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The endogenous dual retinoic acid receptor agonist 9-cis retinoic acid downmodulates antigen-presenting cell functions to control immune responses

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Andreas Kislat

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from the Department of Dermatology, Medical Faculty, Heinrich-Heine-University Düsseldorf

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Supervisor: Univ.-Prof. Dr. med. Bernhard Homey

Co-supervisor: Univ.-Prof. Dr. rer. nat. Rainer Kalscheuer

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1 Summary

Retinoids, vitamin A derivatives, have shown beneficial effects in the treatment in a variety of diseases. The effects of retinoid acids are mediated by binding to transcription factors, the retinoic acid receptors (RAR) and the retinoic X receptors (RXR) and thereby regulating gene expression, proliferation, differentiation and survival of target cells. In dermatology, RAR-agonists are used for the treatment of acne, pustular psoriasis and ichthyosis. In oncology, all-*trans* retinoic acid (ATRA) is used to treat acute promyelocytic leukemia, whereas RXR-ligand bexarotene shows therapeutic benefits in cutaneous T cell lymphoma. Recently, the endogenous dual RAR and RXR agonist 9-*cis* retinoic acid (9-*cis* RA; alitretinoin) demonstrated therapeutic efficacy in chronic hand eczema (CHE). However, the precise effects of endogenous 9-*cis* RA on the human immune system, especially the skin immune system, is so far unknown. This thesis aimed at elucidating the molecular effects underlying the therapeutic outcome of 9-*cis* RA by analyzing specimens from patients suffering from chronic hand eczema before and during 9-*cis* RA treatment.

9-*cis* RA acts on keratinocytes as well as leukocyte subsets *in vitro*. First, 9-*cis* RA alters the expression of genes regarding different keratinocyte functions, including dysregulated barrier genes and genes regulating water loss, wound healing and inflammation thereby altering the recruitment of lymphocytes, especially monocytes and lymphocytes. Second, on monocyte-derived dendritic cells (moDCs) 9-*cis* RA inhibits the up-regulation of maturation markers as well as co-stimulatory molecules and induces chemokine receptor CC-chemokine receptor (CCR)6 and CCR7. Also, cluster of differentiation (CD)73 and interleukin (IL-)10 are activated and may in turn support immunomodulatory functions. 9-*cis* RA-treated moDCs show significantly impaired T cell activating properties by suppressing allogenic proliferation of leukocytes in mixed leukocyte reactions. *In vitro* findings could be confirmed in CHE patients mimicking the decreased CD86 and an increased CD73 expression. Moreover, 9-*cis* RA treatment significantly decreased the proliferation of patient-derived leukocytes following allogenic stimulation when compared with the proliferation before treatment. Furthermore, 9-*cis* RA modulates the chemokine receptor profile of circulating effector memory T cells. Taken together, these results suggest that 9-*cis* RA modulates innate as well as adaptive immune responses by suppression of chemokine-induced leukocyte recruitment and inhibition of dendritic cell-mediated T cell activation.

Zusammenfassung

Retinoide, Vitamin A Derivate, werden seit Jahren erfolgreich für eine Reihe von Erkrankungen eingesetzt. Retinoide vermitteln dabei ihre Effekte durch die Bindung von Transkriptionsfaktoren, den Retinsäure- (RAR) und Retinoid-X-Rezeptoren (RAR bzw. RXR) und regulieren dadurch Proliferation, Differenzierung und die Apoptose ihrer Zielzellen. RAR-Agonisten werden in der Dermatologie für die Behandlung von Akne, pustulöser Psoriasis und Ichthyose eingesetzt. In der Krebstherapie wird zur Behandlung der akuten Promyelozytenleukämie die All-*trans* Retinsäure (ATRA) eingesetzt, während der RXR-Ligand Bexaroten Wirkung bei der Behandlung des kutanen T-Zell Lymphoms zeigt. 2008 konnte gezeigt werden, dass der duale RAR und RXR Agonist 9-*cis* RA signifikante Erfolge bei der Behandlung des chronischen Handekzems (CHE) vorweist. Die genaue Effekte von 9-*cis* RA sind bisher jedoch unbekannt. Diese Arbeit will die zugrundeliegenden Effekte beleuchten und nutzt ein Patientenkollektiv, das systemisch 9-*cis* RA zur Behandlung des CHE erhielt.

In vitro wirkt 9-*cis* RA vor allem auf Keratinozyten und Leukozyten. Bei Keratinozyten beeinflusste 9-*cis* RA die Expression verschiedener Gene, die die Barrierefunktion, den Wasserverlust, die Wundheilung und die Inflammation steuern. Weiterhin wirkte 9-*cis* RA auf Dendritische Zellen, welche aus Monozyten generiert wurden (MoDCs) und inhibierte deren Reifung. Dabei wurde die Expression co-stimulatorischer Moleküle reduziert, während die Chemokinrezeptoren CCR6 und CCR7 induziert wurden. Zusätzlich wurden die immunmodulatorischen Proteine CD73 und IL-10 aktiviert. Diese MoDCs zeigten ein erniedrigtes Aktivierungspotential bei allogenen T-Zellen, welches sich in einer signifikant erniedrigten Proliferation der T-Zellen bei einer gemischten Leukozyten-Reaktion ausdrückte. Eine Untersuchung von CHE-Patienten, welche 9-*cis* RA erhielten, bestätigten die *in vitro* beobachtete Reduktion von CD86, eine erhöhte Expression von CD73 und eine signifikante Erniedrigung der allogenen Proliferation der Patientenleukozyten unter Therapie im Vergleich zur Aktivierungsfähigkeit vor Therapie. Zusätzlich wirkte sich die Therapie auf das sogenannte „Homing“-Verhalten von Effektor-Gedächtnis-T-Zellen aus.

Zusammengefasst deuten die Ergebnisse darauf hin, dass 9-*cis* RA durch die Dendritische Zelle sowohl angeborene als auch erworbene Immunität durch Chemokin-induzierte Leukozytenrekrutierung und Inhibierung der T-Zellaktivierung moduliert.

2 Introduction

2.1 Retinoids

Vitamin A and its derivatives (retinoids) have been characterized as essential for mammalian health and survival in the early 20th century (Hall et al., 2011b). Supplementation of vitamin A improved the survival of children in endemic regions with malnutrition (Rahmathullah et al., 1990, Sommer, 2008, Sommer et al., 1986). Studies on the pathologies associated with vitamin A deficiency and developmental defects occurring after administration of supra-physiological doses of all-*trans* retinoic acid (ATRA) further clarified the function of vitamin A (Means and Gudas, 1995). Vitamin A is ingested naturally with food containing retinyl esters or vitamin A precursors (Harrison, 2005, Yeum and Russell, 2002) and stored in the liver or directly transported to target cells (Blomhoff and Blomhoff, 2006). Retinol, the active form of vitamin A, is oxidized by alcohol dehydrogenases and retinal dehydrogenases *in vivo* to yield retinoic acid (RA) (Vivat-Hannah and Zusi, 2005). RAs exist in several isoforms but the predominant one is ATRA (Mic et al., 2003). These naturally occurring RAs belong to the group of so-called retinoids which also include synthetic compounds with structural or biological similarities to retinol (Uray et al., 2016). Retinoids can be divided into three subgroups (Fig. 1); 1st generation retinoids: natural or endogenous, including ATRA, 9-*cis* RA, and 13-*cis* RA (Vivat-Hannah and Zusi, 2005), 2nd generation retinoids: synthetic monoaromatic with acitretin and etretinate and 3rd generation retinoids: synthetic polyaromatic, including bexarotene, tazarotene and adapalene (Beckenbach et al., 2015).

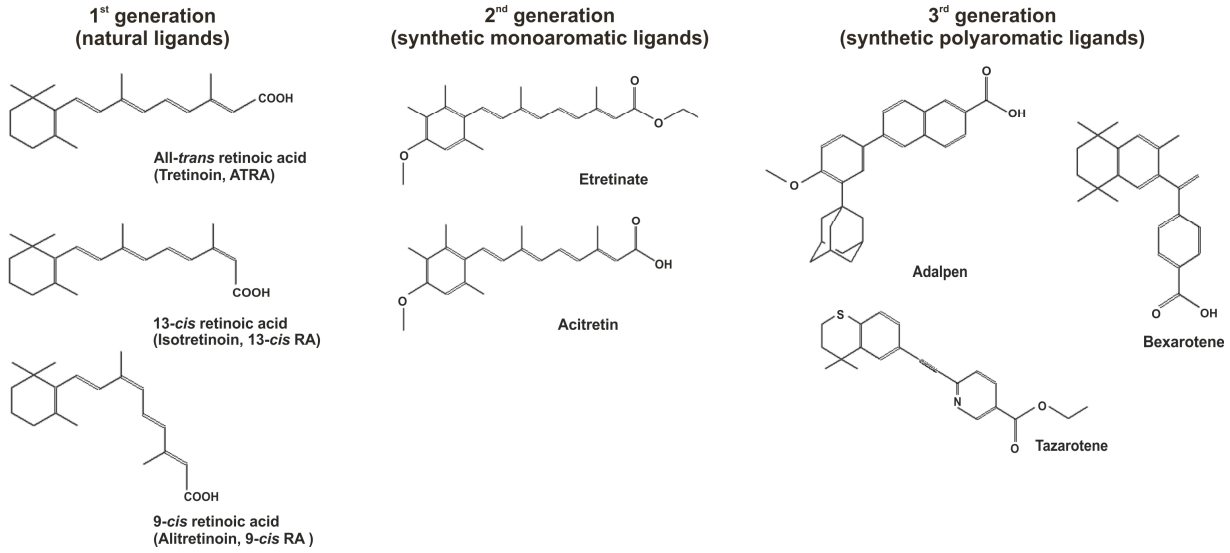

FIGURE 1 Examples for the different generations of retinoids. Natural (1st generation), synthetic monoaromatic (2nd generation) and synthetic polyaromatic specimens (3rd generation).

2.2 Retinoid biology

Retinoids regulate diverse processes including embryonic development, metabolism, immunity, inflammation, proliferation, differentiation, apoptosis, wound healing and keratinization (Nagy et al., 2012, Uray et al., 2016, Means and Gudas, 1995). Moreover, retinoids are known to have functions in both innate and adaptive immunity (Iwata, 2009a, Iwata, 2009b, Mora et al., 2003, Stephensen, 2001, Hall et al., 2011a, Hall et al., 2011b). The effects of retinoids are mediated by binding to two distinct nuclear receptor families: the retinoic acid receptor (RAR) and retinoid X receptor (RXR). Multiple isotypes (α, β and γ) and several isoforms for both RAR and RXR are known (Vivat-Hannah and Zusi, 2005). Both RA receptors bind to DNA predominantly as RAR-RXR heterodimers, which recognize specific short DNA sequences, the so called retinoic acid response elements (RARE) (Gronemeyer and Moras, 1995). The mechanisms underlying the regulation of gene expression by retinoids are different depending on the ligand status controlling transactivation or transrepression (Beckenbach et al., 2015). Transactivation is initiated by ligand binding, dimerization of the receptors, DNA binding and recruitment of coactivators (Dilworth and Chambon, 2001) (Fig. 2). In the transrepression state, the RAR-RXR heterodimer is bound to its ligand and complexed with corepressor proteins and associated factors which inhibit transcription

(Beckenbach et al., 2015). RARs are mainly targeted in cancer therapy and prevention while RXRs have been implicated in metabolic diseases (de Lera et al., 2007).

RARs have been demonstrated to have a comparable response to ATRA, 9-*cis* RA and a very weak response to 13-*cis* RA. RXR agonists show exclusive binding to RXR alone, which include bexarotene and CD3254 (de Lera et al., 2007, Nahoum et al., 2007). 9-*cis* RA is one of the known pan-receptor agonists, which binds with high affinity to both, RAR and RXR (Gronemeyer and Moras, 1995). This binding ability may be explained by the "L" shaped form of 9-*cis* RA (Fig. 1), fitting in both receptors (de Lera et al., 2007). The affinity of 9-*cis* RA for RXRs is about 5–10 fold and its transactivation potential approximately 10–30 times higher than for RARs (Allenby et al., 1993).

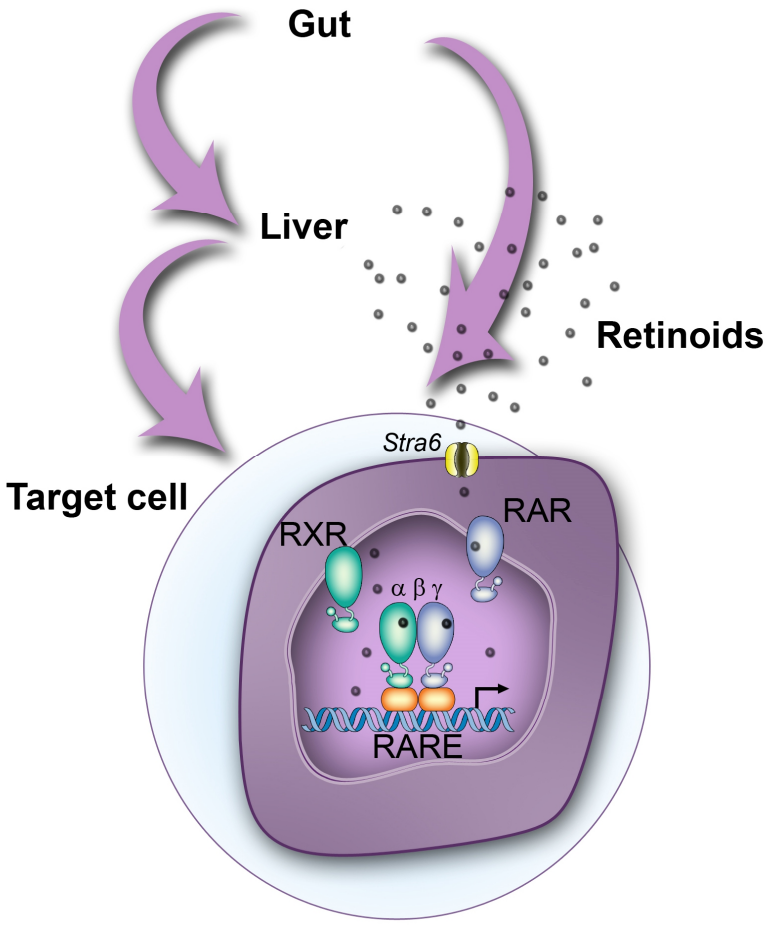

FIGURE 2 Retinoid-Signaling. Retinoids derived from diet are transported and stored in the liver or directly transported and imported into the target cells via retinol transporters (e.g. STRA6). Retinoids bind to their receptors: retinoic acid receptor (RAR) or retinoid X receptor (RXR). Receptors activate or block transcription at certain DNA binding sites, the retinoic acid response elements (RARE).

2.3 Retinoids in dermatology

Retinoids have not only been described as chemo-preventive and chemo-therapeutic agents, but they have also been recognized as regulators of skin functions by altering and controlling epidermal keratinization and proliferation (Dilworth and Chambon, 2001, Beckenbach et al., 2015). Since the 1960s, endogenous retinoids ATRA, 9-*cis* RA, and 13-*cis* RA are used in therapy of skin disorders, including severe acne, psoriasis, or ichthyosis (Orfanos et al., 1979, Kligman et al., 1969, Stuettgen, 1962). Later, synthetic retinoids with a higher safety potential were also introduced for therapy of pre-cancerous and cancerous skin disorders (Beckenbach et al., 2015), for cutaneous T cell lymphoma (bexarotene) or acne (adapalene) (Dilworth and Chambon, 2001). Treatment options include topical as well as systemic applications.

In topical treatment, endogenous retinoids 13-*cis* RA (RAR agonist), ATRA (RAR agonist) and 9-*cis* RA (RAR and RXR agonist) as well as adapalene (RAR agonist), tazarotene (RAR agonist) and bexarotene (RXR agonist) (Beckenbach et al., 2015) are used. 13-*cis* RA, ATRA and adapalene are used for the treatment of inflammatory and non-inflammatory acne (Beckenbach et al., 2015). 13-*cis* RA reduces sebum production, return follicular keratinization to a steady state and reduces bacterial colonization with *Propionibacterium acnes* (Beckenbach et al., 2015). Both, 13-*cis* RA and ATRA have an anti-inflammatory effect, which normalizes immoderate immune reactions via reduction of toll-like receptor 2 (TLR2) on antigen-presenting cells (APCs) (Dispenza et al., 2012, Liu et al., 2005). Further, topical ATRA is prescribed for the treatment of photoaging, post-inflammatory hyperpigmentation or melasma (Kang et al., 2009, Bulengo-Ransby et al., 1993). Tazarotene is in use to treat Psoriasis vulgaris (Beckenbach et al., 2015), whereas bexarotene is used for the treatment of stage IA–IB cutaneous T-cell lymphoma (Schadt, 2013). Finally, 9-*cis* RA, the third member of the endogenous retinoids, is used topically to treat Kaposi's sarcoma (Walmsley et al., 1999) and photoaging (Baumann et al., 2005).

Systemic retinoids include also 13-*cis* RA, 9-*cis* RA, bexarotene as well as the synthetic acitretin (RAR agonist) (Beckenbach et al., 2015). Systemic 13-*cis* RA is not only used for severe acne but also for the treatment of rosacea, where it reduces erythema, hypertrophy and papules and pustules (Erdogan et al., 1998, Kligman et al., 1969, Schaller and Belge, 2013, Reinholz et al., 2013). Additionally, it is used for pityriasis rubra pilaris (Beckenbach et al., 2015). Acitretin is used to treat severe therapy-resistant keratinization disorders like pus-

tular, palmoplantar, erythrodermic psoriasis or hyperkeratosis palmoplantaris (Beckenbach et al., 2015, Thestrup-Pedersen et al., 2001) as well as lichen ruber planus, Darier's disease, pityriasis rubra pilaris, and ichthyosis (Ormerod et al., 2010). Systemic bexarotene is effective in $CD30^+$ cutaneous T-Cell Lymphoma (Stadler et al., 2013). Oral 9-*cis* RA shows positive effects in pityriasis rubra pilaris (Pampin et al., 2013, Amann et al., 2015), Darier's disease (Letule et al., 2013, Barnstedt, 2012), palmoplantar pustular psoriasis (Irla et al., 2012), lichen planus (Brehmer et al., 2011, Alsenaid et al., 2014, Molin and Ruzicka, 2010), alopecia areata (Kolesnik et al., 2013), or cutaneous T-cell lymphoma (Molin and Ruzicka, 2009, Kolesnik et al., 2013) and inconsistent effects in congenital ichthyosis (Ganemo et al., 2012). In 1999, 38 patients with chronic hand eczema were treated with 9-*cis* RA demonstrating a response rate higher than 50%. Side effects were very mild (Bollag and Ott, 1999). Notably, a recent study by Ruzicka et al. demonstrating therapeutic efficacy of oral alitretinoin treatment of patients suffering from chronic hand eczema attracted significant interest. In 2008, a randomized double-blind, placebo-controlled, multicentered trial, including 1032 patients at 111 sites receiving 10 or 30 mg 9-*cis* RA daily for 12–24 weeks confirmed effectiveness of the treatment of chronic hand dermatitis in patients refractory to standard treatment. Clear or almost clear hands were achieved in up to 48% of 9-*cis* RA treated patients, compared with 17% for placebo pointing out a superior treatment effect. Up to 75% of the patients showed median reduction in initial disease signs and symptoms and an absence of eczema for 5.5 – 6.5 months. Treatment was well tolerated, but included adverse effects such as headache, mucocutaneous events, hyperlipidemia, and decreased free thyroxine and thyroid-stimulating hormones (Ruzicka et al., 2008).

Hand eczema is defined as a non-infectious eczematous skin inflammation restricted to the hands (Molin et al., 2009). Its prevalence and incidence can only be estimated because many who are affected do not seek medical attention (Elston et al., 2002). The clinic ranges from mild forms to severe skin changes (Molin et al., 2009). An estimated 6.4 to 14% in the general population is affected by hand eczema, severe hand eczema even ranges from 1.1 - 9.7% (Ruzicka et al., 2008, Crane et al., 2016). Hand eczema is characterized by signs of erythema, oedema, vesicles, papules, scaling, fissures, hyperkeratosis, as well as pain and itch (Diepgen et al., 2007). Patients suffering from hand eczema are very heterogeneous regarding disease manifestations and may be distinguished on the basis of the clinical phenotype, presence of allergies and causative environmental factors (Diepgen et al., 2009). A

combination of an endogenous predisposition with environmental triggers seems to be responsible for disease development in most cases. These endogenous factors include a T cell-mediated immune response against allergens that have penetrated the skin. Environmental aspects imply allergen exposure, cumulative toxic damage by irritants, or a combination of both. Skin naturally acts as a barrier to the exterior environment and protects the body from friction and impact wounds with its flexibility and toughness. Harmful chemicals, bacteria, viruses and ultraviolet light are prevented from entering the body by the skin. Further, skin regulates water loss and body temperature by blood flow and evaporation of sweat. Secretion of sweat and skin lipid cause the elimination of a number of harmful substances resulting from metabolic activities in the intestines and the liver. To protect the body from exterior factors keratinocytes differentiate from the basal layer (stratum basale) while migrating towards the stratum corneum (Candi et al., 2005). The major changes in keratinocytes appear in the stratum granulosum where keratohyalin granules, packed with proteins, and lamellar bodies containing lipids, are formed. Together with keratins, a group of intermediate filaments, the keratohyalin granules form aggregates within the keratinocytes and cause a collapse of the cell, thereby creating the typical corneocyte. During development, structural proteins, including involucrin, loricrin, trichohyalin, small proline-rich proteins (SPRRs) and late cornified envelope proteins (LCE), are produced and subsequently crosslinked by transglutaminases to form the cornified envelope beneath the plasma membrane. These insoluble protein structures later replace the plasma membrane forming a mechanical and a water barrier (Wickett and Visscher, 2006, Candi et al., 2005). It was reported that mutations and/or changes in expression of proteins forming the cornified envelope can cause a barrier dysfunction or defect (Elias and Wakefield, 2014) and lead to several skin disorders (Ovaere et al., 2009, Harding et al., 2003, Feldman et al., 2005, Mahler, 2016, Liang et al., 2015, de Koning et al., 2012). Molin and colleagues identified 185 differentially expressed proteins in CHE samples compared to healthy controls, including barrier genes and antimicrobial peptides (AMPs) (Molin et al., 2015). Interestingly, it was recently demonstrated that barrier genes were up-regulated in skin from CHE patients during systemic 9-*cis* RA-treatment (Kumari et al., 2016). Beside the physical barrier of corneocytes AMPs build the second line of protection. These host defense molecules show a broad antibacterial, antiviral and antifungal activity by binding to the surface of invading pathogens and forming pores to perish their membranes. AMPs are produced by epithelial and immune cells, including keratinocytes,

neutrophils and macrophages. There are two major groups of AMPs: cathelicidins and defensins (Schauber and Gallo, 2008). In case of an acute infection, AMPs can cause pro-inflammatory responses by directly attracting leukocytes or indirectly stimulate cells to produce chemokines. Further, antimicrobial peptides can induce reactive oxygen species and can tribute to phagocytosis (Agier and Brzezinska-Blaszczyk, 2016, Rashid et al., 2016, Hemshekhar et al., 2016). The third component of skin protection are cells of the innate immunity and their components. Skin epithelium, which is composed mainly of keratinocytes, is also interspersed with cells of the innate and adaptive immune system that are highly committed to host defense: dendritic cells, melanocytes, rare T lymphocytes and monocytes. Physical, chemical, or immune-specific insults rapidly evoke an epidermal response characterized by the increased expression of a system of proinflammatory mediators, including chemotactic factors, which initiate the orientated migration of distinct leukocyte subpopulations. In turn, activated monocytes, dendritic cells, and T cells release potent cytokines that act on cells in the local environment to boost the inflammatory response. In particular, tumor necrosis factor alpha (TNF-α) induces the expression of numerous chemokines, including CC-chemokine ligand (CCL)2, CCL5, CCL20 and CCL27 in keratinocytes.

Chemokines belong to a superfamily of cytokine-like proteins, ranging from 7-15 kDa. They can be subdivided into smaller groups due to the relative position of Cysteine (C) residues, subsequently following or separated by other amino acids (X) at the NH_2 terminus of the protein. The superfamily of chemokines consists of two major classes, called CC- and CXC-chemokines and two minor classes, the XC- and the CX_3C-chemokines. To date, more than 40 chemokines have been identified in humans and mice. Chemokines control cellular trafficking under steady state as well as under inflammatory conditions. Moreover, chemokines can directly activate and initiate differentiation of lymphocytes. These functions are carried out by binding to their receptors, which belong to the γ-superfamily of rhodopsin-like-seven-transmembrane receptors. The differentially expressed receptors signal either through G-proteins, which represent the common chemokine receptor or in a G-protein independent manner, so called atypical chemokine receptors. In total, more than 20 chemokine receptors have been characterized, including 5 atypical receptors, and are classified by their bind to CC-, CXC-, XC- and CX_3C-chemokines. Further, some chemokine receptors are able to bind to several chemokines, whereas others only bind one chemokine (Zlotnik et al., 2006, Griffith et al., 2014, Koelink et al., 2012, Homey et al., 2002c).

Disruption of the skin barrier, cellular and epidermal changes stimulates skin resident-tial cells to release various cytokines including pro-inflammatory cytokines Interleukin-1 β (IL-1β) or TNF-α, chemokines (i.e. CCL13, CCL17, CCL18, CCL20 and CCL27) and AMPs (Spiekstra et al., 2005, Cipriani et al., 2014, Homey et al., 2007). In inflammatory skin diseases, like atopic dermatitis (AD), several chemokines have been demonstrated to be elevated in serum of affected patients (Kakinuma et al., 2003, Shimada et al., 2004). Epidermal chemokines are involved in the early response to injury or irritants, and in T cell-mediated skin disorders, and control the recruitment of monocytes/macrophages, dendritic cells, and T cells to lesional sites of eczema (Hanifin and Rajka, 1980). Active immigration of T cells, monocytes, as well as neutrophils is also supported by the increased expression of CCL5. However, type 1 T cells are attracted into the skin by keratinocyte release of Interferon gamma (IFN-γ) induced CXCR3 ligands: CXCL9, CXCL10, and CXCL11. These CXCR3 ligands appear deeply involved in type 1 T cell-mediated diseases such as allergic contact dermatitis and psoriasis but are not relevantly expressed during skin response to irritants (Meller et al., 2007).

According to current concepts, an impaired skin barrier function may be essential for the development of both allergic and irritant contact dermatitis of the hands (Molin et al., 2009, Proksch et al., 2006, Spiekstra et al., 2005). Notably, hand eczema is more common in persons with a history of AD or nickel allergy (Mortz et al., 2001). Hand eczema can be a result of several individual factors or a combination of factors. Eczematous skin diseases, including AD, contact dermatitis or CHE, are characterized by similar pathophysiological concepts (Sampson, 1990). Correspondingly, AD is often utilized as a model for this class of dermatoses (Incorvaia et al., 2008). Only few details of the molecular pathogenesis of the development of chronic hand eczema (CHE) are known. Models like atopic dermatitis, allergic and irritant contact dermatitis may help to understand the mechanism, but some individuals develop CHE independent from trigger factors. Also, atopic risk factors do not always cause eczema. A series of events may lead to the loss of epidermal barrier function, inflammation and eczematization. Atopic eczema arises from an interplay between different players, including genetic, immunological and environmental factors. Genetic defects often cause barrier dysfunction or disruption. These genetic defects can lead to the penetration of antigens (environmental, bacterial, viral) and promote the activation of the innate immune system resulting in an up-regulation of several pro-inflammatory cytokines and chemokines (Cipriani

et al., 2014, Homey et al., 2007). These chemokines and cytokines activate and direct the recruitment of different subsets of leukocytes, including monocytes, dendritic cells (DCs) and circulating memory T cells, to skin lesions. Resident or recruited antigen presenting cells (APCs), activated at site of inflammation, have to migrate to the lymph node and present the antigens to T cells (Park and Kupper, 2015). To stimulate an effective T cells response, an adequate expression of specific co-stimulatory/ adhesions molecules is critical: The B7 family members B7-1 (CD80) and B7-2 (CD86) together with the costimulatory receptor CD28 have been identified as effective T cells activators (Boussiotis et al., 1996). Activated T cells are directed to their target sites by APCs. This process is called “homing” (Clark, 2010) and is seen in many chronically relapsing inflammatory diseases, e.g. psoriasis and allergic-contact dermatitis (Martin et al., 2011). Recruitment of T cells is also very important in the etiopathology of hand eczema and “skin-homing” plays a crucial role throughout the entire pathogenesis of inflammatory skin diseases. Several more homing markers have been discovered. For example effector T cells which have encountered antigens before, become memory T cells and patrol the periphery after clearance of antigens (Michalek and Rathmell, 2010). These memory T cells are key players in the genesis of cutaneous inflammatory responses and provide a high protective immunity. They can be broadly divided into central memory, effector memory and tissue resident subsets (Park and Kupper, 2015), which are endowed with different capacities to home to lymphoid or non-lymphoid tissues, to proliferate in response to antigen or cytokines and to perform effector functions (Lanzavecchia and Sallusto, 2005). $CD4^+$ and $CD8^+$ central memory T (T_{CM}) cells preferentially migrate to draining lymph nodes, dependent on the expression of CD62L (L-selectin) and CCR7, two homing molecules required for T cells to cross high endothelial venules and to enter the lymph node from the bloodstream (Martin-Fontecha et al., 2008, Lefrancois, 2006). In addition, $CD62L^+CCR7^+$ T cells with following markers: CD45RA CD27, CD28, IL-7RA are called stem memory T cells. These cells have also encountered and responded to antigens previously (Soler et al., 2006, Gattinoni et al., 2011). Nevertheless, the lymph node homing marker CCR7 is also found on naïve T cells and is not altered upon differentiation into skin- or gut-homing T cells (Campbell et al., 2001). Only upon antigen encounter and turn over from central memory T cells into effector memory T cells CCR7 expression is lost (Klebanoff et al., 2006). Almost all $CCR7^+$ CD8 positive T cells are naïve T cells, only a small part shows an effector-phenotype (Campbell et al., 2001). Apart from T cells, CCR7 is also differentially ex-

pressed on the surface of lymphocytes and dendritic cells, depending of their stage of differentiation and activation, allowing these cells to change their homing capacity and prospective traffic routes. Another homing marker is the cutaneous leucocyte-associated antigen (CLA) which is present on a skin-associated population of memory T cells, migrating into normal (Bos et al., 1993) and inflamed skin (Santamaria et al., 1995, Picker et al., 1990). CLA binds to endothelial-cell selectin (E-selectin) and platelet selectin (P-selectin). Both are present on non-inflamed dermal microvessels, but expression becomes markedly increased during cutaneous inflammation to attract CD4 and CD8 positive T cells (Agace, 2006). An additional homing marker is CCR9. $CCR9^+$ T cells, which also express the integrin $\alpha_4\beta_7$, preferentially home into the intestine (Papadakis et al., 2003). T cells presented with antigen-pulsed dendritic cells from mesenteric lymph nodes express CCR9 and high levels of $\alpha_4\beta_7$ (Svensson et al., 2008).

To better understand the pathogenesis of relapsing inflammatory skin diseases and the process of homing the next part will focus on some details: The initial phase of relapsing inflammatory diseases, like AD, is characterized by the polarization of naïve T cells into T_H2 cells (Weidinger and Novak, 2016). In the acute inflammatory phase, T_H2-type cytokines IL-4, IL-13 and IL-25 are produced and overexpressed in lesional skin (Cipriani et al., 2014) and many allergic reactions are based on the release of these cytokines (Ueno et al., 2007). Simultaneously, T_H2 cytokines also orchestrate the clearance of extracellular pathogens and inhibit the T_H1 responses (Sallusto et al., 2000). The chemokine receptor repertoire of T_H2 cells includes CCR3, CCR4 and CCR8, as well as the chemo-attractant receptor homologous molecule (CRTh2) (Bettelli et al., 2008, Sallusto et al., 2000). Among these, CCR4 is important for regulating immune balance (Ishida and Ueda, 2006). Its ligand CCL22 was shown to be increased in serum of patients with atopic dermatitis or contact dermatitis and correlated with disease-severity (Shimada et al., 2004). CCR4 is also expressed on the most skin-homing CLA-positive T cells in the circulation and on lymphocytes directly isolated from skin (Soler et al., 2003). The percentage of $CD4^+CCR4^+$ T cells in atopic individuals is preferentially higher as in control subjects, whereas CXCR3 positive T cells are negatively correlated to the severity of AD (Nakatani et al., 2001, Yamamoto et al., 2000). CCR8 bearing T cells have been associated with T_H2 responses and can be attracted by CCL8 (Debes and Diehl, 2011, Islam et al., 2011). Increased $CCR8^+$ cell counts have been found in allergic asthma and atopic dermatitis. Here, they may play a role in the induction and amplification phase of inflammatory responses.

Interestingly, CCR8 positive T cells are also found in healthy skin and may control the balance of effector and regulatory cells (Soler et al., 2006). Moreover, skin-infiltrating lymphocytes in patients diagnosed with psoriasis, atopic dermatitis or allergic-contact dermatitis often express CCR10, also a marker for T_H22 in addition to CCR4 and CCR6 (Yssel and Pene, 2009). Furthermore, CCL27 expression of epidermal basal keratinocytes is directing the recruitment of $CCR10^+$ lymphocytes (Homey et al., 2002a). CCL27 is also increased in serum of patients suffering from inflammatory skin diseases like AD or psoriasis vulgaris (Kakinuma et al., 2003). Both CCR4 and CCR10 are required for the localization of CD4 effector T cells to the site of delayed-type hypersensitivity-induced skin inflammation (Homey et al., 2002b, Reiss et al., 2001). Beside CD4 positive effector cells, recent studies showed $CD8^+$ cells as a main effector T cells in allergic contact dermatitis (Vocanson et al., 2009, Akiba et al., 2002).

Further, AD patients show infiltration of T_H17 and T_H22 cells in its initial phase (Weidinger and Novak, 2016). T_H17 cells produce a distinct set of cytokines including IL-17A, IL-17F and IL-22. T_H17 play an important role in host defense against specific pathogens and have been promoted as a potent inducer of autoimmunity and tissue inflammation (Bettelli et al., 2008). Interleukin-6 (IL-6), transforming growth factor beta (TGF-β) and the transcription factor Retinoid-related orphan receptor γ t (RORγt) induce T_H17 cell differentiation in naïve T cells. A T_H17 response can also be promoted by IL-23. Singh et al. proposed that up-regulation and stable expression of CCR6 is a fundamental feature of T_H17 differentiation (Singh et al., 2008). Moreover, CCR6 is highly expressed on CLA positive memory T cells and up-regulated in psoriasis (Homey et al., 2000). Next to CCR6, human T_H17 cells co-express CCR4 (Acosta-Rodriguez et al., 2007, Zhu and Paul, 2008). Notably, CCR6 is also expressed by most B cells, subsets of CD4 and CD8 memory T cells, and subsets of dendritic cells (DCs) and plays an important role in directing the trafficking of activated T cells into the skin (Paradis et al., 2008). Further, in maturing dendritic cells CCR6 is down-regulated, while CCR7 is up-regulated (Sallusto et al., 2000). Psoriasis shows a marked up-regulation of CCR6 together with its exclusive ligand CCL20 demonstrating that human tissue-infiltrating T_H17 cells not only express CCR6 but also produce CCL20 (Homey et al., 2000). These results suggest that these cells create a particular inflammatory environment favoring their own migration and sequestration which cause the maintenance of chronic inflammatory disease (Pene et al., 2008). Interestingly, Koga et al. demonstrated that the percentage of T_H17 cells in peripheral blood of AD patients is increased and associated with the severity of AD. In the same study,

it was revealed that IL-17$^+$ cells infiltrated acute lesions of the papillary dermis of atopic eczema compared to chronic lesions (Koga et al., 2008).

The late, chronic phase of AD is characterized by a switch from the T_H2- to a T_H1-dominance and hence the production of T_H1 related cytokines, like INF-γ (Avgerinou et al., 2008). Nevertheless, T_H2 cells are still present, whereas T_H17 and T_H22 are almost vanished (Weidinger and Novak, 2016). IFN-γ activates macrophages, NK cells, and CD8$^+$ T-cells. Moreover, T_H1 cytokines induce the secretion of opsonizing antibody isotypes in B-cells, additionally enhancing antigen uptake and -presentation to T-cells and mediating protection against intracellular pathogens. Notably, the chemokine receptors CXCR3 and CCR5 show a striking preferential expression on T_H1 cells as opposed to T_H2 cells (Zhu and Paul, 2008, Bettelli et al., 2008). Expression of the IFN-γ-induced CXCR3 ligands CXCL9, CXCL10 and CXCL11 as in allergic contact dermatitis (ACD), lead to a preferential recruitment of T_H1 cells into lesional skin (Moed et al., 2004). Furthermore, CXCL9 was demonstrated to be significantly elevated in serum of patients with atopic dermatitis and contact dermatitis. Protein levels are positively correlated with disease severity (Shimada et al., 2004). Finally, expression of CXCL9 and CXCL10, were up-regulated in chemical-induced allergic skin responses when compared with irritant skin responses (Meller et al., 2007).

There is one more subtype of T cells, which have been shown to play a critical role in controlling inflammatory skin diseases: T_{regs}. Two main groups of T_{reg} cells have been identified so far. The first group is determined by their CD4$^+$ CD25$^+$ Forkhead Winged-Helix Transcriptional Factor Box p3 (Foxp3$^+$) phenotype and is also referred to as "natural T_{reg} cells", with Foxp3 being the most definitive marker (McGee and Agrawal, 2006). Natural T_{reg} cells are important in preventing inflammation and mediation of self-tolerance. They are normally generated in the thymus upon contact with self-Ag or are induced in the peripheral immune compartment. TGF-β promotes the conversion of CD4$^+$ naïve T cells into Foxp3$^+$ cells. Interestingly, retinoids have been recently shown to increase Foxp3$^+$ cells and inhibit the development of T_H17 cells by enhancing TGF-β-driven Smad3 signaling and inhibiting IL-6 and IL-21 receptor expression (Xiao et al., 2008). T-regulatory type 1 (Tr1) cells represent the second group of T_{regs} and are characterized by their secretion of high levels of IL-10 with or without TGF-β (Verhagen et al., 2006). It was found that human Tr1 cells, IL-10 and TGF-β, as well as receptors for these cytokines were significantly expressed in lesional and in atopy patch test atopic dermatitis or psoriasis skin (Verhagen et al., 2006).

2.4 Retinoids in immunity

Beside structural cells, retinoids have profound and diverse effects on the immune system depending on cell type, body compartment and cytokine milieu. Both monocytes/ dendritic cells (DCs) and T cells have been reported as targets of retinoids. Dendritic cells have an important role in the host defense network of the skin. These cells display antigens to T and B cells and thereby initiate adaptive immune responses. Immature dendritic cells (iDCs) act as a guardian which can sense pathogens directly or indirectly (Pulendran et al., 2001). DCs are found in peripheral tissues, lymphoid organs but also in the blood circulation. Upon activation or antigen encounter DCs mature. This maturation process is accompanied with changes in morphology, cytoskeleton structure, the loss of endo- and phagocytic molecules, secretion pattern of chemokines and cytokines, the up-regulation of co-stimulatory molecules such as CD80, CD86 and CD40, as well as other maturation markers like CD83. Moreover, major histocompatibility complex (MHC) II class molecules are translocated to the cell surface and the expression of chemokine receptors are altered (Ueno et al., 2007). For instance in maturing dendritic cells, CCR6 is down-regulated, while CCR7 is up-regulated (Sallusto et al., 2000). The micromilieu is very critical for the maturation of dendritic cells. Different cell types produce different cytokines, which control the maturation and thereby the development of the dendritic cell subtype. For example, keratinocytes in allergic skin release interleukin (IL)-15, Granulocyte-macrophage colony-stimulating factor (GM-CSF) and thymic stromal lymphopoietin (TSLP) (Ueno et al., 2007).

Activated mature DCs (mDCs) migrate to the draining lymph organs and interact with lymphocytes by B7-family members, MHC II class molecules, Tumor necrosis factor (TNF) family members and cytokines (Vocanson et al., 2009, Ueno et al., 2007). IL-12 is produced by DCs and controls the differentiation into T_H1 cells (Heufler et al., 1996). DC-secreted IL-23 promotes a differentiation of T cells into a T_H17 type (Langrish et al., 2005). The molecules of the B7 family are important for the activation of T cells and can either induce immunity or tolerance. This B7 family consists of 7 members: CD80, CD86, inducible costimulator ligand (ICOSL), programmed death ligand 1 (PD-L1; B7-H1), PD-L2 (B7-DC), B7-H3, and B7-H4, which are all expressed on dendritic cells (Greenwald et al., 2005). Most common known markers are CD80 and CD86, which bind to CD28 or cytotoxic T-lymphocyte antigen-4 (CTLA-4) on T cells. Binding to CD28 signals the T cell to become an effector cell, but CTLA-4 has an inhibitory effect (Caux et al., 1996, Ueno et al., 2007). PD-L1 and PD-L2 seem to play a role in in-

ducing tolerance, binding to PD-1 delivers an inhibitory signal. PD-L1 can be found on immune cells (Greenwald et al., 2005, Freeman et al., 2000). Regarding the TNF family members, one notable interaction is important. The CD40/CD40L complex activates DCs, leading to the up-regulation of CD80 and CD86 and the release of IL-12 and the expression of OX40L, which in turn promote a T cell differentiation into T_H2-type cells (Caux et al., 1994, Soumelis et al., 2002, Flynn et al., 1998, Coquerelle and Moser, 2010).

There are two major types of dendritic cells, the myeloid DCs, which express CD11c, CD13, CD33 and CD11b but lack CD14 or CD16 and the plasmacytoid DCs (pDC) with the following markers: CD123, CD303 and CD304. Moreover, they can be divided by location. During inflammation, a vast majority of dendritic cells migrate into the sites of inflammation. These cells include granulocytes, pDCs and classical $CD14^+$ monocytes. These monocytes are a potential source of inflammatory DCs or monocyte-derived dendritic cells (MoDCs). To generate functional DCs from $CD14^+$ monocytes, they are treated with Granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 (Collin et al., 2013, Pickl et al., 1996). To fully mature these DCs, a wide range of cytokines and molecules can be used, generating potent stimulators of $CD4^+$ and $CD8^+$ T cells and producers of cytokines, like IL-1, -6, -12, -23 and TNF-α (Castiello et al., 2011). Also, TNF-α itself, as part of the innate immunity and a primary cytokine responsible for inflammatory responses in skin, has the potential to induce maturation and migration of DCs (Sallusto and Lanzavecchia, 1994, Yarosh et al., 2000, Lapteva et al., 2001). Additionally, in allergy T cells are differentiated into T_H2-type cells by dendritic cells in inflammatory lesions (Huh et al., 2003, van Rijt et al., 2005, Lambrecht and Hammad, 2003). These dendritic cells produce IL-4, -5, -13 and TNF-α under skin-derived factors like TSLP, while T_H1 cytokine IFN-γ and anti-inflammatory IL-10 are repressed (Soumelis et al., 2002). Skin resident dendritic cells respond to CCL20 (Vanbervliet et al., 2002) and can induce the differentiation of naïve $CD4^+$T cells into T_H1 (IFN-γ) or T_H2 (IL-4, -5, -13) type T cells (Ueno et al., 2007). To direct T cells to the inflammation site, DCs have the capability to instruct them due to their own originating site. DCs of the Peyer‘s patches or the mesenteric lymph node can direct $CD8^+$ T cells into the gut by induction of $\alpha4\beta7$ integrin and CCR9, a receptor for the gut-associated CCL25, on T cells. This mechanism depends partly on retinoids (Mora et al., 2003, Iwata et al., 2004, Iwata, 2009a). Another example is the expression of 1,25-dihydroxyvitamin D_3 in UVB exposed epidermis, which in-

duces CCR10 on T cells, the receptor of keratinocyte-derived CCL27 (Sigmundsdottir et al., 2007).

Dendritic cells also have an important role in immunosupression (Steinman et al., 2003). Peripheral DCs seem to play a part in this process. Immature dendritic cells, which have not been activated yet, are believed to present self-antigens to autoreactive T cells and induce anergy or deletion in the absence of co-stimulatory molecules (Steinman et al., 2000). Further, there are hints that also mature dendritic cells have immunosuppressive potential, i.e. expansion of regulatory T cells (T_{regs}) (Kretschmer et al., 2005, Banerjee et al., 2006, Yamazaki et al., 2003). Moreover, immunosuppressive DCs may actively sustain tolerance by promoting regulatory or suppressor T cells (Moser, 2003, Battaglia et al., 2004, Sakaguchi, 2005). Immunomodulatory or tolerogenic DCs have no specific marker yet, but the leukocyte immunoglobulin-like receptor (LIR) family might be a common receptor. Leukocyte immunoglobulin-like receptor subfamily B member 4 (LILRB4/ ILT3) for instance is expressed upon IL-10 exposure to dendritic cells (McGuirk et al., 2002, Manavalan et al., 2003). In addition, IL-10 from tumors alters differentiation and maturation of dendritic cells and induce a tolerogenic phenotype. The resulting DCs induce antigen-specific anergy (Steinbrink et al., 1997, Enk and Katz, 1992, Enk et al., 1997, Steinbrink et al., 1999)

Retinoids, mostly ATRA, seem to have a general inhibitory effect on production of cytokines that favor T_H1- type T cell generation on monocytes and macrophages, whereas T_H2-generating cytokines are enhanced in response to retinoids (Pino-Lagos et al., 2008). As an example, activated macrophages produce pro-inflammatory cytokine TNF-α, whereas ATRA blocks the transcription of TNF in LPS-activated macrophages (Mehta et al., 1994). In cord blood cells and the monocytic cell line THP-1, RA enhances IL-10 and similarly inhibits pro-inflammatory TNF-α and IL-12 (Kang et al., 2000). Another group could demonstrate the same effect in retinoid-pre-treated and activated mouse macrophages *in vitro* and *in vivo*. These macrophages were then used as APCs together with primed $CD4^+$ T cells and enhanced the T cell production of IL-4 and while INF-γ was reduced (Kim et al., 2004, Kang et al., 2007). The effects of retinoids on DCs are various. In immature DCs retinoids induce apoptosis, while this is not the case in mature DCs while inflammatory signals (TNF-α and IL-1β) are present (Geissmann et al., 2003). Further, retinoid treatment induced the differentiation of immature DCs into mature DCs accompanied by up-regulation of MHCII, costimulatory molecules and enhanced allogeneic antigen-specific T cell proliferation (Geissmann et al.,

2003). Additionally, ATRA and GM-CSF differentiated monocytes into dendritic-like cells, with a DC morphology and a phenotype of immature DCs. This included the expression of CD1a, the up-regulation of adhesion and co-stimulatory molecules as well as a secretion of IL-12. Further, ATRA-DCs could induce a proliferation in naïve $CD4^+$ T cells accompanied by a differentiation into T_H1-type T cells with IFN-γ secretion (Mohty et al., 2003). Opposingly, Zapata-Gonzalez and colleagues reported that retinoids have a rather inhibitory effect on the expression of CD80 and CD86 on LPS-matured DCs. Additionally, LPS-matured 9-*cis* RA treated DCs also reduced allogeneic T cell proliferation (Zapata-Gonzalez et al., 2007). Furthermore, retinoids induce a DC phenotype which enhances gut-homing in T cells by inducing CCR9 and $\alpha_4\beta_7$ integrin on their surface. These T cells migrate then into the intestinal compartment (Johansson-Lindbom et al., 2003, Johansson-Lindbom et al., 2005, Mora et al., 2003, Iwata et al., 2004). In gut associated DCs, retinoids induce CD103 expression and allow these cells to produce RA themselves (Bakdash et al., 2015). ATRA-treated DCs are potent stimulators of T cell proliferation, although there was no evidence of enhanced CD80, CD86 or MHCII expression (Saurer et al., 2007). Darmanin and colleagues could show enhanced lymph node migratory properties of ATRA-treated bone marrow derived DCs (BMDCs) in mice resulting from an increased expression and secretion of matrix metalloproteinases (MMPs) and a reduced expression of tissue inhibitors of metalloproteinases (TIMPs) (Darmanin et al., 2007). Patients with metastatic kidney cancer undergoing an ATRA treatment had reduced cell counts for immature myeloid-suppressive cells (APCs supporting the impairment of immunity), but no change in white blood cells. Further, ATRA-treated patients showed decreased myeloid DC proportions, which contributed to the tolerogenic microenvironment in tumors. Additionally, DCs derived from these patients had a pronounced stimulatory effect on T cell proliferation compared to DCs isolated from untreated cancer patients. Stimulated T cells responded with a release of T_H1 cytokines and lesser T_H2 cytokines secretion (Bronte et al., 2001, Melani et al., 2003, Pino-Lagos et al., 2008, Mirza et al., 2006).

Effects of retinoids on T cells have been described *in vitro* and *in vivo* with impacts on ontogeny, function and differentiation (Pino-Lagos et al., 2008). In immature T cells ($CD4^+CD8^+$ thymocytes), ATRA and 9-*cis* RA induced proliferation by inhibition of apoptosis via RARα activation (Szondy et al., 1998a, Szondy et al., 1998b). 9-*cis* RA was more effective than ATRA and the authors speculated about an involvement of RXR in this process (Szondy et al., 1998b). *In vitro* experiments demonstrated that addition of ATRA to immature T cells

reduce the number of $CD8^+$ maturing T cells, while $CD4^+$ T cell count is increased (Zhou et al., 2008). This is further supported by the observation that children with vitamin A supplementation had increased $CD4^+$ T cell numbers in the periphery (Pino-Lagos et al., 2008). Generally, retinoids have vast effects on the different T cell subtypes T_H1, T_H2, T_H17 and T_{reg} (Fig. 3). Vitamin A-deficient mice showed decreased T helper populations and thereby an impaired immunity, including delayed type hypersensitivity responses and antibody responses (Smith et al., 1987, Carman and Hayes, 1991, Carman et al., 1989). T cells of vitamin A-deficient mice produced high amounts of IFN-γ and APCs from these mice induced an IFN-γ response in T cells. RA supplementation of these vitamin A-deficient mice antagonized this process (Stephensen et al., 2002). The authors therefore speculated that retinoids down-regulate T_H1 activity, reduce APC function and induce T_H2 T cell growth and differentiation (Stephensen et al., 2002). Supporting to this, addition of retinoids to T_H1-favoring conditions to T cells blocked T_H1 development while RA enhanced T_H2 development under T_H2 polarizing conditions demonstrating a direct effect of retinoids on the T_H1/T_H2 balance (Cantorna et al., 1994, Cantorna et al., 1995). Iwata et al., demonstrated that ATRA, 9-*cis*-RA and the RAR agonists, but not RXR agonists, suppressed T_H1 development and enhanced T_H2 development (Iwata et al., 2003). In addition to T_H1/T_H2, T_H17 cells with its signature transcription factor orphan nuclear receptor RORγt can also be affected by retinoids (Ivanov et al., 2006, Pino-Lagos et al., 2008). T_H17 cell differentiation is inhibited by retinoids due to their ability to repress RORγt *in vitro* and *in vivo* (Mucida et al., 2007, Mora et al., 2006). Moreover, a study by Xiao et al. demonstrated that retinoids inhibited the development of T_H17 cells by interfering with signaling events in the transforming growth factor beta (TGF-β) and IL-6/IL-21/IL-23 pathways both *in vitro* and *in vivo* (Xiao et al., 2008). Retinoids also seem to be an important cofactor for the generation of regulatory T cells (T_{reg}) (Xiao et al., 2008, Sun et al., 2007). They induce the gut-homing integrins α4β7 allowing regulatory T cells to migrate and exert their suppressive effects in the gut (Pino-Lagos et al., 2008). Adaptive T_{reg} can be generated in the periphery from mature $CD4^+CD25^-$ T cells under the influence of immature dendritic cells or specific DC subsets (Pino-Lagos et al., 2008). Retinoids enhance the expression of forkhead box p3 transcription factor (Foxp3) (Mucida et al., 2007, Schambach et al., 2007, Coombes et al., 2007, Benson et al., 2007) together with other factors like TGF-β (Walker et al., 2003, Zheng et al., 2004a), IL-2 and/ or IL-10 (Horwitz et al., 2003, Zheng et al.,

2004b) or in situations of specific antigenic presentation (Hoyne et al., 2001, Jonuleit et al., 2000, Apostolou et al., 2002, Horwitz et al., 2003).

Beside their influence on T cell differentiation, retinoids also have an impact on the homing abilities of T cells, namely the induction of CCR9 and $\alpha_4\beta_7$ as described above due to conditioned DCs from the mesenteric lymph nodes and the Peyer's patches (Mora et al., 2003). Iwata *et al*. demonstrated a suppressive effect of ATRA on skin-homing receptors E-lig, P-lig (CLA), CCR4 and CD62L on cultured cells *in vitro* in untreated conditions and under T_H1 conditions. 9-*cis* RA treatment reduced homing marker E-lig *in vitro* (Iwata et al., 2004). It was not observed that retinoid treatment alters the expression of CCR10 on T cells (Sigmundsdottir et al., 2007). Further, ATRA decreased the CLA expression of $CD3^+CLA^+$ T cells *ex vivo*. The authors also found out that CCR10 is down-regulated, CCR9 is up-regulated and CCR4, CCR6 and CD62L are not affected by ATRA (Yamanaka et al., 2008). In mice with a oxazolone-induced contact hypersensitivity response, the number of $CD4^+$ cells which migrated into the inflamed skin were significantly reduced upon ATRA treatment. The authors stated that retinoid treatment diminished the CLA expression in three atopic dermatitis patients in comparison to controls but data was not shown (Yamanaka et al., 2008). Finally, 13-*cis* RA treatment reduced infiltrating T cells in patients with chronic/ subacute cutaneous lupus erythematosus (Newton et al., 1986, Kim, 2013).

In summary, retinoids modulate the T_H1/T_H2 balance, reduce T_H17 and induce T_{reg} differentiation and also modulate the migration of T cells into the gut and to inflammatory sites (Fig. 3).

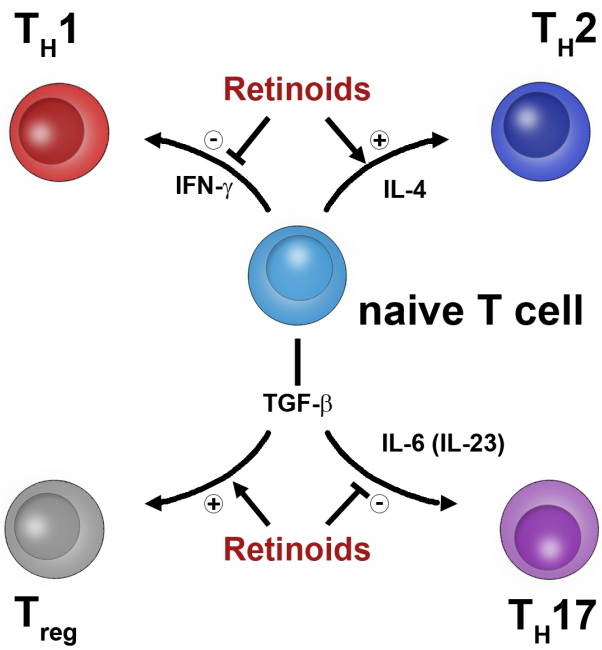

FIGURE 3 Retinoid effect on T cell differentiation. Retinoids participate in differentiation of naïve T cells in helper T cells (T_H1, T_H2, T_H17) or regulatory T cells (T_{reg}).

3 Aim of this thesis

Most studies using all-*trans* retinoic acid as a model-substance for their studies on the molecular effects of retinoic acids. However, as 9-*cis* RA is not only binding to the retinoic-acid receptor (RAR) but also to the Retinoid-X receptor (RXR) it has an exceptional position among all RAs. 9-*cis* RA is effective during the treatment of chronic hand eczema; still, the underlying effects on the skin immune system remain largely unknown. This thesis evaluates the *in vivo* effect of a 9-*cis* RA treatment to provide data for further *in vitro* analyses. Based on this, keratinocytes and dendritic cells were treated with 9-*cis* RA compared to acitretin, a synthetic retinoid only binding to RAR. The aim of the present study was to unravel the underlying effects on the immune system by addressing the following questions:

- What are the effects of 9-*cis* RA on structural cells (i.e. keratinocytes) of the skin? How does 9-*cis* RA alter the gene expression and cytokine release of these cells?
- Which leukocyte subsets are major targets of 9-*cis* RA? What are the phenotypic and functional consequences?
- What are the effects of 9-*cis* RA on circulating human T cell subsets and antigen presenting cells *in vivo*?

4 Material & Methods

4.1 Buffers and solutions

PBS (phosphate buffer saline), 20 x	160 g NaCl 4 g KCl 28.8 g Na_2HPO_4 + 2 H_2O 4.8 g KH_2PO_4	
	ad 1000 ml ddH_2O	adjust to pH 7.4
Ammonium chloride lysis buffer	8.29 g NH_4Cl 1 g $KHCO_3$ 0.0375 g Na_2-EDTA	
	ad 1000 ml ddH_2O	adjust to pH 7.4

Chemicals were from Sigma-Aldrich, Saint Louis, MO, USA except as noted.

4.2 Patient specimens and cell culture

4.2.1 Human skin specimens, blood, serum samples and primary cells

In this basic science study, diagnostic skin specimens, PBMCs and serum samples from chronic hand eczema patients (n=29) that were obtained during routine monitoring were used for further analyses after securing informed consent. During routine monitoring a small amount of blood was taken for the extraction of peripheral blood mononuclear cells (PBMCs) and serum. Skin specimens not further used for diagnostic purposes subsequently were utilized for the extraction of RNA and DNA microarrays. The severity of eczema was routinely monitored using the modified Total Lesion Symptom Score (mTLSS), a specific scoring system introduced by Ruzicka and co-workers (Ruzicka et al., 2008). For all human samples in this thesis the guidelines of the Declaration of Helsinki were deployed and the study was approved by the appropriate local Institutional Review Boards (study number: 5718R; registry ID: 2016095669 and study number: 1928). All subjects provided written informed consent before participation.

Human primary keratinocytes were cultured in keratinocyte medium (GIBCO, Invitrogen, Carlsbad, CA) supplemented with recombinant epidermal growth factor (EGF, 0.1-0.2 ng/ml) and bovine pituitary extract (20-30 µg/ml), L-Glutamate (2mM) (PAA, Pasching, Austria) and 1% of a mixture of antibiotics (penicillin 100 U/ml, streptomycin 100 µg/ml) (PAA, Pasching, Austria).

Human primary monocytes/dendritic cells were cultured in RPMI 1640 GlutamMax (GIBCO, Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS) (Biochrome AG, Berlin, Germany) and 1% of a mixture of antibiotics (penicillin 100 U/ml, streptomycin 100 µg/ml) (PAA, Pasching, Austria) and stimulated with granulocyte macrophage colony-stimulating factor (GM-CSF) (100 ng/ml) (GenScript, Piscataway, NJ, USA) and interleukin (IL-4) (50 ng/ml) (R&D Systems, Minneapolis, MN, USA).

Human primary T cells were cultivated in X-Vivo 15™ media (Lonza, Basel, Switzerland).

Cells were routinely cultured in an incubator at 37°C with 95% humidity and 5% CO_2 (INCO 2, Memmert, Schwabach, Germany). All cytokines were reconstituted in 1% BSA/PBS to a final concentration of 100 µg/ml.

9-*cis* RA stock solution (100 mM) (Basilea Pharmaceuticals, Basel, Switzerland) was prepared by dissolving 100 mg of 9-*cis* RA (molecular weight (MW): 300.435 g/mol) powder in 3.328 ml dimethyl sulfoxide (DMSO, Sigma-Aldrich, Saint Louis, MO, USA). Aliquots were stored at -20°C. Acitretin stock solution (10 mM) (Basilea Pharmaceuticals, Basel, Switzerland) was prepared by dissolving 82,5 mg of acitretin (molecular weight (MW): 326.429 g/mol) powder in 25.27 ml DMSO (Sigma-Aldrich, Saint Louis, MO, USA). Aliquots were stored at -20°C. Both RAs were freshly diluted to 1 mM in DMSO and further diluted to final concentrations in medium.

Keratinocytes were pre-treated in the presence or absence of 9-*cis* RA (9-*cis* retinoic acid) or acitretin (1, 100 nM) for 48 h. Subsequently, cells were treated with rhTNF-α (10 ng/ml) (AbD Serotec, Oxford, UK) plus rhIL-1β (5 ng/ml) (R&D Systems, Minneapolis, MN, USA), rhIL-4 (50 ng/ml) (R&D Systems, Minneapolis, MN, USA), rh interferon gamma (IFN-γ) (10 ng/ml) (R&D Systems, Minneapolis, MN, USA) or medium in the presence or absence of 9-*cis* RA (9-*cis* retinoic acid) or acitretin (1, 100 nM).

4.2.2 Isolation of peripheral blood mononuclear cells (PBMCs)

"Buffy coats" are concentrated suspensions of leukocytes obtained during procession of total blood. PBMCs can be isolated from buffy coats by Ficoll density-gradient centrifugation. Lymphocytes and monocytes will concentrate at the interphase between the upper phase (plasma, thrombocytes) and the lower phase (Ficoll) according to their specific density. Erythrocytes and granulocytes of higher density will form a cell pellet.

Buffy coats were obtained from the Institute of Hemostasis and Transfusion Medicine, University Hospital, Düsseldorf, Germany. Briefly, the buffy coat cell suspension was diluted 1:2 with RPMI 1640 (GIBCO, Invitrogen, Carlsbad, CA, USA). 37.5 ml of cell suspension was layered on top of 12.5 ml Ficoll-Paque Plus™ solution (GE Healthcare, Buckinghamshire, UK) and was separated by centrifugation (1150 rpm, 20 min, room temperature (RT)) (Rotina 46 R, Hettich, Bäch, Switzerland) without break. The interphase, containing the PBMC, was carefully transferred into a fresh tube, washed in RPMI 1640 (GIBCO, Invitrogen, Carlsbad, CA), and centrifuged again. The resulting pellet was resuspended in 25 ml of ammonium chloride lysing buffer to lyse erythrocytes and incubated for 10 min at 4°C. Thereafter, the remaining cells were washed in PBS (Merck, Darmstadt, Germany), and subsequently centrifuged (1150 rpm, 10 min, 8°C).

During routine monitoring of chronic hand eczema patients treat with 9-*cis* RA peripheral blood cells were isolated after securing informed consent using BD Vacutainer® CPT™ Cell Preparation Tube with Sodium Citrate (BD Biosciences, San Jose, CA, USA) according to the manufacturer's protocol.

4.2.3 Isolation of monocytes

CD14-positive monocytes were isolated according to the manufacturer's protocol using magnet beads (R&D Systems, Minneapolis, MN, USA). Briefly, PBMCs were labelled with CD14 biotinylated antibody followed by the addition of MagCellect Streptavidin Ferrofluid (magnetic beads). Monocytes were separated using the MagCellect Magnet (R&D Systems, Minneapolis, MN, USA).

4.2.4 Generation of monocyte-derived dendritic cells (MoDCs)

CD14-positive monocytes were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA, USA), 10% fetal calf serum (FCS) (Biochrome AG, Berlin, Germany) and penicillin/streptavidin (PAA,

Paching, Austria) and stimulated with rhGM-CSF (100 ng/ml) (GenScript, Piscataway, NJ, USA) and rhIL-4 (50 ng/ml) in the presence or absence of 9-*cis* RA or acitretin (10, 100, 1000 nM). Medium was changed after 3 days. At day 6, one half of the medium was replaced with rhGM-CSF (100 ng/ml) and rhIL-4 (50 ng/ml) and rhTNF-α (100 ng/ml) in the presence or absence of 9-*cis* RA or acitretin (10, 100, 1000 nM). At day 9, dendritic cells were harvested and subjected to flow cytometry or for further analyses.

4.2.5 Isolation of T cells

Isolated PBMCs were resuspended in RPMI 1640. Depletion of monocytes and macrophages was carried out in 175 cm^2 cell culture flasks (Greiner Bio-One GmbH, Kremsmünster, Austria) at standard cell culture conditions as monocytes and macrophages will adhere to the wall of the plastic flask. Therefore, the non-adherent cells were suspended in 20 ml of RPMI 1640, centrifugated as above, and resuspended in 4 ml of 1 x column wash buffer (R&D Systems, Inc., Minneapolis, MN). T cell isolation was performed by the use of human T cell enrichment columns (R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's instructions. Finally, T cells were resuspended in appropriate medium or chemotaxis buffer.

4.2.6 Mixed leukocyte reaction

The influence of 9-*cis* retinoic acid and acitretin on allogenic stimulation of monocyte-derived dendritic cells was analyzed. Briefly, $5x10^3$ monocyte-derived immature and mature dendritic cells (DC) pre-treated in the presence or absence of 9-*cis* RA or acitretin (10, 100, 1000 nM) and $1x10^5$ purified T cells were co-cultured for 6-7 days in 96-well round-bottom-plates in X-Vivo 15 media (Lonza, Basel, Switzerland). Proliferation of leukocytes was measured by BrdU-incorporation (Roche, Basel, Switzerland) after 6-7 days in a 96-well plate reader (Multiskan Ascent, Thermo Fisher Scientific, Wilmington, DE, USA).

$1x10^5$ isolated Patient-PBMCs before and 12 weeks after initiation of 9-*cis* RA treatment were co-cultured with $1x10^5$ enriched T cells for 6 days. Proliferation was measured by BrdU-incorporation after 6 days in a 96-well plate reader.

4.3 Flow cytometry analysis

Lymphocyte subset analysis was done on FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA) using CellQuest software (BD Biosciences, San Jose, CA, USA) and visualized in

a histogram plot (counts over fluorescence). In each of the tube at least 50,000 events were counted.

To analyze the activation of T lymphocytes and the activation status of monocytes, PBMCs were stained with mAbs listed in table 2. Subsequently, cells were analyzed using a FACScan and CellQuest software (Becton Dickinson, Mountain View, CA, USA) or FlowJo (FLOWJO, LLC, Ashland, OR, USA).

TABLE 1 Used antibodies for flow cytometry.

Target	Isotype	Conjugation	Company
CD4	Mouse IgG1	PerCP	EXBIO Praha, a.s., Vestec, Czech Republic
CD8a	Mouse IgG1	APC	eBioscience, Inc., San Diego, CA, USA
CD14	Mouse IgG2a	APC	BD Pharmingen, San Diego, CA, USA
CD62L	Mouse IgG1	PE-Cy5	BD Pharmingen, San Diego, CA, USA
CD73	Mouse IgG1	PE	Biolegend, San Diego, CA, USA
CD80	Mouse IgG1	FITC	eBioscience, Inc., San Diego, CA, USA
CD86	Mouse IgG1	PE	EXBIO Praha, a.s., Vestec, Czech Republic
CD86	Mouse IgG2b	PE-Cy5	R&D Systems, Minneapolis, MN, USA
CD83	Mouse IgG1	APC	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
CCR4	Mouse IgG1	PE	BD Pharmingen, San Diego, CA, USA
CCR6	Mouse IgG1	PE	BD Pharmingen, San Diego, CA, USA
CCR7	Mouse IgG2a	PE	R&D Systems, Minneapolis, MN, USA
CCR8	Rat IgG2b	PE	R&D Systems, Minneapolis, MN, USA
CCR9	Mouse IgG2a	PE	R&D Systems, Minneapolis, MN, USA
CCR10	Rat IgG2a	PE	R&D Systems, Minneapolis, MN, USA
CLA	Rat IgM	FITC	BD Pharmingen, San Diego, CA, USA
CXCR3	Mouse IgG1	PE	BD Pharmingen, San Diego, CA, USA
HLA-DR	Mouse IgG2a	PE-Cy5	BD Pharmingen, San Diego, CA, USA
PD-L1	Mouse IgG1	FITC	BD Pharmingen, San Diego, CA, USA

4.4 Protein quantification in fluids

4.4.1 ELISA

Cytokine and chemokine concentrations in the supernatants of retinoic acid stimulated human keratinocytes, dendritic cells or serum were measured by enzyme-linked immunosorbent assay (ELISA DuoSet, R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Briefly, monoclonal capture antibody (2 µg/ml) was incubated overnight in the wells of an immunosorbent 96-well plate (NUNC, Rochester, NY, USA). After blocking with reagent diluents (1% BSA in PBS) for 1 h at RT, wells were aspirated and rinsed with wash buffer (0.05% Tween® 20 in PBS). Samples were then incubated for 2 h. Following

another aspiration and wash step, appropriate biotinylated detection antibodies were incubated for 2 h. After another aspiration and wash step, streptavidin-horseradish peroxidase P was incubated in the wells for 20 min. Following a final aspiration and wash step, substrate solution was incubated in the wells for 20 min. Finally, stop solution was added. Optical densities were measured at 450 nm by use of a microplate reader (Multiskan Ascent, Thermo Fisher Scientific, Wilmington, DE, USA). Sample concentrations were calculated against standard curves and with the Graphpad Prism software (version 5.03, GraphPad Software, La Jolla, CA, USA).

4.4.2 Luminex screening assay

Serum samples were analyzed for the expression of chemokine with a bead-based multiplex Luminex® Screening Assay (R&D Systems, Minneapolis, MN, USA) using polystyrene beads according to the manufactures' protocol. Events were measured and quantified with the Bio-Plex® 200 System (Bio-Rad, Hercules, CA, USA).

4.4.3 Cytokine array

Supernatants of monocyte derived dendritic cells matured with TNF-α and treated with or without 9-*cis* RA [100 nM] were analyzed using Human Cytokine Array C5 (Ray Biotech, Norcross, GA, USA) according to the manufactures' protocol. Sample concentrations were calculated against internal standards as percentage of positive control using the ImageJ software (National Institutes of Health, USA, https://imagej.nih.gov/ij/). Overall high expression and a fold $\log_2$ difference of more than 1.5 were considered relevant.

4.5 Enzymatic activity assays

4.5.1 CD73 enzymatic activity

Dendritic cells ($5x10^4$) were incubated in RPMI 1640 GlutaMaxII (Invitrogen, Carlsbad, CA, USA), 10% fetal calf serum (FCS) (Biochrome AG, Berlin, Germany) and penicillin/streptavidin (PAA, Paching, Austria) and incubated as described previously (Burghoff et al., 2014). Briefly, cells incubated for 1h with or without 50 µM AOPCP (Tocris, Bristol, UK). The reaction was started with 50 µM Etheno-AMP (Biolog, Bremen, Germany). After 10, 20, 30, 40, 50, and 60 minutes, 15 µl of the supernatant was taken. The reaction was stopped by mixing the supernatant with the same volume 1 M perchloric acid and stored at −20°C until further analysis. For HPLC analysis, samples were neutralized with 1 M K_3PO_4. Etheno-AMP and etheno-

adenosine amounts were quantified using a 1525 Binary HPLC pump (Waters GmbH, Eschborn, Germany), connected to Waters 2475 multi wavelength fluorescence detector (λEm = 410 nm, λEx = 280 nm). Separation of the two analytes was accomplished on an XTerra MSC18 Column (Waters GmbH, Eschborn, Germany, 5 µm, 4.5 × 100 mm). Solution A contained 6% (v/v) acetonitrile/5.7 mM tetrabutylammonium bisulfate/30.5 mM KH_2PO_4, pH 5.8. Solution B consisted of 66% (v/v) acetonitrile/5.7 mM tetrabutylammonium bisulfate/30.5 mM KH_2PO_4, pH 5.8. At a flow rate of 0.75 ml/min separation was achieved by a linear gradient from 0 to 34% (v/v) solution B for 5.6 min and 34% (v/v) solution B for 1.4 min. Areas from the appropriate chromatograms were used for determination of reaction rate which is given as enzymatic activity per 1×10^6 cells by using Waters Breeze software (Waters GmbH, Eschborn, Germany). Each sample was measured in triplicate. All used chemicals from Sigma-Aldrich, Saint Louis, MO, USA or stated otherwise. The measurement of the CD73 enzyme activity was done in the lab of the Institute of Cardiovascular Physiology, Medical Faculty, Heinrich-Heine University, under supervision of Prof. Dr. J. Schrader and Dr. S. Burghoff.

4.6 Nucleic acids

4.6.1 Total ribonucleic acid (RNA) isolation using TRIzol® Reagent

RNA extraction was done from cells by using TRIzol® Reagent, containing phenol and guanidine isothiocyanate, which lyses the cells. Addition of chloroform separates the organic and clear (containing RNA) phases, whereas phenol removes the proteins from nucleic acid samples during isolation.

Briefly, to lyse cells 0.5-1 ml of TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) was added to the well of cell culture plate and was put into a reaction tube (Eppendorf, Hamburg, Germany). After adding 0.2 volume of chloroform (Merck, Darmstadt, Germany), cells were vortexed and subsequently centrifuged (15 min, 12000 rpm, 4°C) (Biofuge 13R, Heraeus Sepatech GmbH, Osterode, Germany). Following centrifugation, the clear upper phase was transferred into a new tube and RNA precipitation was achieved by adding 0.5 volume isopropyl alcohol (Merck, Darmstadt, Germany). The solution was mixed by vortexing, incubated overnight at -20°C, and finally centrifugated (12000 rpm, 30 min, 4°C). The RNA precipitate was visible as a pellet on the bottom and on the side of the tube. To remove residual salt, the pellet was washed in 1 ml of 80% ethanol (Merck, Darmstadt, Germany) after su-

pernatant had been carefully removed. Again, the sample was centrifugated for (12000 rpm, 30 min, 4°C). After removal of supernatant, the pellet was dried for 10 min at RT and subsequently dissolved in an appropriate amount of H_2O (DEPC) (Roth, Karlsruhe, Germany). Samples were stored at -80°C.

4.6.2 Total RNA isolation using RNeasy Mini Kit

RNA extraction from tissue samples was isolated with the RNeasy Mini Kit (QIAgen, Hilden, Germany) according to the manufacturer's protocol.

4.6.3 Optical density measurement

RNA yield was determined using NanoDrop™ 2000 (Thermo Fisher Scientific, Wilmington, DE, USA) photometer. RNA concentration was measured according to the manufacturer's manual. An OD 260/280 ratio between 1.8 and 2.1 indicated that extracted RNA was devoid of any appreciable protein, salt, or solvent contamination.

4.6.4 Quantitative polymerase chain reaction (qPCR) analysis

PCR-based analysis was performed to study gene expression of candidate genes. As quantitative PCR is the most sensitive application it was used to quantitate transcription levels of different genes of interest.

4.6.5 Complementary deoxyribonucleic acid (cDNA) synthesis

cDNA was synthesized from different messenger RNA (mRNA) templates using reverse transcriptase enzyme Superscript II (Invitrogen, Carlsbad, CA, USA).

Since DNA removal is necessary for subsequent applications DNase digestion was performed. Therefore, 4 µg of total RNA was mixed with the following reagents:

- 1.5 µl 5 x first strand buffer (Invitrogen, Carlsbad, CA, USA)
- 1 µl RNasin Plus (40 units/µl, Promega, Madison, WI, USA)
- 1 µl DNase I recombinant (Roche, Basel, Switzerland)

RNase-free water was added to a final volume of 16 µl and prepared mixes were incubated for 20 min at 37°C, 10 min at 70°C, and thereafter held at 4°C.

RNA was primed with 1 µl of anchored oligo$(dt)_{12\text{-}18}$ (0.5 µg/µl, Invitrogen, Carlsbad, CA, USA) and 0.4 µl random hexamer primers (500 µg/ml, Promega, Madison, WI, USA). RNase-free

water was added to a final volume of 20 µl and samples were incubated for 10 min at 70°C in order to reduce RNA secondary structures. Then, the following reagents were added for first strand synthesis:

4.5 µl	5 x first strand buffer (Invitrogen, Carlsbad, CA, USA)
1 µl	0.1 M DTT (Invitrogen, Carlsbad, CA, USA)
1 µl	dNTP mix (10 mM, Bioline USA Inc., Taunton, MA, USA)
0.5 µl	RNasin Plus (40 units/µl, Promega, Madison, WI, USA)
1 µl	Superscript II (200 units/µl, Invitrogen, Carlsbad, CA, USA)
30 µl	Total volume (add RNase-free water)

The reaction was gently mixed and after an initial incubation step of 2 min at 42°C for optimal primer annealing cDNA synthesis was carried out for 50 min at 42°C followed by an incubation step of 15 min at 70°C to inactivate the enzyme (Trio-Thermoblock, Biometra, Göttingen, Germany). Thereafter samples were stored at -20°C.

4.6.6 Primer design

Gene specific oligonucleotides for qPCR were either designed on mRNA sequences deduced from GenBank (table 3) or obtained as a TaqMan® Gene Expression Assays by Applied Biosystems.

TABLE 2 Oligonucleotides for qPCR. Oligonucleotides were obtained from MWG (Biotech, Ebersberg, Germany) or AOD (Assays on Demand): (Applied Biosystems Inc., Foster City, CA, USA).

Gene	*System*	*Sequence forward*	*Sequence revers*
STRA6	TaqMan® Primer	agaccaggtccacactga	ttcataatagccaaaggcataaaa
STRA6	TaqMan® Probe	ctgcccacactcgagagccagat	
SPRR3	SYBR® Green	tcaggagcttagaggattcttca	ttctgctggtaagaactcatgc
SPINK6	SYBR® Green	gtgccttctgtaaggccatagt	ttttccaggatgctttaggc
FLG	SYBR® Green	aaatggaagaaatcaatatcatgg	tttatatttttggctccttcg
OCLN	SYBR® Green	agccggtctaggacgcagca	aggcctggatgacatggctga
CLDN1	SYBR® Green	ggctgtcattggggctgcga	gcctgaccaaattcgtacctgga
HAS2	SYBR® Green	gtccattatgtacaggtttgtga	tccaaccatgggatcttctt
HAS3	SYBR® Green	gagatgtccagatcctcaacaa	cccactaatacactgcacac
HYAL1	SYBR® Green	ccaggaatcatgtcaggccatcaa	cccactggtcacgttcagg
HYAL2	SYBR® Green	ggcttagtgagatggacctc	ccgtgtcaggtaatctttgag
DEFB103A	TaqMan® Primer	tgaggatccattatcttctgtttgc	tgtgtttatgattcctccatgacc
DEFB103A	TaqMan® Probe	ttgctcttcctgtttttggtgcctgtt	
DEFB4A	TaqMan® Primer	cctcttcatattcctgatgcctct	ggctccactcttaaggcaggt
DEFB4A	TaqMan® Probe	ccaggtgtttttggtggtataggcgatcc	
RNASE7	SYBR® Green	ggagtcacagcacgaagacca	catggctgacttgcatgcttga
HRNR	SYBR® Green	ttcgtcttccagctatggtcagca	agtaacttgagccagccccgtgtt
S100A7	SYBR® Green	agacgtgatgacaagattgac	tgtccttttctcaagacgtc
CCL5	AOD	Hs00174575_m1	
CCL20	AOD	Hs00171125_m1	
CCL22	AOD	Hs00171080_m1	
CXCL14	AOD	Hs00171135_m1	
CCL7	AOD	Hs00171147_m1	
CCL17	AOD	Hs00171074_m1	
MMP12	SYBR® Green	agttttgatgctgtcactaccg	cactggtctttggtctctcagaa
AQP9	SYBR® Green	gcaaccgtctttggcattta	ttttctcccacgatcagca
S100A9	SYBR® Green	tcggctttgacagagtgcaa	gccccagcttcacagagtat

4.6.7 Quantitative real time (qPCR) analysis

To quantitate differences in mRNA expression, qPCR was performed. qPCR consists in "real time" detection of a specific product as quantitation is carried out after each round of amplification by measuring fluorescence emission. SYBR® Green is a fluorescent dye which intercalates into double-stranded DNA. The amount of PCR product is directly correlated with the fluorescence emission. The fluorescent signal during the thermal PCR program is detected by an optical detector. In an amplification plot, the logarithmic fluorescent signal is plotted against cycle number. The initial phase is characterized by low PCR amplification; therefore, a low fluorescent signal is detected. The following phase, the geometric phase PCRs product is amplified exponentially. When reaction reagents are depleted, the amplification curve reaches a final plateau. When a threshold is set intersecting the curve in its geometric phase,

the resulting crossing point is called threshold cycle (C_t)-value. Genes expressed at higher rates have higher starting copy numbers and, therefore, appear earlier during the amplification resulting in lower C_t-values. As an internal standard gene expression analysis of 18S RNA was used since it is expressed at relatively constant levels throughout different cells. Quantitative PCR was performed using the Applied Biosystems 7000 System/ Quantstudio 6 and Power SYBR® Green PCR Master Mix or TaqMan® Universal PCR Master Mix (Applied Biosystems Inc., Foster City, CA, USA). For a final reaction volume of 25 µl per well, the following gene specific mixes were prepared.

	Primer forward	***Primer reverse***	***Detection Mix***	***Target Probe***	***cDNA (25ng/µl)***	**dH_2O**
SYBR® Green	2.5 µl [2 µM] *		12.5 µl	X	10 µl	x
TaqMan®	0.6 µl [45 µM]	0.6 µl [45 µM]	12.5 µl	0.75 µl [10 µM]	10 µl	0.55 µl
Eukaryotic 18S rRNA (TaqMan®)	0.15 µl [10 µM]	0.15 µl [10 µM]	12.5 µl	0.15 µl [10 µM]	10 µl	2.05 µl
TaqMan® Gene Expression Assays (Assay on demand) **	1 µl		10 µl	X	10 µl	x

*Primer mix [2 µM]: 20 µl Forward primer [45 µM], 20 µl Reverse primer [45 µm], 410 µl dH_2O

**Final reaction volume: 21 µl

The following PCR program was applied:

Temperature	Time	
95°C	10 min	
50°C	2 min	40 cycles
60°C	1 min	
95°C	15 s	

To prove whether primer-dimer artefacts had affected the reaction in case of SYBR® Green detection, a dissociation protocol was carried out after termination of the PCR program. In this protocol, the temperature is gradually increased to melt the products formed during the PCR reaction. The melting point can be easily detected since the fluorescent signal decreases as DNA double strands separate and therefore intercalated SYBR® Green is released. Differ-

ent PCR products obtained with the same primer pair should have approximately the same melting point. In fact, different DNA templates primed with a particular primer pair give rise to amplification products of the same size.

All qPCR samples were run in singles and final raw data was exported as .csv/.xls files for further statistical analysis in Excel spreadsheets.

For absolute quantification ΔC_t-values were determined: $\Delta C_t = 2^{C_{t,18S} - C_{t,sample}}$, all samples were multiplied by 10^5, resulting in the final formula of: $RU = 10^5 \cdot \Delta C_t$

For fold quantification $\Delta\Delta C_t$-values were determined: $\Delta\Delta C_t = 2^{-(\Delta C_t - mean_{\Delta Ct,18S})}$

Where $C_{t,18S}$ is the C_t-value obtained for the 18S RNA and $C_{t,\ sample}$ is the C_t-value for a specific gene in a specific sample. Mean $_{\Delta Ct,\ 18S}$ is the average of ΔC_t-values obtained for the technical replicates of 18S RNA of the medium control of one experiment.

4.6.8 DNA microarray

RNA preparations were checked for RNA integrity by Agilent 2100 Bioanalyzer quality control. All samples in this study showed high quality RNA Integrity Numbers (RIN; median = 8). DNA microarray was done at the BMFZ, University of Düsseldorf. Data was processed with the help of P. Oláh, Dept. of Dermatology, University of Pecs Hungary and will be described in 4.6.9.

4.6.9 RNA sequencing and analysis

RNA preparations were checked for RNA integrity by Agilent 2100 Bioanalyzer quality control. All samples in this study showed high quality RNA Integrity Numbers (RIN; median = 8). RNA was labelled and sequenced by Exiqon (Exiqon, Vedbaek, Denmark). RNAseq reads were quality controlled and filtered with FastQC (Babraham Bioinformatics group, Cambridge, UK). Low-quality sequences were trimmed with the fastx toolkit (Hannon Lab - Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA). Data was mapped with the splicing-aware aligner TopHat (Trapnell et al., 2009) and mapped alignments were quantified with htseq-count (Anders et al., 2015) in strand-specific mode, whereas non-unique and low mapping alignments were discarded. Expression differential expressed genes were carried out with the DESeq2 (Love et al., 2014), normalized between samples according to the size factor. Per-gene dispersion estimates were conducted to reduce the false discovery rate, while reduc-

tion of spurious fold change estimations due to moderate sample size was achieved by discarding genes exhibiting the lowest 5% of expression, $\log_2$ fold-change <1 and the false discovery rate >0.05. Interaction networks were created with ReactomeFIViz Cytoscape plugin (Wu et al., 2010). To clear the graph up, only network nodes ≥ 3 degrees, or belonging to the top 75% of $\log_2$ fold-change ratios were considered in the network visualizations. Module clusters were annotated by gene ontology (GO) biological functions, clusters were signed with GO terms corresponding to the highest number of genes and he highest significance. Heatmaps were done with the gplots R package (https://cran.r-project.org/web/packages/gplots/index.html). This was done with the help of P. Oláh, Dept. of Dermatology, University of Pecs Hungary.

4.7 Patients

The routine monitoring included 29 patients diagnosed with chronic hand eczema in the Department of Dermatology, University Hospital Düsseldorf (Tab. 1). They received 10[1] or 30 mg 9-*cis* RA orally, daily for 12 weeks. Chronic hand eczema patients treated with 9-*cis* RA are routinely monitored before (PRE) and during the treatment at week four (Wk4), week eight (Wk8) and at week twelve (Wk12) (s. leaflet for Toctino®). Severity of hand eczema was determined using the modified Total Lesion Symptom Score (mTLSS), a specific scoring system introduced by Ruzicka and co-workers (Ruzicka et al., 2008) and by physician global assessment (PGA). Briefly, the mTLSS is a combined skin score of the severity of 7 different aspects of the hand eczema (color of erythema, scaling, lichenification⁄ hyperkeratosis, vesiculaton, edema, fissures and Pruritus ⁄pain with a balanced score of 0-3 each), the affected palmar area involved. The score value may range between 0 (no skin affection) and 3 (maximal skin affection) (Ruzicka et al., 2008). The advantage of this monitoring was taken and serum as well as blood samples for further analysis were secured after obtaining the informed consent of the patients. The guidelines of the Declaration of Helsinki were deployed and the study was approved by the appropriate local Institutional Review Boards (study number: 5718R; registry ID: 2016095669 and study number: 1928) and all subjects provided written informed consent before participation.

[1] one of 29 patients received 10 mg 9-cis RA in the first four weeks of treatment

TABLE 3 Characterization of the group studied.

Characteristics of the patients	
Age (yr)	
Mean:	53.76
Range:	34-73
Sex	
Total:	29
Male: female ratio	14:15
Female (%)	48.28%
mTLSS (initial admission)	
Mean:	10.38
Range:	6-18

4.8 Statistics

Data were expressed at mean +/- standard deviation (SD) or +/- standard error of the mean (SEM). Statistical significance was assessed by either paired or unpaired Student's t-test or Mann-Whitney-U-test using Graphpad Prism software (version 5.03, GraphPad Software, La Jolla, CA, USA). P-values below 0.05 were considered as statistically significant (*P ≤ .05, **P ≤ .01, ***P ≤ .001).

5 Results

5.1 Effects of retinoids on skin

5.1.1 Systemic 9-*cis* RA treatment alters gene expression in the skin of patients

Expression profile between lesional skin of patients with chronic hand eczema (CHE) before treatment with systemic 9-*cis* RA (n=3 independent donors) was compared to gene expression after twelve weeks (Wk12; n=3 independent donors). In total 244 differentially expressed genes (DEG) were significantly regulated ($p < 0.05$) with a fold change (FC) of > 1.5 clustering in three groups: A, extracellular matrix (ECM) organization and skin barrier; B, cell surface signaling and C, genes involved in chemokine signaling and immunity (Fig. 4, Tab. 4). Additionally, a gene ontology (GO) analysis, a categorization of expressed genes into larger groups regarding biological processes, molecular functions and cellular components, was done (Ashburner et al., 2000). This analysis showed regulations in keratinization, defense responses, epidermal cell differentiation, epidermis development and inflammatory responses (Tab. 5). Finally, a FANTOM 5 analysis, a comprehensive databank containing RNA expression in different cell types (The FANTOM Consortium and the RIKEN PMI and CLST (DGT), 2014), revealed that most regulated transcripts were expressed by skin and monocyte-derived cells.

TABLE 4 Expression profile between lesional skin of patients with CHE before treatment with systemic 9-*cis* RA was compared to gene expression after twelve weeks (Wk12) (n=3 independent donors). TOP 100 genes up-regulated (UP) and down-regulated (DOWN), their fold changes and p-values are listed.

RANK	Gene	Fold change (log_2) UP in Wk12	p value	Gene	Fold change (log_2) DOWN in Wk12	p value
1	CCDC144AandFAM106C	3.891	3.558E-01	LCE3A	-4.660	4.329E-02
2	GSDMC	3.232	3.676E-01	CXCL9	-4.038	4.307E-02
3	LCE5A	3.042	1.787E-03	OR1E1	-3.741	3.468E-01
4	CYP26A1	2.926	5.134E-02	CCL18	-3.621	2.138E-03
5	PHGR1	2.606	1.458E-01	DEFB4A	-3.607	1.623E-01
6	ANXA9	2.373	2.028E-02	CXCL10	-3.504	3.367E-02
7	IL37	2.225	3.781E-03	PI3	-3.495	1.733E-02
8	GJB4	2.160	6.141E-03	RHCG	-3.293	2.067E-03
9	FLG2	2.157	2.214E-02	OASL	-3.283	1.409E-02
10	PSAPL1	2.144	3.018E-02	KRT6C	-3.047	1.111E-01
11	PDGFRL	2.118	2.467E-02	LOC401463	-3.015	3.244E-01
12	ANGPTL7	2.110	6.650E-02	KRT16	-3.015	6.397E-02
13	NDUFV1	2.089	3.686E-01	MMP12	-2.962	9.859E-03
14	HRNR	2.083	8.324E-02	KRT6B	-2.806	6.807E-02
15	MYOC	2.032	1.888E-02	DSC2	-2.679	1.164E-03
16	CYB5RL	2.031	5.509E-01	PRSS53	-2.616	9.693E-03
17	BTC	1.984	8.427E-02	AKR1B15	-2.563	1.146E-02
18	FIBIN	1.932	1.750E-02	AKR1B10	-2.554	2.819E-03
19	AQP9	1.922	3.084E-02	MMP9	-2.383	6.840E-02
20	PI16	1.893	8.659E-03	GZMB	-2.331	2.203E-02
21	AADAC	1.889	8.700E-02	KLHDC7B	-2.263	1.356E-01
22	CADM3	1.886	6.230E-02	CHAC1	-2.263	2.160E-02
23	MGP	1.839	1.153E-02	LOC642947	-2.247	1.204E-02
24	ENST00000479981	1.815	3.382E-01	GAL	-2.195	6.061E-02
25	IL17D	1.801	1.310E-02	CCR7	-2.105	1.311E-01
26	CHRDL1	1.791	1.908E-02	LTF	-2.081	1.448E-01
27	CPZ	1.764	5.405E-02	RGS1	-2.066	6.598E-02
28	CILP2	1.756	4.270E-02	KRT85	-2.062	1.078E-02
29	BDNFOS	1.751	5.540E-02	CD1B	-2.054	1.029E-01
30	H19	1.720	2.232E-01	CHI3L2	-2.005	2.515E-01
31	CILP	1.718	6.952E-03	KRT75	-2.004	9.500E-02
32	CYP2J2	1.710	5.787E-02	CCL27	-1.994	3.311E-02
33	PTN	1.657	3.038E-02	IFI6	-1.983	1.118E-01
34	PIP	1.651	6.852E-02	LCE3E	-1.944	1.102E-01
35	CTHRC1	1.637	6.877E-02	CCL17	-1.932	7.308E-02
36	OGN	1.632	1.034E-01	COL29A1	-1.913	8.976E-02
37	MFAP4	1.630	1.164E-02	IDO1	-1.891	6.889E-02
38	FAM180B	1.625	7.049E-03	KRT6A	-1.798	1.165E-01
39	FMO2	1.615	3.235E-02	BATF2	-1.762	2.229E-01
40	PCOLCE2	1.609	9.776E-02	LCE3D	-1.757	8.979E-02
41	DYRK4	1.595	3.190E-01	CCL13	-1.717	6.991E-03
42	CARD18	1.577	1.480E-01	BIRC3	-1.699	1.293E-01
43	MUCL1	1.566	6.339E-03	CXCL11	-1.696	1.423E-01
44	GAS2	1.560	1.501E-01	PRH2	-1.693	2.220E-02
45	FBLN1	1.542	4.014E-02	KLK12	-1.687	1.355E-01
46	CRNN	1.536	5.002E-02	GML	-1.684	2.675E-02
47	IGFBP6	1.536	9.166E-02	PYHIN1	-1.672	3.220E-01
48	LHFP	1.534	1.767E-02	GNLY	-1.659	3.022E-02
49	GSTM5	1.532	3.784E-03	C15orf48	-1.645	1.365E-01
50	HIC1	1.532	1.736E-02	CCL19	-1.645	2.295E-01

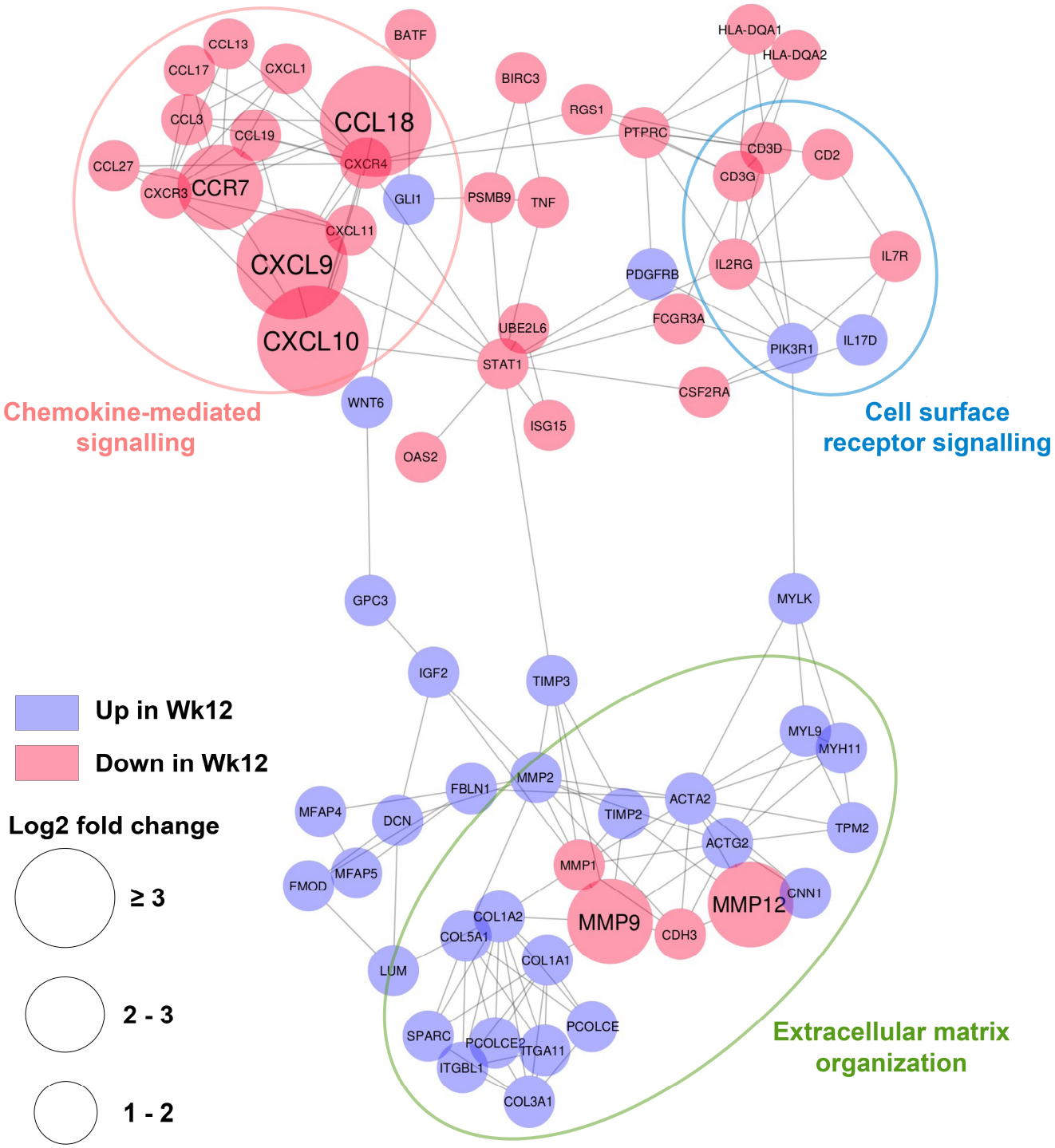

FIGURE 4 Top regulated genes in biopsies analyzed with DNA microarray. Red: down-regulated (DOWN) genes; blue: up-regulated (UP) genes during treatment with 9-*cis* RA using a $\log_2$ fold change > 1 and a cutoff p value of < 0.05.

TABLE 5 TOP 50 Gene ontology (GO) terms, regarding biological processes, molecular functions and cellular components are listed between gene expression in lesional skin of patients with chronic hand eczema (CHE) before treatment with systemic 9-*cis* RA and after twelve weeks (Wk12).

GO Term	*Description*	*P-value*	*nr. of genes*
GO:0002376	immune system process	8.06E-11	32
GO:0006952	defense response	1.02E-10	25
GO:0072676	lymphocyte migration	1.35E-10	8
GO:0019221	cytokine-mediated signaling pathway	3.76E-10	17
GO:0048247	lymphocyte chemotaxis	6.39E-10	7
GO:0060326	cell chemotaxis	1.16E-9	11
GO:0070098	chemokine-mediated signaling pathway	3.08E-9	8
GO:0006955	immune response	4.13E-8	20
GO:0006935	chemotaxis	5.94E-8	12
GO:0042330	taxis	6.18E-8	12
GO:0007166	cell surface receptor signaling pathway	7.97E-8	31
GO:0006954	inflammatory response	9.59E-8	14
GO:0030595	leukocyte chemotaxis	1.99E-7	8
GO:0031424	keratinization	2.77E-7	6
GO:0006950	response to stress	4.59E-7	35
GO:0050896	response to stimulus	1.05E-6	49
GO:0032103	positive regulation of response to external stimulus	1.48E-6	10
GO:0009607	response to biotic stimulus	2.6E-6	16
GO:0010647	positive regulation of cell communication	5.16E-6	23
GO:0023056	positive regulation of signaling	5.41E-6	23
GO:0043207	response to external biotic stimulus	7.4E-6	15
GO:0045071	negative regulation of viral genome replication	8.04E-6	5
GO:1903901	negative regulation of viral life cycle	8.25E-6	6
GO:0048525	negative regulation of viral process	9.47E-6	6
GO:0050900	leukocyte migration	1.08E-5	9
GO:0051707	response to other organism	1.49E-5	12
GO:0043950	positive regulation of cAMP-mediated signaling	1.52E-5	3
GO:0009967	positive regulation of signal transduction	1.57E-5	21
GO:0048584	positive regulation of response to stimulus	1.75E-5	25
GO:0060337	type I interferon signaling pathway	2.06E-5	5
GO:1902533	positive regulation of intracellular signal transduction	2.08E-5	16
GO:0090218	positive regulation of lipid kinase activity	2.89E-5	4
GO:0002687	positive regulation of leukocyte migration	3.01E-5	6
GO:0072610	interleukin-12 secretion	3.28E-5	2
GO:0038111	interleukin-7-mediated signaling pathway	3.28E-5	2
GO:0098542	defense response to other organism	3.53E-5	10
GO:0002548	monocyte chemotaxis	4.2E-5	4
GO:1903900	regulation of viral life cycle	4.87E-5	7
GO:0016477	cell migration	5.04E-5	14
GO:0050921	positive regulation of chemotaxis	5.05E-5	6
GO:0070613	regulation of protein processing	5.51E-5	5
GO:0009605	response to external stimulus	5.78E-5	19
GO:0009615	response to virus	5.8E-5	8
GO:1903317	regulation of protein maturation	5.89E-5	5
GO:0050792	regulation of viral process	6.09E-5	7
GO:0002690	positive regulation of leukocyte chemotaxis	7.64E-5	5
GO:0045069	regulation of viral genome replication	7.64E-5	5
GO:2000106	regulation of leukocyte apoptotic process	7.64E-5	5
GO:0051797	regulation of hair follicle development	8.01E-5	3
GO:0048583	regulation of response to stimulus	8.59E-5	36

TABLE 6 TOP 20 UP or DOWN regulated genes from out of Tab. 4 analyzed with the FANTOM 5 data base. Genes are analyzed by their promotor activity in cells and tissues. RANK of genes represents the corresponding activity in the total of 1829 cells/ tissues.

Gene (UP)	*associated with skin/ monocytes*	*RANK from 1829*	*Gene (DOWN)*	*associated with skin/ mono-cytes*	*RANK from 1829*
CCDC144A	Skin, palm	46	LCE3A	No data availible	-
GSDMC	No data availible	-	CXCL9	**Monocyte-derived cells**	**1**
LCE5A	**Skin, adult**	**1**	OR1E1	No data availible	-
CYP26A1	Monocyte-derived cells	120	CCL18	**Monocyte-derived cells**	**1**
PHGR1	Skin, palm	40	DEFB4A	Fingernail	2
ANXA9	Skin, fetal	146	CXCL10	**Monocyte-derived cells**	**1**
IL37	No data availible	-	PI3	Monocyte-derived cells; Keratinocyte, epidermal	9; 45
GJB4	Keratinocyte, epidermal	4	RHCG	Monocyte-derived cells; Keratinocyte, epidermal	39; 50
FLG2	**Skin, adult**	**1**	OASL	**Monocyte-derived cells**	**1**
PSAPL1	**Skin, adult**	**1**	KRT6C	Fingernail; Keratinocyte, epidermal	1; 11
PDGFRL	Fibroblasts; Monocyte-derived cells	1; 12	LOC401463	**Monocyte-derived cells**	**1**
ANGPTL7	Skin, palm	26	KRT16	Fingernail; Keratinocyte, epidermal	1; 9
NDUFV1	Monocyte-derived cells	8	MMP12	**Monocyte-derived cells**	**1**
HRNR	No data availible	-	KRT6B	**Keratinocyte, epidermal**	**1**
MYOC	Skin, palm; monocytes	11; 63	DSC2	Fingernail; Keratinocyte, epidermal	3; 16
CYB5RL	Keratinocyte, epidermal	67	PRSS53	**Monocyte-derived cells**	**1**
BTC	No data availible	-	AKR1B15	Fingernail	6
FIBIN	Skin, adult	32	AKR1B10	Skin, adult	19
AQP9	**Monocyte-derived cells**	**1**	MMP9	**Monocyte-derived cells**	**1**
PI16	Skin, adult	16	GZMB	Dendritic Cells, plasmacytoid	2

5.1.2 Biological activity of retinoids in cultured human primary keratinocytes

To identify relevant retinoid-responsive pathways, the epidermal compartment was brought into focus. Since epidermal barrier function, the abundance of antimicrobial peptides and the production of chemokines critically regulate cutaneous homeostasis and initiate cutaneous inflammation, expression analyses of well-known target genes in resting or activated cultured human primary keratinocytes in the presence of absence of retinoids was performed. Human primary keratinocytes were pretreated with varying doses of 9-*cis* RA (n=7 independent donors) or with the synthetic and immunomodulatory retinoid acitretin

(n=4 independent donors) for 48h and for additionally 24h with different activating cytokines, i.e. proinflammatory with TNF-α/ IL-1β, IL-4 and IFN-γ in the presence or absence of retinoids. Vehicle treatment was done in a final concentration of 1:10000 and had no effect on gene expression. Using a PCR array system, expression of different genes seen regulated in the microarray analysis of patients' skin, including AMPs, chemokines and genes participating in ECM functions was assessed. Data (except for *STRA6*) are expressed as a ''fold change'' considering untreated keratinocytes (Medium without cytokine) of each donor (value=1) and tested with an unpaired two-tailed Mann-Whitney U-test.

First, as a reality check retinoid-induced retinol transporter *STRA6* was analyzed. Its expression is inducible by retinoids in keratinocytes (Szeto et al., 2001, Bouillet et al., 1997). There was an up-regulation of *STRA6* expression in resting and in stimulated cultured human primary keratinocytes in combination with 100 nM 9-*cis* RA or acitretin (Fig. 5) and showed that retinoids are active in keratinocytes.

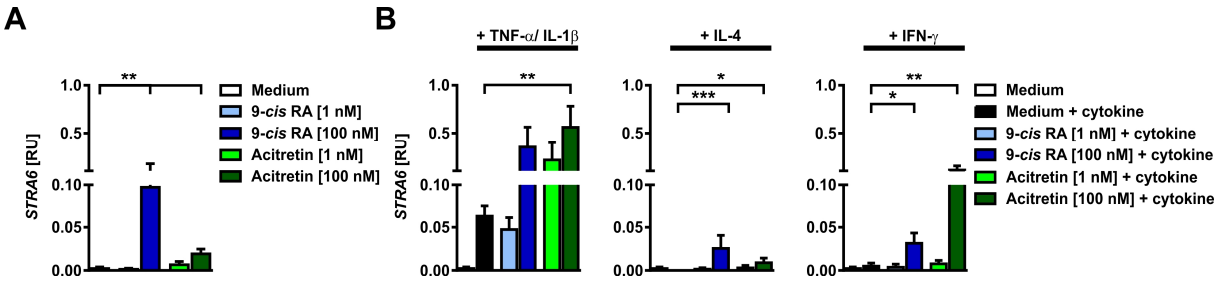

FIGURE 5 Effects of 9-*cis* RA or acitretin on retinol transporter *STRA6* gene expression in cultured human primary keratinocytes. Cells were left without cytokine (A) or treated with primary pro-inflammatory cytokines TNF-α plus IL-1β or T cell-derived effector cytokines IL-4 or IFN-γ (B) in the presence or absence of 9-*cis* RA (1, 100 nM; n=7 independent donors) or acitretin (1, 100 nM; n=4 independent donors). Twenty-four hours later cells were harvested, RNA was extracted and gene expression was analyzed using quantitative RT-PCR. Results were normalized to 18S ribosomal RNA expression. Values represent as mean + SEM in relative units of target gene in 25 ng of total cDNA. Mann-Whitney U test was used to evaluate significant differences (*p<0.05, **p<0.01, ***p<0.001).

The cornified envelope builds the first line of defense against chemicals, pathogens and ultraviolet light. It is the result of the keratinization process, where living keratinocytes are transformed into corneocytes, which involves several proteins and molecules, including proteases and several proteins like filaggrin (FLG), involucrin (IVL), loricrin (LRC), the late cornified envelope proteins (LCEs) and the small proline rich proteins (SPRRs). A common feature of eczema is a dysregulated barrier function resulting in an increased transepidermal water loss (Kamsteeg et al., 2010). Impaired skin barrier function leads to the infiltration of

allergens and irritants into the skin and further stimulates immunological and inflammatory processes (Proksch et al., 2006). Further, disruption of the skin barrier leads to a massive release of cytokines like interleukin-1 beta (IL-1β) or tumor necrosis factor-alpha (TNF-α) (Wood et al., 1992, Smith et al., 2002). As part of the skins extracellular matrix, hyaluronan, a high-molecular-weight glycosaminoglycan, participates in different functions including migration, adhesion, proliferation and moreover with tissue regeneration, repair and hydration (Tammi et al., 2002, Manuskiatti and Maibach, 1996). Hyaluronan is synthesized by hyaluronan acid synthases HAS1, HAS2 and HAS3 (Weigel et al., 1997). Hyaluronan degradation is controlled by two major forms, hyaluronidases HYAL1 and HYAL2, in somatic tissues (Stern and Jedrzejas, 2006).

First, genes that directly contribute to the skin barrier including small proline rich protein 3 (*SPRR3*), the specific serine protease inhibitor of Kazal type 6 (*SPINK6*), filaggrin (*FLG*), occludin (*OCLN*) and claudin 1 (*CLDN1*) (Fig. 6) were tested for their responsiveness to retinoid treatment in keratinocytes. SPRR3 overexpression and FLG mutations have been associated with defects in skin barrier (Liang et al., 2015, Mahler, 2016). SPRR3 is induced by 1 nM acitretin in resting cells as well as in TNF-α/IL-1β stimulated cells in comparison to the corresponding control. 9-*cis* RA treatment reduced *SPRR3* expression levels dose-dependently in TNF-α/IL-1β stimulated cells (9-*cis* RA, p=0.0023) compared to cytokine treatment alone. A direct comparison of 9-*cis* RA- and acitretin-induced gene expression in resting and TNF-α/IL-1β activated cells showed a general higher expression of *SPRR3* by acitretin in equimolar doses. SPINK6 inhibits desquamation and might be important for epidermal barrier function (Fischer et al., 2013). As shown in mouse keratinocytes with ATRA (Fischer et al., 2014), 9-*cis* retinoic acid was also able to reduce the expression of *SPINK6* in resting keratinocytes in comparison to medium control (p=0.0006). Acitretin induced the expression of *SPINK6* at a concentration of 1 nM (p=0.0061), while 100 nM acitretin reduced the expression (p=0.0061) directly compared to medium without cytokine. In TNF-α/IL-1β-activated cells, 9-*cis* RA prevented the up-regulation of TNF-α/IL-1β-induced expression of *SPINK6* (p=0.007) while acitretin further increased *SPINK6* (p=0.0424) compared to pro-inflammatory activated cells. Moreover, 9-*cis* RA is more capable to reduce *SPINK6* expression than acitretin in equimolar doses (p=0.0424). Regarding the tight junctions and barrier genes, overall expression changes were low. *CLDN1* and *FLG* mRNA expression were not affected by retinoids. A direct comparison of 100 nM 9-*cis* RA treated and 100 nM acitretin

treated in TNF-α/IL-1β-activated keratinocytes revealed a higher expression of *FLG* by acitretin (p=0.0061). *OCLN* expression is decreased by 9-*cis* RA at a concentration of 1 nM (p=0.026) compared to the medium control.

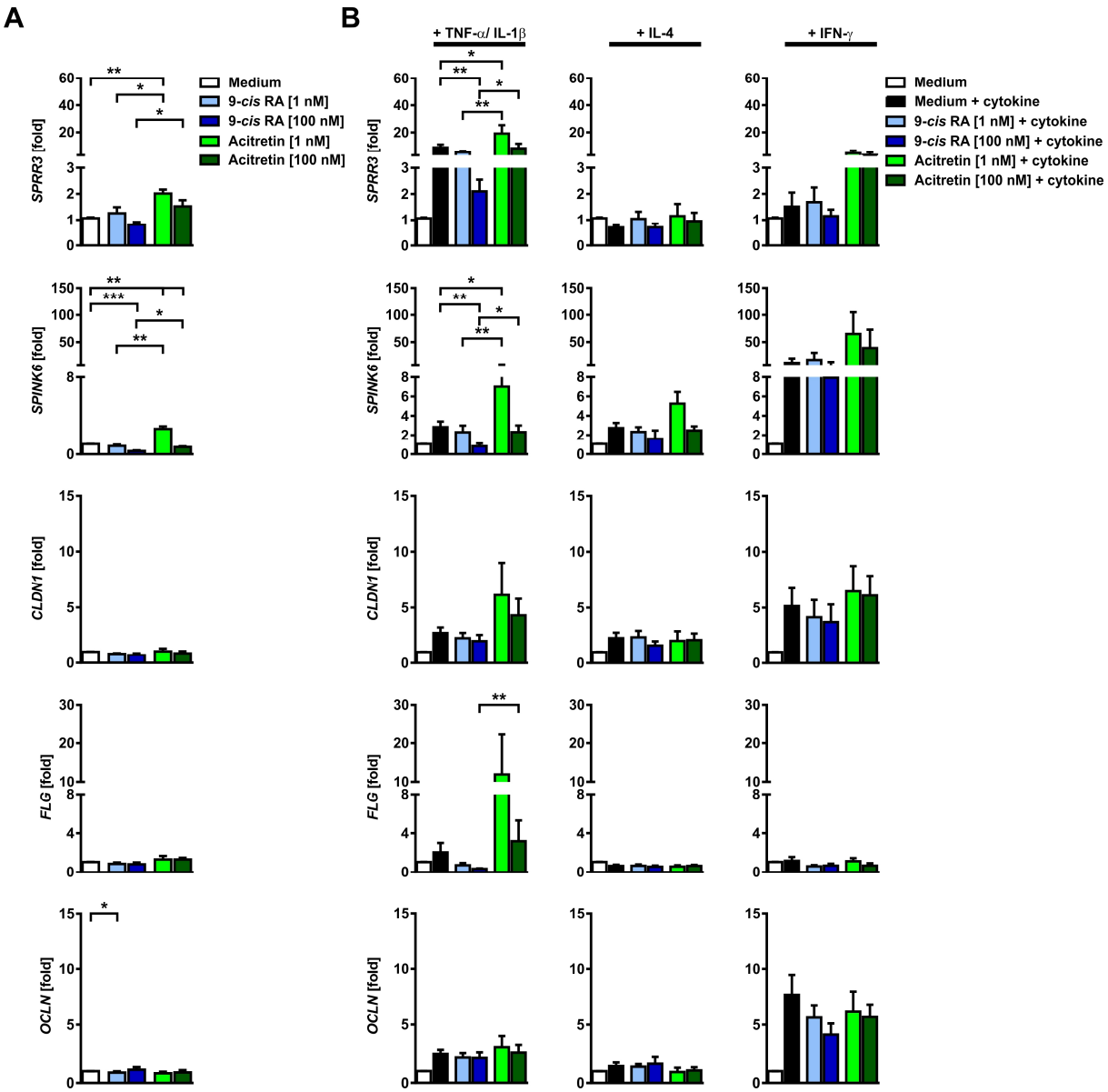

FIGURE 6 9-*cis* RA reduced the TNF-α/IL-1β induced expression of barrier genes *SPRR3* and *SPINK6*. Keratinocytes were left without cytokines (A) or treated with primary pro-inflammatory cytokines TNF-α plus IL-1β or T cell-derived effector cytokines IL-4 or IFN-γ (B) in the presence or absence of 9-cis RA (1, 100 nM; n=7 independent donors) or acitretin (1, 100 nM; n=4 independent donors). Twenty-four hours later cells were harvested, RNA was extracted and gene expression was analyzed using quantitative RT-PCR. Results were normalized to 18S ribosomal RNA expression. Values represent as mean + SEM of the fold change to the untreated medium control without cytokines. Mann-Whitney U test was used to evaluate significant differences (*p<0.05, **p<0.01, ***p<0.001).

Second, tissue regeneration in keratinocytes in response to retinoid treatment was studied using epidermal growth factor receptor ligand betacellulin (*BTC*), hyaluronan synthases 2 and 3 (*HAS2*, *HAS3*) and hyaluronidases 1 and 2 (*HYAL1*, *HYAL2*) (Fig. 7). *BTC* mRNA expression was induced by 100 nM 9-*cis* RA (p=0.0006) and 100 nM acitretin in resting cells (p=0.0061), in TNF-α/IL-1β stimulated (9-*cis* RA, p=0.007; acitretin, p=0.0242) and IL-4 stimulated cells (9-*cis* RA, p=0.007; acitretin, p=0.0121) compared to the untreated medium control, pro-inflammatory or T_H2 activated cells respectively. *HAS2* expression was up-regulated by 9-*cis* RA [100 nM] in resting cells in comparison to medium control (p=0.007) while acitretin did not alter *HAS2* expression. Expression of *HAS3* was increased significantly by 9-*cis* RA treatment in resting cells (p=0.0006) compared with untreated control, in IL-4 treated keratinocytes (p=0.0041) and IFN-γ-activated cells (p=0.0023) in comparison to IL-4 or IFN-γ alone, respectively. *HYAL1* and *HYAL2* were not regulated by 9-*cis* RA, whereas 100 nM acitretin resulted in a marked induction of *HYAL1* in resting (p=0.0061), in TNF-α/IL-1β–activated cells (p=0.0061), in IFN-γ-treated (p=0.0242)) and further of *HYAL2* under resting conditions (p=0.0061) compared to their corresponding medium controls. Expression of *HYAL1* in TNF-α/IL-1β ([100 nM] p=0.0242)) and IFN-γ ([1 nM] p=0.0061; [100 nM] p=0.0061)) as well as expression of *HYAL2* in TNF-α/IL-1β ([100 nM] p=0.0424)) and IFN-γ ([1 nM] p=0.0424) stimulated structural cells significantly directly compared to equimolar doses of 9-*cis* RA.

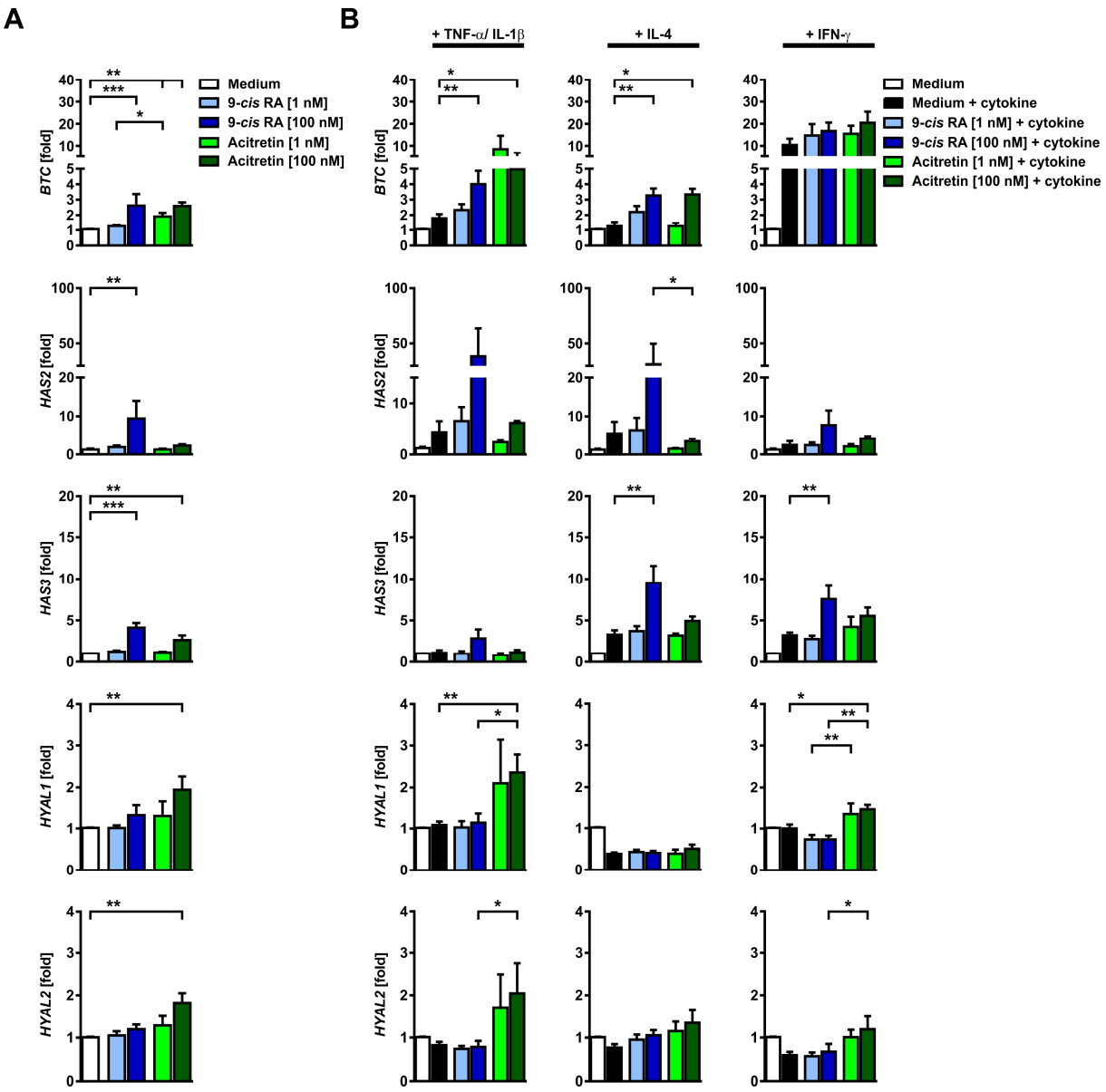

FIGURE 7 9-*cis* RA induced genes involved in tissue regeneration in resting and activated human primary keratinocytes, whereas hyaluronan degradation is not affected. Keratinocytes were left without cytokines (A) or treated with primary pro-inflammatory cytokines TNF-α plus IL-1β or T cell-derived effector cytokines IL-4 or IFN-γ (B) in the presence or absence of 9-cis RA (1, 100 nM; n=7 independent donors) or acitretin (1, 100 nM; n=4 independent donors). Twenty-four hours later cells were harvested, RNA was extracted and gene expression was analyzed using quantitative RT-PCR. Results were normalized to 18S ribosomal RNA expression. Values represent as mean + SEM of the fold change to the untreated medium control without cytokines. Mann-Whitney U test was used to evaluate significant differences (*p<0.05, **p<0.01, ***p<0.001).

Antimicrobial peptides (AMPs) play a crucial role in the innate immune system of the skin. AMPs are classified based on their structures into cathelicidins and defensins. In the human system, one cathelicidin (LL-37) and four β-defensins are known (Agier and Brzezinska-Blaszczyk, 2016, Schauber and Gallo, 2008). These peptides directly kill gram-negative and gram-positive bacteria, fungi and several viruses (Gallo et al., 2002). AMPs are

induced by physical barrier disruption and under inflammatory conditions e.g. in psoriasis or contact dermatitis (Proksch and Brasch, 2012, Proksch et al., 2006). To analyze whether the retinoids have an effect on the expression of AMPs in keratinocytes *in vitro*, representing members of human β-defensins *DEFB4A* (hBD-2), *DEFB103A* (hBD-3), *RNASE7*, hornerin (*HRNR*) and S100A7 (psoriasin) were analyzed (Fig. 8). Acitretin reduces *DEFB4A* in resting cells ([100 nM] p=0.0061) in comparison to the untreated medium control. Further, all molecules with antimicrobial activity except for *HRNR* were induced by TNF-α/IL-1β-treatment compared to medium without cytokine. Both, 9-*cis* RA and acitretin were able to inhibit the up-regulation of the TNF-α/IL-1β-induced expression of *DEFB4A* (9-*cis* RA [100 nM] (p=0.0012); acitretin [100 nM] (p=0.0121)) and *DEFB103A* (9-*cis* RA [100 nM] (p=0.0006); acitretin [100 nM] (p=0.0242)) as against the TNF-α/IL-1β-treated control. 9-*cis* RA and acitretin decreased *DEFB103A* in resting cells compared to medium (9-*cis* RA [100 nM] p=0.0262); acitretin [100 nM] p=0.0061). *DEFB103A* expression was also reduced by both retinoids in a concentration of 100 nM in IL-4 activated cells directly compared to IL-4 control (9-*cis* RA (p=0.0379); acitretin (p=0.0424) and in keratinocytes stimulated with IFN-γ (9-*cis* RA [100 nM] p=0.0379); acitretin [100 nM] p=0.0424) as against the IFN-γ control. *RNASE7* expression was also up-regulated by TNF-α/IL-1β activation and significantly decreased by a co-treatment with 9-*cis* RA [100 nM] (p=0.0379). *HRNR* expression was induced by acitretin [100 nM] (p=0.0242) in resting keratinocytes compared to medium control. Expression of *S100A7* was not regulated by 9-*cis* RA or acitretin.

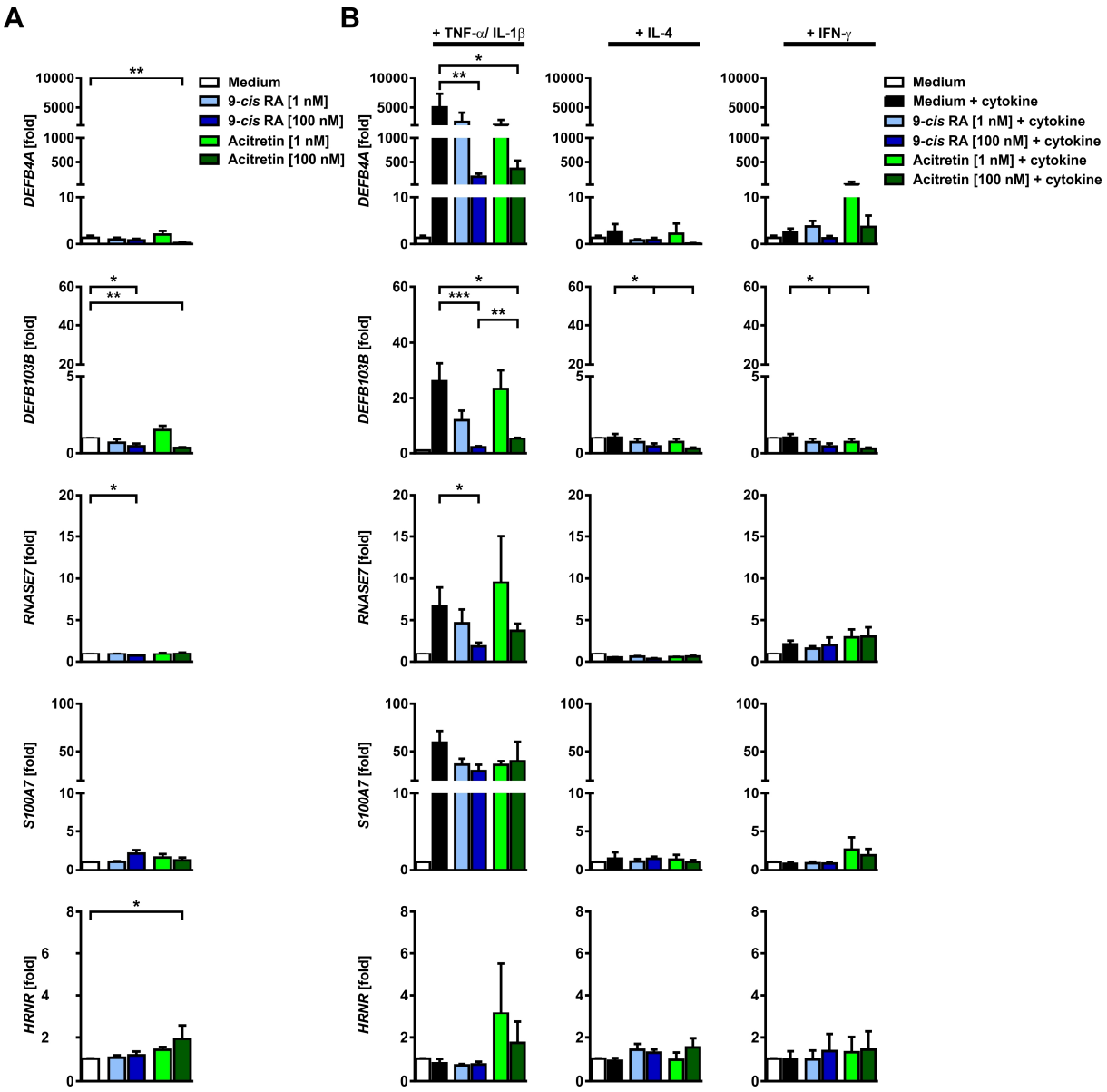

FIGURE 8 9-*cis* RA reduced the expression of antimicrobial peptides DEFB4A, DEFB103A, RNASE7, hornerin (HRNR) and S100A7. Keratinocytes were left without cytokines (A) or treated with primary pro-inflammatory cytokines TNF-α plus IL-1β or T cell-derived effector cytokines IL-4 or IFN-γ (B) in the presence or absence of 9-cis RA (1, 100 nM; n=7 independent donors) or acitretin (1, 100 nM; n=4 independent donors). Twenty-four hours later cells were harvested, RNA was extracted and gene expression was analyzed using quantitative RT-PCR. Results were normalized to 18S ribosomal RNA expression. Values represent as mean + SEM of the fold change to the untreated medium control without cytokines. Mann-Whitney U test was used to evaluate significant differences (*p<0.05, **p<0.01, ***p<0.001).

Histopathological analyses of lesional hand eczema show a dense inflammatory infiltrate, mainly effector $CD4^+$ T cells expressing the T_H1 cytokines IL-2 and IFN-γ, monocytes/macrophages and dendritic cells (Willis et al., 1989, Hoefakker et al., 1995, Hanifin and Rajka, 1980). These cells are recruited via chemokines receptors responding to chemokines released by keratinocytes and fibroblasts at inflammatory sites (Muller et al., 2001, Homey

et al., 2002a, Charo and Ransohoff, 2006). To analyze the *in vitro* effect of retinoids on expression and release of chemokines, keratinocytes were activated with inflammatory cytokines (Fig. 9). CCL5 plays an important role in recruiting type 1 memory T cell and migration of Langerhans cells to sites of inflammation (Ouwehand et al., 2013). IFN-γ induced *CCL5* mRNA up-regulation was significantly inhibited by 9-*cis* RA (p=0.0023). ELISA data confirmed that levels of CCL5 were significantly reduced by 9-*cis* RA in supernatants of keratinocytes activated with IFN-γ (n=4; p=0.0286) as compared to IFN-γ activated cells. CCL22 is important for the recruitment of T_H2 cells to sites of inflammation and for the regulation of T_H2-related immune responses (Soumelis et al., 2002). *CCL22* was induced in both TNF-α/IL-1β and IFN-γ activated cells but not regulated by 9-*cis* RA. Acitretin was capable to repress the transcription of *CCL22* significantly in TNF-α/IL-1β activated cells (p=0.0242) compared to medium with pro-inflammatory cytokines. The chemokine CCL20 is induced by pro-inflammatory cytokines and recruits T_H17 cells, as well as monocytes (Griffith et al., 2014). *CCL20* expression was increased by TNF-α/IL-1β but not regulated by retinoids. The expression of the homeostatic chemokine *CXCL14* was decreased by 9-*cis* RA in resting conditions compared to medium ([1 nM] p= 0.0379, [100 nM] p= 0.0006).

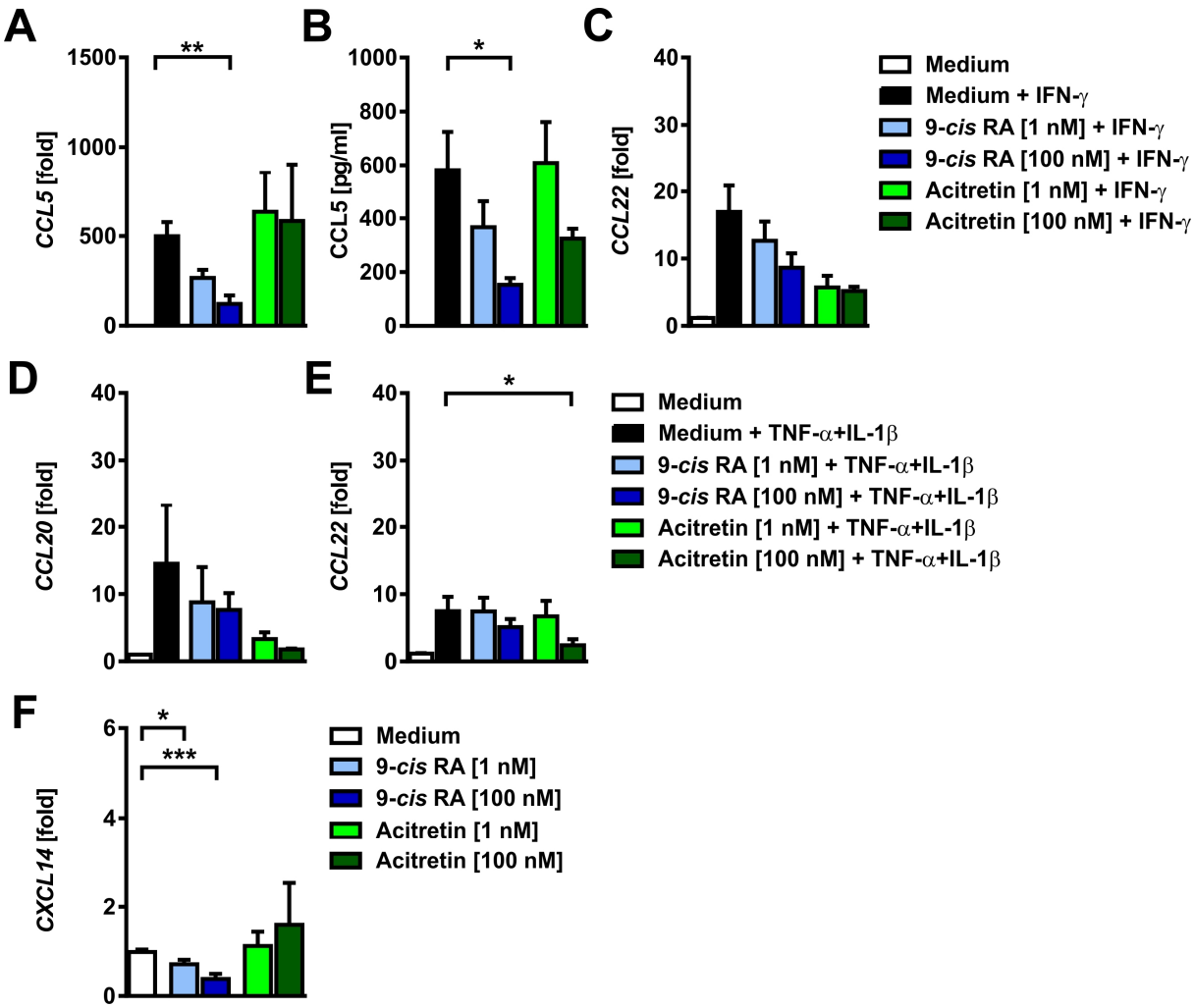

FIGURE 9 9-*cis* RA regulates the chemokine profile in resting and activated human primary keratinocytes. Effects of 9-*cis* RA and acitretin on the gene expression of CCL5 in keratinocytes in response to T cell-derived effector cytokine IFN-γ in the presence or absence of 9-*cis* RA or acitretin [1, 100 nM] (A) and the release of CCL5 into the supernatant of IFN-γ-activated human primary keratinocytes (B). Regulation of CCL22 in TNF-α/IL-1β (D) and IFN-γ-activated keratinocytes (C) in the presence or absence of 9-*cis* RA or acitretin. Expression of CCL20 TNF-α/IL-1β-treated (E), and of CXCL14 in resting keratinocytes (F) in the presence or absence of 9-*cis* RA and acitretin. qPCR-values represent the mean + SEM of seven (9-*cis* RA) and four (acitretin) individual experiments. ELISA-values represent the mean + SEM of four experiments. Mann-Whitney U test was used to evaluate significant differences (*p<0.05, **p<0.01, ***p<0.001).

5.2 Effect of retinoids on human monocyte derived dendritic cells (MoDCs)

5.2.1 9-*cis* RA changes the transcriptome of MoDCs

Overall the retinoid-induced regulation of target genes in keratinocytes was moderate. Hence, the attention was directed towards retinoid-induced modulation of leukocyte functions. With regard to retinoid-induced immunoregulation, the effect of retinoids on dendritic cells *in vitro* were analyzed. CD14 positive monocyte were treated with GM-CSF and IL-4 to induce dendritic cells in the presence or absence of the endogenous retinoid 9-*cis* RA. To analyze the effect of the pan-receptor agonist 9-*cis* RA, synthetic acitretin as a RAR-agonist was used as a control retinoid. After 6 days DCs were stimulated with TNF-α for maturation, hereafter referred to as mature MoDCs (mMoDCs), or left untreated immature MoDCs (iMoDCs). MoDCs were further matured in the presence of 9-*cis* RA or acitretin and referred to as "retinoid-DCs" or specifically as "9-*cis* RA-DC" or "acitretin-DC". At first, changes of the morphology of 9-*cis* RA treated monocyte-derived dendritic cells were visible after day three in culture. Cells changed from a rather round shaped cell with pseudopodia into longer shaped cells connecting to each other (Fig. 10).

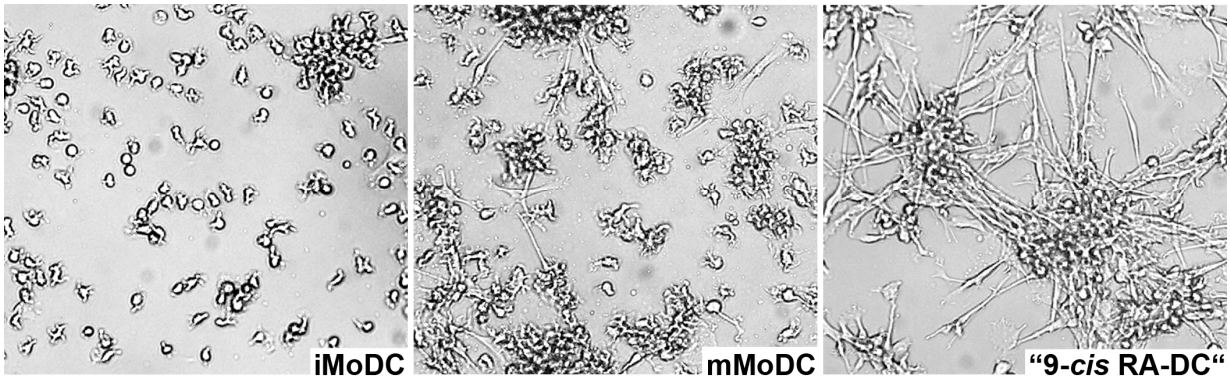

FIGURE 10 Morphological changes of 9-*cis* RA treated monocyte-derived dendritic cells (MoDCs). Representative microscopical images of immature monocyte-derived DC (iMoDC), maturated (mMoDC) and 9-*cis* RA [100 nM] treated TNF-α maturated cell ("9-*cis* RA-DC") cultures after 9 days. Phase contrast light microscopy, magnification: 200x.

To take a more global approach, the transcriptome of dendritic cells was analyzed. RNA of iMoDCs, mMoDCs and "9-*cis* RA-DCs" (n=6 individual donors each) were harvested, 200 ng of each donor was pooled with the others from the same group and analyzed by whole transcriptome RNA sequencing. Transcription was quality controlled and filtered, mapped alignments were quantified in strand-specific mode, whereas non-unique and low mapping alignments were discarded. Expression of differential expressed genes were nor-

malized between samples according to the size factor. A Venn-diagram between all three pairs (mMoDCs vs "9-*cis* RA-DC, iMoDCs vs "9-*cis* RA-DC, iMoDCs vs mMoDCs) showed a larger group of genes common in the comparison iMoDCs vs "9-*cis* RA-DCs (Fig. 11).

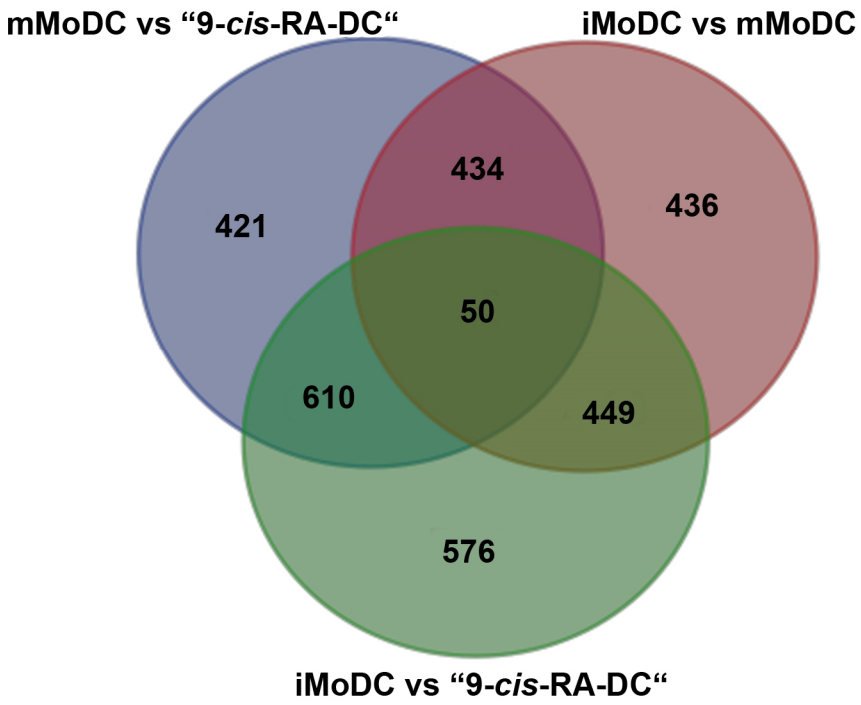

FIGURE 11 Venn diagram comparing the gene expression between the groups of mMoDCs vs "9-cis RA-DC", iMoDCs vs "9-cis RA-DC and iMoDCs vs mMoDCs

TOP 100 regulated genes in mMoDCs and "9-*cis* RA-DCs" are shown in Table 7.

TABLE 7 RNA of mMoDCs and "9-*cis* RA-DCs" (n=6 individual donors each) were analyzed by whole transcriptome RNA sequencing. TOP 100 genes up-regulated (UP) and down-regulated (DOWN) genes in "9-*cis* RA-DCs" vs mMoDCs with their fold changes and p-values are listed.

RANK	Gene	Fold change (log_2) UP in 9-*cis* RA-DCs	p value	Gene	Fold change (log_2) DOWN in 9-*cis* RA-DCs	p value
1	CXCR5	4.589	2.759E-04	AIRE	-4.252	1.584E-04
2	CTD-2201I18.1	4.562	3.998E-04	LONRF2	-4.208	1.620E-04
3	CP	4.449	1.114E-04	RAB27B	-4.206	1.088E-04
4	EPB41L1	3.853	1.219E-04	IL12B	-4.126	7.593E-04
5	AC011899.9	3.735	4.728E-04	PDZD2	-4.117	1.137E-04
6	S100A9	3.685	1.207E-04	ABHD17C	-4.095	5.581E-04
7	CCL7	3.680	1.922E-04	SRSF12	-4.012	9.866E-04
8	IGHE	3.571	7.478E-04	INSM2	-3.993	1.530E-03
9	GCNT3	3.559	6.109E-04	CEP55	-3.966	1.676E-04
10	SARDH	3.541	3.057E-04	NCKAP5	-3.954	2.364E-04
11	DBN1	3.497	2.387E-04	KCNN1	-3.930	2.896E-04
12	LYNX1	3.474	3.106E-04	TUBB2A	-3.907	1.020E-03
13	CR1	3.447	1.351E-04	IFI44L	-3.867	1.180E-04
14	PITPNM3	3.379	1.952E-03	FERMT2	-3.846	3.850E-04
15	SPOCD1	3.372	3.266E-04	ADCY5	-3.840	1.244E-03
16	FCAR	3.317	8.736E-04	ALPK3	-3.839	2.253E-04
17	RHBDD2	3.312	1.939E-04	DEPTOR	-3.734	1.128E-04
18	LPO	3.312	7.125E-03	HAPLN3	-3.716	1.051E-04
19	COL4A2	3.240	4.168E-03	TMEM200A	-3.696	4.143E-04
20	GNGT2	3.221	3.626E-03	TMEM97	-3.661	1.696E-04
21	PAPSS2	3.214	3.571E-04	ANO9	-3.611	1.082E-04
22	PGBD5	3.207	4.692E-03	GPR27	-3.606	5.476E-04
23	CHDH	3.194	5.203E-04	GPC5	-3.563	8.266E-04
24	ITGB8	3.193	5.207E-04	MAPK11	-3.558	1.961E-04
25	CTA-363E6.1	3.189	2.215E-03	VAT1L	-3.549	1.016E-03
26	MIR3648	3.177	6.785E-03	SH3BP4	-3.547	8.062E-04
27	LILRA1	3.177	2.668E-03	RASL10B	-3.541	3.695E-03
28	GPR114	3.163	2.702E-03	CCSER1	-3.535	3.829E-04
29	CD300A	3.144	4.604E-04	LAD1	-3.512	1.326E-04
30	COL5A3	3.126	3.547E-03	SLC16A9	-3.502	1.366E-04
31	LILRA3	3.124	4.377E-04	IL32	-3.484	2.938E-04
32	ABI3	3.099	1.007E-03	XKR4	-3.450	8.788E-04
33	G0S2	3.098	4.147E-04	CD1A	-3.442	1.044E-04
34	ITGA6	3.089	6.320E-04	ABTB2	-3.431	2.568E-04
35	SPARC	3.077	4.431E-04	MMP12	-3.429	1.033E-04
36	NRIP3	3.064	5.194E-04	FSTL1	-3.425	1.562E-04
37	DHRS3	3.048	6.438E-04	CD1E	-3.409	1.190E-04
38	ARHGEF10L	3.011	6.429E-04	SLC25A23	-3.383	3.509E-04
39	NOTCH3	3.009	2.245E-03	GRAMD1B	-3.378	6.106E-04
40	FHAD1	3.001	1.745E-03	PTPRG	-3.357	6.147E-04
41	CCL2	2.994	6.406E-04	MIR503HG	-3.328	8.143E-04
42	GLDN	2.976	1.334E-02	ZDHHC23	-3.328	3.197E-03
43	AL161626.1	2.971	1.007E-03	TNC	-3.320	2.201E-04
44	UBXN11	2.954	1.137E-03	FUT8	-3.312	1.395E-03
45	CA12	2.947	4.646E-03	RNF150	-3.294	2.352E-03
46	JAKMIP2	2.925	4.961E-03	HIST1H2BB	-3.286	1.913E-03
47	MMP7	2.906	3.128E-03	MKI67	-3.274	6.966E-04
48	CRB2	2.899	5.540E-03	SPEF2	-3.242	6.688E-04
49	MS4A4A	2.897	8.860E-04	GP1BA	-3.202	8.967E-04
50	GALNT12	2.873	9.771E-04	PDGFRA	-3.196	3.965E-03

A set of genes regulated during maturation, like maturation marker CD83, co-stimulatory molecules CD80 and CD86, as well as others previously described (Schinnerling et al., 2015, Imai et al., 1997, Yamamoto et al., 2000, Satyam et al., 2009, Decker et al., 2008, Lapteva et al., 2001, Korthals et al., 2007) was analyzed in the RNAseq data set as well. Most maturation markers followed TNF-α stimulation of DCs were down-regulated in "9-*cis* RA-DC" (Tab. 8).

TABLE 8 RNA of iMoDCs, mMoDCs and "9-*cis* RA-DCs" (n=6 individual donors each) were analyzed by whole transcriptome RNA sequencing. Key gene expression of genes regulated during maturation of DCs and fold changes between mMoDC vs "9-*cis* RA-DC" were analyzed, whereas positive regulations means up-regulation in "9-*cis* RA-DC".

Gene_name	***Expression iMoDC***	***Expression "9-cis RA-DC"***	***Expression mMoDC***	***Fold change (log_2) mMoDC vs "9-cis RA-DC" (+ = UP in "9-cis RA-DC")***
CD83	9662.66	17778.15	65980.74	-1.89
CD86	11962.49	17346.12	16024.35	0.11
CD80	1230.79	913.75	5509.05	-2.59
CCL22	9376.23	146243.14	213922.84	-0.55
CCL17	10379.57	81983.65	335675.08	-2.03
HLA-DQA1	46767.44	10150.61	29288.70	-1.53

Bioinformatic analyses showed that several genes clustered in the following Gene ontology terms (GO terms): A) the antigen processing presentation, B) Chemokine mediated signaling, C) negative regulation of cell proliferation and D) extracellular matrix regulation (Fig. 12).

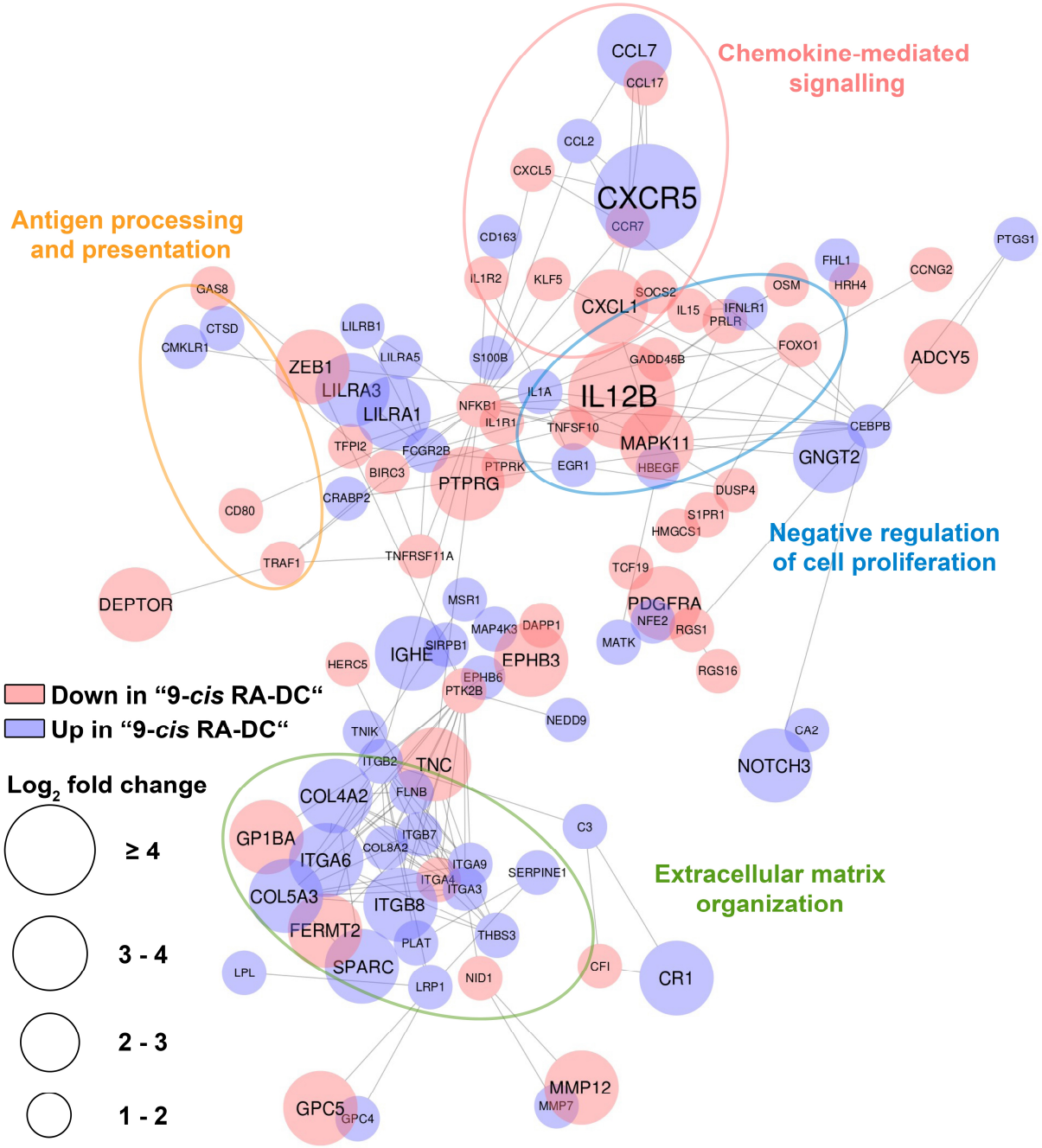

FIGURE 12 Cluster analysis of differential expressed genes in "9-*cis* RA-DCs" vs mMoDCs. Colors indicate up-regulated genes in "9-*cis* RA-DCs" (UP, blue) and down-regulated genes (Down, red). Scales of circles indicate the fold change of the differential expressed genes. Clusters are shown in colors.

To validate the RNAseq data, a set of genes were tested by qPCR including chemokines *CCL17* and *CCL7*, matrix metalloproteinase 12 (*MMP12*), S100-family member 9 (*S100A9*), aquaporin (*AQP9*), with *CCL7*, *MMP12* and *S100A9* being among the 50 highest differentially regulated genes under the tested conditions. Also, the FANTOM5 databank revealed that these genes are up-regulated during activation and maturation of monocytes. *CCL17* and *MMP12* have been associated with maturation of monocytes to dendritic cells

(Castiello et al., 2011). Both genes showed an increase in expression upon maturation (both p=0.0022) compared to iMoDCs, while retinoids diminished the maturation-induced up-regulation of *CCL7* and *MMP12* (*CCL17* 9-*cis* RA: p=0.0022; acitretin: p=0.0714/ *MMP12* 9-*cis* RA: p=0.0043; acitretin: p=0.0303). For aquaporin, both retinoids induced the expression of this *AQP9* (9-*cis* RA: p=0.0022; acitretin: p=0.0043) as against mMoDCs. *CCL7* and *S100A9* expressions are up-regulated in "retinoid-DCs" (both p=0.0022) in comparison to mMoDCs. The data obtained by RNA sequencing was in good accordance to qPCR data from single donors (Fig. 13).

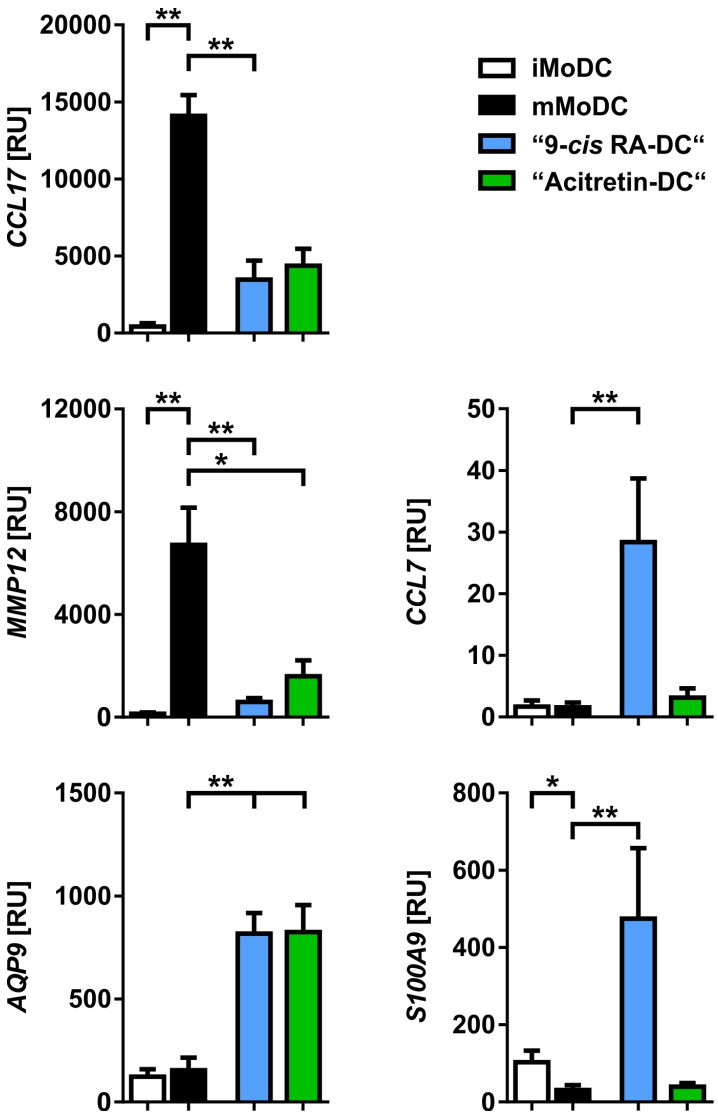

FIGURE 13 9-*cis* RA changes the expression profile of monocyte derived dendritic cells (moDCs). Validation of chosen genes in single donors of iMoDCs, mMoDCs, "9-*cis* RA-DCs" (100 nM; all n=6 individual donors) and "Acitretin-DCs" (100 nM; n=4 individual donors). qPCR-values represent the mean + SEM. Mann-Whitney U test was used to evaluate significant differences (*p<0.05, **p<0.01).

5.2.2 Retinoids have an inhibitory effect on the maturation of MoDCs

More common expressed genes between iMoDCs and "9-*cis* RA-DC" (Fig. 11, Tabs. 7 + 8), a RNAseq (Fig. 12) as well as qPCR data (Fig. 13) suggested a rather immature phenotype of "9-*cis* RA-DCs. To determine whether 9-*cis* RA has an immunomodulatory outcome, its effect on DC differentiation was analyzed. A new set of dendritic cells was stimulated under the same conditions as described above and analyzed by flow cytometry. To characterize the maturation of DCs, different markers were used. First, CD83, a well-known maturation marker for human DCs, was tested. Together with co-stimulatory molecule CD86, it was strongly up-regulated during maturation of monocyte derived dendritic cells. iMoDCs expressed low levels of CD83 and CD86. Maturation markers were induced upon TNF-α-treatment (mMoDCs) and reduced upon 9-*cis* RA-treatment to a level comparable to iMoDCs (Fig. 14). DMSO had no effect on the expression of the tested markers and is not shown further.

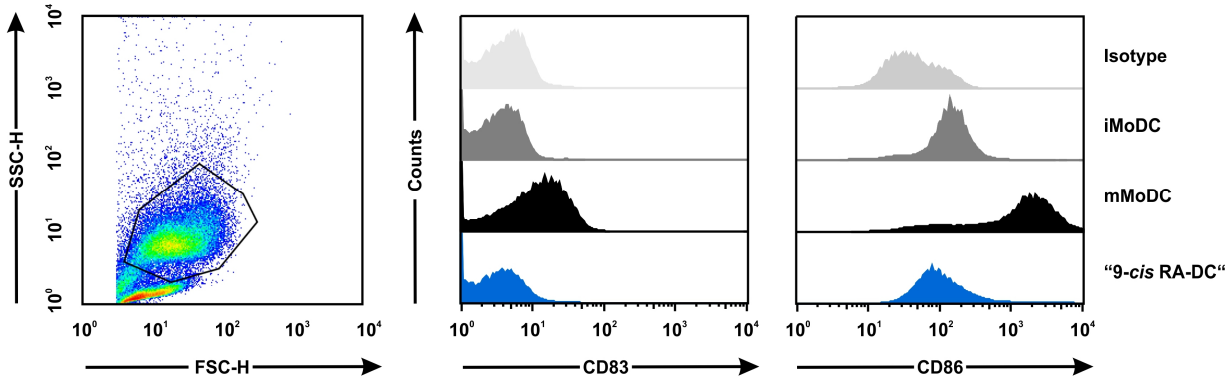

FIGURE 14 Surface expression of MoDC maturation markers CD83 and CD86. Positively selected CD14$^+$ monocytes were cultured in medium supplemented with GM-CSF and IL-4 to generate immature DCs. Retinoids were added at culture initiation and cells were fed fresh medium every 3 days. Maturing was initiated by TNF-α at day 6. Representative half offset histograms of one donor show the expression of surface markers CD83 and CD86 and the corresponding isotype controls (light grey) on immature (iMoDC, dark grey), TNF-α maturated (mMoDC, black) and" 9-*cis* RA-DCs" (blue) after 9 days of culture. 9-*cis* RA strongly down-regulates CD83 and CD86 compared to mMoDCs.

In total, the following markers were analyzed for surface expression: CD83, CD80, CD86, chemokine receptors CCR6 and CCR7, major histocompatibility complex molecule class II HLA-DR, Programmed death-ligand 1 (PD-L1) and ecto-5'-nucleotidase CD73. Data is expressed as a ''fold change'' considering immature MoDCs (iMoDC) of each individual donor (value=1) and tested with an unpaired two-tailed Mann-Whitney U-test. mMoDCs expressed high levels of CD83, while with all doses of 9-*cis* RA and acitretin [100 and 1000 Nm]

treatment MoDCs were low positive for CD83 (n=11-28 independent individual donors). Analysis of co-stimulatory molecule CD80 (n=11-12 independent donors) showed an up-regulation in mMoDCs compared to iMoDCs. Addition of 100 and 1000 nM 9-*cis* RA or 100 nM acitretin significantly reduced CD80 expression in comparison to mMoDCs. After exposure to TNF-α, "retinoid DCs" were not able to up-regulate surface expression of CD86 (n=11-25 independent donors) compared to mMoDCs (Fig. 15). Individual p-values for the expression of different markers are shown in table 9.

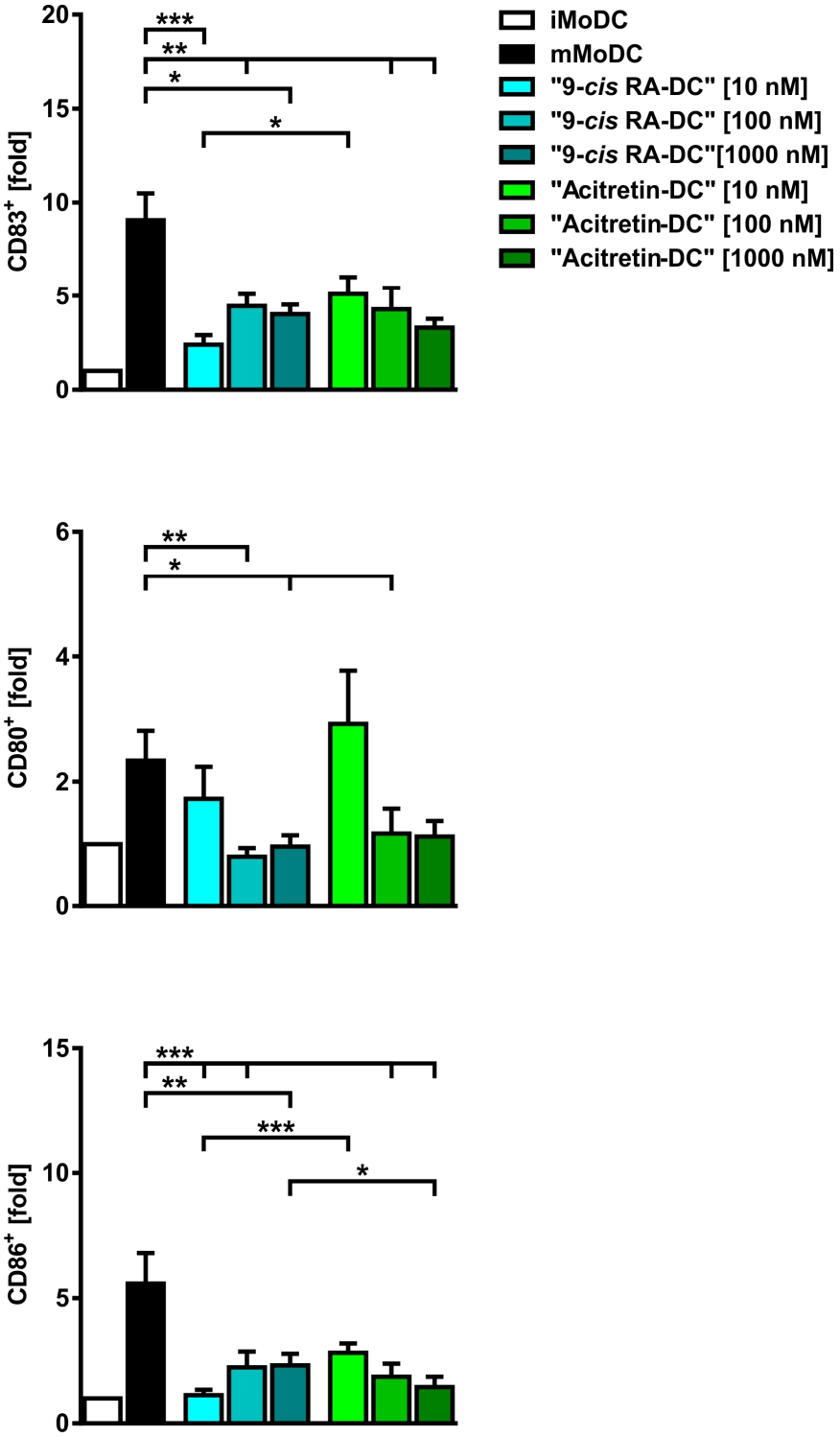

FIGURE 15 "Retinoid-DCs" are not sensitive to maturation stimuli. Positively selected CD14$^+$ monocytes were cultured in medium supplemented with GM-CSF and IL-4 to generate immature DCs. Retinoids were added at culture initiation and cells were fed fresh medium every 3 days. Maturing was initiated by TNF-α at day 6. Surface expression of maturation marker CD83 (n=11-28 independent donors), co-stimulatory molecules CD80 (n=11-12 independent donors) and CD86 (n=11-25 independent donors). Fold changes are shown as mean +SEM of the surface expression of CD83, CD80 and CD86. The means were significantly different using unpaired two-tailed Mann-Whitney U-test (*p<0.05, **p<0.01, ***p<0.001).

Moreover, molecules involved in antigen presentation like major histocompatibility complex (MHC) molecule class II, HLA-DR (n=4-5 individual donors) and inhibitor programmed death-ligand 1 (PD-L1) (n=3-6 independent donors) were tested by FACS analysis (Fig. 16). HLA-DR was already highly expressed in iMoDCs and mMoDCs. All retinoid doses except for 10 nM acitretin significantly lowered HLA-DR expression compared to mMoDCs.

PD-L1 expression was induced in mMoDCs by maturation in comparison to iMoDCs. Addition of 10 nM 9-*cis* RA inhibited the expression of PD-L1 as against mMoDCs.

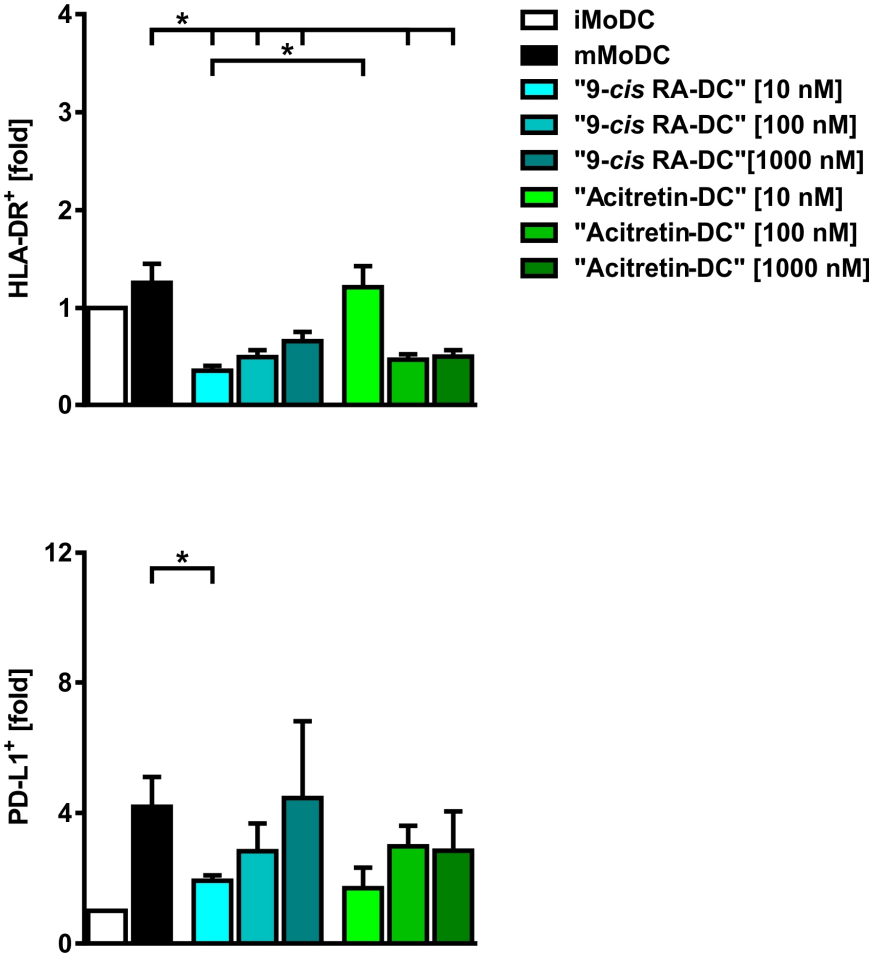

FIGURE 16 Retinoid-DCs treatment down-regulates antigen presentation in MoDCs. Surface expression of HLA-DR (n=4-5 individual donors), and inhibitory PD-L1 (n=3-6 independent donors) on the surface of MoDCs during maturation. Fold changes are shown as mean +SEM of the surface expression of HLA-DR and PD-L1. The means were significantly different using unpaired two-tailed Mann-Whitney U-test (*p<0.05).

5.2.3 Retinoids change the chemokine receptor profile of MoDCs

To further study the effects of 9-*cis* RA and acitretin on maturation of DCs, cells were stained for the CC-chemokine receptors CCR6 and CCR7 (Fig. 17). In maturing DCs, CCR6 is down-regulated whereas CCR7 is up-regulated (Caux et al., 2000, Yanagihara et al., 1998, Ohl et al., 2004). The overall regulation of CCR6 and CCR7 in mMoDCs compared to iMoDCs was marginal, but expression of CD83 and CD86 confirmed their maturation status. “Retinoid-DCs” had a higher CCR6 and CCR7 expression compared to mMoDCs. Addition of 1000 nM 9-*cis* RA significantly increased CCR6 expression compared to mMoDCs. Higher doses of both retinoids significantly up-regulated expression of CCR7 in comparison to mMoDCs.

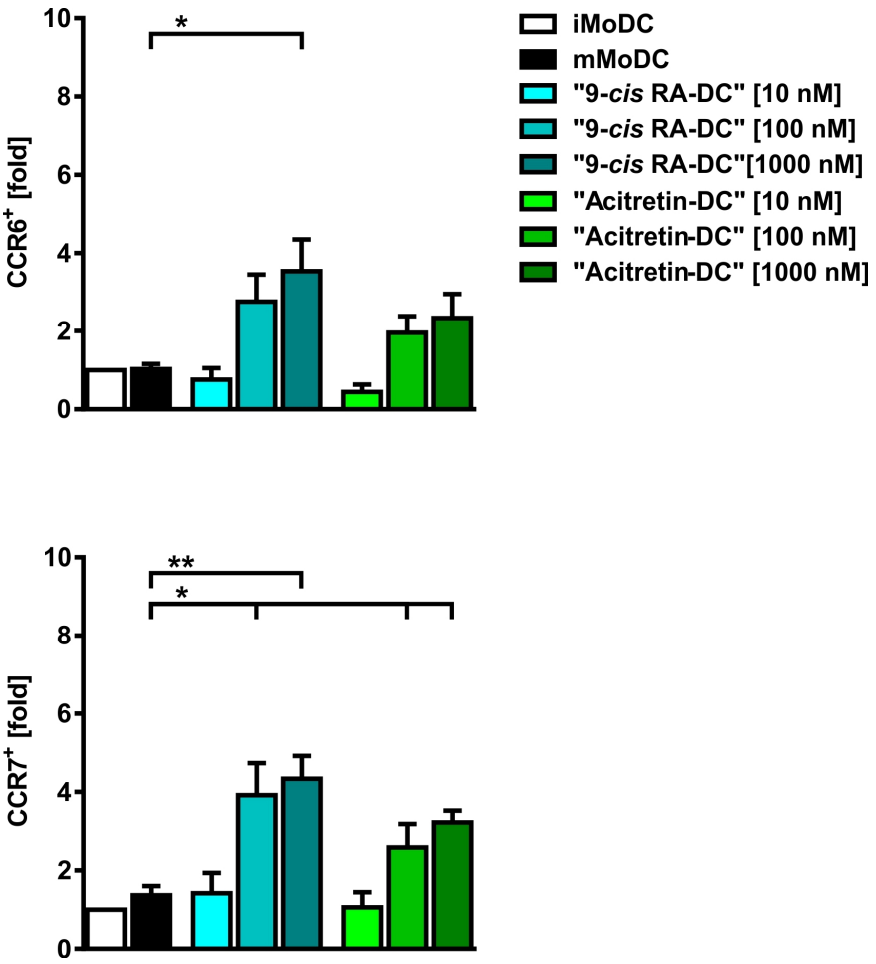

FIGURE 17 Retinoids increase chemokine receptor expression in MoDCs. Expression of chemokine receptors CCR6 (n=4-13 independent donors) and CCR7 (n=3-10 independent donors. Fold changes are shown as mean +SEM of the surface expression of CCR6 and CCR7. The means were significantly different using unpaired two-tailed Mann-Whitney U-test (*p<0.05, **p<0.01).

5.2.4 Retinoids induce the expression and activity of ecto-5'-nucleotidase (NT5E/CD73) on MoDCs

To study the immune-modulatory functions of these less maturated “retinoid-DCs”, ecto-5'-nucleotidase CD73 expression and activity was analyzed. CD73 is known to produce adenosine from adenosine monophosphate (AMP). Adenosine itself has immune-modulatory functions on T-cells by binding to adenosine receptors (ADORA1, 2A, 2B and A3) (Sevigny et al., 2007, Raskovalova et al., 2005, Koshiba et al., 1997, Huang et al., 1997, Fredholm, 2007). Maturation had no effect on mMoDCs as against iMoDCs. Addition of 100 and 1000 nM 9-*cis* RA or 100 nM acitretin significantly up-regulated the expression of CD73 (n=7-22 independent donors) on the cell surface of MoDCs (Fig. 18A). To test the enzyme activity of CD73, etheno-AMP degradation to etheno-adenosine was measured. CD73 was not only expressed on the cell surface but also active with enzymatic activity increasing with addition of 100 nM 9-*cis* RA by 1.75-fold and with 1000 nM 9-*cis* RA (p=0.0286) by 4.3-fold compared to mMoDCs (Fig. 18B).

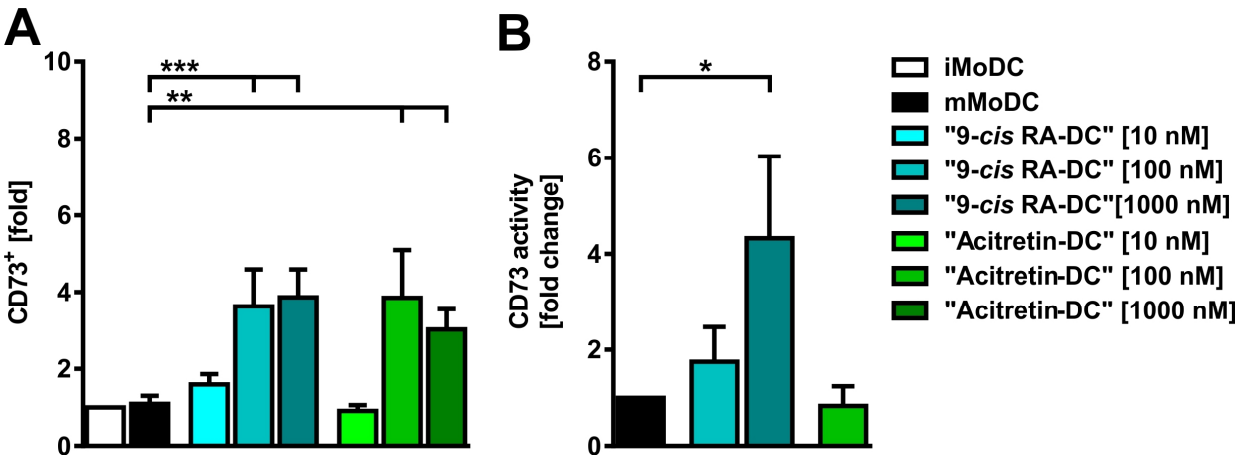

FIGURE 18 9-*cis* RA and acitretin induces the expression and activity of ecto-5'-nucleotidase CD73 on monocyte derived dendritic cells (MoDCs). A) Expression of CD73 (n=7-22 independent donors). Fold changes are shown as mean +SEM of the surface expression of CD73. The means were significantly different using unpaired two-tailed Mann-Whitney U-test (**p<0.01, ***p<0.001). B) CD73 enzyme activity of MoDCs. Data is expressed as a ''fold change'' considering mature MoDCs (mMoDC) of each individual donor (value=1). The means were significantly different using unpaired two-tailed Mann-Whitney U-test (*p<0.05).

TABLE 9 P-values of fold expression of TNF-α matured dendritic cells vs TNF-a matured and retinoid co-treated DCs regarding the fold expression of CD83, CD80, CD86, chemokine receptors CCR6 and CCR7, HLA-DR, PD-L1 and CD73

Marker	*mMoDC vs "9-cis RA-DC" [10 nM]*	*mMoDC vs "9-cis RA-DC" [100 nM]*	*mMoDC vs "9-cis RA-DC" [1000 nM]*	*mMoDC vs "acitretin-DC" [10 nM]*	*mMoDC vs "acitretin-DC" [100 nM]*	*mMoDC vs "acitretin-DC" [1000 nM]*
CD83	*** 0.0002	* 0.011	* 0.026	ns 0.1061	** 0.0015	** 0.0021
CD80	ns 0.1149	** 0.0018	** 0.007	ns 0.8633	* 0.0241	ns 0.0786
CD86	*** < 0.0001	*** 0.0002	** 0.0016	ns 0.0721	*** 0.0002	*** < 0.0001
CCR6	ns 0.6411	ns 0.1167	* 0.0145	0.0196	ns 0.0754	ns 0.1802
CCR7	ns 0.5035	* 0.0279	** 0.0028	ns 0.9343	* 0.0314	* 0.0112
HLA-DR	* 0.0159	* 0.0159	* 0.0317	ns 0.8254	* 0.0159	* 0.0159
PD-L1	* 0.0476	ns 0.1797	ns 0.4476	ns 0.0667	ns 0.474	ns 0.1714
CD73	ns 0.1351	*** 0.0002	*** 0.0004	ns 0.6348	** 0.0048	** 0.0021

5.2.5 Retinoids regulate the release of cytokines and chemokines of MoDCs

As 9-*cis* RA seemed to have an infect on the maturation of DCs, the influence of 9-*cis* RA on cytokine/ chemokine production was evaluated by a cytokine array (Fig. 19). With the addition of 100 nM 9-*cis* RA, supernatants demonstrated an increase in IL-1β (Fold change (FC): 1.6), IL-10 (FC: 1.68), CCL5 (FC: 1.86), CCL7 (FC: 2.3), CCL8 (FC: 1.77), CCL20 (FC: 2.33) compared to mMoDCs.

A

Control (pos.)	Control (pos.)	Control (pos.)	Control (pos.)	Control (neg.)	Control (neg.)	CXCL5	GCSF	GM-CSF	GRO α/β/γ	CXCL1
CCL1	IL-1α	IL-1β	IL-2	IL-3	IL-4	IL-5	IL-6	IL-7	IL-8	IL-10
IL-12 (p40/p70)	IL-13	IL-15	IFN-γ	CCL2	CCL8	CCL7	M-CSF	CCL22	CXCL9	CCL4
CCL15	CCL5	SCF	CXCL12	CCL17	TGF-β1	TNF-α	TNF-β	EGF	EGF1	Angiogenin
OSM	TPO	VEGF-A	PDGF-BB	Leptin	BNDF	CXCL13	CCL23	CCL11	CCL24	CCL26
FGF-4	FGF-6	FGF-7	FGF-9	FLT-3 Ligand	CX3CL1	CXCL6	GDNF	HGF	IGFBP-1	IGFBP-2
IGFBP3	IGFBP4	IL-16	CXCL10	LIF	TNFSF14	CCL13	MIF	CCL20	CXCL7	NT3
NT-4	SPP1	TNFSF11	CCL18	PLGF	TGFB2	TGFB3	TIMP1	TIMP2	Control (pos.)	Control (pos.)

B

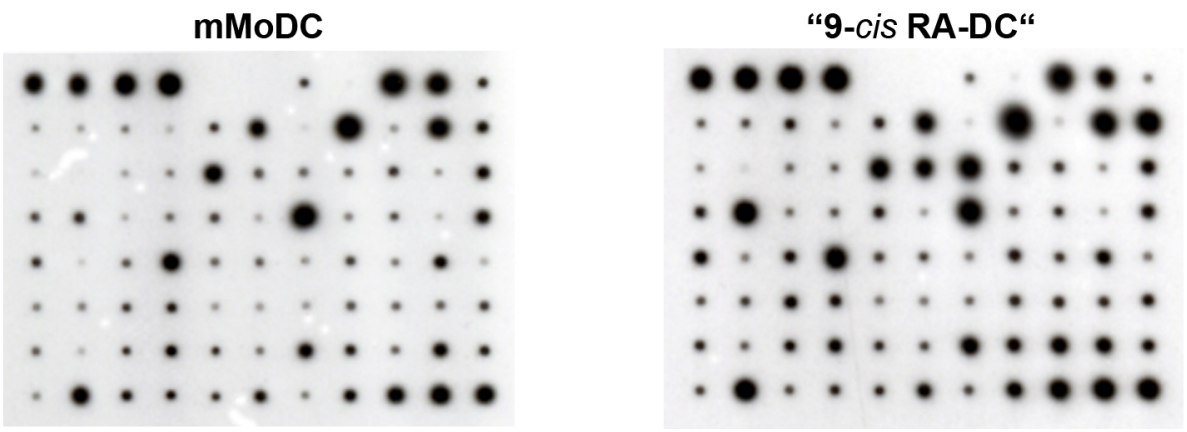

FIGURE 19 Effect of 9-*cis* RA on cytokine and chemokine production of MoDCs. A, Panel of the used cytokine array. B, Scans of the chemiluminescence exposed films from the membranes treated with supernatants of mMoDCs and "9-*cis* RA-DCs" [100 nM] from a representative donor.

IL-10, CCL5 and CCL20 were further analyzed by ELISA (Fig. 20). IL-10 production (n=10-12) was significantly ($p<0.001$) elevated with supplementation of 100 nM "9-*cis* RA-DCs" compared to both iMoDCs and mMoDCs. Addition of 100 9-*cis* RA also significantly increased expression of IL-10 in MoDCs compared to an equimolar dose of acitretin ($p=0.0006$). CCL5 and CCL20 (n=3 each) also demonstrated an up-regulation of the tested chemokines in 9-*cis* RA-DCs.

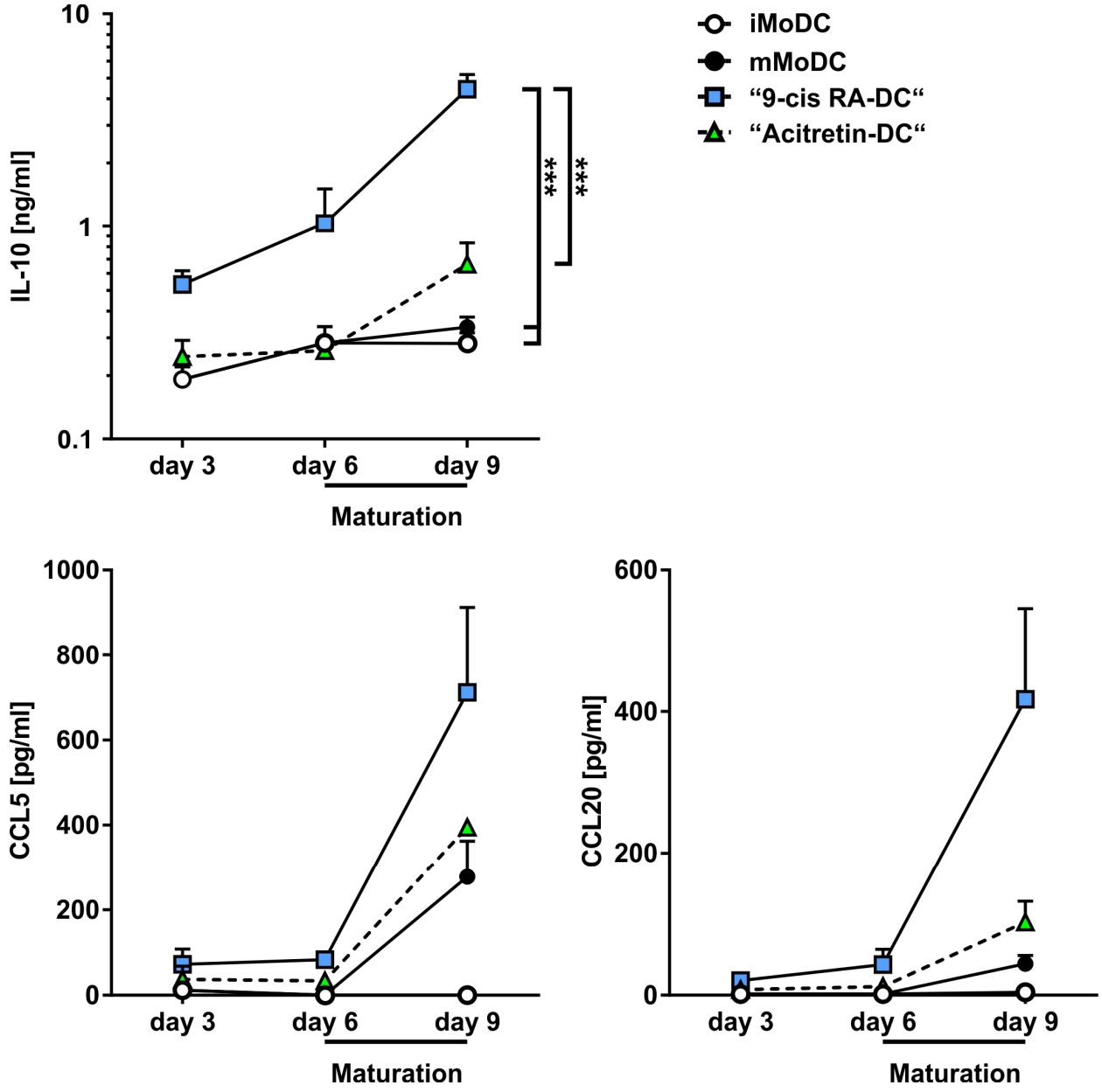

FIGURE 20 The influence of 9-*cis* RA and acitretin on IL-10, CCL5 and CCL20 release of MoDCs. DCs were stimulated with GM-CSF and IL-4 in the presence or absence of 9-*cis* RA or acitretin (100 nM). At day 6, cells were treated with GM-CSF/IL-4 in the presence or absence of 9-*cis* RA or acitretin (100 nM) and pro-inflammatory cytokine TNF-α or left TNF-α untreated. Supernatants were collected at day 3, day 6 and day 9 and tested by ELISA for IL-10 (n=6-12), CCL5 (n=3) and CCL20 (n=3). Mann-Whitney U test was used to evaluate significant differences (*p<0.05, **p<0.01, ***p<0.001).

5.2.6 Retinoids inhibited proliferative responses in allogenic CD3⁺ T cells

Monocyte-derived dendritic cells treated with 9-*cis* RA or acitretin show significant changes in maturation. The altered expression of the surface maturation marker CD83 as well as co-stimulatory molecules CD80 and CD86 obtained with 9-*cis* RA- or acitretin -treated DCs suggested an inhibitory role on the DCs´ ability to activate T cell proliferation. Allogenic $CD3^+$ T cells were co-cultured with retinoid-treated MoDCs, and proliferation of leukocytes was measured by BrdU-incorporation after 6-7 days (n=28 individual donors). iMoDCs showed a low capability to induce T-cell proliferation, whereas mMoDCs induced a pronounced proliferation of allogenic T cells. Monocytes differentiated in the presence of retinoids had significantly reduced capacity to stimulate $CD3^+$ T cell proliferation in a primary MLR assay compared to mMoDCs. Moreover, "9-*cis* RA-DCs" were more effective in much lower (10-fold) concentrations compared to acitretin in equimolar doses of 10 and 100 nM (Fig. 21).

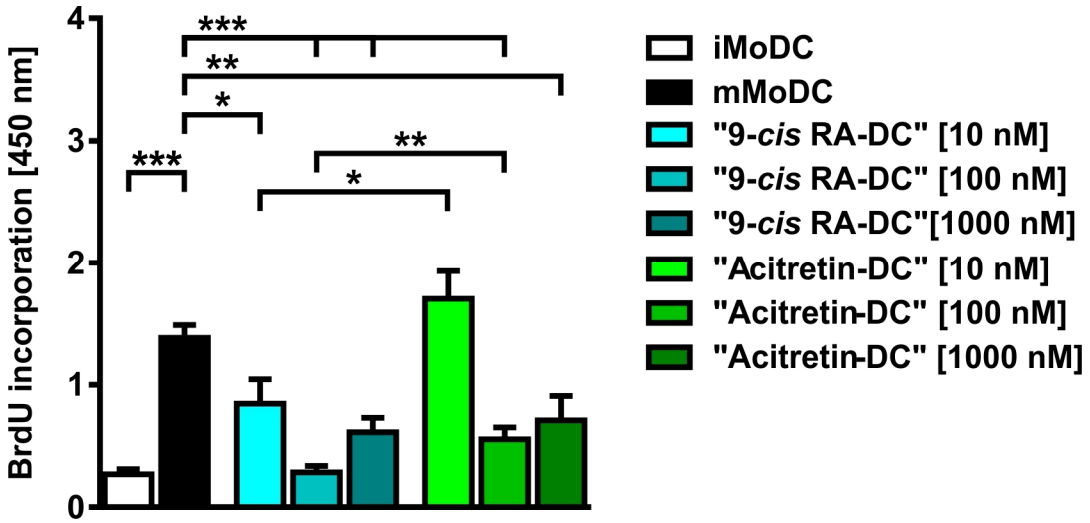

FIGURE 21 $CD3^+$ T cell proliferation and expansion with MoDCs stimulated with retinoids. The effect of graded doses of 9-*cis* RA and acitretin on dendritic cells on the allogenic stimulation of T cell proliferation assessed by BrdU-incorporation after 6-7 days at a low MoDC : T cell ratio (1:200). Results are expressed as mean +SEM, data represents the optical density (OD) of 6-28 independent donors. The means were significantly different using unpaired two-tailed Mann-Whitney U-test (*p<0.05, **p<0.01, ***p<0.001).

5.3 Effects of systemic 9-*cis* RA in patients suffering from chronic hand eczema (CHE)

5.3.1 Systemic 9-*cis* RA treatment

Having established the inhibitory effects of 9-*cis* RA on the maturation (i.e. down-regulation of CD83, CD 86, CD80) and T cell proliferation *in vitro*, I looked for evidence of these features *in vivo*. To this end, the effect of systemic 9-*cis* RA treatment on circulating $CD14^+$ monocytes, effector memory T cells, chemokine release and allogenic T cell proliferation was studied. The subjects were treated with a daily dose of 30 mg 9-*cis* RA for treatment of a chronic hand eczema. Subjects had a routinely blood exam to test liver functions and fat levels. At baseline (PRE) and after four (Wk4), eight (Wk8) and 12 weeks (Wk12) during treatment, the numbers and proliferative capacity of different circulating cells (Tab. 10) and the serum levels of different chemokines were determined.

TABLE 10 Chosen markers to identify cell populations

	Monocytes	**T cells**
Population markers	CD14	CD4, CD8
Skin homing marker	-	CLA
Chemokine receptors	-	CCR4, CCR6, CCR7, CCR9, CCR10, CXCR3
Lymph node homing marker	-	CD62L
Co-stimulatory molecules	CD80, CD86	-
Ectonucleidase System	CD73	CD73

Patients with at least 50% reduction in mTLSS (Ruzicka et al., 2008) were considered responders. From 29 patients, 23 individuals (79.3%) met these criteria, six patients (20.7%) were non-responders. Characteristics of the responder group are listed in table 11. Responders showed overall reduction of 78.63% of symptoms during treatment. Figure 22 shows the hand as well of lesional palmar skin of a 41-year patient before and after 12 weeks of 9-*cis* RA. Before treatment, the patient presented with an overall inflamed palm, represented by a prominent redness and dermal swelling. Further, the skin showed a number of fissures (Fig. 22A). H&E demonstrated a vast infiltrate (Fig. 22B). In general, the patient collectively showed an overall reduction of symptoms during treatment with 9-*cis* RA

(10[2] or 30 mg/d) to an mTLSS of 2.23 ± 1.82 ($p < 0.001$), achieving an improvement of 78.63% (Fig. 22C + Tab. 11).

TABLE 11 Responders characteristics of the monitored group

Group characteristics	Intention to treat	23
	Male/female	13:11
	age	53.76 ± 11.19
Hand eczema type	dyshidrosiform	2 (8.7%)
	hyperkeratotic-rhagadiform	18 (78.3%)
	Dyshidrosiform + hyperkeratotic-rhagadiform	3 (13 %)
mTLSS	PRE	10.48 ± 3.37
	Wk4	6.33 ± 3.3
	Wk8	4.02 ± 2.22
	Wk12	2.24 ± 1.82
	Reduction PRE vs Wk12	78.63 %

[2] One patient out of 29 was treated with 10 mg 9-cis RA in the first four weeks

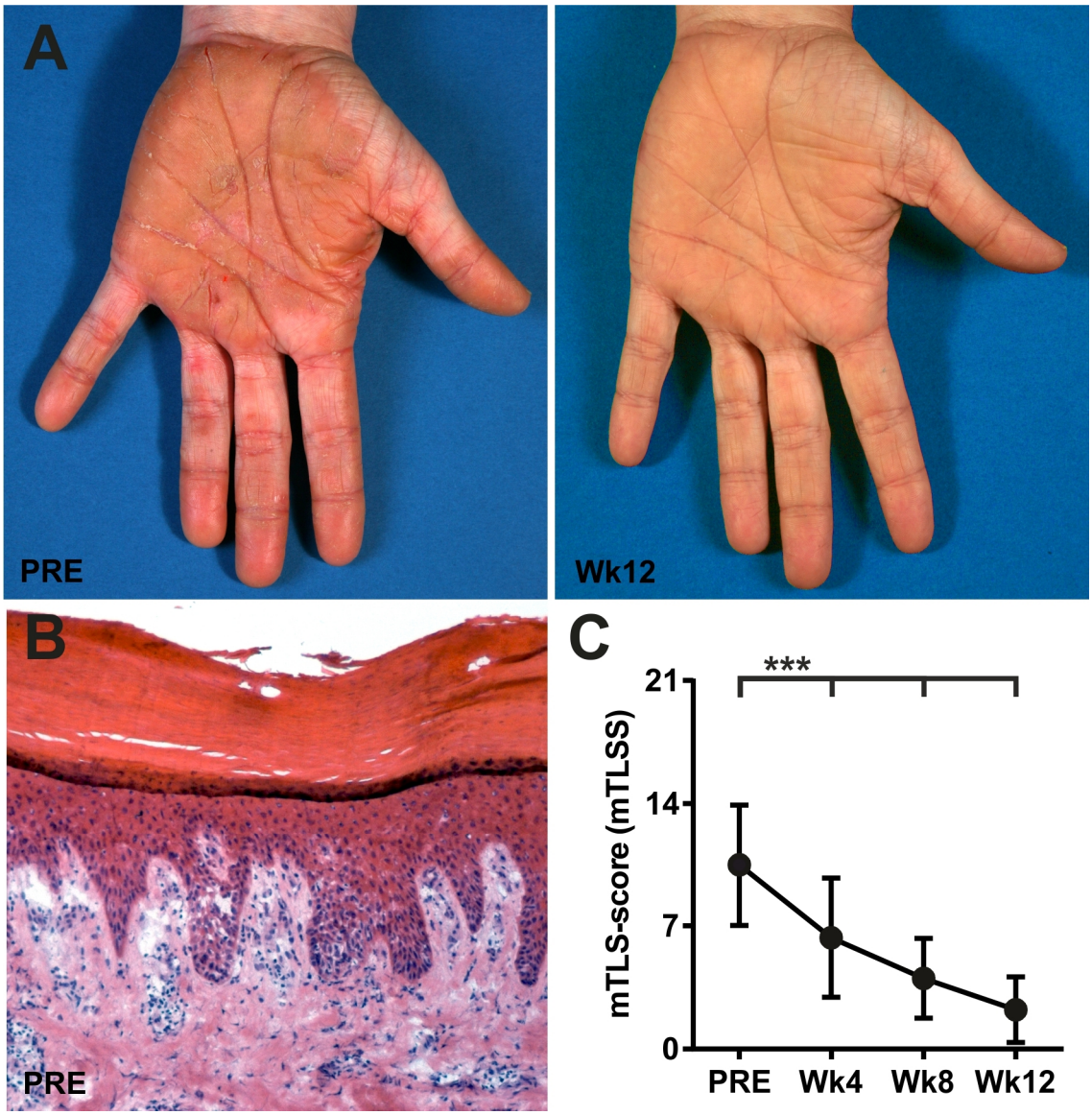

FIGURE 22 9-*cis* RA in patients with chronic hand eczema. A, 41-year-old patient diagnosed with hand eczema before (PRE) and 12 weeks (Wk12) under 9-*cis* RA. B, haematoxylin and eosin (H&E) staining of lesional palmar skin of a patient prior to 9-*cis* RA (Original magnification: 100x). C, mTLS-score evaluated from patients before and under 9-*cis* RA (10 or 30 mg/d). Values represent the mean +/- SD of 23 patients (responders). Student's t-test was performed to evaluate differences (***p<0.001).

5.3.2 Oral 9-*cis* RA modulates surface expression of monocytes and CD4 positive effector memory T cells

Blood cells were isolated with using BD Vacutainer® CPT™ Cell Preparation Tube with Sodium Citrate, washed and stained for different markers (Tab. 11). The percentage of positive, gated cells in patients at baseline and during treatment were analyzed using a paired student's T-test, whereas every patient served as its own control. The number of pairs for each time point and the individual p-values are given at the end of this chapter (Tab. 12). Upon treatment of 9-*cis* RA, the percentage of $CD14^{+}CD80^{+}$-monocytes were not changed.

The percentage of $CD14^+CD86^+$ -monocytes were significantly reduced after 12 weeks treatment with oral 9-*cis* RA compared to baseline (p = 0.027). Moreover, $CD14^+CD73^+$ cells were up-regulated during treatment at week four (p = 0.0043) and week twelve (p = 0.016) directly compared to baseline levels (Fig. 23).

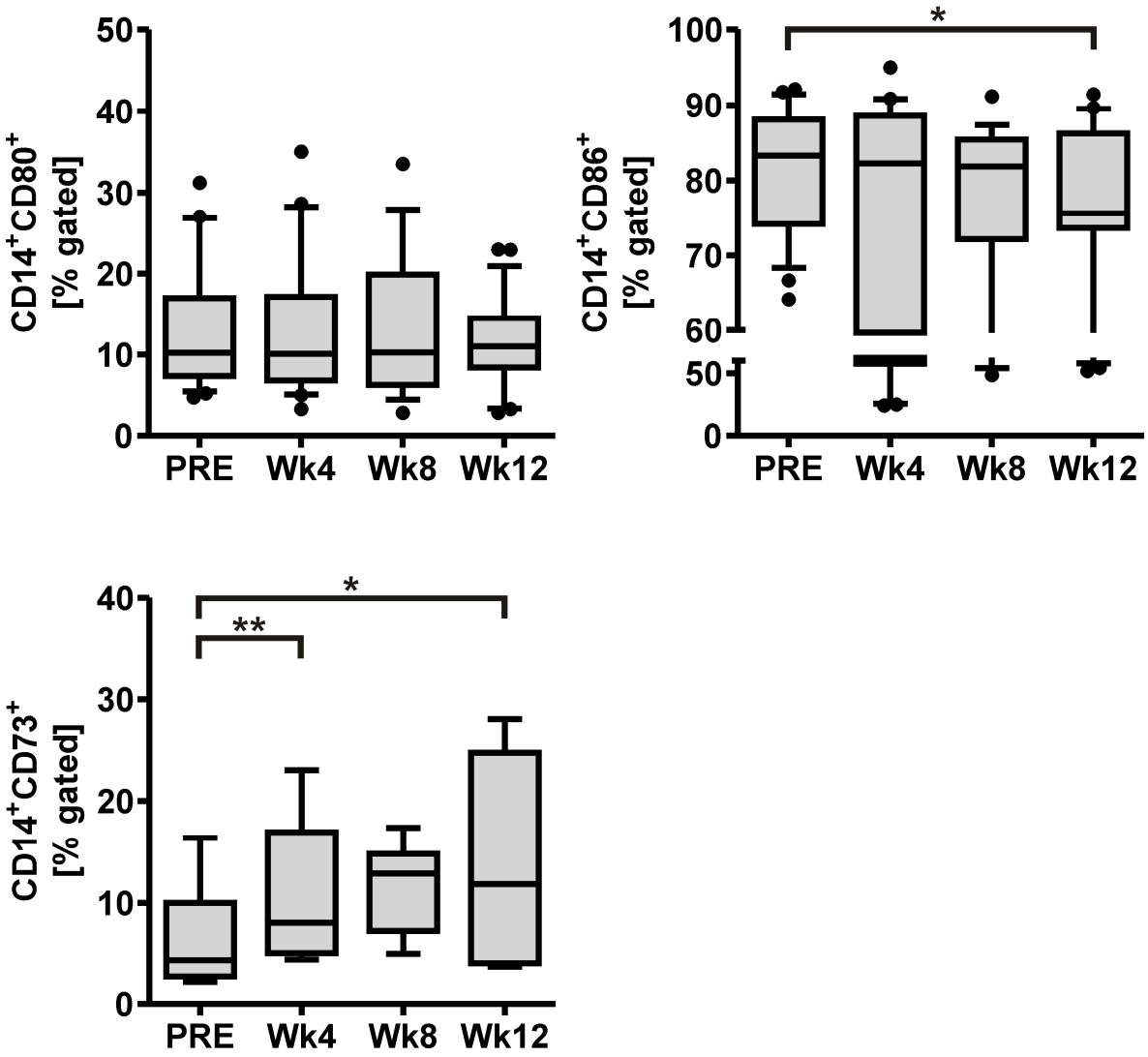

FIGURE 23 Regulation of pathogenically relevant cell populations in patients with chronic hand eczema. Effects of 9-*cis* RA on the phenotype of CD14+ monocytes. Results are shown for patients regarding $CD14^+CD86^+$-, $CD14^+CD80^+$- and $CD14^+CD73^+$ -monocytes before treatment with 9-*cis* RA and after four, eight and twelve weeks. Graphs show the percentage of gated cells as Box and whiskers. The Whisker lines indicate 10% and 90% values, the black vertical lines represent median values, the outliers are represented as filled circles. The means were significantly different using a paired Student's t-test (*p<0.05, **p<0.01).

To further determine the expression profile of circulating effector memory T cells, their chemokine receptor expression and their "skin-homing" phenotype was studied (Fig. 24). The migration of memory T cells, referred to as "skin-homing", is a crucial event throughout the entire pathogenesis of CHE and other inflammatory skin diseases. The cutaneous lymphocyte-associated antigen (CLA), which characterizes a memory T cell subpopula-

tion, as well as numerous chemokine receptors play a crucial role in the homing process (Homey et al., 2006).

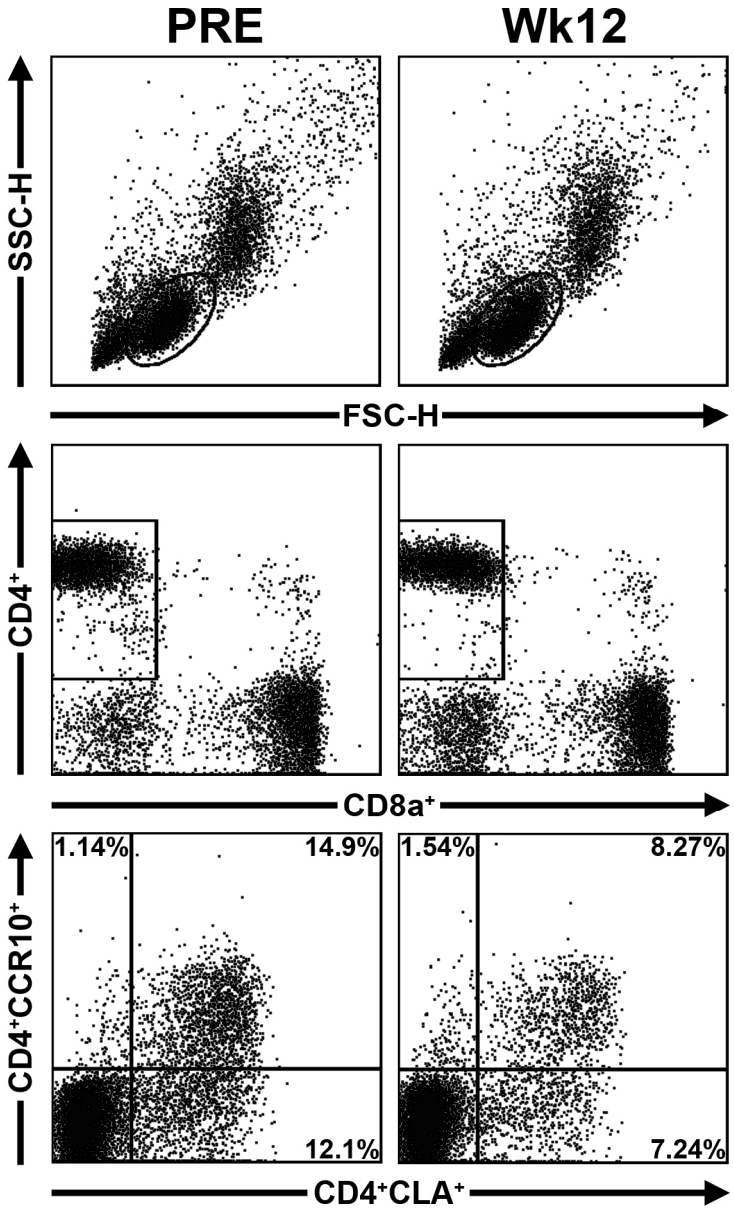

FIGURE 24 Gating strategy and FACS-analysis of $CD4^+CLA^+CCR10^+$-T-cells. PBMCs were stained with CD4, CD8a, CLA and CCR10 antibodies. CD4 positive cells were gated within the lymphocyte gate. Thereafter CLA-positive cells were plotted against CCR10 positive cells within the $CD4^+$ T cells. A representative patient before treatment of 9-*cis* RA (PRE) and after 12 weeks is shown.

Interestingly, the percentage of effector $CD4^+CLA^+$ (Wk12: $p<0.0001$), $CD4^+CCR9^+$ (Wk12: $p=0.0027$) and $CD4^+CLA^+CCR9^+$ (Wk12: $p=0.0033$) as well as the percentage of $CD4^+CCR10^+$ (Wk8: $p=0.0146$; Wk12: $p=0.0113$) and $CD4^+CLA^+CCR10^+$ T cells (Wk8: $p=0.0109$; Wk12: $p=0.0008$) under systemic 9-*cis* RA treatment were significantly lowered after week 12 compared to baseline percentages. Moreover, $CD4^+$ $CXCR3^+$ population was significantly decreased ($p=0.0101$) during 9-*cis* RA treatment after twelve weeks as against basement levels (Figs. 25 and 26).

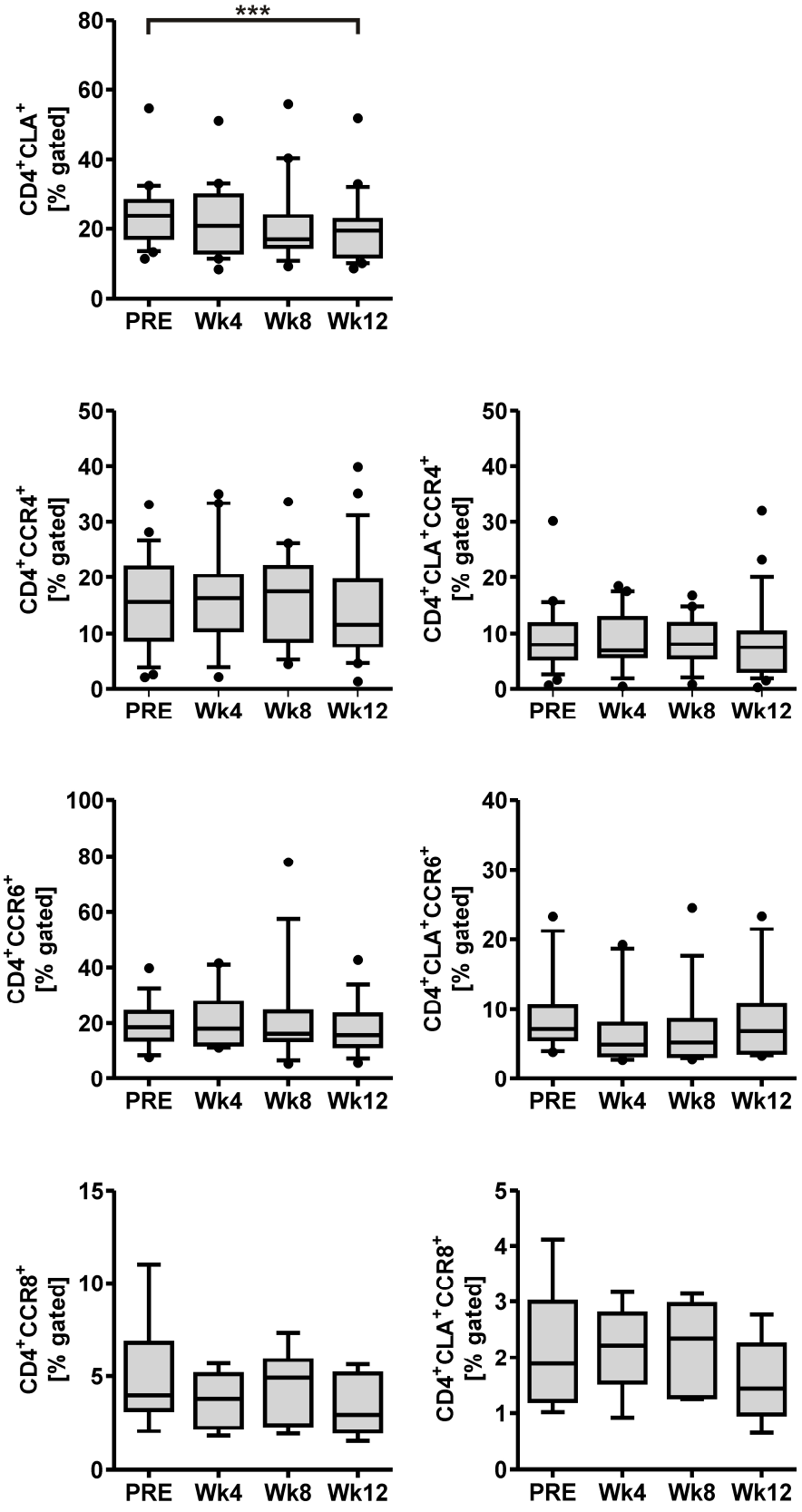

FIGURE 25 Retinoid-induced regulation of pathogenically relevant T cell subpopulations in patients with chronic hand eczema. Effects of 9-*cis* RA on the phenotype of $CD4^+$-T cells. Results are shown for patients regarding CLA^+-, $CCR4^+$-, $CCR6^+$-, $CCR8^+$- and CLA^+CCR4^+-, CLA^+CCR6^+- and CLA^+CCR8^+-T cells before treatment with 9-*cis* RA and after four (Wk4), eight (Wk8) and twelve weeks (Wk12). Graphs show the percentage of gated cells as Box and whiskers. The Whisker lines indicate 10% and 90% values, the black vertical lines represent median values, the outliers are represented as filled circles. The means were significantly different using a paired Student's t-test (*p<0.05, **p<0.01, ***p<0.001).

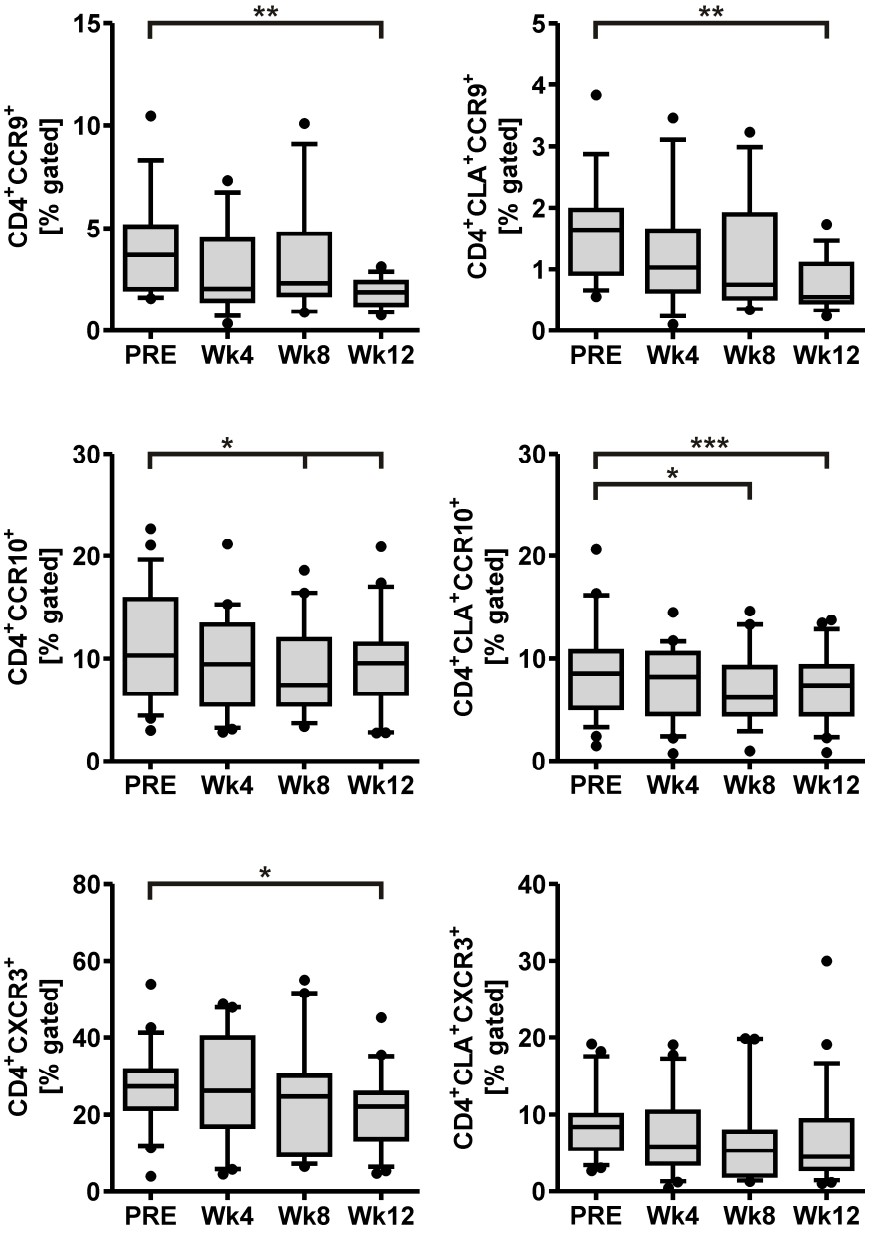

FIGURE 26 Retinoid-induced regulation of pathogenically relevant T cell subpopulations in patients with chronic hand eczema. Effects of 9-*cis* RA on the phenotype of $CD4^+$-T cells. Results are shown for patients regarding $CCR9^+$-, $CCR10^+$-, $CXCR3^+$- and CLA^+CCR9^+-, CLA^+CCR10^+- and CLA^+CXCR3^+ T cells before treatment with 9-*cis* RA and after four (Wk4), eight (Wk8) and twelve weeks (Wk12). Graphs show the percentage of gated cells as Box and whiskers. The Whisker lines indicate 10% and 90% values, the black vertical lines represent median values, the outliers are represented as filled circles. The means were significantly different using a paired Student's t-test (*p<0.05, **p<0.01, ***p<0.001).

With regard to CD8-positive T cells (Figs. 27 and 28), chemokine receptors CCR10 was down-regulated on circulating CD8 positive cells during week four (p=0.0463) compared to baseline expression. Expression of $CCR9^+$ (p=0.0026) and CLA^+CCR9^+ (p=0.019) were decreased in the course after week twelve of 9-*cis* RA treatment in comparison to expression at the start of the treatment. Further, $CD8^+CLA^+CCR6^+$ T cells were down-regulated significantly (p = 0.0378) at week eight of 9-*cis* RA treatment as against the percentage of positive cells before the treatment. Further, percentage of $CD8^+CCR7^+$ positive T cells were significantly increased after eight weeks of 9-*cis* RA (p = 0.0294) and at week twelve (p = 0.0355) compared to baseline percentages.

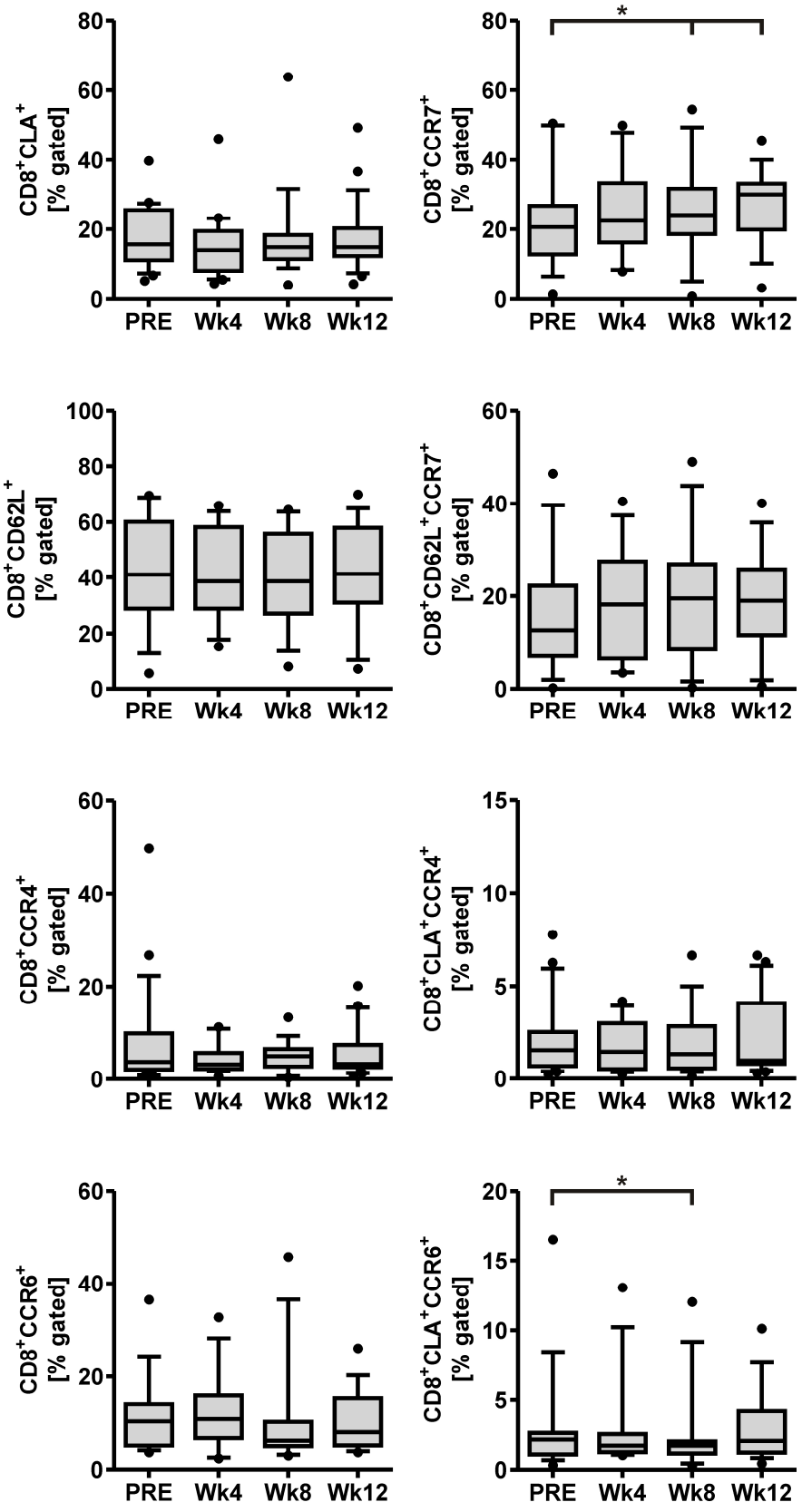

FIGURE 27 Retinoid-induced regulation of pathogenically relevant T cell subpopulations in patients with chronic hand eczema. Effects of 9-*cis* RA on the phenotype of $CD8^+$-T cells. Results are shown for patients regarding CLA^+-, $CCR7^+$-, $CD62L^+$, $CCR7^+$ $CD62L^+$-, $CCR4^+$, $CCR6^+$-, CLA^+CCR4^+- and CLA^+CCR6^+-T cells before treatment with 9-*cis* RA and after four (Wk4), eight (Wk8) and Graphs show the percentage of gated cells as Box and whiskers. The Whisker lines indicate 10% and 90% values, the black vertical lines represent median values, the outliers are represented as filled circles. The means were significantly different using a paired Student's t-test (*p<0.05, **p<0.01, ***p<0.001).

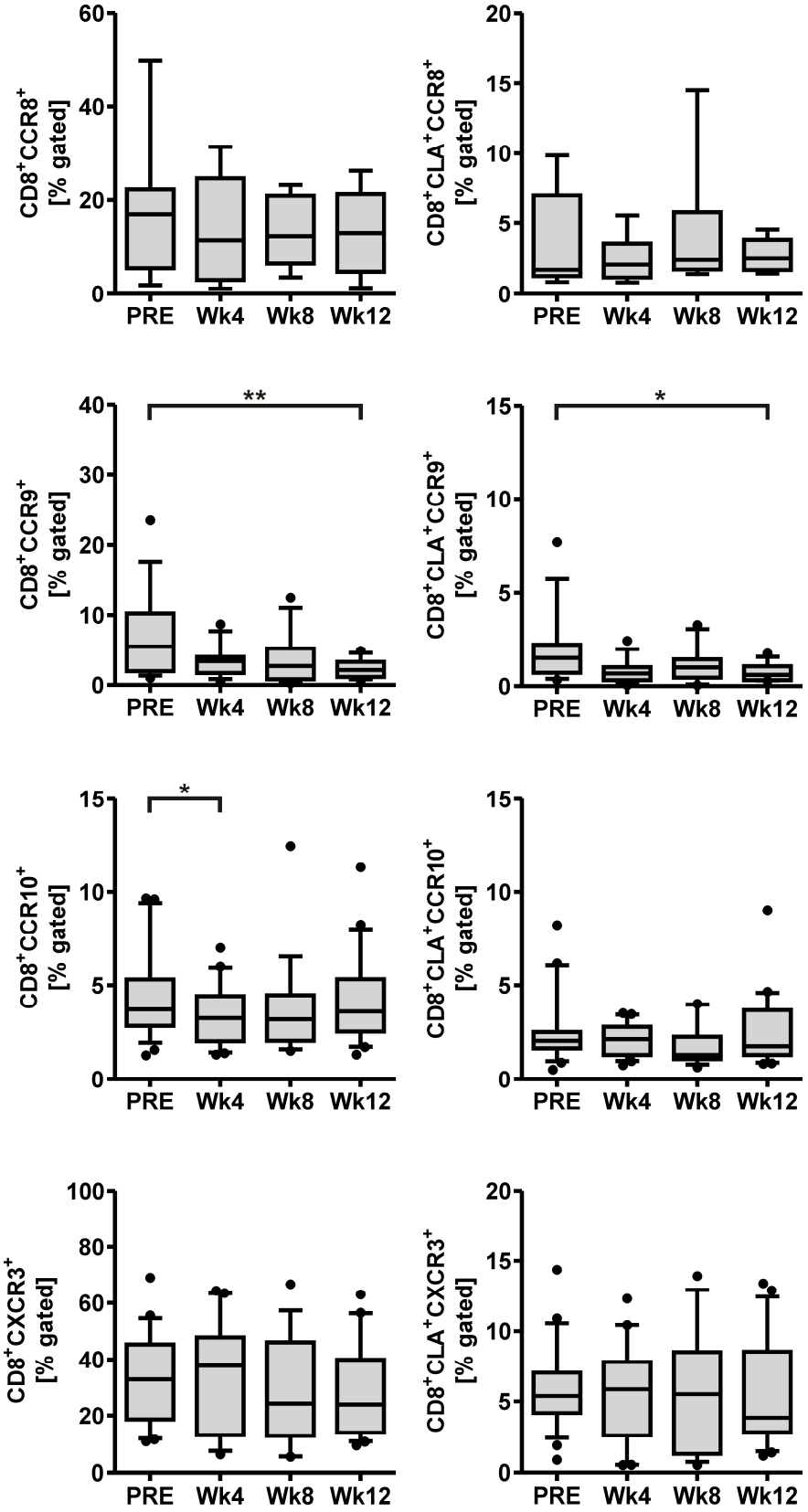

FIGURE 28 Retinoid-induced regulation of pathogenically relevant T cell subpopulations in patients with chronic hand eczema. Effects of 9-*cis* RA on the phenotype of $CD8^+$-T cells. Results are shown for patients regarding $CCR8^+$-, $CCR9^+$-, $CCR10^+$-, $CXCR3^+$-, CLA^+CCR8^+- and CLA^+CCR9^+- and CLA^+CCR10^+- and CLA^+CXCR3^+-T cells before treatment with 9-*cis* RA and after four (Wk4), eight (Wk8) and twelve weeks (Wk12). Graphs show the percentage of gated cells as Box and whiskers. The Whisker lines indicate 10% and 90% values, the black vertical lines represent median values, the outliers are represented as filled circles. The means were significantly different using a paired Student's t-test (*p<0.05, **p<0.01, ***p<0.001).

5.3.2 Serum markers during 9-*cis* RA treatment

To measure the effect of 9-*cis* RA on chemokine expression in the serum of patients before and under treatment, a Luminex screening assay (marked with #) and ELISAs were performed. The following chemokines were tested: CCL2#, CCL3#, CCL4#, CCL5, CCL8#, CCL13#, CCL17#, CCL20#, CCL27, CXCL1#, CXCL4#, CXCL5#, CXCL8#, CXCL9#, CXCL10#, CXCL11#, and CXCL13#. Due to the Luminex plate layout not all time points and all patients could have been tested. CXCL8, CXCL9 and CXCL11 were under the detection limit whereas CXCL4 was above. Chemokines CCL8, CCL13 and CCL27 were significantly reduced after four weeks of 9-*cis* RA treatment compared to corresponding expressions before the treatment (Fig. 29). Moreover, CCL8 and CCL13 remained reduced after twelve weeks in comparison to expression at the baseline, whereas CCL27 returned to its expression levels before treatment.

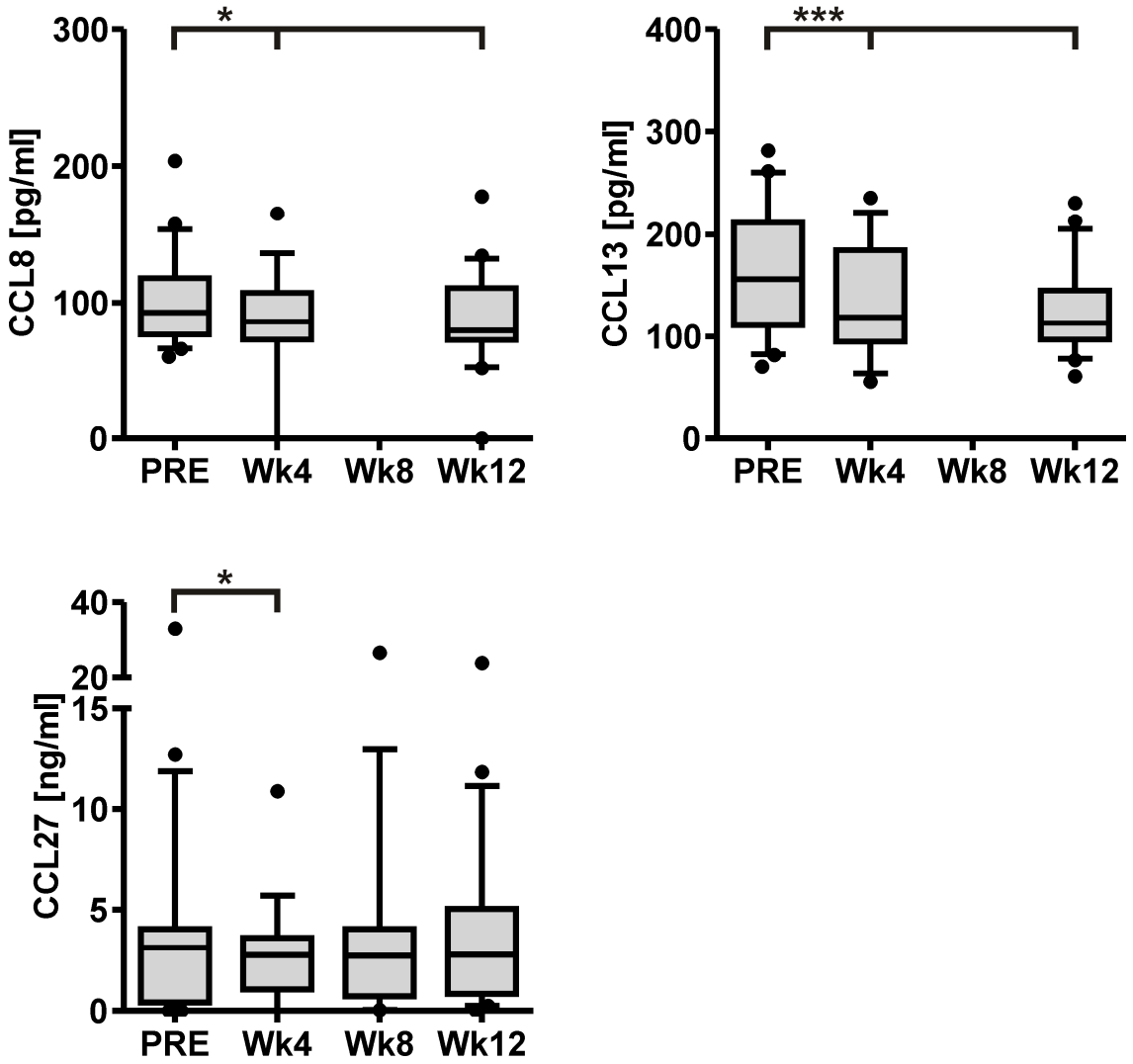

FIGURE 29 Regulation of chemokines during 9-*cis* RA treatment in patients with chronic hand eczema. Results are shown for patients CCL8 and CCL13 and CCL27 before treatment with 9-*cis* RA and after four and twelve weeks, and for CCL27 also after eight weeks. Graphs show the concentration of chemokines in serum as Box and whiskers. The Whisker lines indicate 10% and 90% values, the black vertical lines represent median values, and the outliers are represented as filled circles. The means were significantly different using a paired Student's t-test (*p<0.05, ***p<0.001).

5.3.3 9-*cis* RA treatment reduced allogenic stimulation of PBMCs of patients suffering from chronic hand eczema (CHE)

To confirm the inhibitory effect on T cell proliferation of 9-*cis* RA treated immune cells *in vivo*, PBMCs from patients suffering from CHE before and after week four, eight and twelve of treatment with 9-*cis* RA were isolated and cultivated with allogenic T cells isolated from healthy donors. At day six, proliferation rate of leukocytes was measured by BrdU-incorporation. BrdU incorporation was analyzed with one-tailed paired Student's t-test. 9-*cis* RA treatment significantly decreased T cell proliferation following allogenic stimulation after week eight ($p = 0.0285$) and week twelve ($p = 0.0346$) when compared with T cell proliferation rates before the treatment (Fig. 30).

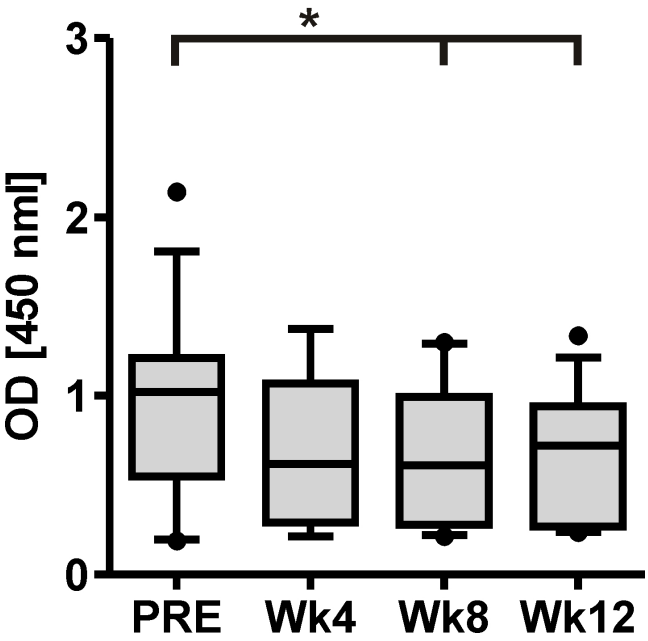

FIGURE 30 9-*cis* RA reduces T cell proliferation of patients[3] *ex vivo*. PBMCs of 9-*cis* RA treated CHE patients were co-cultivated with allogenic T cells from healthy donors. Graph shows BrdU-incorporation as extinction coefficient [450 nm] as Box and whiskers. The Whisker lines indicate 10% and 90% values, the black vertical lines represent median values, the outliers are represented as filled circles. The means were significantly different for week eight ($p=0.0285$) and week twelve ($p=0.0346$) compared to baseline proliferation using a one-tailed paired Student's t-test (*$p<0.05$).

[3] It was not possible to obtain enough PBMCs from all donors to conduct both FACS-analysis and MLR.

TABLE 12 Statistics of the paired-T-Test (Number of pairs and p-values) of the analyzed surface expression of $CD14^+$, $CD4^+$ and $CD8^+$ cells, serum expression CCL8, CCL13 and CCL27 and of the mixed leukocyte reaction in before treatment (PRE) in comparison to week four (WK4), week eight (Wk8) and week twelve (Wk12)

		*Number of pairs; p-Value to **PRE***		
	Marker	**week 4 (Wk4)**	**week 8 (Wk8)**	**week 12 (Wk12)**
$CD14^+$ monocytes	**$CD14^+CD80^+$**	20; 0.621	17; 0.7921	23; 0.2114
	$CD14^+CD86^+$	21; 0.054	17; 0.3143	23; **0.027 ***
	$CD14^+CD73^+$	8; **0.0043 ******	6; 0.0602	8; **0.016 ***
$CD4^+$ T cells	**$CD4^+CLA^+$**	20; 0.4238	19; 0.119	23; < **0.0001 *****
	$CD4^+CCR4^+$	19; 0.9602	19; 0.8138	23; 0.422
	$CD4^+CCR6^+$	12; 0.8335	13; 0.8022	15; 0.3474
	$CD4^+CCR8^+$	8; 0.1174	6; 0.5371	8; 0.1975
	$CD4^+CCR9^+$	13; 0.1979	11; 0.2154	15; **0.0027 ******
	$CD4^+CCR10^+$	20; 0.3893	19; **0.0146 ***	23; **0.0113 ***
	$CD4^+CXCR3^+$	20; 0.5492	19; 0.2896	23; **0.0101 ***
	$CD4^+CLA^+CCR4^+$	19; 0.3463	19; 0.6109	23; 0.4132
	$CD4^+CLA^+CCR6^+$	12; 0.1458	14; 0.1056	15; 0.4821
	$CD4^+CLA^+CCR8^+$	8; 0.9957	6; 0.9269	8; 0.1906
	$CD4^+CLA^+CCR9^+$	13; 0.4411	11; 0.1432	15; **0.0033 ******
	$CD4^+CLA^+CCR10^+$	20; 0.1752	19; **0.0109 ***	23; **0.0008 *****
	$CD4^+CLA^+CXCR3^+$	20; 0.0506	19; 0.0636	23; 0.1132
$CD8^+$ T cells	**$CD8^+CLA^+$**	20; 0.1626	18; 0.937	23; 0.9785
	$CD8^+CCR4^+$	19; 0.1425	18; 0.1402	23; 0.309
	$CD8^+CCR6^+$	12; 0.7508	12; 0.2918	15; 0.4065
	$CD8^+CCR7^+$	12; 0.8085	12; **0.0294 ***	15; **0.0355 ***
	$CD8^+CCR8^+$	8; 0.1912	6; 0.5126	8; 0.265
	$CD8^+CCR9^+$	13; 0.0711	11; 0.0674	15; **0.0026 ******
	$CD8^+CCR10^+$	20; **0.0463 ***	18; 0.4141	23; 0.5047
	$CD8^+CXCR3^+$	19; 0.8323	18; 0.258	23; 0.1732
	$CD8^+CD62L^+$	12; 0.1215	12; 0.178	15; 0.8547
	$CD8^+CLA^+CCR4^+$	19; 0.1669	18; 0.2638	23; 0.9016
	$CD8^+CLA^+CCR6^+$	12; 0.2166	12; **0.0378 ***	15; 0.8941
	$CD8^+CLA^+CCR8^+$	8; 0.1932	6; 0.6704	8; 0.3509
	$CD8^+CLA^+CCR9^+$	13; 0.0729	11; 0.1652	15; **0.019 ***
	$CD8^+CLA^+CCR10^+$	20; 0.3319	18; 0.1061	23; 0.8746
	$CD8^+CLA^+CXCR3^+$	19; 0.1488	18; 0.5922	23; 0.5845
	$CD8^+CD62L^+CCR7^+$	12; 0.5804	12; **0.0401 ***	15; 0.1351
Serum levels	**CCL8**	18; **0.0271** *	x	21; **0.0147** *
	CCL13	18; **0.0008 *****	x	21; **0.0006 *****
	CCL27	18; **0.0383 ***	18; 0.1197	21; 0.6459
Allogeneic Stimulation	**MLR**	8; 0.1862	11; **0.0285 ***	13; **0.0346 ***

6 Discussion

This thesis aimed to unravel the molecular and cellular responses to 9-*cis* RA in different cell types. The advantage of chronic hand eczema patients treated with 9-*cis* RA was taken to investigate retinoid biology and in particular the effects of a dual retinoid receptor agonist 9-*cis* RA in the human system *in vivo*. 9-*cis* RA altered the expression of genes expressed in keratinocytes regarding different functions *in vitro* and *in vivo*. Known genes overexpressed or dysregulated participating in barrier functions were normalized. Pro-inflammatory defensins and lymphocyte-attracting chemokines were down-regulated. This was further supported by a lower expression of keratinocyte-associated CCL27 as well as allergy/atopy-associated CCL8 and CCL13 into the serum of treated patients *in vivo*. Also, 9-*cis* RA modulated the activation processes and interfered with the up-regulation of co-stimulatory molecules *in vitro*. RA shaped monocytes/ MoDCs into an immunomodulatory phenotype characterized by lower expression of maturation markers CD83, CD80, CD86 and a high secretion of IL-10. Moreover, the present study demonstrates for the first time the upregulation of expression and activity of CD73 on the surface of APCs accompanied by a suppression of the allogenic stimulation of leukocytes in MLR assays. *In vitro* observations were validated *in vivo*, showing down-regulation of CD86, up-regulation of CD73 and a down-modulation of the allogeneic stimulated proliferation potential in patients receiving 9-*cis* RA. Finally, 9-*cis* RA treatment changed the phenotype of skin-homing memory T cells in the periphery.

9-*cis* RA alters gene expression of structural cells in the skin *in vitro* and *in vivo*

Even though retinoids in general are known to regulate several immune functions, the effects of 9-*cis* RA on the skin immune system remain largely unknown. Most studies using all-*trans* retinoic acid (ATRA) as a model-substance to study the effects of retinoic acids, a RA binding to only to one of the retinoic acid receptors (i.e. the retinoic-acid receptor (RAR)) quite contrary to 9-*cis* RA which binds to both retinoic acid receptors, to the RAR and to the Retinoid-X receptor (RXR). As 9-*cis* RA is effective during the treatment of chronic hand eczema the present study aimed to unravel the effects of the dual retinoid receptor agonist 9-cis RA on the skin immune system *in* vivo taking advantage of CHE-patients treated with 9-*cis* RA. First, response to 9-*cis* RA was documented and scored using the modified

total lesion symptom score (mTLSS). The mTLS-score reduction of the patient monitoring was in the line with previous controlled and uncontrolled trials with 9-*cis* RA (Ruzicka et al., 2008, Kumari et al., 2016) and demonstrate that oral 9-*cis* RA induces significant responses in patients with CHE. The collective was therefore useful to study the effects of 9-*cis* RA *in vivo*.

A genome wide microarray analysis showed an impact of 9-*cis* RA treatment on 244 genes including genes regarding extra cellular matrix (ECM), skin barrier and chemokine regulation revealing two distinct cell types as targets of the 9-*cis* RA treatment: keratinocytes and activated monocytes/ dendritic cells. Molin and colleagues identified 185 differentially expressed proteins in CHE samples compared to healthy controls, including barrier genes and AMPs (Molin et al., 2015). The shown impact on genes regarding skin barrier in this thesis was further supported by the observation of Kumari et al., which showed that barrier genes were normalized in skin from CHE patients during systemic 9-*cis* RA-treatment (Kumari et al., 2016). Previous findings are in good accordance to the shown microarray results, as treated patients demonstrated the regulation of structural proteins, chemokines and AMPs. The presented findings here broaden the view of regulated genes and further gave a more detailed insight and impact on gene expression under the influence of 9-*cis* RA *in vivo*. Therefore, mRNA levels of different genes seen in the microarray data and known to participate during epidermal differentiation, skin barrier formation, hyaluronan turnover, defensins and chemokines were analyzed. These included genes known to be overexpressed or mutated and factors disturbing the formation of the cornified envelope lead to disrupted barriers and skin disorders (Ovaere et al., 2009, Harding et al., 2003, Feldman et al., 2005, Mahler, 2016, Liang et al., 2015, de Koning et al., 2012). Barrier impairment is a key feature of CHE (Kumari et al., 2016) leading to the expression of antimicrobial peptides. These AMPs in turn activate the innate immunity and a release of proinflammatory cytokines and chemokines including CCL13, CCL17, CCL18 and CCL27 (Cipriani et al., 2014, Homey et al., 2007). In 2009, Lee and colleagues pointed out that 3346 genes were affected by ATRA in epidermal keratinocytes including genes involved at keratinocyte differentiation, formation of epidermal barrier function, pro- and anti-apoptotic genes (Lee et al., 2009) One of known effects of ATRA is the inhibition of keratinocyte differentiation (Fuchs and Green, 1981, Jetten, 1990). Keratinocyte differentiation involves ceramides, proteases and several proteins like FLG, involucrin (IVL), loricrin (LRC), late cornified envelope proteins (LCEs) and the SPRRs. In this study, it is shown

for the first time that SPRR3 is downregulated in keratinocytes by 9-*cis* RA. Previously, this effect was shown for ATRA (Fischer et al., 2014, Hohl et al., 1995, Lee et al., 2009). Interestingly, it has been demonstrated that dysregulation or overexpression of barrier genes, including SPRR3 could lead to an impaired supramolecular organization of lamellar body–derived lipids into normal bilayer structures (Liang et al., 2015). Therefore, 9-*cis* RA might regulate the normal organization of the skin barrier and restore a normal bilayer structure. Water loss and invasion of pathogens might be reduced or impaired reconstituting the physiological state of the skin. Additionally, 9-*cis* RA was more effective than acitretin maybe hinting to its ability binding to both retinoid receptors in contrast to acitretin.

Another regulated gene *in vitro* and *in vivo* was SPINK6 which has been demonstrated be a human skin as a specific inhibitor of KLKs, especially in formation of very resistant palmoplantar skin (Meyer-Hoffert and Schroder, 2011) and for epidermal barrier functions (Fischer et al., 2013, Molin et al., 2015). SPINK6 has been shown to be down-regulated by ATRA or TNF-α/IFN-γ in cultured differentiating keratinocytes (Fischer et al., 2014, Hohl et al., 1995, Lee et al., 2009). Here, it is demonstrated to be up-regulated in inflammatory keratinocytes *in vitro* and in the skin of CHE patients *in vivo*, indicating to an overall up-regulation in inflamed skin. The discrepancy between the mentioned literature and the presented results regarding the up-regulation of SPINK6 may be due to complexity of inflammatory system of the skin. Its rank in the microarray analysis and its down-regulation by 9-*cis* RA indicates that SPINK6 is one of the major factors involved in CHE and that 9-*cis* RA is able to down-regulate this factor to an expression state restoring the barrier functions.

Barrier genes are discussed controversially in literature regarding their involvement and function in eczema formation. FLG is known to be reduced in the presence of ATRA (Asselineau et al., 1989, Hohl et al., 1991, Lee et al., 2009). Further, the filaggrin promotor region has a retinoic acid response element (RARE) with suppressor functions (Okano et al., 2012). In 2015 Molin and colleagues identified 185 candidate proteins in hand eczema samples compared to healthy volunteers, which included a down-regulation of FLG (Molin et al., 2015). Surprisingly, Kumari et al. demonstrated an up-regulation of *FLG* and *CLDN1* mRNA and protein levels in skin with a systemic 9-*cis* RA treatment in patients with chronic hand eczema while OCLN was not altered (Kumari et al., 2016). Opposingly, immortalized oral mucosal epithelial cells (mouse oral keratinocytes, GE1) treated with ATRA have been shown to down-regulate *CLDN1* and to up-regulate wound healing associated *OCLN* (Okano et al.,

2012, Hatakeyama et al., 2010). The presented data show that these genes were altered only marginally *in vitro* and *in vivo*. It is likely that mRNA levels are only regulated for a short time and regulations cannot be observed for the long time of three months as in the presented cohort. Kumari et al. also had a higher number of samples included into their study representing a higher statistical safety. At last, the primary human keratinocytes might differ in their reaction to RA as in the complex setting of cells in skin and are therefore not suitable to see effects in these barrier genes. *In vitro,* 2D keratinocytes models contain only one cell type. The 3D *in vivo* situation in the skin is more complex. Here, keratinocytes interact with their proximate neighboring cells i.e. other keratinocytes, fibroblasts, immune cells, and the extracellular matrix. This complex interaction also determines the reaction to stimuli and drugs in terms of differentiation, apoptosis, proliferation.

Betacellulin (BTC) is a ligand for the epidermal growth factor receptor and directly activates it (Schneider et al., 2008). BTC is involved in epidermal homeostasis and wound angiogenesis (Schneider et al., 2008) and is expressed in suprabasal keratinocytes (Piepkorn et al., 2003, Rittie et al., 2006). Previously, BTC was shown to be down-regulated in skin of patients treated with topical ATRA (Rittie et al., 2006). Interestingly, the microarray data of skin biopsies after 9-*cis* RA treatment and the *in vitro* keratinocyte data presented here demonstrated an up-regulation of BTC for the first time. 9-*cis* RA seems to directly up-regulate BTC expression and thereby induce wound healing and regeneration especially in palmoplantar skin by cell proliferation via EGFR. As a result, differentiation and proliferation processes might return to a homeostatic level and a down-modulation of pro-inflammatory processes (Lichtenberger et al., 2013). This effect of 9-*cis* RA is supported by the observations of Molin and colleagues (Molin et al., 2015) or Kumari et al. (Kumari et al., 2016).

The wound healing aspect is further supported by the expression of hyaluronan metabolism-associated genes. Hyaluronan is a high molecular weighted glycosaminoglycan which participates in different functions including, migration, adhesion, proliferation and moreover with tissue regeneration, repair and hydration. It is synthesized by hyaluronan synthases (HAS) 1-3 (Tammi et al., 2002, Manuskiatti and Maibach, 1996, Chen and Abatangelo, 1999). HAS3 is expressed by keratinocytes and mostly regulates hyaluronan synthesis and can be induced by β-carotene (Sayo et al., 2013). The hyaluronan synthesis are also regulated by several factors like epidermal growth factor (Saavalainen et al., 2005), T_H2 or T_H1 cytokines (Sayo et al., 2002, Malaisse et al., 2014). Results from previous studies sug-

gested that HAS2-synthesized hyaluronan also controls keratinocyte migration in *in vitro*-scratch/ in vitro wound repair assays as well as the epidermal proliferation and differentiation process (Rilla et al., 2002, Pasonen-Seppanen et al., 2003). Several studies linked hyaluronan and inflammatory skin diseases: In atopic dermatitis, the expression of HAS2 and HAS3 are increased in lesional skin compared to non-lesional or healthy skin. HAS3 was up-regulated in keratinocytes in response to stress and in lesional skin of AD patients (Malaisse et al., 2014). Hyaluronidases (HYAL) 1 and 2 are the major hyaluronan degrading enzymes. Contact allergens induce the hyaluronan degradation in the NCTC2544 keratinocyte cell line, by increasing the expression of HYAL1 and HYAL2. Further, the produced hyaluronan fragments could in turn induce a IL-18 dependent response (Nikitovic et al., 2015). In lesional skin of atopic patients, the expression of HYAL1 and HYAL2 are significantly up-regulated compared to healthy skin (unpublished data). A regulation for the HAS-system was also seen with ATRA in human and rat skin organ cultures (Pasonen-Seppanen et al., 2003, Pasonen-Seppanen et al., 2008, Saavalainen et al., 2005) and in a mouse model using retinaldehyde (Kaya et al., 2005). Here, it was confirmed that 9-*cis* RA is able to regulate the HAS expression, showing that HAS2 and HAS3 are up-regulated while hyaluronidases are not affected *in vitro*. Therefore, wound healing and tissue regeneration might be induced through the regulation of the hyaluronan system. Also, a lower amount of hyaluronan fragments due to the imbalance of hyaluronan production and lower degradation may also lead to impaired inflammatory responses and a thickening of the epidermis as indicated by Pasonen-Seppanen in rat keratinocytes (Pasonen-Seppanen et al., 2008). Interestingly in this context is the induction of hyaluronidases by acitretin, a RA only binding to RAR, demonstrated that 9-*cis* RA due its dual retinoid receptor affinity might result in the seen expression pattern.

Supporting these observations, a regulation of a set of pro-inflammatory defensins was seen *in vitro* and *in vivo* which are demonstrated to be regulated in eczema (Proksch and Brasch, 2012). DEFB104 (hBD-2) was shown to induce the migration of immature dendritic cells and memory T cells via CCR6 (Yang et al., 1999). Moreover, DEFB104 stimulates mast cells to release histamine and prostaglandin D2, which also implies a role for defensins in allergic reactions (Niyonsaba et al., 2001). Harder et al. have shown that ATRA is a potent inhibitor of the β-defensins DEFB104 and DEFB103A (hBD-3) in TNF-α, IL-1β and IFN-γ activated keratinocytes *in vitro* (Harder et al., 2004). Here, 9-*cis* RA and acitretin reduced *DEFB104* and *DEFB103A* on the mRNA level in a pro-inflammatory context. Moreover, the

presented gene expression not only mimics the findings from Harder et al. (Harder et al., 2004), but for the first time show that 9-*cis* RA is more effective in reducing DEFB103A in comparison to acitretin. These findings demonstrate the anti-inflammatory effect of 9-*cis* RA in keratinocytes. Allergic reactions, which are also seen in CHE patients, might be reduced and leukocyte recruitment might be diminished.

RNASE7 is a skin-derived protein, which is present in the stratum corneum and shows antimicrobial properties. RNASE7 could be isolated from supernatants of primary keratinocytes and can be induced by inflammatory cytokines, e.g. TNF-α, IL-1β and IFN-γ (Harder and Schroder, 2002). *RNASE7* was also reduced by 9-*cis* RA and acitretin in a pro-inflammatory context, demonstrating this effect for RAs for the first time, which may indicate a reduced inflammatory response of cells resulting in a lower recruitment of effector cells as seen for DEB104 and DEFB103A.

Hornerin (HRNR), a member of the S100 family, is expressed in normal and wounded skin and moreover in lesional atopic dermatitis and psoriasis. Further, HRNR protein could be detected in palmoplantar skin and is expressed in cultured keratinocytes (Wu et al., 2009). HRNR does not only participate in skin barrier formation, it also has antimicrobial activity (Takaishi et al., 2005, Wu, 2007, Elias, 2007). As *HRNR* was not affected in keratinocytes by inflammatory cytokines or by 9-*cis* RA a stable expression might be important for survival of cells and is therefore not affected easily. Also, the down-regulation of inflammation-induced defensins to a homeostatic-like level and the unaffected level of hornerin may demonstrate that not all defensins are reduced and skin is not unprotected at all. Supporting this hypothesis, 9-*cis* RA treated patients do not present a significantly increased frequency of infections.

Other S100-family members, like S100A7, are part of the epidermal differentiation complex, can be induced by barrier disruption for example by tape stripping of the skin. Molin et al., demonstrated an up-regulation of S100A7 in skin specimens of chronic hand eczema vs healthy skin (Molin et al., 2015). Additionally, Molin et al. demonstrated a significant up-regulation of S100A7 in the palmar skin of patients with CHE, which might function as an alarm signal (Molin et al., 2015). The regulation of S100A7 by 9-*cis* RA may function as a negative regulator of this alarm signal and further supports that RA reduce pro-inflammatory signals.

As a result of the expression pattern of defensins *in vitro* I concluded that proinflammatory mediators, including chemotactic factors, which initiate the orientated migration of distinct leukocyte subpopulations, might also be reduced. Physical, chemical, or immune-specific insults rapidly evoke an epidermal response characterized by the increased expression of chemokines. In the past decade, numerous studies identified chemokines associated with atopic dermatitis, including CCL5, CCL20 and CCL22 (Homey et al., 2007) or particular by tumor necrosis factor-alpha (TNF-α) in keratinocytes (Banno et al., 2004). Active immigration of T cells, monocytes, as well as neutrophils is also supported by the increased expression of CCL5. CCL5 has been reported to be neosynthesized in keratinocytes of AD- and psoriasis-patients under different proinflammatory stimuli and thereby modulate the recruiting of immune cells (Giustizieri et al., 2001). A down-regulation of lymphocyte-attracting molecules was also seen *in vitro* and *in vivo* with RA treatment. It was reported earlier that 13-*cis* RA is capable of CCL5 inhibition in rat fibroblasts (Adams et al., 2005). It was also shown that acitretin reduced the production of CCL5 in a dose dependent manner in human epidermal keratinocytes cell line HaCaT under inflammatory conditions (Zhang et al., 2008). However, the effect of 9-*cis* RA on the expression of CCL5 and CXCL14 in human primary epidermal keratinocytes has not been reported, yet. These findings further supported the microarray data and the hypothesis that 9-*cis* RA directly and indirectly regulates chemokine expression and thereby leukocyte recruitment.

9-*cis* RA impairs maturation of MoDCs, induce immunomodulatory/ immunosuppressive effects and thereby inhibits T cell activation *in vitro* and *in vivo*

Since the overall retinoid-induced regulation of target genes in keratinocytes was moderate, the attention was directed towards antigen presenting cells, in particular dendritic cells, as the second target of 9-*cis* RA. Based on this first set of observations and findings from the literature, the hypothesis that RA-treated DCs are instructed into an immune-regulatory type of DCs was deduced. The presented findings indicate that dendritic cells treated with 9-*cis* RA remain in an immature state. The mMoDC phenotype presented here resembled the "TNF-α/CD40L" phenotype with an up-regulation of key genes previously described (Schinnerling et al., 2015, Imai et al., 1997, Yamamoto et al., 2000, Satyam et al., 2009, Decker et al., 2008, Lapteva et al., 2001, Korthals et al., 2007, Castiello et al., 2011). Based on previous studies, it was concluded that TNF-α matured-MoDCs would rather in-

duce and recruit T_H2 phenotype T cells. Instead, "9-*cis* RA-DCs" demonstrated a decreased expression of maturation-associated genes *CD83*, *CD80*, *CD40*, *CCL17* and *CCL22* in comparison to mMoDCs. Markers like *CD14* or *CD163* expression were up-regulated compared to mMoDCs. Total, on the transcriptional level, the "9-*cis* RA-DC" demonstrated a more iMoDCs expression pattern and supported the hypothesis that RA-treatment shapes monocytes/ DCs into immuno-modulatory DCs which have the ability to down-modulate T cells responses and control inflammatory processes. These observations were further supported by results obtained by flowcytometry of MoDCs which showed that 9-*cis* RA as well as acitretin have an inhibitory effect on the differentiation and maturation of DCs, matching the RNA sequencing data. Earlier studies have demonstrated an inhibitory effect of retinoids on the maturation of DCs and observed that ATRA has inhibitory effects on the differentiation, maturation and function of DCs derived from human monocytes *in vitro* and also primes naïve T cell to differentiate into T_H2 type and T_{reg} (Jin et al., Yao et al., 2006, de Sousa-Canavez et al., 2009). These results align with studies that showed a down-regulation of CD83, CD86 and CD80. ATRA is capable to reduce CD80 and CD86 *in vitro* in pharmacological doses (10 µM – 1 µM), whereas a physiological dose (10 nM) induce rather a differentiation not observed in other doses. De Sousa-Canavez et al. observed an inhibitory effect on the maturation and differentiation with ATRA (1 µM and 10 nM). CD80 and CD86 were down-regulated in both concentrations upon GM-CSF + IL-4 stimulation and additional TNF-α maturing (de Sousa-Canavez et al., 2009). Also, ATRA reduced the percentage of $CD83^+HLA\text{-}DR^+$ mature dendritic cells (Tao and Yang, 2004). Zapata-Gonzalez et al. reported that 9-*cis* RA also interferes with differentiation process and modulates lymphoproliferative induction of lipopolysaccharide (LPS)-activated monocyte-derived dendritic cells. Remarkably, the same group further matured DCs with TNF-α and observed a similar effect in the reduction of CD80, but not a reduction of CD86 (Zapata-Gonzalez et al., 2007). Of note, they used a relatively low dose of 9-*cis* RA compared to the chosen dose here. Overall, these results confirmed data on other RAs and showed that 9-*cis* RA inhibited the maturation of dendritic cells by reducing co-stimulatory molecules CD80 and CD86. Impairment of CD86 and CD80 expression on macrophages and dendritic cells lead to a reduced activation and a lower cytokine production of T cells (Lanier et al., 1995). As a result of 9-*cis* RA, "retinoid-DCs" have a lower activation potential and might function as an immune modulating cell rather than an inflammatory cell. Further, 9-*cis*-RA is not only effective in reducing maturation of TNF-α stimulated DCs *in*

vitro, but it is more effective than synthetic RAR-ligand acitretin in lower doses. There are also groups showing no influence of retinoids during the maturation process using LPS, poly IC (Bakdash et al., 2015) or TNF-α (Jin et al., 2010) supporting a special role for the dual receptor agonist 9-*cis* RA. Since "retinoid-DCs" stay in an immature phenotype, it was concluded that 9-*cis* RA directly or indirectly inhibits maturation of dendritic cells.

To further characterize "9-*cis* RA-DCs", the chemokine expression of these cells was analyzed. Immature dendritic cells normally express CCR6, which is down-regulated during maturation, whereas CCR7 is up-regulated (Zapata-Gonzalez et al., 2007, Gordon et al., 2014). Surprisingly, chemokine receptors CCR6 and CCR7 were regulated against the speculation. Up-or down-regulation was not demonstrated in the literature before in MoDCs, although CCR6 and CCR7 were up-regulated in human promyelocytic leukemia cell line NB4 treated with ATRA (Park et al., 2004). Bakdash et al. could not demonstrate an up-regulation of CCR6 using ATRA during the generation of MoDCs (Bakdash et al., 2015). As ATRA binds only to RAR, the upregulation of CCR6 might be an effect of 9-*cis* RA binding to both receptors. ATRA-treated bone marrow-derived DCs from mice exhibited an enhanced migration towards CCR7 ligands CCL19 and CCL21, but CCR7 expression was not altered (Darmanin et al., 2007). Haileselassi et al. could show an increased expression of CCR7 in MoDCs upon retinoic acid treatment (Haileselassie et al., 2016). It was confirmed that CCR6 can be up-regulated by 9-*cis* RA in immature dendritic cells but was down-regulated upon LPS induced maturation (Zapata-Gonzalez et al., 2007, Villablanca et al., 2008). The results suggest a new effect of 9-*cis* RA allowing retinoid-DCs to stay in the periphery but also possess the capacity to migrate into the lymph node to regulate immune reactions at both sites.

As mentioned before, RNA expression profiles and the expression of surface molecules of "retinoid-DCs" in comparison to mMoDCs revealed rather an immature or "immunomodulatory/suppressive" phenotype. Additionally, immature dendritic cells are known to mediate tolerance rather than the activation of leukocyte subsets (Li et al., 2012, Yoo and Ha, 2016, Hawiger et al., 2001). "9-*cis* RA-DCs" showed indeed transcriptional changes as well as protein expressions that are similar to immunomodulatory DCs. Based upon this observations that immunomodulatory molecules may support a repressive DC phenotype and "ecto 5'-nucleotidase" (NT5E/CD73) attracted the attention. ENTPD1 (CD39) and the downstream CD73 are able to break down the adenosine triphosphate into adenosine monophosphate and finally into adenosine. Adenosine concentration has shown to be up-regulated in

inflamed or remodeling tissues (Driver et al., 1993, Blackburn et al., 2003). Extracellularly produced or secreted adenosine can be sensed by the adenosine receptors ADORA1, ADORA2A, ADORA2B and ADORA3, which can have pro- or anti-inflammatory properties. In dendritic cells upon maturation, ADORA2A becomes up-regulated and may act inhibitory on the immune response (Fredholm, 2007). The activation of ADORA2A results in the accumulation of intracellular cAMP, which in turn inhibits NF-κB (Lappas et al., 2006, Morello et al., 2006, LaPar et al., 2011, Lukashev et al., 2004). Further, the expression of ADORA2A on DCs an T cells had a suppressive effect in allograft rejection and prevent T cell proliferation, expansion and T cell receptor activation (Sevigny et al., 2007, Raskovalova et al., 2005, Koshiba et al., 1997, Huang et al., 1997). In addition, the group of Li et al. demonstrated that the ADORA2A agonist ATL313 induced tolerized phenotype in DCs (Li et al., 2012). In this thesis, CD73 was indeed induced by RA in the concentrations of 100 and 1000 nM. Further, the enzyme activity was tested, demonstrating not only the presence of CD73 but also the increased activity of the molecule on the surface of "retinoid-DCs". It has been pointed out that CD73 can be induced by transforming growth factor and RA on T cells in mice (Francois et al., 2015). Findings of the present study demonstrate for the first time that 9-*cis* RA and acitretin can induce CD73 on the surface of moDCs and that the molecule is active on these cells. Interestingly, supporting to the presented data, $CD73^{-/-}$ mice treated with 1% 2,4,6-trinitrochlorobenzene as a model for contact hypersensitivity show a three times higher ear swelling and an increased T cell infiltration. While injection of hapten-pulsed $CD11c^{+}$ DCs or *in vitro* generated bone-marrow-derived dendritic cells did not have an effect on ear swelling, the authors speculated that epicutaneous sensitization is amplified in the absence of CD73 and analyzed the different DC subsets in the draining lymph node after sensitization. They found significantly increased numbers of skin derived $MHCII^{++}CD11c^{+}CD207^{+}$ DCs in KO mice, which had a high capacity to induce T cell proliferation *in vitro*. Their conclusion was that absence of CD73 lead to an augmented sensitization and the modulation of the adenosine monophosphate-adenosine balance could improve the contact hypersensitivity (Pushkakevskaya et al., 2016). Another mechanism of adenosine could be the mediation of ingestion of apoptotic cells by macrophages. This mechanism is thought to be part of the tissue remodeling process, the immune homeostasis and the control of inflammations. In 2011, Koroskenyi et al. demonstrated that adenosine is one of the factors produced by macrophages in the process of ingesting apoptotic cells (Koroskenyi et al., 2011). These findings support the role of CD73

in the regulation of immune functions of DCs and findings indicate a new role for RA by inducing CD73 and resulting in an immunomodulatory response. CD73 is could be a factor regulating the adenosine monophosphate-adenosine balance in dendritic cells critically regulated by 9-*cis* RA.

A immunomodulatory effect is even further supported by a high release of anti-inflammatory IL-10 (Mosser and Zhang, 2008) by 9-*cis* RA-treated DCs. This observation is in line with other findings demonstrating IL-10 induction in ATRA-treated DCs using stimulation with CD40L-transfected J558 cells (Jin et al., 2010). Further, IL-10 expression is elevated in supernatants (Jin et al., 2010, Tao et al., 2006, Haileselassie et al., 2016) and on the RNA level (Yao et al., 2006, Tao and Yang, 2004) of DC generated in the presence of ATRA. Here, findings demonstrate, that 9-*cis* RA is capable to induce anti-inflammatory IL-10 in moDCs and that 9-*cis* RA is superior to synthetic RAR-ligand acitretin. This further supports the hypothesis that RA is regulating the immune responses of DCs. Additionally, several protocols use IL-10 to establish immunosuppressive DCs (Schinnerling et al., 2015). And even more, DC models with increased IL-10 release induce immunosuppressive responses and reduces autoimmune processes or allograft rejection (Li et al., 2012, Ueno et al., 2007). Results indicate that the high release of IL-10 shapes monocytes into immunomodulatory DCs. There is also data linking adenosine and adenosine receptors to the production of IL-10 (Koroskenyi et al., 2011, Panther et al., 2012). Monocytes treated with adenosine and TNF-α up-regulate IL-10 (Le Moine et al., 1996) and incubation of LPS-activated DCs with adenosine monophosphate or adenosine also increased the IL-10 production in a dose dependent manner (Panther et al., 2012, Panther et al., 2003). “9-*cis* RA-DCs” may be influenced in maturation and differentiation by the expression and activity of CD73 accompanied by the up-regulation and release of IL-10. Consequently, these cells DCs may develop an immunomodulatory and/or an anti-inflammatory phenotype.

9-*cis* RA DCs-treatment was able to reduce T cell proliferation at all doses significantly, acitretin-treated DCs also decreased T cell proliferation. These results were in line with the reduced allostimulatory capacity shown by Zapata-Gonzales in an assay using lymphoproliferation triggered by 9-*cis* RA-treated SEB pulsed and irradiated DCs (Zapata-Gonzalez et al., 2007). Similar, ATRA-treated DCs also inhibited T cell proliferation compared to controls (Tao and Yang, 2004). Additionally, IL-10-treated DCs exhibit also a lower allogeneic activation potential (Torres-Aguilar et al., 2010a). Here, 9-*cis* RA is not only capable to

reduce allogenic responses in T cells but is also more effective than acitretin. This might also be due the fact that 9-*cis* RA is a dual retinoid-receptor agonist, including RXR with its broad spectrum of binding partners. Previous studies showed that 9-*cis* RA has a similar effect on DCs like PPARγ activators, also a binding partner of RXR, concluding that 9-*cis* RA mechanisms of actions depend on the RXR-heterodimer partner (Zapata-Gonzalez et al., 2007, Gosset et al., 2001).

The advantage of chronic hand eczema patients treated with 9-*cis* RA was taken to investigate retinoid biology and in particular the effects of a dual retinoid receptor agonist 9-*cis* RA in the human system *in vivo*. *In vitro* data indicated that RA, especially 9-*cis* RA, is altering differentiation and maturation of dendritic cells, i.e. decreased CD80 and CD86 expression and increased CD73 expression. This thesis confirms that CD86 was down-regulated, while CD73 was up-regulated during 9-*cis* RA treatment. This is the first time showing these effects for 9-*cis* RA *in vivo*, confirming the *in vitro* data. This indicates that monocytes differentiating and maturating in the milieu of 9-*cis* RA could rather induce immunomodulatory effects than allergy. The phenotypic change could also be a mechanism to alter the fate and chemokine receptor profile of T cells. Retinoids are known for the induction of gut homing (Iwata et al., 2004) while vitamin D is able to induce skin homing (Sigmundsdottir et al., 2007) of T cells. These T cell conversions are carried out by means of dendritic cells. The "9-*cis* RA-DC" might be actually involved in the conversion of T cells into an impaired skin-homing and/or to a preferentially gut-homing phenotype.

As indicated above, 9-*cis* RA treatment alters the secretion of chemokines of keratinocytes and DCs. Here, it is demonstrated that CCL27, CCL8 and CCL13 were significantly reduced during 9-*cis* RA treatment. CCL8 and CCL13 attract immune cells, including monocytes, T cells, eosinophils and basophils (Blanpain et al., 1999, Proost et al., 1996). Both chemokines have been associated with atopic/allergic reactions: CCL8 by recruiting T_H2 cells into IL-5–mediated chronic allergic inflammation sites (Islam et al., 2011) and CCL13 attracts T_H1 and T_H2 cells via CCR1 and CCR3 and has key functions in atopic dermatitis (Mendez-Enriquez and Garcia-Zepeda, 2013). Reduction of eczema-associated chemokines strongly suggests a decreased recruitment of leukocytes to inflamed skin, supporting the anti-inflammatory role for 9-*cis* RA.

To see if patient-derived cells under a 9-*cis* RA treatment are mimicking a reduced allogeneic stimulation capacity as seen *in vitro*, the allogenic mixed leukocyte reaction system was used. *Ex vivo* cells exhibited a significant reduced ability to stimulate allogeneic T cells to proliferate matching the data seen with RA-treated DCs, demonstrating this effect *in vivo* for 9-*cis* RA for the first time. This finding is contractional to the results from Mirza et al. in 2006. While mononuclear cells from cancer patients receiving less than 135 ng/ml ATRA showed a no change in proliferation in response to an allogeneic stimulation during treatment, a higher dose (> 150 ng/ml) increased the proliferation (Mirza et al., 2006). This could be an effect of 9-*cis* RA binding to both RAR and RXR while ATRA is only binding to RAR. A mouse transplantation model using acyclic retinoid NIK-333, an analogue of RA could reveal a suppressive effect on allogeneic immune responses (Ohsawa et al., 2010). The presented findings here demonstrate a reduced activation and allogeneic stimulation of peripheral mononuclear cells in patients receiving 9-*cis* RA. This might result in a reduced activation-dependent T cell proliferation. Additionally, immunomodulatory, tolerogenic and semi mature DCs have been shown to reduce T cell proliferation (Jiga et al., 2004) which could be due to a high expression of IL-10 and a down-regulation of costimulatory molecules as seen *in vitro* generated Retinoid-DCs. In 2010 Torres-Aguilar and coworkers demonstrated that autoreactive T cells from type 1 diabetes patients showed a lower proliferation cocultured with IL-10/TGF-β DCs in comparison to conventional DCs. T cells acquired antigen-specific tolerance by the treatment with IL-10/TGF- β DCs (Torres-Aguilar et al., 2010b). The results indicate a general higher immunomodulatory capacity of these cells against maturation stimuli and thereby a potential mechanism to reduce activation-dependent proliferation in T cells.

9-*cis* RA treatment alters the phenotype of "homing" T cells *in vivo*

Regarding the modification of the T cell phenotype, the presented findings align with several studies showing effects of RA *in vitro* and *ex vivo*. A first hint on the effects of retinoids came from Iwata et al. in 2004. The group demonstrated a suppressive effect of ATRA on skin-homing receptors E-lig, P-lig (CLA), CCR4 and CD62L on cultured cells *in vitro* in untreated conditions and under T_H1 conditions. Also, 9-*cis* RA treatment reduced homing marker E-lig *in vitro* (Iwata et al., 2004). It was also noted that RA did not alter the expression of CCR10 on T cells, however vitamin D induced CCR10 expression on T cells *in vitro*

(Sigmundsdottir et al., 2007). In 2008, it was shown that *ex vivo* $CD3^+CLA^+$ T cells lost the CLA expression upon treatment with ATRA. The authors also analyzed the expression of CCR4, CCR6, CCR7, CCR9, CCR10 and CD62L on these cells and found out that CCR10 is down-regulated, CCR9 is up-regulated and CCR4, CCR6 and CD62L are not affected by ATRA (Yamanaka et al., 2008). These findings are in line with the data on the patients receiving 9-*cis* RA, as there were no effects on CCR4, CCR6 and CD62L. However, this is the first time demonstrating that 9-*cis* RA modifies the "skin-homing" phenotype of circulating $CD4^+$ and $CD8^+$ T cells in the periphery *in vivo*. Due to a decreased expression of CCR10 on $CD4^+$/ $CD8^+$ cells as well as a downmodulation of CLA on $CD4^+$ cells, it is very likely that numbers of cells migrating to skin are decreased. Supporting to this in a mice model with a oxazolone-induced contact hypersensitivity response, ATRA treatment reduced the number of $CD4^+$ cells which migrated into the inflamed skin. Further, the authors stated that RA treatment diminished the CLA expression in three atopic dermatitis patients in comparison to controls (Yamanaka et al., 2008). Moreover, isotretinoin treatment reduced infiltrating T cells in patients with chronic/ subacute cutaneous lupus erythematosus (Newton et al., 1986, Kim, 2013). This data is confirmed in patients receiving systemic 9-*cis* RA showing decreased inflammatory infiltrate in skin specimens after treatment.

In contrast to findings of the present study, it was shown that retinoids are capable of altering the homing specificity of T cells by increasing the expression of $\alpha_4\beta_7$-integrin and CCR9 on activated T cells (Iwata et al., 2004). Upon ATRA treatment, CCR9 is induced on CD4 and CD8 positive T cells (Iwata et al., 2004, Kim, 2013, Sigmundsdottir et al., 2007). Moreover, ATRA was reported to imprint gut-homing of gut-DCs activated naïve CD8 T cells (Eksteen et al., 2009). In the presented patient group here, the percentage of peripheral CCR9 positive $CD4^+$ and $CD8^+$ T cells was significantly reduced. As for the expression of CCR9 on $CD4^+$ and $CD8^+$ T cells in the periphery, there was no clue if these cells home into the intestine or if these cells are lost in the periphery in the analyzed patients. On the one hand, vitamin A-deficient mice are not capable to induce CCR9 and $\alpha4\beta7$ on T cells, hinting that retinoids are crucial for gut-homing (Iwata et al., 2004). Also, CCR9 KO mice have profound defects in oral tolerance (Cassani et al., 2011). In patients treated with 9-*cis* RA it is possible that $CCR9^+$ T cells increasingly home into the gut and thereby maybe induce oral tolerance. On the other hand, there are some indications that treatment with retinoids, like isotretinoin, are probably associated with induction inflammatory bowel diseases (Crockett et al.,

2010, Reniers and Howard, 2001). This is maybe promoted by a higher infiltration of CCR9 positive cells into the gut.

Regarding the reduction of $CD4^+CXCR3^+$ cells it was reported that vitamin A-deficient mice have a higher number of T_H1 cells (Cantorna et al., 1995). Additionally, ATRA and 9-*cis* RA promote the polarization into T_H2 cells by enhancing IL-4 production and inhibiting IFN-γ production (Hoag et al., 2002, Stephensen et al., 2002, Iwata et al., 2003). On the other hand, in low doses ATRA are inhibiting T_H2 polarization in a repetitive stimulation setting (Wansley et al., 2013). Also, spleen cells from mice with collagen-induced arthritis treated with ATRA tended to down-regulate the expression of CXCR3 (Nozaki et al., 2006). Since CXCR3 is highly present on T_H1 cells, it is likely to hypothesize that 9-*cis* RA has an effect on $CD4^+$ T_H1 cells, in general, although the preferentially skin homing population $CD4^+CLA^+CXCR3^+$ was not affected significantly by the treatment. However, T_H2 cells recruitment (CCR4) maybe altered indirectly by the lower secretion of CCL8 as seen in the serum of patients but this is not clear, yet.

Xiao et al. showed a 9-*cis* RA-induced T_H17 inhibition by interfering with TGF-β and IL-6/IL-21/IL-23 signaling. The same group also found that 9-*cis* RA induce regulatory T cells (T_{reg}) (Xiao et al., 2008). Moreover, systemic 9-*cis* RA treatment reduced the percentage of IL-17-producing cells in $CD154^+CD4^+$ cells upon stimulation, while an observed reduction of regulatory T cells in the periphery during treatment with 9-*cis* RA was due to their recruitment to the skin (Schindler et al., 2014). In the analyzed patients here, there was no change in the percentage of circulating $CD4^+CCR6^+$ cells. This observation might be due to an absent stimulus as the group of Schindler stimulated cells from patients with staphylococcal enterotoxin B and anti-CD28 followed by a down-regulation of IL-17 (Schindler et al., 2014).

While antigen encounter promotes the turnover of central memory T cells into effector T cells leads to the down-regulation of CCR7 (Klebanoff et al., 2006), the analyzed patients show an increase in $CD8^+CCR7^+$, thereby maybe indicating a higher percentage of non-polarized central memory T cells. Moreover, CCR7-deficient mice have been demonstrated to be more susceptible to autoimmunity (Worbs and Forster, 2007). ATRA treatment increased the percentage of $CD8^+$ central memory T cells ($CCR7^+CD62L^+$) in the context of vaccination. The authors speculated that RA may promote an improvement in vaccine efficiency by enhancing mucosal immunity. This effect is partly mediated by resident memory $CD8^+$ T cells and partly by memory cells recruited from lymph nodes (Tan et al., 2011).

Conclusions

Findings of the present study demonstrate, that 9-*cis* RA interferes at distinct steps, time points and sites with the skin immune system (Fig. 31):

I. At an early step 9-*cis* RA interferes with keratinocytes. Here, 9-*cis* RA alters the expression of genes regarding different keratinocyte functions. Known genes overexpressed or dysregulated participating in barrier disruption are normalized. While water loss may be reduced, wound healing and tissue regeneration might be induced through the regulation of the hyaluronan system. Differentiation and proliferation processes might return to a homeostatic level. Pro-inflammatory defensins and lymphocyte attracting chemokines are also down-regulated and thereby the recruitment of infiltrating lymphocytes, especially monocytes and T cells might be diminished. This is further supported by a lower release of keratinocyte-associated CCL27 as well as atopy/allergy-associated CCL8 and CCL13.

II. At a later phase 9-*cis* RA inhibits MoDCs maturation and interferes with the up-regulation of co-stimulatory molecules *in vitro* and *in vivo*. RA is shaping monocytes/ monocyte-derived dendritic cells into an immunomodulatory phenotype. *In vitro*, this process is characterized by lower expression of maturation markers CD83, CD80, CD86, a high secretion of IL-10 into the supernatant, the expression of CD73 and LIR-family members on RNA levels and on the surface. This phenomenon is supported by the observation that 9-*cis* RA significantly suppressed the allogenic stimulation of leukocytes in MLR assays. “Retinoid-DCs” also have an increased expression of CCR6 and CCR7 allowing them to stay in the periphery or migrate into the lymph node to potentially induce an immunosuppressive reaction at both sites. *In vivo*, the $CD14^+$ monocyte surface expression of CD86 is down-regulated, whereas CD73 is up-regulated under 9-*cis* RA treatment *in vivo*. Moreover, a reduced activation and allogeneic induced proliferation of peripheral mononuclear cells in patients under 9-*cis* RA treatment further supports the immune modulating effects of 9-*cis* RA *in vivo*. 9-*cis* RA treatment also modifies the expression profile of chemokine receptors/homing markers in the periphery. Expression of CCR10 on $CD4^+$/ $CD8^+$ cells as well as CLA on $CD4^+$ cells is down-regulated during treatment. 9-*cis* RA might diminish the presence of T_H1 cells ($CXCR3^+$) in the periphery in general, whereas there is no evidence of an altered T_H2 cell recruitment ($CCR4^+$).

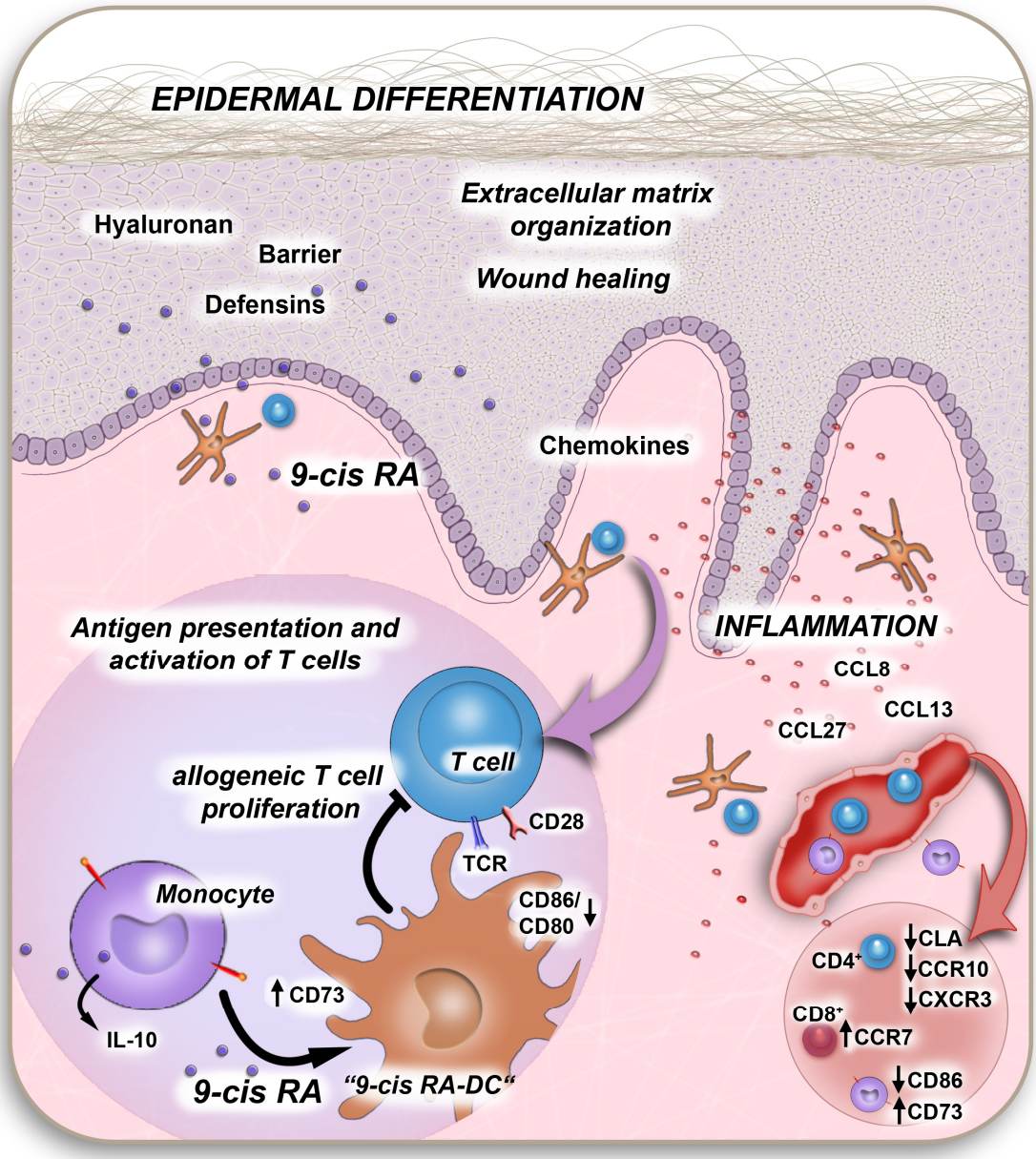

Figure 31 Proposed model of the effects of 9-*cis* RA on the immune system of the skin. First, 9-*cis* RA alters the gene expression in keratinocytes, including dysregulated barrier genes and genes regulating water loss, wound healing and inflammation thereby altering the recruitment of lymphocytes, especially monocytes and lymphocytes. Second, in dendritic cells (MoDCs) 9-*cis* RA inhibits the up-regulation of maturation markers and co-stimulatory molecules and induces chemokine receptor CCR6 and CCR7. Also, the immunomodulatory factors CD73 and IL-10 are activated, in turn, an immunomodulatory DCs phenotype is generated. As a result, reduced antigen presentation might be leading towards impaired leukocyte activation, proliferation and expansion. Moreover, the percentage of circulating skin-homing effector cells is decreased *in vivo*. Taken together, 9-*cis* RA may interfere with different steps in the inflammatory process of the skin.

7 List of abbreviations

°C	Degree Celsius
%	Percent
9-*cis* RA	9-*cis* retinoic acid, alitretinoin
"9-*cis* RA-DC"	Mature monocyte-derived dendritic cells treated with 9-*cis* RA
13-*cis* RA	13-*cis*-retinoic acid
"Acitretin-DC"	Mature monocyte-derived dendritic cells treated with acitretin
AMPs	Antimicrobial peptides
AD	Atopic dermatitis
ATRA	*All-trans* retinoic acid
c	Centi
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CCL	CC-chemokine ligand
CCR	CC-chemokine receptor
CHE	Chronic hand eczema
Cl	Chlor
CXCL	CXC-chemokine ligand
CXCR	CXC-chemokine receptor
C_t	Threshold cycle
DC	Dendritic cells
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EGF	Epidermal growth factor
FCS	Fetal calf serum
g	Gram
H_2O	Water
HAS	Hyaluronan synthase
HYAL	Hyaluronidase
GM-CSF	Granulocyte macrophage colony-stimulating factor
IFN-	Interferon
iMoDC	Immature monocyte-derived dendritic cell
IL-	Interleukin-
l	Liter
LCE	Late cornified envelope
m	Meter
µ	Micro
m	Milli
M	Molar
min	Minute
MLR	Mixed leukocyte reaction
mMoDC	Mature monocyte derived dendritic cell
MoDCs	Monocyte-derived dendritic cells
mRNA	Messenger ribonucleic acid
mTLSS	Modified total lesion symptom score
n	Nano

Na	Natrium
PBMCs	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PBS	Phosphate buffer saline
PRE	Before treatment
qPCR	Quantitative polymerase chain reaction
RAR	Retinoic acid receptor
RARE	Retinoic acid response elements
“Retinoid-DC”	Mature monocyte-derived dendritic cells treated with retinoids
rh	Recombinant human
rpm	Rounds per minute
RT	Room temperature
RXR	Retinoic X acid receptor
SD	Standard deviation
SEM	Standard error mean
SPRRs	Small proline-rich proteins
T_H	T helper cells
TNF-α	Tumor necrosis factor-alpha
TGF	Transforming growth factor
T_{reg}	Regulatory T cells
VAD	Vitamin A deficiency
Wk	Week

8 Citations

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■ Conferences

- 2011: Sixth European workshop on immune mediated inflammatory diseases (EwIMID) – Nice, France
- 2012: 45th Annual European Society for Dermatological Research (ESDR) meeting – Venice, Italy
- 2013: International Investigative Dermatology (IID) meeting – Edinburgh, Scotland
- 2014: 41. ADF-Tagung - Arbeitsgemeinschaft Dermatologische Forschung – Köln, Germany

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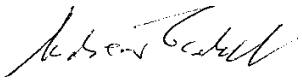
11 Declaration

I, Andreas Kislat, declare under oath that I have compiled my dissertation independently and without any undue assistance by third parties under consideration of the 'Principles for the Safeguarding of Good Scientific Practice at Heinrich Heine University Düsseldorf'. This dissertation has not been submitted in the same or similar form to other institutions. I have not previously failed a doctoral examination procedure.



Andreas Kislat

Mönchengladbach, 05.04.2018