/-}$ mice exhibit no histological changes upon rIL-23 treatment

Here, the ear skin of rIL-23-treated mice was histologically analyzed. Representative H&E images of the psoriasiform phenotype of *wt* (left panel) and *Rage*$^{-/-}$ (right panel) skin sample are illustrated in Figure 14A. H&E staining revealed a mild psoriatic phenotype represented by moderate acanthosis, parakeratosis, and dermal infiltrating cells. Furthermore similar development of hyperkeratosis was observed in *wt* and *Rage*$^{-/-}$. An analysis of the cellular infiltration showed no significant alterations in the amount of infiltrating cells into the dermis between both genotypes investigated (Figure 14B). Moreover, the mild epidermal thickening due to hyperproliferation of keratinocytes was formed equally in *wt* and *Rage*$^{-/-}$ mice upon rIL-23 injections (Figure 14C). Noteworthy, despite the low level of acanthosis keratinocytes exhibited a proliferative phenotype. Figure 14D displays representative pictures of immunofluorescence staining using an anti-Ki-67 antibody, showing epidermal cells in a proliferative state and furthermore the presence of proliferative cells in the dermal compartment. Statistical analysis revealed unaltered amount of Ki-67^{+} keratinocytes in *wt* and *Rage*$^{-/-}$ mice (Figure 14E).

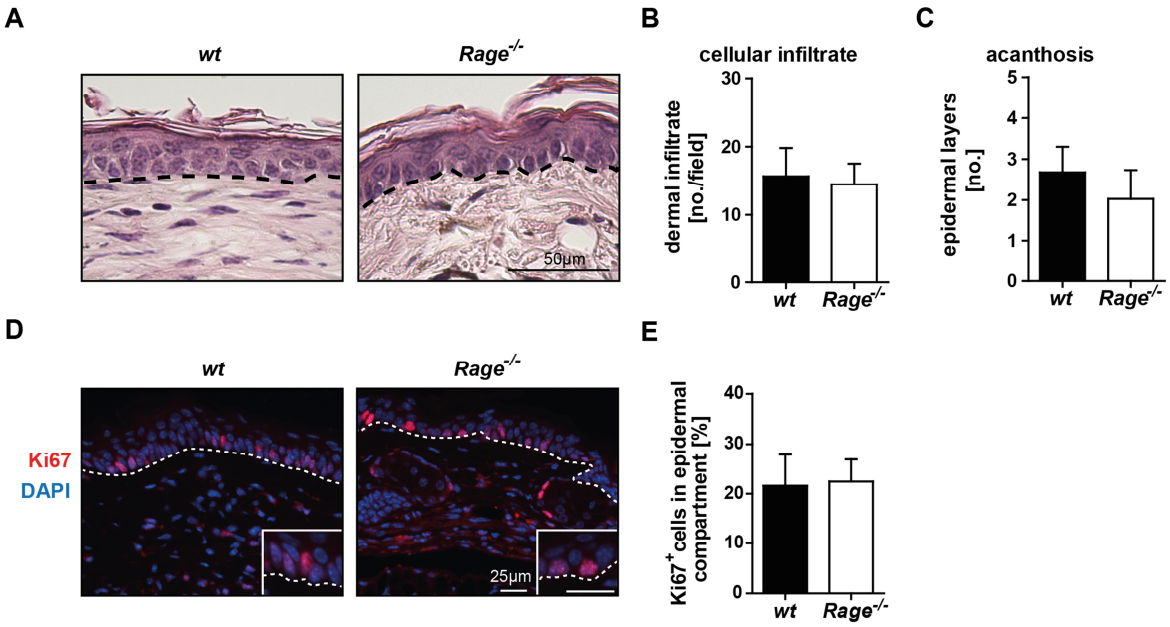

Figure 14 Psoriasiform features occur in *wt* as well as in *Rage*$^{-/-}$ mice upon rIL-23 injections.
Brightfield microscopic pictures of hematoxylin-eosin stained (H&E staining) sections of *Rage*$^{-/-}$ (n=9) and *wt* (n=9) back skins, sacrificed 24 h after the last rIL-23 treatment (**A**). Cellular infiltration was measured by counting the dermal infiltrating cells (**B**) and acanthosis by counting epidermal layers (**C**). Proliferative cells in the epidermis of treated skins are detected by immunofluorescence staining using anti-Ki67 (red staining; nuclei were stained with DAPI, blue signal) (**D**) and statistically analyzed by expressing the number of Ki-67^{+} cells as percentage of DAPI^{+} cells in the epidermal compartment of treated skins. The dotted lines represent the dermal-epidermal junction (**E**). P values were calculated by two-tailed, unpaired sample t-test.

VIII.7.3. Composition of the inflammatory infiltrate is unchanged in *Rage*$^{-/-}$ mice upon rIL-23 treatment

The investigation of infiltrating immune cells into the dermal compartment of rIL-23-induced inflamed ear skin revealed similar numbers for *wt* and *Rage*$^{-/-}$ mice as demonstrated by H&E staining (Figure 14A, B). Further analyses were performed to characterize the composition of dermal infiltrating cells in both genotypes. Therefore, immunofluorescence stainings using anti-Ly-6B.2 antibody for neutrophils, anti-F4/80 antibody for macrophages, and anti-Cd11c antibody for DCs were performed on *wt* and *Rage*$^{-/-}$ skin sections. Representative images of detected neutrophils (left panel), macrophages (middle panel), and DCs (right panel) within the dermis of inflamed tissue are illustrated in Figure 15A. Moreover, statistical analyses demonstrated that each population accounts for approximately 3 to 5 % of the infiltrating cells and that the individual cell types showed no significant difference between *wt* and *Rage*$^{-/-}$ mice (Figure 15B).

DCs are known to be key players in the interface between the innate and adaptive immune system and thereby in immune mechanisms contributing to psoriasis [3], [395]. For this reason, next to dermal DC amounts the number of DCs in cervical lymph nodes, located in the nearest destination to the site of inflammation, was analyzed *via* flow cytometric analysis (Figure 15C). Isolated cells from lymph nodes of *wt* and *Rage*$^{-/-}$ mice were stained with anti-Cd11c antibody conjugated with the fluorescent dye allophycocyanin (APC) (Figure 15C) or with the respective APC-conjugated IgG isotype control (data not shown). Differences in eGFP levels of *wt* and *Rage*$^{-/-}$ mice arise from characteristics of Rage-deletion construct. Rage deletion was confirmed by the expression of eGFP resulting in detectable green fluorescent signals [392]. The analysis verified the results of the immunofluorescence stainings by showing unaltered numbers of resting Cd11c^{+} DCs in inflamed lymph nodes *wt* and *Rage*$^{-/-}$ mice (Figure 15D).

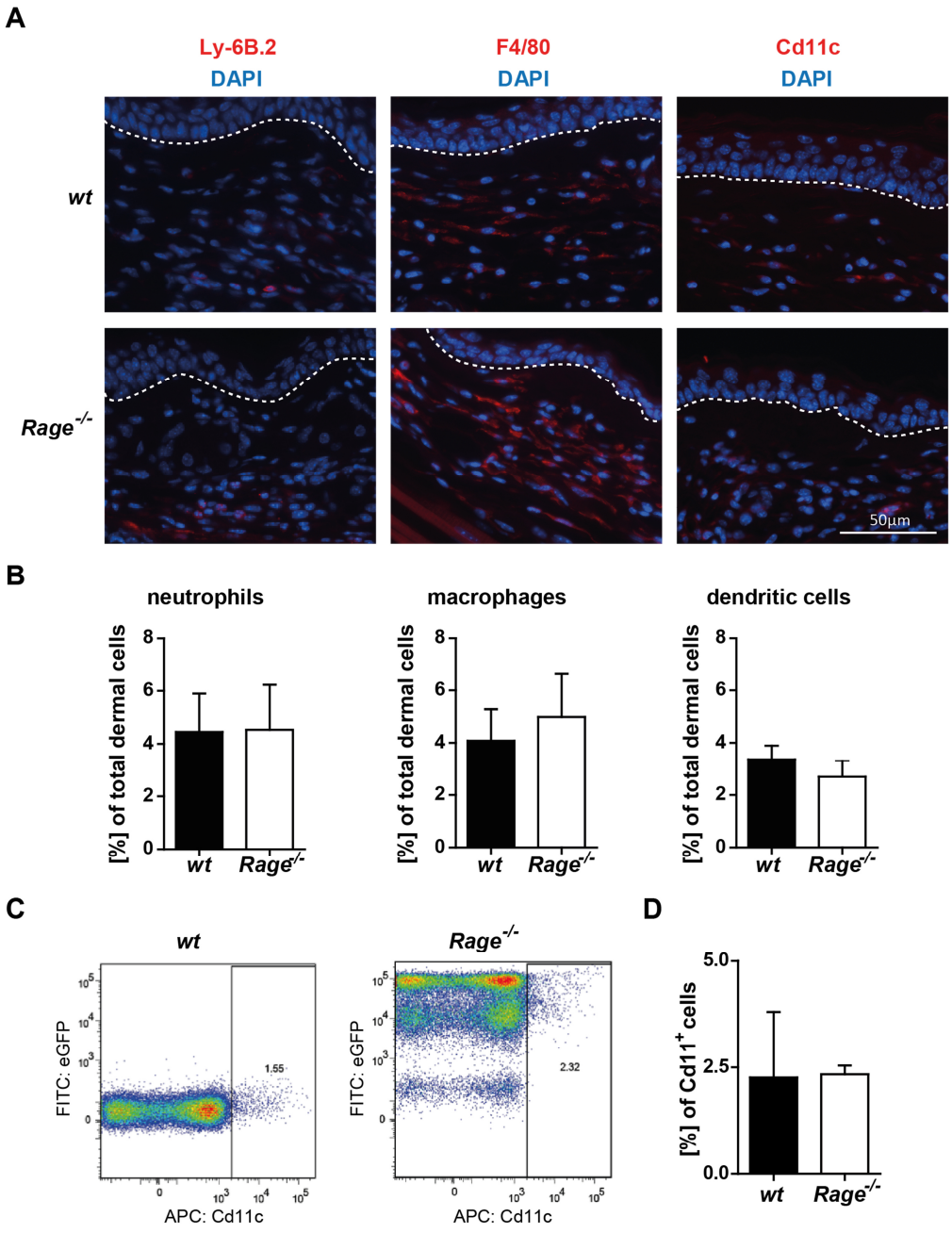

Figure 15 Psoriasis-associated immune cell infiltration occurs in *Rage*$^{-/-}$ mice upon rIL-23 injections.

Infiltrating immune cells in the dermis of rIL-23 treated skins (n=9 per genotype) were detected by immunofluorescence stainings using anti-Ly-6B.2 for neutrophils, anti-F4/80 for macrophages, and anti-Cd11c for dendritic cells (red stainings; nuclei were stained with DAPI, blue staining). The dotted lines represent the dermal-epidermal junction (**A**). Statistical analyses of immunofluorescence pictures were accomplished by expressing the number of staining-positive cells as percentage of DAPI^{+} cells (**B**). *Wt* and *Rage*$^{-/-}$ cells from lymph nodes were isolated and stained with an anti-Cd11c-APC antibody. Illustrations of a flow cytometric analysis of eGFP$^{+/-}$ Cd11c^{+} subpopulation of *wt* and *Rage*$^{-/-}$ cells (**C**). Comparison of the revealed percentages of Cd11c^{+} dendritic cells (**D**).

As described in VIII.2.2, S100A8 and S100A9 expression during a chronic inflammation is important for the development of a sustained activation of RAGE signaling. Immunofluorescence images exhibited an accumulation of S100a8- as well as S100a9-positive cells in the dermal compartment, principally in the upper part of the dermis of rIL-23 treated ear skins. Of note, also keratinocytes in the epidermis express S100 proteins as illustrated in Figure 16A. Moreover, the amount of S100 protein-expressing cells did not differ between *wt* and *Rage*$^{-/-}$ mice (Figure 16B).

Next, *wt* and *Rage*$^{-/-}$ ear skins were analyzed in regard to their expression profile of psoriasis-associated proinflammatory cytokines. Members of the interleukin cytokine family were described to play important roles in the initiation and maintenance of psoriasis. For instance, IL-23 predominantly secreted by DCs is a key cytokine in the survival and proliferation of T_H17 cells. T helper cell migration into the psoriatic lesion has been characterized as the beginning of disease maintenance [55]. In turn, IL-17 and IL-22 released by these T cells lead to increased keratinocyte production of antimicrobial peptides (AMPs), characterized as another important event in psoriasis [55]. Therefore, further immunofluorescence-based analyses focused on the distribution of these interleukins. Representative images of the immunofluorescence using anti-IL-17, anti-IL-22, as well as anti-IL-23 antibodies exhibited a uniform distribution of all investigated interleukins within the dermis of rIL-23 treated mice. However, differences between the diverse interleukins were observed regarding the respective amounts of cells producing these cytokines, especially when comparing IL-17$^+$ and IL-23$^+$ cell numbers (Figure 16C, D). However, no significant alterations neither regarding quantity nor distribution of investigated interleukin-positive cells between *wt* and *Rage*$^{-/-}$ mice were found (Figure 16 D).

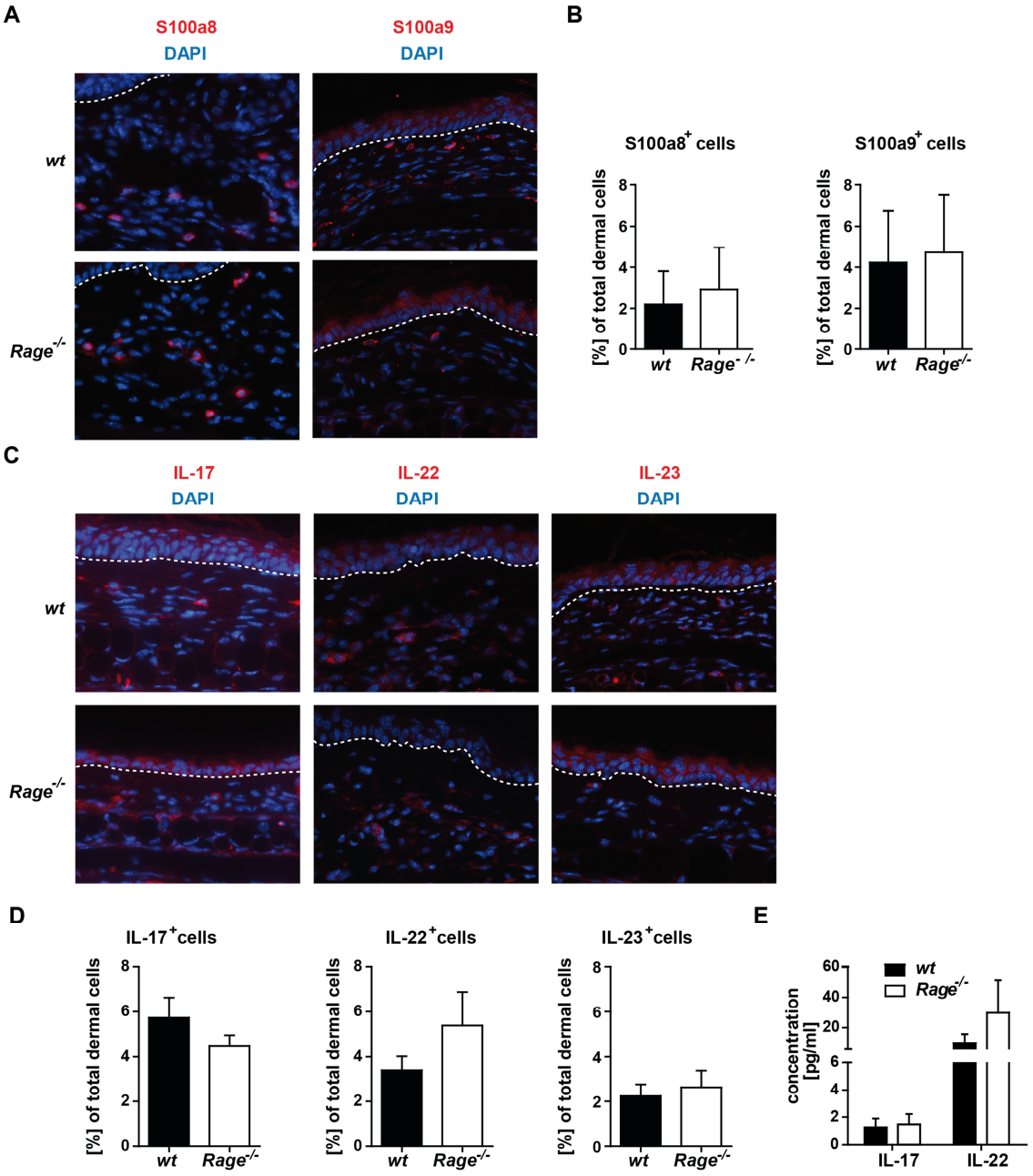

Figure 16 Similar S100 protein expression and interleukin levels upon rIL-23 in *Rage*$^{-/-}$ and *wt* mice.

S100a8- and S100a9-positive cells of rIL-23-treated ears of *wt* (n=9) and *Rage*$^{-/-}$ (n=9) mice were detected using immunofluorescence with (red staining; nuclei are stained with DAPI, blue staining). The dotted line represents the dermal-epidermal junction (**A**). Immunofluorescence stainings for the detection of interleukin-positive cells in the dermal compartment of psoriasifrom ears were performed using anti-IL-17, anti-IL-22, and anti-IL-23 antibodies (red staining; nuclei were stained with DAPI, blue staining) (**C**). Statistical analysis of immunofluorescence pictures was performed by expressing the number of staining-positive cells as percentage of DAPI^{+} cells in the dermal compartment (**B**, **D**). Interleukin (IL-17, IL-22) protein levels in mice sera and leach out samples of tissue biopsies were measured by immunoassay (**E**).

Moreover, concentrations of secreted interleukins were measured in the sera of rIL-23-treated mice *via* multiplex immunoassay. *Wt* and *Rage*$^{-/-}$ mice did not differ in the concentration of IL-17. In contrast, IL-22 serum concentration was markedly higher and furthermore exhibited an upregulation in *Rage*$^{-/-}$ mice in comparison to the *wt* controls (Figure 16E).

Taken together, the results from the *in vivo* rIL-23 model demonstrated that the injections of recombinant IL-23 lead to a psoriasiform inflammation of the skin. However, the quality and quantity of the inflammatory response is unaltered in *Rage*$^{-/-}$ mice compared to wildtypes, suggesting that rIL-23 induces psoriasiform inflammation independently of RAGE.

IX. Discussion

Psoriasis is a common cutaneous chronic inflammation and difficult to treat. Importantly, despite tremendous progress in the past years underlying molecular mechanisms of psoriasis remain still unknown. In this thesis it was hypothesized that RAGE might play a central role in the initiation and maintenance of chronic inflammations such as psoriasis. *In vivo* data presented in this thesis revealed that Rage is involved in psoriasis pathogenesis and, furthermore, pointed out a role for Rage on innate immune cells. Finally, data are provided indicating that RAGE signaling on dendritic cells contributes to the development of cutaneous inflammatory diseases including psoriasis.

IX.1. RAGE signaling initiates and maintains chronic inflammation

The receptor for advanced glycation end-products (RAGE) has been implicated in the development of many different inflammatory diseases such as atherosclerosis, rheumatoid arthritis, inflammatory bowel disease, and cancer [224], [356], [401], [402]. The expression of RAGE by immune and cutaneous cells, the high stability of RAGE-ligand complexes, as well as the existence of a positive feed-forward loop of RAGE signaling suggested a potential role of RAGE in the initiation and maintenance of a cutaneous chronic inflammation *inter alia* psoriasis.

Indeed, in this thesis RAGE was found to be highly expressed in the epidermal as well as in dermal compartments of human psoriatic plaques in comparison to matched non-lesional specimens (Figure 3A). Moreover, the overexpression of RAGE during inflammation was also observed in the murine system. Mouse models of psoriasis revealed increased Rage mRNA as well as protein levels (Figure 3B, C). In line with this investigation, an upregulation of RAGE has been reported for many other inflammatory diseases, as elevated RAGE levels in different tissues outside the lung are frequently associated with pathological manifestations [232], [252], [253]. For instance, atherosclerotic plaques in humans and mice were shown to highly overexpress RAGE [403], [404], and *vice versa,* Rage deficiency in a murine atherosclerosis model was associated with reduced MAP kinase and NF-κB activation leading to delayed plaque progression [238], [404], [405]. The same study showed that the expression of Rage correlated with the amounts of Rage ligands and, additionally, with the severity of the disease. This illustrates the high importance of the RAGE axis for the maintenance of some inflammations.

Inflammatory responses can be classified as either acute or chronic, while the acute inflammation also displays the initial phase of the chronic process. In contrast to an acute

inflammation, which is terminated by a resolution phase, a chronic inflammation is constantly active. In the here-presented *in vivo* study, loss of Rage delayed and dampened the initiation of the IMQ-induced inflammation (Figure 4D). Moreover, in the absence of Rage the chronic state of the psoriasiform disease, which was defined as the phase when the inflammatory response plateaued, reached a significantly lower level, indicating a reduced chronic inflammatory response (Figure 4E). In terms of numbers, *wt* mice developed chronic inflammatory conditions designated with an adaptive PASI of almost 60, while IMQ-induced inflammation in *Rage*$^{-/-}$ mice plateaued at an approximately 3-fold lower value. Previous *in vitro* studies demonstrated that the initial phase of a developing cutaneous inflammation is dependent on the activation and recruitment of immune cells into the inflamed tissue [55]. The here-performed study showed for the first time that the loss of Rage during chronic inflammation *in vivo* leads to a reduction of infiltrating cells (Figure 5C) and thereby to a dampened development of psoriasis (Figure 4D).

A role for Rage in acute inflammatory responses of the skin was already described by Gebhardt and coworkers. They revealed that Rage-deficient mice failed to develop an acute inflammation upon single treatment with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and a prolonged inflammation upon triple TPA treatment every 48 h [263]. However, as the inflammation induced by this model resolved within three to four days, the settings did not allow the investigation of the role of Rage in more chronic inflammatory conditions as seen in human pathology. This was underscored by the fact that no T cells were detectable within the dermis, indicating that the adaptive immune system was not activated, thereby limiting inflammatory responses to innate immune cells [384]. In contrast, the IMQ model comprises the activation of innate and adaptive immune cells, as shown by infiltrating T cells, thus allowing the investigation of Rage in fully implemented chronic inflammatory conditions [54]. Acute inflammation, as the initial process occurring in chronic inflammation, is mainly characterized by increased blood flow, increased vascular permeability for plasma proteins, and the emigration of leukocytes, predominantly neutrophils [18]. In the applied IMQ model, inflamed tissue of *wt* mice exhibiting a psoriasiform phenotype showed significantly higher neutrophil infiltrations compared to those of IMQ-treated *Rage*$^{-/-}$ mice (Figure 6A, D). In contrast to acute inflammations, in which predominantly neutrophils emigrate to sides of inflammation, macrophages take over their functions in chronic inflammation [24]. Indeed, data revealed that macrophage recruitment into inflamed tissue is dependent on the expression of Rage, thereby providing evidence that Rage plays a role in acute as well as in chronic inflammatory conditions (Figure 6A, C). Previous studies described dendritic cells (DCs) as key players in the development of a chronic inflammation [171]. These cells are the connecting link between the innate and adaptive immune system by being responsible for antigen presentation to T cells and thus for their differentiation into diverse T_H cells [3]. For

instance, DC-secreted IL-23 has been reported to be involved in determining the fate of naïve $CD4^+$ T cells into the T_H17 cell lineage [8]–[10]. In fact, the applied IMQ model demonstrated that Rage loss was accompanied with significantly reduced amounts of infiltrating DCs into the psoriasiform skin (Figure 6A, B). Maturation of these DCs and their migration into draining lymph nodes, where antigen presentation takes place, is facilitated by RAGE signaling as shown by others [261]. This is of high importance for their functionality and will be discussed later in more detail. Data provided in the present study exhibited a significant difference in the quantity and quality of the response to IMQ in the chronic phase of the inflammation (Figure 4D).

Psoriatic plaques are characterized by diverse histopathological changes of the skin, including acanthosis, papillomatosis, parakeratosis, as well as immune cell infiltration and keratinocyte hyperproliferation [55]. Analysis of IMQ-treated mice revealed the existence of all these psoriatic symptoms in *wt* mice (Figure 4D, 5). In contrast, *Rage*$^{-/-}$ mice showed diminished acanthosis as well as a reduced number of proliferating epidermal cells (Figure 5B, E). These findings indicate that RAGE contributes to the development of the histopathological hallmarks of psoriasis.

The positive feed-forward loop of RAGE is known to promote an inflammation by transactivating the expression of its own and of RAGE ligands [263]. On top, upregulated expression of the receptor and its ligands leads to sustained activation and thereby to the maintenance of an inflammatory response. The S100 family members *S100a8* and *S100a9* mRNA levels were highly increased in IMQ-treated *wt* mice (data not shown), while the loss of Rage under psoriasiform conditions limited the expression of these two proteins significantly (Figure 6E, F). Besides S100a8/a9, many of the S100 protein family members including S100A7 are described as RAGE ligands [302]. Madsen and colleagues were able to demonstrate that S100A7 is upregulated in psoriatic plaques and released during inflammatory responses [386]. Secreted S100A7 proteins may potentiate the inflammatory response *via* its RAGE-mediated leukocyte-chemotactic function [302]. Using genetically modified mice that express elevated amounts of the human ortholog S100A7/A15, mS100a7a15, they concluded that the S100A7-RAGE interaction activates the MAP kinase pathway leading to an enhanced secretion of proinflammatory cytokines and to the upregulation of RAGE [303]. Activation of MAP kinases lead to subsequent NF-κB activation, a transcription factor known to regulate genes associated with stress response including inflammation [406]. In fact, Chen and colleagues were able to show that a constitutive activation of NF-κB due to deficiency in IκB-α, establishes severe psoriasiform dermatitis in adult mice [407]. In line with this data, the milder phenotype of Rage-deficient mice was accompanied with downregulation of *NF-κB* mRNA and upregulation of the inhibitor gene

Nfκbia under inflammatory conditions (IMQ treatment) in this thesis (Figure 7A), indicating that elevated levels of RAGE and its ligands S100A8/A9 activate the NF-κB signaling pathway in psoriasis. Moreover, key cytokines for establishing a proinflammatory milieu like *Il-6*, *Il-1β* and *Tnf-α* were downregulated in psoriasiform *Rage*$^{-/-}$ mice (Figure 7A). These cytokines are known to activate the canonical NF-κB pathway by binding their respective receptors and have been implicated in chronic inflammatory diseases such as rheumatoid arthritis, inflammatory bowel disease and asthma [408]–[410]. The exact signaling cascade downstream of RAGE for gene transactivation remains largely unclear and needs further investigations. However, the present data indicate that RAGE regulates the NF-κB pathway and subsequent gene induction in psoriasis.

In addition to *NF-κB* induction, the presented data revealed a Rage-dependent activation of *Stat-3* transcription (Figure 7A). STAT-3 has been reported to be involved in the pathogenesis of psoriasis, as epidermal keratinocytes in psoriatic plaques are characterized by elevated cytokine levels that promote STAT-3 activation [52], [411]. For instance, IL-22 a cytokine released by activated T_H17 and T_H22 cells, phosphorylates STAT-3 in keratinocytes, thereby contributing to the crosstalk between keratinocytes and immune cells in psoriasis [52], [412], [413]. IMQ-treated *Rage*$^{-/-}$ mice displayed significant reduced numbers of IL-22 positive cells in the dermal compartment as well as less *Il-22* transcripts in total skin (Figure 7C, D). This indicates that besides transcriptional regulation of STAT-3, RAGE is also involved in posttranscriptional mechanisms controlling STAT-3 and maybe also NF-κB activation. Among the target genes of these transcription factors several cell cycle control and proliferation genes are listed [414]. Furthermore, Chen and colleagues reported a keratinocyte hyperplasia under constitutive NF-κB activation, indicating that RAGE-mediated activation of these transcriptional regulators results in enhanced proliferation capacity. Indeed, the proliferative activity of epidermal keratinocytes is enhanced by Rage signaling in the IMQ model (Figure 5D, E). Deregulated keratinocytes contribute to the development of typical skin aberrations of psoriatic patients [71]. In fact, the number of proliferative keratinocytes is highly upregulated in psoriatic plaques and is known to correlate with the clinical severity of psoriasis [394]. Such psoriatic hallmarks have been described for the convenient IMQ model, which induces a psoriasiform chronic inflammatory response *via* the IL-23/T_H17 axis and which allows the elucidation of pathogenic mechanisms occurring between cutaneous and immune cells of the innate and adaptive immune components [54]. A number of alternative psoriasis mouse models exists, however, these models are often associated with genetic manipulations [43]. Therefore, the IMQ model has the advantage to induce psoriasiform inflammation without genetic alterations. IMQ initiates the inflammatory responses upon binding to TLR-7/8 on monocytes, macrophages, plasmacytoid dendritic cells, B cells as well as on differentiated keratinocytes. Repetitive topical treatment leads to

the development of chronic inflammation including the whole orchestra of cells involved in psoriasis. The examination of *Tlr7* and *Tlr8* transcript levels of in *wt* and *Rage*$^{-/-}$ mice revealed with Tlr-7 being slightly down- and Tlr-8 slightly upregulated only negligible differences (Figure 4C). This clarifies that the IMQ application can equally initiate an inflammatory response in both mice strains.

Activation of the IL-23/T_H17 pathway, characterized by IL-23 secreting DCs and accumulation of T_H17 cells, plays a major role in the pathogenesis of IMQ-induced psoriasis-like skin inflammation [54]. It has been shown that IL-23- or IL-17R-deficient mice fail to develop a chronic inflammation. In fact, the IMQ-treated skin of *Rage*$^{-/-}$ mice showed reduced T_H17-related cytokine transcript levels including *Il-17* and *Il-22* (Figure 7C), along with the suppression of histopathological hallmarks (Figure 5) and inflammation-related genes (Figure 7A, B). Additionally, IL-23 expressing cells were highly decreased (Figure 7D). These observations suggest that Rage activation leads to induction of IL-23/T_H17 pathway resulting in psoriasiform skin inflammation in this model.

Taken together, Rage promotes the initiation and maintenance of a chronic inflammation by activating the recruitment of innate immune cells into the inflamed tissue. Moreover, induction of RAGE by its ligands resulted in the activation of psoriasis-related transcription factors, thereby leading to the secretion of proinflammatory cytokines known to be involved in the establishment of chronic inflammations. However, Rage-deficient mice could not fully abolish the inflammatory response. This is in line with the observations from Ueyama and coworkers, who demonstrated that the loss of Tlr-7 diminished but not abrogated the development of a chronic inflammation upon IMQ application [415]. In summary, these data give a first hint for redundant functions of the PRR family members in promoting and maintaining inflammatory conditions.

IX.2. The RAGE axis - a novel pattern recognition receptor signaling in chronic inflammation

As discussed before, RAGE initiates and maintains chronic responses in the context of psoriasis. The consideration of all examined parameters showed that the inflammatory response is indeed significantly reduced in *Rage*$^{-/-}$ compared to *wt* mice (Figure 5, 6). However, the absence of RAGE cannot entirely suppress the development of a chronic inflammation. Indeed, Rage-independent upregulations of various inflammatory markers, *e.g.* interleukin amounts, were observed upon induction with inflammatory stimuli (Figure 7C-E). This leads to the conclusion that RAGE plays an important role in chronic disorders but raises the question what other receptors contribute to the development and maintenance of a

chronic inflammation. Besides RAGE, also TLRs belong to the pattern recognition receptor (PRR) family and their signaling result in the activation of immune and inflammatory responses [416]–[419]. RAGE and TLRs share several common ligands such as HMGB1 as well as S100A8/A9 and various more [278], [296], [353], [372], [381], [420]–[422]. For instance, under inflammatory conditions HMGB1 builds complexes with DNA and signals *via* binding to membrane-bound RAGE and/or TLR-9 on endosomes [257], [285]. Moreover, HMGB1–nucleosome complexes activate APCs *via* TLR-2, thereby contributing to the pathogenesis of systemic lupus erythematosus (SLE) [319]. Additionally, the functional association of HMGB1, RAGE and TLRs is known to enhance inflammatory responses in diabetes [423]. Ligand binding is known to enhance the heterodimerization, *e.g.* S100B stimulus leads to the stabilization of RAGE-TLR-2 heterodimers [235]. The existence of common ligands and heterodimer formation as well as the secretion of similar proinflammatory cytokines also point towards redundant functions between these two PRR family members. This is further indicated by the observed upregulation of different Tlrs such as Tlr-2 and Tlr-4 in IMQ-treated *Rage*$^{-/-}$ mice during chronic inflammation (Figure 4C).

Similarities between RAGE and TLRs do not only exist with regard to pathway induction but also concerning downstream signaling. Stimulation of TLR signaling pathways requires in most cases the adaptor protein MyD88, and in case of TLR-2/TLR-4 also the presence of the adaptor molecule TIRAP and leads to the recruitment and induction of IRAK-4, IRAK-1, as well as TRAF-6 and subsequent to the formation and activation of the TGF-β activates kinase-1 (TAK-1) complex [181]. Activated TAK-1 phosphorylates the IKK complex and MAP kinases, such as c-Jun N-terminal kinase (JNK), thereby inducing the activation of NF-κB and AP-1, and the secretion of proinflammatory cytokines, respectively [181]. Although the complete signaling pathway of RAGE is still not fully understood and several different adaptor proteins of RAGE are described, it has been shown that TIRAP and MyD88 bind to the phosphorylated cytoplasmic domain of RAGE [424]. The interaction of RAGE with these adaptor proteins further induces the recruitment of IRAK-4, which subsequently activates downstream effector kinases such as MAP kinases and IKK complexes, leading to induction of proinflammatory cytokines *via* the activation of transcription factors including NF-κB and AP-1 (Figure 2). The presence of shared ligands as well as the similarities found by comparing downstream signaling provided first evidence that RAGE and some members of the TLR family functionally interact in order to regulate inflammatory responses. Functional support has been provided by Ueyama and coworkers, who showed that *Tlr-7*$^{-/-}$ mice demonstrated reduced levels of acanthosis and infiltrating inflammatory cells as well as amounts of T_H17-related cytokines compared to *wt* mice. This indicates diminished inflammatory responses to IMQ, leading to the conclusion that Tlr-7 deficiency was not able to abolish the inflammation completely [415]. Interestingly, the upon IMQ treatment exhibited

phenotype of *Rage*$^{-/-}$ mice used in this thesis was comparable to that described for *Tlr-7*$^{-/-}$ mice (Figure 5A-C, 7C-E). These data along with the similarities of the two receptor families regarding their signaling pathways suggest that RAGE and TLRs possess a joint role in the pathogenesis of psoriasis. In order to verify the assumed redundant functions of RAGE and TLR-7 in psoriasiform inflammation and whether the receptors can compensate each other, it would be of high interest to generate Rage-Tlr-7 double knockout mice and apply the IMQ-induced inflammation model.

Furthermore, also other PRR such as RIG-I-like (RLRs), NOD-like (NLRs), and C-type lectin receptors are involved in the transcriptional regulation of inflammatory mediators [4]. This is consistent with the study from Rabeony and coworkers from 2015 showing that IMQ-induced skin inflammation was abolished in the absence of MyD88, the adaptor protein shared by RAGE, IL-1R and TLR signaling [425]. Major inflammatory cytokines released by activated DCs as a direct consequence of PRR signaling include IL-1, TNF-α, IFNs, IL-4, IL-5, IL-6, IL-13, and IL-17 [426]. Gene expression data revealed that all of these cytokines are downregulated in *Rage*$^{-/-}$ mice (Figure 7A), suggesting an important contribution of RAGE in the PRR signaling on DCs.

All these PRR share several downstream targets with RAGE and lead to the secretion of different subsets of proinflammatory cytokines, dependent on pathophysiological conditions, cell type, and ligand concentrations. The variety of influences hampered the investigation of a distinct receptor under pathophysiological conditions. Nevertheless, the data provided in this thesis proved that RAGE contributes and extends the inflammatory response of PRR in the context of psoriasis.

IX.3. RAGE impacts immune response in psoriasis

Despite a plethora of studies, the pathogenesis of psoriasis is still not completely understood. This is attributed, to some extent, to the complexity of the disorder. Psoriasis is driven by various cells, regulatory molecules of the innate and adaptive immune system as well as by skin epithelium and connective tissue [131]. The combination of environmental and potential genetic predisposition contributes to the development of psoriasis by promoting secretion of proinflammatory cytokines and antimicrobial peptides as well as the release of DNA/RNA molecules by keratinocytes [133]. These factors have chemoattractant and activating effects on different subsets of dendritic cells [55]. Plasmacytoid dendritic cells, when activated, secrete high amounts of IFN-α, which in turn leads to the maturation and activation of dermal dendritic into inflammatory dendritic cells [137], [138], [427]. Activated myeloid dendritic cells recognize and capture antigens, migrate to local draining lymph nodes and present antigens

to T cells, thereby inducing T cell differentiation [139]. Differentiation of T cells is dependent on the cytokine composition released by DCs; for instance IL-23 secreted by some DC subsets is responsible for the generation of T_H17 cells and subsequent for the production of proinflammatory cytokines like IL-17 and IL-22. The IL-23/T_H17 axis has been claimed to have an important role in the induction of psoriasis [167]. In addition, IL-12-triggered differentiation to T_H1 cells contributes to the establishment of an inflammatory milieu *via* the secretion of proinflammatory cytokines including IFN-γ and TNF-α [97]. In turn, all these proinflammatory cytokines function on keratinocytes leading to a sustained activation of these cells, consequently to the secretion of other proinflammatory cytokines. These feed-forward loops involving keratinocytes, innate and adaptive immune cells are responsible for the maintenance psoriasis [55].

As mentioned before, IMQ is known to induce a psoriasiform phenotype in mice including histopathological changes and recruitment of $CD4^+$ T cells, $CD11^+$ DCs and plasmacytoid dendritic cells; cell types known to be important for the IL-23/T_H17 axis of psoriasis [54]. An *in vitro* study from Li and colleagues showed that IMQ forces proinflammatory cytokine production by differentiated keratinocytes [166], indicating that IMQ induces early events of psoriasis pathogenesis. This thesis extends these findings by demonstrating that the IMQ-induced activation of keratinocytes is independent of RAGE signaling (Figure 9G, H). Therefore, Rage-dependent induction of a psoriasiform inflammation in mice upon IMQ treatment must involve other cell types. Recently, Ueyama and colleagues were able to demonstrate that the low proinflammatory cytokine production by bone marrow-derived DCs upon stimulation with IMQ could be enhanced when DCs were co-stimulated with IFN-α. Taken these data and the results of this thesis into account, one can suggest two things: First, keratinocytes contribute in a RAGE-independent manner to IMQ-induced psoriasiform conditions. Second, that other cell types, including dendritic cells, must be responsible for the RAGE-dependent effects of IMQ-treatment in the mouse model.

IX.3.1. RAGE expressed by innate immune cells predominantly drives chronic inflammation

By using the IMQ model, we could show that the absence of Rage decreased the number of infiltrating DC in dermal compartments of mice (Figure 6A, B), and that Rage was indeed expressed by human and murine DCs under inflammatory conditions (Figure 10A). Comparing human psoriatic plaques with high PASI to lesions with low PASI indicated a correlation of number of RAGE-expressing dendritic cells as well as RAGE expression itself and clinical severity of the disease (Figure 10B). This is in line with an observation gained by the generation of bone marrow chimeras, showing that Rage expression on immune cells, but not keratinocytes, was required for innate immune cell recruitment, a critical step in the

pathogenesis of psoriasis [55], [263]. Moreover, not only the expression of RAGE is upregulated on DCs, also the expression of genes associated with maturation and activation of DCs including *Cd80*, *Cd86*, and *Cd40* depends on Rage (Figure 7A). CD80 and CD86 as well as CD40 are expressed on mature DCs, whereas immature DCs express only low levels of these co-stimulatory molecules, which are needed for the activation of T cells [3], [428], [429]. Manfredi showed that the RAGE-HMGB1 axis is involved in the homing of DCs to lymph nodes, where antigen presentation takes place [261]. Together with the data provided in this thesis, this indicates that Rage disrupts the interaction of DC and T cells, thereby inhibiting the differentiation of naïve T cells into T helper subsets. These data alone could not serve as an evidence for the role of RAGE on DCs, as the functions of co-stimulatory molecules are complex and depend, for instance, on the receptors they engage. More specifically, binding of CD80/CD86 to respective receptors can have contrarian effects: While engagement of CD28 activates T cells, the interaction with cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) induces the inhibition of T cell differentiation [430]. However, taking a closer look onto the gene expression data revealed an enormous downregulation of the T_H cell subset marker Cd4 in mice lacking Rage (Figure 7A), thereby confirming the data from Manfredi and coworkers. These results indicate a role of RAGE on DCs in the pathogenesis of psoriasis; which was confirmed by the results from the rIL-23 mouse model. This *in vivo* model rescued the impaired inflammatory phenotype upon Rage loss by sustained addition of rIL-23, a cytokine secreted by activated DCs (Figure 14A). Taken together, this provides evidence that Rage expression by cells ‘upstream’ of T cells, but as discussed in the previous section, ‘downstream’ of keratinocytes is of enormous importance for the development of a chronic inflammation such as psoriasis.

A small subpopulation of DCs, namely plasmacytoid dendritic cells (pDCs), is described to infiltrate the mouse back skin after IMQ administration [431], [432]. Additionally, in patients exacerbation of psoriasis is accompanied by an induction of IFN-α secretion and infiltration of pDCs in lesional skin, whereas pDCs are not detectable in healthy skin [433]. As RAGE expression was shown to be upregulated on DCs during psoriasis in humans or psoriasiform inflammation in mice (Figure 10), and as pDCs infiltrate psoriatic plaques, an investigation of RAGE on pDCs was indispensable. Furthermore, TLR-9, which is known to be involved in the activation of pDCs and which is exclusively expressed on B cells, keratinocytes and pDCs, was found to be downregulated in IMQ-treated *Rage*$^{-/-}$ mice compared to the controls (Figure 4C). This indicates that the PRR-mediated pDC activation plays a critical role in the pathogenesis of psoriasis. TLR-9 shows endosomal localization and provides protective immune functions by binding to viral or bacterial DNA [434]. However, extracellular mammalian self-DNA captured in immune complexes has been shown to contribute to autoimmune diseases *via* inducing the production of IFN-α [435]. This led to the hypothesis

that a surface-expressed receptor has to be involved in the recognition of self-DNA immune complexes. *In vitro* analysis of *Rage*$^{-/-}$ and *wt* pDCs revealed that Rage plays a crucial role in the activation of pDCs *via* HMGB1-CpG complexes in the context of SLE [257]. Furthermore, recent studies provided evidence that cell-free DNA can even be directly linked to psoriasis severity. Indeed, anti-psoriasis therapies were effective by just decreasing cell-free-DNA amounts [397]. The here-presented thesis provided evidence that two RAGE ligands, HMGB1 and S100B, in complex with self-DNA activate pDCs to secrete high amounts of IFN-α, and that RAGE itself is involved in the initiation of this process.

More specifically, high IFN-α concentrations were detected in the supernatants of alarmin-self-DNA complexes-stimulated pDCs; but both, HMGB1 and S100B alone, could not trigger IFN-α secretion by pDCs (Figure 11C-F). CpG dinucleotides are known to have stimulatory effects on psoriasis-related pDCs without being complexed with other proteins *in vitro* [436]–[438]. As CpG concentrations used in these experiments showed no alterations, the observed increased in IFN-α secretion by pDCs is not mediated by a single administration of CpG; instead, it is induced by CpG-complex formation (Figure 11B). In addition, RAGE expression on pDCs was upregulated due to the presence of alarmin-CpG complexes (Figure 12A). Despite the fact, that the RAGE ligands, HMGB1 and S100B, are additionally described as ligands of the TLR family [235], [353], [421], we identified RAGE as a major receptor for the alarmin-CpG complexes by using a RAGE blocking antibody (Figure 12B). Consistent with our data, Tian and colleagues revealed that upon binding to DNA molecules the affinity of HMGB1 to RAGE is increased, and no binding to TLR-2 or TLR-4 on pDCs was observable [257]. Surprisingly, results from the same study showing that S100B in complex with self-DNA failed to activate pDCs could not be verified in this thesis [257]. Instead, S100B-CpG complexes were found to have similar stimulatory effects on pDCs as HMGB1-CpG (Figure 11 D, F). An interplay of RAGE and TLR-9 upon HMGB1-CpG engagement is described for murine pDCs, and leads to the recruitment of the adaptor protein MyD88 [257]. This is in line with the observation that additionally to *Tlr-9,* transcript levels of *Myd88* are downregulated in IMQ-treated *Rage*$^{-/-}$ mice in comparison to the inflamed *wt* mice (Figure 4C, 7A). This suggests that the absence of Rage could lead to a blockade of pDC activation *in vivo*. The missing existence of a Rage-Tlr-9 axis might suppress the recruitment of Myd88, thereby preventing an upregulation of Tlr-9 on endosomes as well as the secretion of proinflammatory cytokines such as Ifn-α and Tnf-α. This could be confirmed by the study from Lande and colleagues, showing that another psoriasis-associated alarmin, LL-37, in complex with self-DNA led to the activation of psoriatic pDCs [136]. LL-37 protects self-DNA from proteolytic degradation und converts it in a potent pDCs activator. A comparable mechanism might be assumed for HMGB1 and S100B. Furthermore, an *in vivo* approach using CpG injections to induce inflammatory conditions in murine airways revealed an

impaired inflammatory response to DNA in Rage-deficient mice [400]. All these data indicate that the ongoing process in psoriatic inflammation is dependent on the alarmin-self-DNA-RAGE axis of pDCs.

As several hallmarks of psoriasis, *e.g.* acanthosis, are dependent on a deregulation of keratinocyte proliferation and differentiation, and since these psoriasiform symptoms were decreased in IMQ-treated *Rage*$^{-/-}$ mice (Figure 5A), one might claim a role of RAGE on keratinocytes in psoriasis. Indeed, under normal conditions RAGE is expressed on keratinocytes *in vitro* (Figure 8B). Moreover, inflammation induced in the absence of Rage was accompanied with a significant reduction of hyperproliferating epidermal cells, mainly keratinocytes (Figure 5 B, E). Interestingly, an impact of RAGE on keratinocyte proliferation and migration in wound healing has already been proposed [439]. Moreover, a study revealed a role of RAGE in keratinocytes during acute inflammation of the skin. RAGE was described to support the maintenance of epidermal keratinocyte activation thereby inhibiting the resolution phase of an acute inflammation *in vivo* [384]. Besides RAGE, also the expression of several other PRRs including members of the TLRs family and NLR family, sensitized keratinocytes to PAMPs and DAMPs [440]. Previous studies identified TLR-9 to be expressed on psoriatic keratinocytes. In contrast, normal keratinocytes express this TLR family member exclusively at basal levels. Modulation of TLR expression in chronic plaque psoriasis led to an enhanced response to bacterial DNA [441]–[443]. Inflammatory keratinocytes are the main source of IL-1β, a cytokine promoting T cell-dependent inflammation in the skin [444]. Investigations of transcripts and protein levels of IL-1β revealed a missing activation potential for alarmin-self-DNA complexes on keratinocytes (Figure 8C-E). These observations are in sharp contrast to the studies from Dombrowski and colleagues showing that self-DNA alone is a potent trigger of AIM2 activation in keratinocytes resulting in IL-1β secretion. Furthermore, they exhibited that LL-37, known to build complexes with self-DNA, thereby activating pDCs, has antiinflammatory effects on keratinocytes. In keratinocytes, binding of LL-37 to self-DNA neutralizes DNA-mediated inflammation [445]. Interestingly, the loss of Rage in keratinocytes *in vivo* had no effect on proinflammatory cytokine secretion, including IL-1β secretion, except TNF-α secretion [384]. This could explain the lack of upregulated IL-1β secretion upon CpG and/or alarmin stimulus (Figure 8C-E).

Despite the conflicting data for self-DNA keratinocyte activation potential, there is no doubt about these cells being activated during chronic inflammation and contributing *via* the secretion of proinflammatory cytokines to the development of chronic inflammation. As Li and colleagues showed keratinocytes could be activated *via* TLR-7. This receptor is expressed only at basal levels by non-differentiated keratinocytes. Ongoing keratinocyte differentiation,

which takes place in psoriasis, is accompanied with a consequent increase in TLR-7 expression and, respectively, with enhanced sensitivity of cells towards stimulation with the TLR-7/8 agonist IMQ. Furthermore, this study showed that IMQ-treated differentiated keratinocytes are able to secrete proinflammatory cytokines, including TNF-α and IL-8, indicating their involvement in IMQ-induced psoriasiform inflammation. Data provided in this thesis revealed that differentiated keratinocytes (Figure 9C) stimulated with IMQ are capable of secreting alarmins such as HMGB1 and S100B (Figure 9D-F). It has been already demonstrated that terminal differentiated keratinocytes release RNA and DNA to the extracellular matrix, molecules that are normally degraded immediately [135]. The additional secretion of alarmins might than lead to a complex formation of self-DNA and alarmins, as it is already described for HMGB1 [257]. As described before, these alarmins in combination with self-DNA are important activators of pDCs (Figure 10). This defines the activation of keratinocytes upon IMQ application as a trigger of pDCs activation by releasing pDC stimulants.

Interestingly, the absence of RAGE on IMQ-treated differentiated keratinocytes did not influence the quantity of keratinocyte alarmin secretion (Figure 9G, H), revealing that the IMQ-induced secretion of HMGB1 and S100B by keratinocytes is independent of RAGE. Furthermore, these data indicate that the loss of Rage did not interfere with early events in the induction of a psoriasiform inflammation in mice.

The *in vivo* IMQ model in line with the *in vitro* data revealed that RAGE is involved in the initiation and maintenance of cutaneous chronic inflammation. The relevance of RAGE in the initial phase of a chronic inflammation is shown by the dependence of pDCs on RAGE for their proper activation, which represents an important event in the establishment of psoriasis. In contrast to the study of Leibold and colleagues, an absence of a resolution phase was observed in the *in vivo* IMQ model [384], which is a hint for the importance of RAGE on these cells during maintenance of psoriasis.

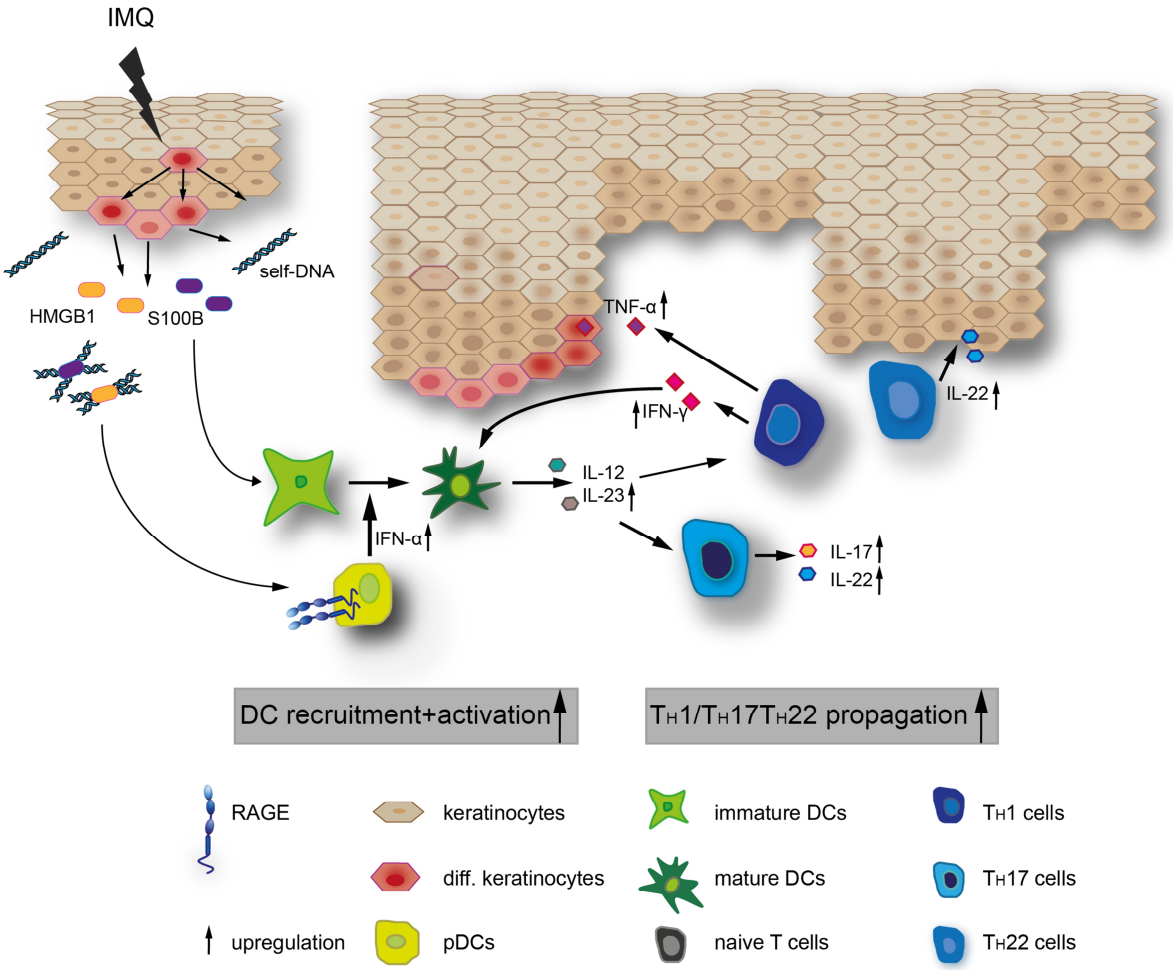

Figure 17 Possible role of RAGE and its ligands in the pathogenesis of psoriasis.
IMQ, a selective TLR-7/8 agonist, leads to the activation of differentiated keratinocytes. These activated keratinocytes secrete proinflammatory cytokines, AMPs, and alarmins *inter alia* HMGB1 and S100B, as well as DNA and RNA molecules. These alarmins build complexes with self-DNA thereby activating pDCs *via* RAGE. Activated pDCs secrete high amounts of IFN-alpha, which leads to the activation and maturation of DCs. In turn, maturated DCs circulate to the lymph nodes and secrete cytokines such as IL-23 and IL-12. IL-23, a potential downstream target of RAGE, is a cytokine known to force the differentiation of naïve T cells into T_H17 cells. Differentiated T_H cells migrate into dermal compartments, where an interaction with innate immune cells and keratinocytes *via* the secretion of cytokines such as TNF-α, IFN-γ, IL-17, IL-22 takes place.

Nevertheless, RAGE does not solely affect the activation of innate immune cells, but also their infiltration into inflamed tissue. Provided data indicate that Rage deficiency decreased the number of infiltrating macrophages, neutrophils, and dendritic cells into inflamed tissue (Figure 6 A-D). Similar observations have been exhibited by Gebhardt and coworkers in their model of TPA-induced skin inflammation [263]. Moreover, a decrease in the accumulation of innate immune cells, here neutrophils and macrophages, was also found in acute peritonitis-like inflammation in *Rage*$^{-/-}$ mice [446]. These data strongly recommend a role of RAGE in mediating innate immune cell recruitment into inflamed tissues *in vivo*.

As aforementioned, the provided data consistently suggest that RAGE functions as a regulatory molecule on innate immune cells, especially on DCs. These cells are known to represent antigens to naïve T and B cells, which results in their activation. This highlights the importance of DCs as key players in the interface between the innate and adaptive immunity [3] and raises questions about potential consequences arising from a loss of RAGE on DCs for adaptive immune responses, especially for psoriasis-associated T cell functions.

IX.3.2. RAGE signaling regulates adaptive immune responses by modulating innate immunity

Apart from cells of the innate immune system, activated $CD4^+$ and $CD8^+$ T cells may also express RAGE [255]. A tremendous downregulation of *Cd4* in the absence of Rage upon IMQ application indicates a reduced number of T helper cells in inflamed tissue (Figure 7A). This subset consists of IL-17 and IL-22-producing T_H17 and/or T_H22 cells as well as of IFN-γ and TNF-α- producing T_H1 cells [97], whereas the role of Treg cells during psoriasis remains controversial. Interestingly, some genes associated with the differentiation of $CD4^+$ T cells into T_H1 or T_H17 cells were found to be deregulated (Figure 7A). It has been shown that the frequency of IL-17^+ and IL-22^+CD4^+ cells in the blood of psoriasis patients is increased [447], revealing that these cells have an important impact on the maintenance of psoriasis. These data support the idea that the loss of RAGE inhibits the recruitment of T helper cells into inflamed tissue. Whether RAGE expression on T_H cells itself plays a role or if the missing maturation and activation of dendritic cells reduces the T cell infiltration requires further investigation. Nevertheless, both subsets, T_H17 and T_H22 cells, are influenced by the cytokine IL-23, which is required for their expansion and maintenance. IL-23 is highly expressed by DCs in the context of psoriasis [167]. Importantly, in the IMQ model Rage-deficiency was accompanied by impaired *Il-23* transcript levels (Figure 7C). As discussed before, this can be explained by the dependency of pDCs activation on RAGE in respect to the secretion of proinflammatory cytokines (Figure 12B, C). Intradermal rIL-23 injections into mice ears lead to the development of a psoriasiform phenotype in mice by mimicking constitutive DC activation resulting in the induction of the IL-23/T_H-17 axis and, consequently, in the differentiation of T cells into T_H17 cells [53]. Histopathological hallmarks of psoriasis were unaltered in *Rage*$^{-/-}$ mice upon rIL-23 injections when compared to the wildtype situation (Figure 14A). Moreover, the progression of the inflammatory reaction was not affected by the absence of Rage (Figure 13) and Rage deficiency had no impact on the amounts of infiltrating cells (Figure 14B, 15A, B). Furthermore, no alterations regarding the number of lymph node-infiltrating DCs in *Rage*$^{-/-}$ mice after rIL-23 injections was observed (Figure 15D) and rIL-23 injections also rescued the impaired infiltration of IL-17^+, IL-22^+, and IL-23^+ cells into inflamed tissue in Rage-deficient mice (Figure 16D, E). This indicates that the presence

of IL-23 rescues the ineffectiveness of *Rage*$^{-/-}$ DCs in modulating the differentiation of naïve T cells into T_H17 and/or T_H22 cells. In other words, these data point towards a role of IL-23 as a downstream target of Rage and suggest that a functional RAGE axis in innate immune cells ensuring a proper differentiation and activation of T cells but not RAGE on T cells itself to be important for the development and maintenance of psoriasis. However, in order to fully prove this hypothesis, an investigation of T cell- or DC-specific Rage-deficient mice would be necessary.

IX.4. RAGE axis as a possible therapeutic target for the treatment of chronic inflammatory diseases

Any psoriasis therapy aims for the amelioration or even full resolution of clinco-pathological symptoms. However, although a range of diverse therapies is available for the treatment of psoriasis, this goal is difficult to reach and still cannot be achieved in several patients. The existing long-term systemic therapies are burdened with the risk of adverse events. Therefore, clinical research mainly focused on the development of new therapeutics, which target molecules involved in the pathogenic mechanisms of psoriasis.

For instance, the IL-23/T_H17 axis was assigned to play an important role in the induction of psoriasis. More specifically, DC-secreted IL-23 seems to be responsible for the production of T_H17-related proinflammatory cytokines such as IL-17 and IL-22, which drives the disease [167]. Specific intervention of the IL-23/T_H17 axis might inhibit the hyperactive immune responses. Therefore, numerous studies including monoclonal antibodies directed against these cytokines were performed.

Consequently, antibodies directly blocking IL-12/IL-23, such as Ustekinumab and Briakinumab, were developed. These antibodies were shown to significantly reduce pathohistological hallmarks of psoriasis and ameliorate the disease burden [448]–[451]. Furthermore, anti-IL-17/IL-17R antibodies including Secukinumab, Ixekizumab, and Brodulumab were developed and represent therapeutics targeting downstream effects of IL-23 [452]–[454]. These interleukin blockers support the idea of T_H17 cells being key players in psoriasis and could perhaps replace the TNF-α pathway as a major therapeutic target in chronic inflammation [455]. Identifying and blocking pathways responsible for the secretion of IL-23 release would represent a great chance to prevent or resolve psoriatic lesions.

Besides highlighting the important influence of RAGE in the pathogenesis of psoriasis the here-presented data identified IL-23 as a downstream target of RAGE. Blocking the RAGE

axis might diminish IL-23 production and therefore decrease the induction of adaptive immune responses leading to disease progression.

In an Alzheimer´s disease (AD) mouse model Deane and coworkers identified a RAGE-specific inhibitor (FPS-ZM1) that exclusively binds to RAGE with a high affinity, inhibiting amyloid β-induced cellular stress, thereby suppressing neuroinflammatory responses [456]. Furthermore the inhibitor was able to block the engagement of RAGE by other RAGE ligands, which have been suggested to contribute to RAGE-mediated tissue damage in models of diabetes, inflammatory disorders and AD [457]. In April 2015 a phase III trial with the small molecule RAGE antagonist TTP488 started in the US [458]. Before, TTP488 was demonstrated to be effective by reducing amyloid plaques in an AD transgenic mouse model and by revealing beneficial effects in a phase II trial comprising 400 patients with mild to moderate AD [459].

These studies along with the results of this thesis point towards a potential use of RAGE as a selective therapeutic target arresting disease progression by blocking basic pathological mechanism of psoriasis.

Taken together, this thesis proposes RAGE as a regulatory molecule in cutaneous chronic inflammations such as psoriasis. For the first time, its role in the manifestation of a chronic inflammation of the skin, including the development of characteristic hallmarks such as acanthosis, parakeratosis, and dermal infiltration of innate immune cells was uncovered. Although RAGE was found to be expressed by keratinocytes and $CD11^+$ dendritic cells, these cell types demonstrated distinct degrees of dependence. The inflammation-associated activation of keratinocytes resulting in the release of two alarmins, HMGB1 and S100B, was found to occur in a RAGE-independent manner. In contrast, RAGE is essential for the alarmin-CpG-induced activation of plasmacytoid dendritic cells, illustrating an existing RAGE-mediated epidermal-innate immune crosstalk in psoriasis.

The rescue of the defective inflammatory phenotype by intradermal injections of rIL-23 pointed towards two things: on IL-23 as a potential target of RAGE as well as on a role of RAGE on dendritic cells driving chronic inflammatory diseases. The hypothesis that RAGE signaling is important for the activation of the IL-23/T_H17 axis was further supported by the observed downregulation of genes involved in the differentiation of T helper cells in the absence of RAGE. The indicated central role of RAGE in the IL-23/T_H17 axis of psoriasis illustrates RAGE as a potential target of new therapeutic strategies intervening ‘upstream’ of IL-23.

X. References

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XI. Abbreviations

°C	Degree Celsius
µ	Micro
%	Percent
7-AAD	7-aminoactinomycin
A	Alanine
A	Adenine
aa	Amino acid
ABIN	A20-binding inhibitor of NF-κB
ACT	Actin
AD	Alzheimer´s Disease
AGE	Advanced glycation end-products
AGER	Gene coding for RAGE
AMP	Antimicrobial peptide
AP	Activator protein
APC	Antigen-presenting cell
Apcs	Gene coding for amyloid P component serum
ATP	Adenosine triphosphate
B.C.	Before Christ
BCR	B cell receptor
BDCA	Blood DC antigen
BSA	Bovine serum albumin
bp	Base pair
C	Cysteine
C	Cytosine
C3/C5a	Complement component 3/5a
Ca^{2+}	Calcium
CCHCR	coiled-coil, α-helical rod
CCL	Chemokine (C-C-motif) ligand
CCR	Chemokine (C-C-motif) receptor
CD	Cluster of differentiation

cDNA	complementary DNA
CDSN	corneodesmosin
CHCl3	Chloroform
CO_2	carbon dioxide
CpG	Cytosine-phosphate-guanine dinucleotide (unmethylated)
Crp	Gene coding for C-reactive protein
CTLA	Cytotoxic T lymphocyte-associated protein
CTTP	Cytoplasmic transductional-transcritional processor
C-type/unit	Constant type/unit
CXCL	Chemokine (C-X-C-motif) ligand
CXCR	Chemokine (C-X-C-motif) receptor
Cy	Cyanine
DAMPs	Danger-associated molecule pattern
DAPI	4’,6-diamidino-2-phenylindole
DC	Dendritic cell
ddH_2O	Double-distilled water
DIA	Diaphanous
DMSO	Dimethylsulfoxid
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide
EDC	epidermal differentiation complex
EDTA	Ethylenediaminetetraacetic acid
eGFP	Enhanced green fluorescent protein
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated protein kinase
EtOH	Ethanol
FACS	Fluorescence-activated cell sorting
FCS	Fetal bovine serum
FDA	Food and Drug Administration
fl	Full-length
Foxp3	Gene coding for forkhead box p3
FPS-ZM1	N-Benzyl-4-chloro-N-cyclohexylbenzamide

FRET	Fluorescence resonance energy transfer
g	Gramm
G	Guanine
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GWAS	Genome-wide association studies
h	Hours
H&E	Hematoxylin and eosin
H_2O	Water
H_2SO_4	Sulfuric acid
HCl	Hydrochloric acid
HKGS	Human keratinocyte growth supplement
HLA	Human leukocyte antigen
HMGB1	High-mobility group box 1
HRP	Horse-radish peroxidase
Hs	Homo sapiens
HSPG	Heparin sulfate proteoglycan
ICAM	Intracellular adhesion molecule
IF	Immunofluorescence
IFN	Interferon
IgG	Immunoglobin G
IκB	nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor
IKK	IκB kinase complex
IL	Interleukin
IMQ	Imiquimod
iNOS	Inducible nitric oxide synthase
IRAK	IL-receptor associated kinase
IRF	IFN regulatory factor
JNK	c-Jun N-terminal kinase
k	Kilo
kb	Kilo base pairs
KCl	Potassium chloride
kDa	Kilodalton

KIR	Killer immunoglobulin-like receptor
l	Liter
LPS	Lipopolysaccharide
µ	Micro
M	Molar
m	Milli
MAC-1	Macrophage-1 antigen
MAP	Mitogen-activated protein
MED	Minimal erythermogenic dose
MeOH	Methanol
MHC	Major histocompatibility complex
min	Minutes
Mm	Mouse musculus
MMP	metallopeptidase
miRNA	Micro RNA
mRNA	Messenger RNA
MTX	Methotrexate
MyD88	Myeloid differentiation factor 88
n	Nano
NaCl	Sodium chloride
NAD^+	nicotinamide adenine dinucleotide
nbUVB	Narrow ultraviolet-B
NF-κB	Nuclear factor κ B
NFKBIA	Gene coding for IκB
NHEK	Normal human epidermal keratinocyte
NK cell	Natural killer cell
NLR	Nucleotide-binding oligomerization domain receptor (NOD-like receptor)
PAMPs	Pathogen-associated molecule pattern
PASI	Psoriasis area and severity index
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate-buffered saline

PD-L	Programmed death ligand
pDC	Plasmacytoid dendritic cell
PFA	Paraformaldehyde
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PRR	Pattern recognition receptors
PSORS	Psoriasis susceptibility (loci)
PUVA	Psoralen combined with ultraviolet-A
Q	Glutamine
qPCR	Quantitative real-time polymerase chain reaction
R	Arginine
RAGE	Receptor for advanced glycation end products
Rage$^{-/-}$	Rage-deficient [mouse]
rcf	Relative centrifugal force
rIL-23	Recombinant interleukin 23
RLR	Retinoic acid-inducible gene-1 like receptors (RIG-I-like receptors)
RNA	Ribonucleic acid
rpm	Rounds per minute
RT	Room temperature
SD	Standard Deviation
SDS	Sodium dodecyl sulfate
sec	Seconds
Slan-DC	6-sulfo LacNAc dendritic cell
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism
SP	Specificity protein
sRAGE	Soluble RAGE
SSC	Squamous cell carcinoma
STAT	Signal transducer and activator of transcription
T	Threonine
T	Thymine
TAK	TGF-β activated kinase

TBS	Tris-buffered saline
T_C	Cytotoxic T lymphocyte
TGF-β	Transforming growth factor-β
T_H	Helper T lymphocyte
Tip-DC	TNF-α/iNOS-producing dendritic cell
TIRAP	Toll-interleukin 1 receptor domain containing adaptor protein
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNFAIP	Tumor necrosis factor α-induced protein
TNFR	TNF receptor
TNIP	TNFAIP3 interacting protein
TPA	12-*O*-tetradecanoylphorbol-13-acetate
TRAF	TNFR-associated factor
TRAF3IP	TRAF-3 interacting protein
Treg	Regulatory T lymphocyte
UTR	Untranslated region
UV	Ultraviolet
V	Volt
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
V-type/unit	Variable type/unit
v/v	Volume/volume
wt	Wildtype [mouse]
w/v	Mass/volume

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