THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Regulation of retinoid-mediated signaling in the skin and its implication for skin homeostasis in mice

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ABBREVIATIONS

Abca12	ATP-binding cassette A12
Acer1	Alkaline ceramidase 1
AD	Atopic dermatitis
Adh7	Alcohol dehydrogenase 7
Aldh1a	Aldehyde dehydrogenase 1a
ATRA	all-*trans* retinoic acid
Bco	Beta-carotene oxygenase
Ccl	Chemokine (CC-motif) ligand
Ccr3	Chemokine (CC-motif) receptor 3
Crabp	Cellular retinoic acid binding protein
C_T	Threshold cycle
Cyclo	Cyclophilin A
Cyp26a1	Cytochrome P450 26a1
Cyp26b1	Cytochrome P450 26b1
Cyp2s1	Cytochrome P450 2s1
Dgat	Diacylglycerol O-acyltransferase
e.c.	Epicutaneous
Fabp5	Fatty acid binding protein 5
Flg	Filaggrin
Gba	Glucocerebrosidase
Hbegf	Heparin-binding EGF-like growth factor
H&E	Haematoxylin + eosin
Hmgcs2	3-Hydroxy-3-methylglutaryl-CoA synthase 2
HPLC	High performance liquid chromatography
Ifng	Interferon γ
IL	Interleukin
Ivl	Involucrin
i.p.	Intraperitoneal
Klk	Kallikrein
Krt	Keratin
Lor	Loricrin
Lrat	Lecithin-retinol acyltransferase

Min	Minute
Mmp9	Matrix metalloproteinase 9
MS-MS	Mass spectrometry – mass spectrometry
NR	Nuclear hormone receptor
NR4A1	Nuclear receptor family 4, group A, number 1 (NUR77)
OVA	Ovalbumin
PBS	Phosphate-buffered saline
PPAR	Peroxisome proliferator-activated receptor
qRT-PCR	Quantitative real time polymerase chain reaction
RA	Retinoic acid
RAR	Retinoic acid receptor
Rarres2	Retinoic acid responder 2
Rbp	Retinol binding protein
Rdh	Retinol dehydrogenase
Rpm	Rounds per minute
RT	Room temperature
RXR	Retinoid X receptor
Sdr16c5	Short chain dehydrogenase / reductase 16C5
Sec	Second
SEM	Standard error of mean
Spink5	Serine peptidase inhibitor, Kazal-type 5
SptIc2	Serine palmitoyltransferase
S100a7a	S100 calcium binding protein A7A / Psoriasin
Tgm	Transglutaminase
Th1	T helper cell 1-type
Th2	T helper cell 2-type
TLDA	TaqMan® low density array
Tslp	Thymic stromal lymphopoietin
UDL	Under detection limit
Ugcg	UDP-glucose ceramide glucosyltransferase
VA	Vitamin A

CONTENTS

1 INTRODUCTION

Retinoids, which comprise endogenous and exogenous vitamin A derivatives, are essential for the regulation of several physiological processes of many organs, tissues, and cell types. Through their involvement in the regulation of various aspects of skin cell proliferation, differentiation, apoptosis, epidermal barrier function, and Th1/Th2 balance (Elias, 1987; Elias *et al.*, 1981; Stephensen *et al.*, 2002), retinoids play particular roles in immune-modulatory events (Rühl, 2007; Rühl *et al.*, 2004; Rühl *et al.*, 2007), and skin physiology (Roos *et al.*, 1998). Retinoids exert their functions mainly via the nuclear hormone receptors retinoic acid receptors (RAR) α, β, and γ and retinoid X receptors (RXR) α, β, and γ. These are ligand-dependent transcription factors which, as RAR-RXR heterodimers, regulate the expression of multiple genes in skin and various other tissues (Mangelsdorf *et al.*, 1995). Retinoid metabolism and concentrations in skin are tightly regulated ensuring sufficient levels of the endogenous pan-RAR activator all-*trans* retinoic acid (ATRA) (Chapellier *et al.*, 2002; Everts *et al.*, 2004; Everts *et al.*, 2007). However, alterations in retinoid metabolism, signaling, and concentrations have been observed in various dermatoses, such as psoriasis (Saurat, 1999), ichthyosis (Mevorah *et al.*, 1996), and atopic dermatitis (AD) (Mihály *et al.*, 2011). Thereby, it remains unclear whether these changes are symptoms or the trigger of such diseases. The aim of this work was to determine how topically applied agonists or antagonists selective for RARs or RXRs might influence retinoid metabolism and signaling in mouse skin, as well as epidermal barrier homeostasis and skin-based immune regulation relevant for skin disorders such as AD. Moreover, it was of interest whether the induction of allergic immune responses by systemic or combined systemic and topical treatments with ovalbumin (OVA) is able to modify retinoid metabolism and retinoid-mediated signaling in the skin of mice and its correlation to receptor specific agonist or antagonist treatments of mice.

2 THEORETICAL BACKGROUND

2.1 Retinoids

Vitamin A (VA) is an essential component of the human diet which can not be synthesized within the human body. The daily recommended allowance for VA corresponds to 0.8 mg for women and 1 mg for men, respectively (e.V., 2008). The term vitamin A (VA) designates all compounds that possess qualitatively the biological activity of the VA-alcohol retinol, such as retinal, retinyl esters, and various pro-VA carotenoids. Furthermore, the group of retinoids comprises several of these natural but also synthetic forms of VA, consisting of four isoprenoid units joined in a head-to-tail manner. In fact, the term retinoids designates all natural and synthetic compounds exhibiting similar structure and/or effectiveness like retinol and therefore also includes retinoic acids (RA) (IUPAC-IUB, 1982; Rühl, 1999).

Active metabolites of VA, such as RA and retinal, play important roles in several physiological processes such as epithelial surface maintenance, immune competence, reproduction, and development (Blomhoff, 1994). Moreover, retinoids are commonly used for the therapy of various skin disorders like psoriasis, ichthyoses, and particularly acne vulgaris (Baron *et al.*, 2008). Acne is the most common human skin disorder which is characterized by comedogenesis, increased sebum production, inflammation, and colonization with Propionibacterium acne. The RA isomer 13-*cis* RA (Isotretinoin) is commonly used for acne therapy and proved to be effective by normalizing keratinization which prevents comedogenesis, by reducing sebum production, and diminishing inflammation (Layton, 2009; Saurat, 1997; Thielitz *et al.*, 2006). However, several adverse effects of retinoids are reported such as teratogenicity, depression, and mucocutaneous side effects (e.g. cheilitis) rendering them a topic of debates (Blasiak *et al.*, 2013; Brelsford and Beute, 2008; Layton, 2009).

2.1.1 Retinoid metabolism

Retinoids are primarily obtained via the diet as retinyl esters from foods of animal origin, such as meat, fish, and dairy products, or they can be generated from plant-derived pro-VA carotenoids like β-carotene. Upon ingestion, retinyl esters are hydrolyzed by pancreatic and intestinal enzymes into free retinol which is then, together with pro-VA carotenoids, taken up by enterocytes (Blomhoff and Blomhoff, 2006). Within enterocytes, centric or eccentric

cleavage of pro-VA carotenoids (Glover and Redfearn, 1954; Karrer *et al.*, 1930) is catalyzed by the enzymes beta-carotene oxygenase (BCO) 1 and 2 (Kiefer *et al.*, 2001; Redmond *et al.*, 2001; von Lintig and Vogt, 2000) to generate retinal (or apo-8'-carotenal and other apo-carotenals) (Goodman and Huang, 1965; Olson and Hayaishi, 1965) which is subsequently reduced to retinol (Figure 1) (Li and Tso, 2003). Following, retinol is bound to the cellular retinol binding protein 2 (Crbp2) which promotes its re-esterification by the enzymes lecithin:retinol acyl transferase (Lrat) (Herr and Ong, 1992) and to a smaller extent by diacylglycerol acyltransferase 1 (Dgat) (Wongsiriroj *et al.*, 2008). In fact, Lrat mRNA expression is increased in response to high RA levels which is thought to be a feedback mechanism (Fisher and Voorhees, 1996; Kurlandsky *et al.*, 1996; Pavez Lorie *et al.*, 2009). Upon esterification, retinyl esters together with further lipids (cholesterol, triacylglycerides) are incorporated into chylomicrons which are secreted into the lymph for transport into the blood stream (Blomhoff *et al.*, 1990; Harrison and Hussain, 2001). Chylomicron triacylglyceride hydrolysis results in the formation of chylomicron remnants which are taken up by hepatocytes or extra-hepatic target cells. Within the liver, retinyl esters are in turn hydrolyzed, the resulting retinol is bound to retinol binding protein 1 (Rbp1) and transferred to perisinusoidal stellate cells where dietary retinoids are stored as retinyl esters (Blomhoff and Blomhoff, 2006; D'Ambrosio *et al.*, 2011; Newcomer and Ong, 2000). Indeed, hepatic stellate cells are the main storage site of VA in the human organism containing 50-80% of the body's total retinol as retinyl esters (Blomhoff *et al.*, 1985; Senoo, 2004). In order to maintain a steady plasma retinol concentration of 1-2 µM, retinol can be mobilized in hepatic stellate cells, associated to Rbp4 and is then secreted into the general circulation (Blomhoff and Blomhoff, 2006; O'Byrne *et al.*, 2005; Soprano and Blaner, 1994). In the blood stream this complex associates with another protein, transthyretin (Peterson, 1971), which reduces glomerular filtration of retinol. Rbp-retinol complexes are thought to be taken up by target cells via the transmembrane-spanning receptor encoded by the Stra6 gene (Kawaguchi *et al.*, 2007). Within peripheral target tissues such as skin, retinol can be stored upon Lrat-catalyzed esterification (Baron *et al.*, 2008) or it is converted into its bioactive metabolite ATRA via a two-step oxidation process. Firstly, all-*trans* retinol is oxidized by alcohol/retinol dehydrogenases and/or short-chain dehydrogenases/reductases (e.g. Adh7, Rdh10, Rdh16, and Sdr16c5) to generate all-*trans* retinal. By means of three different aldehyde dehydrogenases 1a (Aldh1a1, 2, and 3) all-*trans* retinal is further oxidized to ATRA (Figure 1), the major biologically active VA metabolite (Blomhoff and Blomhoff, 2006; Conaway *et al.*, 2013).

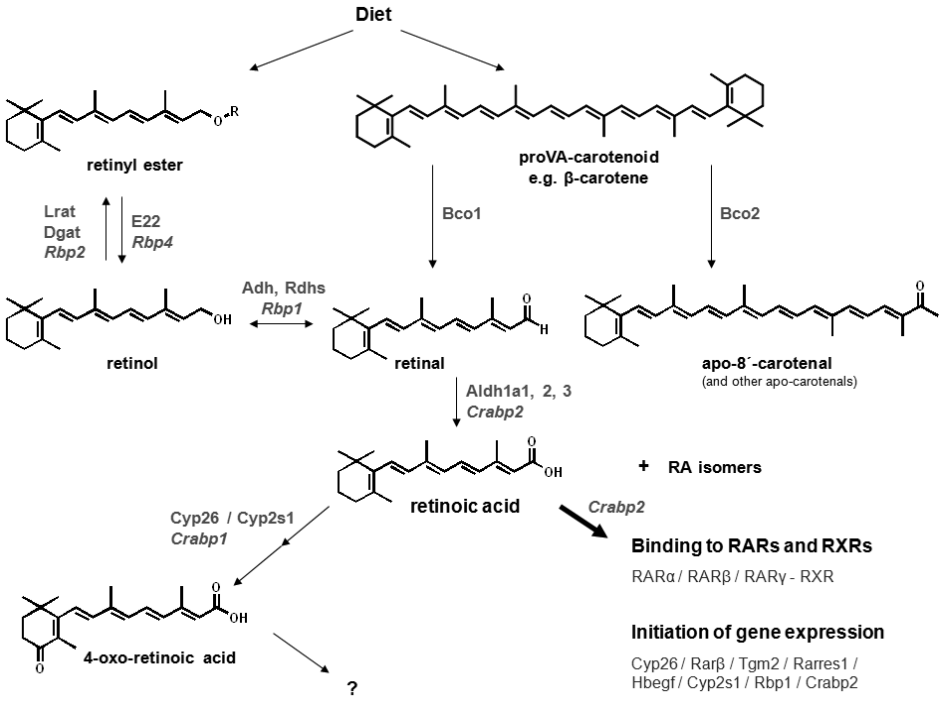

Figure 1. Retinoid metabolism and function following dietary uptake of retinyl esters and proVA carotenoids. Involved enzymes and retinoid transport proteins (*italic*) are indicated in grey.

ATRA is then bound by the cellular RA binding protein 2 (Crabp2) and shuttled to nuclear hormone receptors (NRs: RAR, RXR, PPARβ/δ) to exert its biological activity via gene expression regulation. Interestingly, Crabp2 itself is a direct target of ATRA and its expression increased upon high RA levels (Astrom *et al.*, 1991; Sanquer and Gilchrest, 1994). However, RA levels are tightly controlled in skin cells and other tissues resulting in its degradation to more polar and less active metabolites by cytochrome P450 enzymes such as Cyp26a1, Cyp26b1, and Cyp2s1 (Blomhoff and Blomhoff, 2006; Roos *et al.*, 1996; Smith *et al.*, 2003; White *et al.*, 1997; White *et al.*, 2000) in case of excess. Another cellular RA binding protein, Crabp1 is believed to promote this degradation pathway via RA transport towards Cyp enzymes (Figure 1) (Donovan *et al.*, 1995). Therefore, the steady-state-system of intracellular retinoid concentrations appears to be regulated by complex feedback mechanisms which involve several of the above mentioned enzymes and retinoid binding proteins. Indeed, despite lower consumption of retinyl esters by mice and direct cleavage of β-carotene in their intestine, retinoid metabolism and homeostasis are comparable within mice and man. Moreover, also topically applied retinoids are partly absorbed by skin cells via slow diffusion (Tavakkol *et al.*, 1994). Depending on the kind of retinoid applied, the compound can be further metabolized into various derivatives (Kang *et al.*, 1995; Lehmann and Malany, 1989) which can potentially influence the expression of several genes in the skin.

2.1.2 Retinoid function in the skin

Retinoids are essential for skin physiology (Roos *et al.*, 1998) through their role in the regulation of several aspects of skin cell proliferation, differentiation, apoptosis, epidermal barrier function, and immune regulation (Elias, 1987; Elias *et al.*, 1981). Noticeably, alterations of retinoid metabolism and signaling were found in the skin of patients with various skin diseases, such as psoriasis (Saurat, 1999), ichthyosis (Mevorah *et al.*, 1996), and AD (Mihály *et al.*, 2011). Thereby, it is unclear whether these alterations are the trigger or if they are consequence of these skin diseases.

Epidermal barrier

Retinoid function in skin barrier maintenance is mainly based on the regulation of epidermal permeability (based on epidermal hyperproliferation), epidermal differentiation (Elias, 1987), sebum secretion (Zouboulis *et al.*, 1998), and apoptosis (Trautmann *et al.*, 2000). Furthermore, disturbance of retinoid receptor-mediated signaling (especially via RXR) in the skin was shown to result in various epidermal alterations such as disturbed keratinocyte proliferation, alopecia, and induction of AD in different mouse models (Feng *et al.*, 1997; Li *et al.*, 2001; Li *et al.*, 2005).

Immune system

Retinoids are important modulators of immune function and immune response (Rühl, 2006; Schuster *et al.*, 2008; Stephensen, 2001). It has been shown recently that VA can modify cell numbers of immune competent cells (Blomhoff *et al.*, 1992; Miller and Kearney, 1998; Ross and Stephensen, 1996; Smith *et al.*, 1987), as well as cytokine and chemokine levels (Aukrust *et al.*, 2000; Cantorna *et al.*, 1995; Nunez *et al.*, 2010), and antibody responses (Ross *et al.*, 2009; Ross and Hämmerling, 1994). Interestingly, RAR- and RXR-mediated pathways have been found to be involved in Th2 development and Th1/Th2 balance (Dawson *et al.*, 2008; Rühl *et al.*, 2004; Stephensen *et al.*, 2007; Stephensen *et al.*, 2002). Thereby, RA is possibly able to directly modify pro- and anti-inflammatory immune responses. Moreover, a role of retinoids in the modification of the immune phenotype of atopic skin diseases like AD has been shown previously (Rühl *et al.*, 2004; Rühl *et al.*, 2007).

2.2 Gene expression regulation by nuclear hormone receptors

Retinoids exert the vast majority of their functions in organs and tissues, such as the skin, via NR-mediated gene expression regulation.

2.2.1 Nuclear hormone receptors

NRs comprise a superfamily of DNA binding, ligand-dependent transcription factors like RAR α, β, and γ, RXR α, β, and γ, or peroxisome proliferator-activated receptors (PPARα, β/δ, and γ). These molecules are present in the cytoplasm or nucleus and play a crucial role in development, homeostasis, and other biological processes in mammals (Whitfield *et al.*, 1999). NRs feature a specific structure consisting of a N-terminal extension which is highly variable in length and sequence, a well-conserved central DNA-binding domain containing two zinc-fingers as structural DNA-binding motif, a hinge region which influences intracellular trafficking and subcellular distribution of the NR, the large C-terminal ligand-binding domain with moderately conserved sequence, and a variable C-terminal extension (Klinge, 2000). The variable sequences allow the discrimination between different NR subfamilies and further between various subtypes within these families.

NRs are sequence specific transcription modulators which regulate the gene expression in a ligand-dependent manner. This implies the plasma membrane penetration of lipophilic ligands, e.g. retinoids, followed by their delivery to the receptor and ligation of the ligand-binding domain of the cognate NR. Upon ligation, RARs and PPARs form heterodimers with RXRs and conformational changes take place. This enables the dimer to bind with high affinity to a specific hormone response element as depicted in Figure 2. This structure is a specific hexanucleotide half-element in the DNA which is arranged in a particular motif, such as palindromes (inverted repeats) or direct repeats, with spacing between these half-sites (De Luca, 1991; Whitfield *et al.*, 1999). It is usually located close to the core promoter, the region where the transcription process starts (Glass, 1994). These elements are referred to as RA-responsive element in the case of RARs and as PPAR-responsive element for PPARs, respectively. Ensuing, in cooperation with several co-regulating proteins (co-activators and co-repressors) the gene expression of respective target genes is modulated (Michalik and Wahli, 2007a).

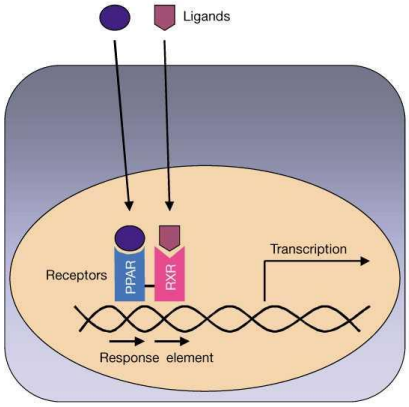

Figure 2. Basic mechanism of action of NRs (modified after Kersten *et al.* 2000).

2.2.2 Nuclear hormone receptor families: RAR, RXR, and PPAR

The RAR was discovered in 1987 (Giguere *et al.*, 1987; Petkovich *et al.*, 1987) and up-to-date 3 subtypes, RAR α, β, and γ, were identified (Chambon, 2004; Evans, 2004). These receptors belong to the same type 2 class as the PPARs α, β/δ, and γ within the NR superfamily (Michalik and Wahli, 2007a) and therefore always act as heterodimers with either RXR α, β, or γ (Levin *et al.*, 1992).

The activated (i.e. liganded) RAR-RXR regulates the expression of multiple genes in skin and various other tissues (Mangelsdorf and Evans, 1995; Mangelsdorf *et al.*, 1995) while its transcriptional activity is dependent on the RAR-activating ligand (Chapellier *et al.*, 2002; Feng *et al.*, 1997; Mangelsdorf and Evans, 1995; Pérez *et al.*, 2012). The transport protein Crabp2 is responsible for the delivery of RAR agonists to the receptor (Dong *et al.*, 1999). On the other hand, the heterodimer is believed to function as a transcriptional silencer in the absence of appropriate ligands (Bertram, 1999; Chambon, 1996). The most abundant RAR and RXR subtypes in the skin are RXRα and RARγ, followed by lower quantities of RARα, while RARβ and RXRγ are not expressed (Fisher *et al.*, 1994). Since retinoid receptors exhibit tissue and cell type-specific distribution patterns, functional specificity of each subtype is suggested (Altucci *et al.*, 2007; de Lera *et al.*, 2007; Dolle *et al.*, 1990; Elder *et al.*, 1992; Elder *et al.*, 1991; Germain *et al.*, 2006; Redfern and Todd, 1992). Moreover, RAR and RXR subtypes differ in ligand specificity and/or affinity (Allenby *et al.*, 1993; Allenby *et al.*, 1994; Altucci *et al.*, 2007; de Lera *et al.*, 2007; Germain *et al.*, 2006), therefore, it can be assumed that their contribution to gene expression patterns in tissues such as skin differs,

depending on quantitative receptor distribution, on the nature and level of co-regulators, as well as on available retinoid receptor-selective agonists and antagonists.

Unsaturated fatty acids and eicosanoids are applicable ligands for PPARα, β/δ, and γ (Bertram, 1999). The most abundant PPAR subtype in the skin is PPARβ/δ (Michalik and Wahli, 2007b). PPAR-mediated pathways are important in skin physiology because they are involved in epidermal barrier recovery, keratinocyte differentiation, and lipid synthesis (Michalik and Wahli, 2007b). For example, overexpression of PPARδ in the epidermis causes a psoriasis-like skin disease featuring hyperproliferation of keratinocytes, dendritic cell accumulation, and endothelial activation (Romanowska *et al.*, 2010).

2.2.3 Gene expression regulation by retinoids

The retinoid ATRA is the well known endogenous ligand of RARs which, through its binding, is able to induce or decrease the expression of various genes. Interestingly, a cross-talk exists between RAR and PPARδ pathways. Indeed, besides RARs, also PPARδ can be activated by ATRA, depending on the ratio of their specific ligand transport proteins. Crabp2 initiates RAR signaling, whereas the fatty acid-binding protein 5 (Fabp5) promotes PPARδ-mediated signaling upon ATRA-binding (Schug *et al.*, 2007; Shaw *et al.*, 2003). Moreover, PPARδ activation has been reported at high ATRA concentrations suggesting that tissue levels of ATRA can determine which NR pathways are up-regulated and thereby influence the gene expression profile (Schug *et al.*, 2007; Shaw *et al.*, 2003). However, these findings are still controversially discussed (Berry and Noy, 2009; Borland *et al.*, 2011; Rieck *et al.*, 2008).

2.3 The skin

The skin of mice and man consists of epidermis, dermis, and subcutis and represents the essential physical barrier between an organism and its surrounding environment which may contain pathogens, allergens, chemicals, etc. Therefore, a functioning epidermal barrier is the precondition to avoid percutaneous penetration and to prevent the development of cutaneous disorders. The epidermis is mainly composed of keratinocytes. Additionally, several structural proteins, enzymes, and lipids are involved in assembly and maintenance of the epidermal barrier. During terminal differentiation, keratinocytes move towards the stratum corneum layer of the epidermis and transform into flattened and anucleated corneocytes (Proksch *et al.*,

2008). Their plasma membrane is replaced by an insoluble protein layer, the cornified envelope, and the cells are locked together by corneodesmosomes. The cornified envelope is predominantly composed of structural proteins such as loricrin (Lor), involucrin (Ivl), and filaggrin (Flg) which are cross-linked by transglutaminase 1 (Tgm1). Its function is to act as a scaffold for the attachment of lipids from the surrounding lipid lamellae matrix. The lipid matrix consists of cholesterol, cholesterol esters, free fatty acids, and most importantly ceramides (Holleran *et al.*, 2006; Jennemann *et al.*, 2007), and it helps to prevent water loss and the penetration of water soluble substances. Several enzymes, such as 3-Hydroxy-3-methylglutaryl-CoA synthase 2 (Hmgcs2) (Proksch *et al.*, 1993), β-Gluco-cerebrosidase (Gba), and UDP-glucose ceramide glucosyltransferase (Ugcg) (Coderch *et al.*, 2003), are involved in the generation of these lipid matrix components.

Furthermore, in a process called desquamation corneocytes are continuously shed from the epidermal surface and replaced by new keratinocytes. Desquamation is regulated by proteases (e.g. kallikrein-related peptidase 5 (Klk5) and Klk7) which break down the extracellular corneodesmosomal adhesion proteins, as well as by protease inhibitors like Kazal-type 5 serine protease inhibitor LEKTI (encoded by the Spink5 gene) (reviewed in: (Cork *et al.*, 2009; Proksch *et al.*, 2008; Proksch *et al.*, 2003).

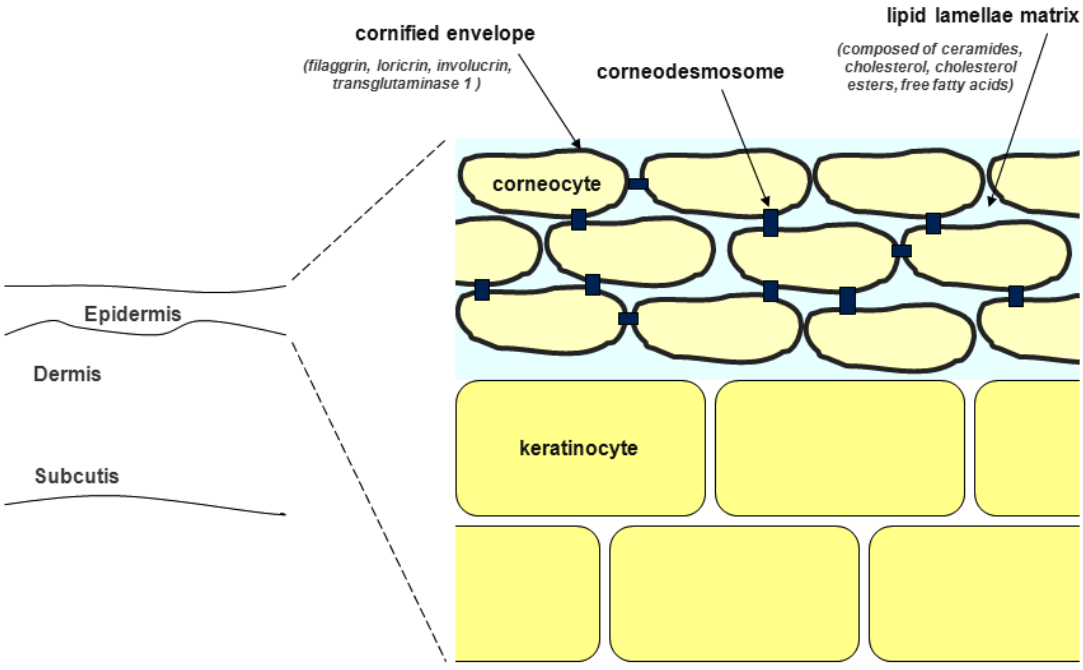

Figure 3. Simplified model of the skin with its epidermal barrier (magnified section).

This epithelial tissue homeostasis and maintenance of the epidermal permeability barrier are tightly regulated processes. Moreover, the skin possesses several active immune surveillance mechanisms providing additional defense against the environment (reviewed by: (Kupper and Fuhlbrigge, 2004). However, disturbances of levels and/or activity of involved proteins, enzymes, and lipids disrupt the epidermal barrier and thereby compromise the physiological function which may predispose the skin to dermatological diseases.

2.4 Atopic dermatitis

The allergic skin disease AD is the most common inflammatory skin condition, predominantly affecting infants and children and it is characterized by a disturbed epidermal permeability barrier, eczematous lesions, epidermal hyperproliferation, and skin dryness (Bieber, 2008; Bieber and Novak, 2009). AD is a Th2-driven disease and therefore described by increased IgE levels, elevated Th2 cytokines (e.g. IL-4, IL-10) and Th2 cell-attracting chemokines (CCL11, CCL17, CCL22, CCL24), as well as enhanced microbial colonization of the skin due to decreased expression of antimicrobial peptides (Boguniewicz and Leung, 2010; Gros *et al.*, 2009; Owczarek *et al.*, 2010). Moreover, the number of T cells expressing the cutaneous lymphocyte-associated antigen for skin homing is increased while the infiltration of regulatory T cells is impaired in AD skin (Boguniewicz and Leung, 2010). AD is commonly associated with other allergic conditions such as allergic rhinitis and asthma. Around 10-30% of children and 2-10% of adults are affected in industrialized countries, with a marked increase in AD prevalence during the past 30 years (Bieber, 2008; Bieber and Novak, 2009; Palmer *et al.*, 2006). Interestingly, alterations of retinoid metabolism and signaling were recently found in the skin of AD patients (Mihály *et al.*, 2011). Furthermore, it has been shown that retinoids are able to modify the immune phenotype of atopic diseases by promoting Th2-mediated immune responses (Rühl *et al.*, 2004; Rühl *et al.*, 2007). Notably, various studies report an "outside-inside-outside" pathogenic mechanism of AD (Cork *et al.*, 2009; Elias *et al.*, 2008; Marsella and Samuelson, 2009) which is initiated by inherited or acquired insults like a mutation of the Flg gene or disturbed lipid synthesis. This will induce alterations of the epidermal structure and function allowing the readily penetration of allergens or bacterial infection leading to immune system activation (the inside). Immune response components such as IL-4 can then reduce Flg expression and ceramide synthesis and thereby further compromise the epidermal barrier to the outside (Elias *et al.*, 2008; Elias and Steinhoff, 2008). However, the exact disease pathogenesis is not yet fully elucidated.

3 MATERIALS AND METHODS

Mice

For topical retinoid applications, 8-12 weeks old female C57BL/6 mice and for OVA treatment, 8-10 weeks old female BALB/c mice were obtained from and housed within the animal facility of the University of Debrecen, Hungary. Animals were maintained in single cages on standard animal chow and water *ad libitum*. All experimental procedures were approved by the Committee of Animal Research of the University of Debrecen, Hungary (Approval number: 25/2006 DEMÁB).

Topical treatment with retinoid receptor specific agonists and antagonists

Mice (n=6-8 per treatment group) were anesthetized and subsequently shaved on dorsal skin sites using an electric razor. Retinoid receptor specific agonists and antagonists were applied topically each other day in 25 µl acetone (vehicle/control; Merck) per treatment for two weeks. According to previous studies by other groups (Calleja *et al.*, 2006; Chapellier *et al.*, 2002) following amounts of agonists and antagonist were applied per treatment (in 25 µL): ATRA, 40 nmol; LG268, 100 nmol; BMS753, 40 nmol; BMS189961, 40 nmol; BMS614, 100 nmol; UVI2041, 100 nmol; BMS493, 100 nmol; UVI3003, 100 nmol. On day 14, four hours after the last treatment, mice were sacrificed, sera and full thickness skin biopsies were collected, skin specimens were shock frozen in liquid nitrogen and all samples were kept at -80 °C until analyses. Skin samples were obtained from equal body sites by means of the same procedure for each mouse in order to control for variability among specimens. Samples were visibly controlled to ensure no excessive adipose tissue remained, though some contamination with remaining adipose tissue cannot be excluded. Animal studies were performed by J. Gericke with assistance (topical treatments) of the medical student J. Ittensohn.

Retinoid receptor specific agonists and antagonists

ATRA was a gift from BASF (Ludwigshafen, Germany) and the synthetic RXR activator LG268 was kindly provided by Ligand Pharmaceuticals (San Diego, California, USA). All other synthetic agonists and antagonists were synthesized by Prof. Ángel de Lera (Vigo, Spain) according to the following protocols. Agonists selective for RARα (BMS753) and RARγ (BMS189961) were produced as described in the original patents (Swann *et al.*, 1996; Zusi *et al.*, 1998) with the yields indicated.

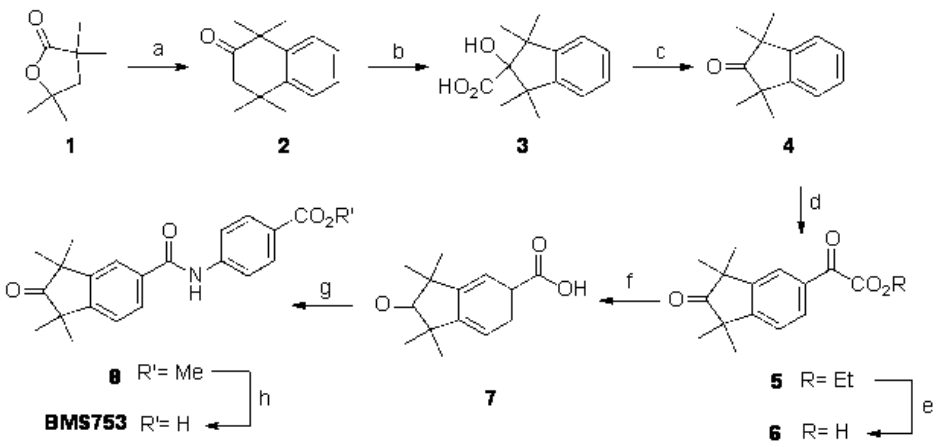

Figure 4. Reagents and conditions of the synthesis of BMS753: a. $AlCl_3$, C_6H_6, 100 ºC, 4h (65%). b. $KMnO_4$, H_2O, NaOH, 100 ºC, 3h (78%). c. CrO_3, AcOH, 25 ºC, 4h (93%). d. $AlCl_3$, $ClCOCO_2Et$, CH_2Cl_2, 25 ºC, 2 h (43%). e. NaOH (1N, aq), MeOH, 25 ºC, 1 h (99%). f. NaOH, MeOH, H_2O_2, 25 ºC, 16 h (96%). g. i) Oxalyl chloride, CH_2Cl_2, DMF, 5 min. ii) Methyl 4-aminobenzoate, pyridine, 25 ºC, 16 h (45%). h. NaOH (1N, aq), MeOH, 70 ºC, 4 h (89%).

The RARγ-selective antagonist (UVI2041) was prepared by the condensation of the ester 15 derived from chalcone 14 (Alvarez *et al.*, 2009) with hydroxylamine (Tsang *et al.*, 2007; Tsang *et al.*, 2005) followed by hydrolysis.

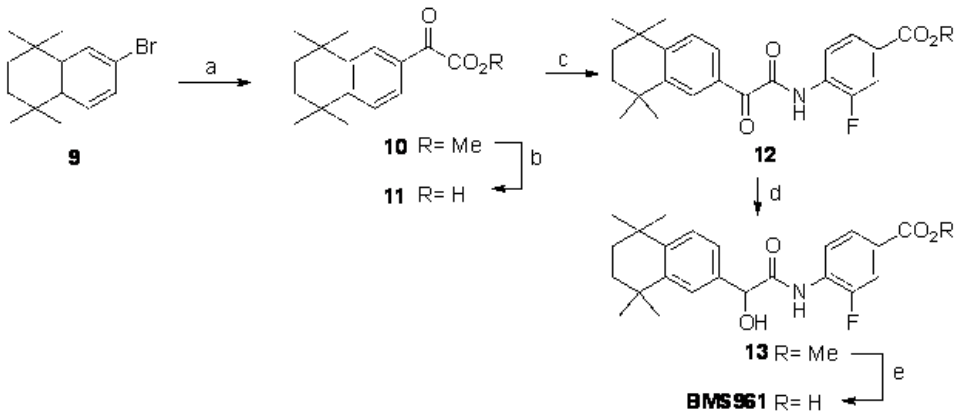

Figure 5. Reagents and conditions of the **s**ynthesis of BMS189961: a. i) *t*-Butyllithium, THF, -78 ºC, 30 min. ii) $(COCO_2Me)_2$, THF, 25 ºC, 16 h (88%) b. $LiOH{\cdot}H_2O$, 4h, 25 ºC (76%) c. i) Oxalyl chloride, DMF. ii) Ethyl 4-amino-3-fluorobenzoate, Et_3N, EtOAc, 16 h, 25 ºC (65%). d. $NaBH_4$, MeOH, 5 min, (79%) e. $LiOH{\cdot}H_2O$, 25 ºC, 4h (64%).

The RAR pan-antagonist/inverse agonist (BMS493) and the RXR pan-antagonist (UVI3003) were synthesized according to reported procedures (Bourguet *et al.*, 2010; Nahoum *et al.*, 2007).

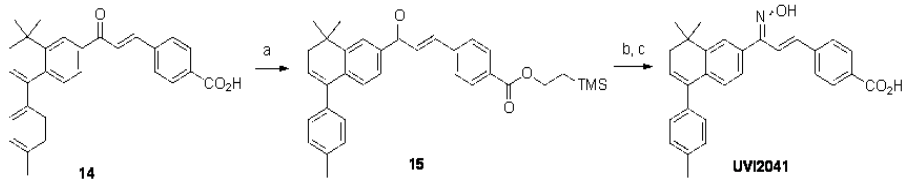

Figure 6. Reagents and conditions of the synthesis of UVI2041: a) EDC (1.1 equiv), DMAP (0.01 equiv), Trimethylsilylethanol (1.1 equiv), CH_2Cl_2, 18 h, 23 ºC, 65%. b) NH_2OH (2 equiv), pyridine (2.2 equiv), EtOH, 70 ºC, 20 h, 66% (*E/Z* isomer mixture at the oxime). c) TBAF (2 equiv), DMSO, 30 min, 63%.

The RARα-specific antagonist (BMS614) was made following the patented procedure developed at BMS (Starrett *et al.*, 1996; Starrett *et al.*, 1998).

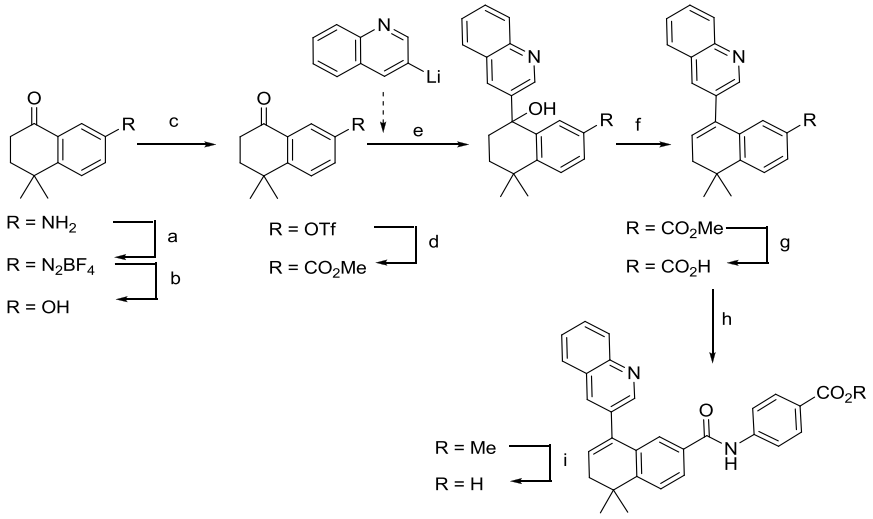

Figure 7. Reagents and conditions of the synthesis of BMS614: a) HBF_4, $NaNO_2$, H_2O, 10 ºC, 89%. b) H_2SO_4, H_2O, reflux, 1h, 88%. c) Tf_2O, Py, 25 ºC, 16h, 100%. d) $Pd(OAc)_2$, dppp, CO, Et_3N, MeOH, DMSO, 70 ºC, 3h, 93%. e) *i.* 3-bromoquinoline, *n*-BuLi, THF, -78 ºC. *ii.* THF, 25 ºC, 2h, 32%. f) *p*-TsOH, toluene, 90 ºC, 2.5h, 83%. g) NaOH (10M), EtOH/H_2O (1:1), 25 ºC, 24h, 88%. h) *i.* $(ClCO)_2$, CH_2Cl_2, DMF, 25 ºC, 2h; *ii.* methyl 4-aminobenzoate, Py, 25 ºC, 2h, 26%. i) NaOH (10M), EtOH/H_2O (1:1), 25 ºC, 24h, 28%.

The purity of the synthesized compounds was determined to be greater than 95% by HPLC after crystallization. We have confirmed that these retinoids are stable when stored as solids or in solution at -78 °C, and during the time frame of biological experiments.

Sensitization with ovalbumin

Sensitization (n=8 mice per treatment group) was performed by repetitive systemic administration of OVA and allergen-induced dermatitis was triggered based on a model previously reported (Dahten *et al.*, 2008; Weise *et al.*, 2011). Briefly, mice were sensitized at days 47, 60 and 67 with 10 µg OVA intraperitoneally (i.p.) (Sigma-Aldrich, Hungary) adsorbed to 1.5 mg aluminum hydroxide ($Al(OH)_3$) (Thermoscientific, Hungary) or with phosphate-buffered saline (PBS; control) (Figure 8).

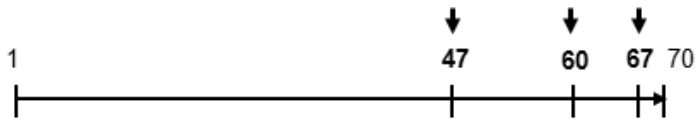

Figure 8. Mice were sensitized i.p. with 10 µg OVA adsorbed to 1.5 mg $Al(OH)_3$ (OVA i.p.) or with phosphate-buffered saline (PBS; control) on days 47, 60 and 67 (black arrows).

For combined treatment (Dahten *et al.*, 2008), mice were sensitized i.p. on days 1, 14 and 21 with 10 µg OVA adsorbed to 1.5 mg $Al(OH)_3$. This was followed by topical application (e.c.) of 100 µg OVA adsorbed to 1.5 mg $Al(OH)_3$ in 100 µl PBS (weekly dose) onto shaved dorsal skin, divided into four applications of 25 µl every other day of one week (Figure 9).

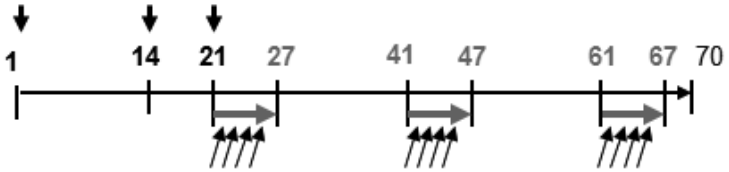

Figure 9. A third group of mice was sensitized i.p. with 10 µg OVA adsorbed to 1.5 mg $Al(OH)_3$ on days 1, 14 and 21 (black arrows) followed by e.c. OVA exposure for three 1-week periods (grey arrows) separated by 2-weeks intervals (OVA i.p.+e.c.). Each mouse received a weekly e.c. dose of 100 µg OVA adsorbed to 1.5 mg $Al(OH)_3$ in 100 µl PBS on shaved back skin divided into four applications of 25 µl every other day of one week (black angular arrows). Three days after the last treatment (day 70), mice were sacrificed and skin and serum samples were collected.

Epicutaneous (e.c.) treatment was repeated for a total exposure of three weeks separated by two-week intervals. Three days after the last treatment (day 70) mice were sacrificed, skin and serum samples were collected and kept at -80 °C until analyses. All animal experiments were conducted by J. Gericke with assistance (topical treatment) of J. Ittensohn (medical student).

RNA preparation

Total RNA was isolated by J. Gericke from frozen full thickness skin biopsies using Tri® reagent (Trizol) following the manufacturer's instructions:

1. A frozen skin sample (ca. 100 mg) is cut into small pieces and homogenized (3 min) with the QIAGEN Tissue Lyser in 1 mL Trizol, using autoclaved QIAGEN metal beads. After short centrifugation (at 13.000 rpm; 4 °C), supernatants are transferred to 1.5 ml Microcentrifuge tubes.
2. 200 µL of chloroform are added to each sample, samples are stirred, incubated for 3 min at room temperature (RT), and centrifuged for 15 min (at 13.000 rpm; 4 °C).
3. The obtained upper aqueous phase is transferred into 500 µL isopropanol containing Microcentrifuge tubes, stirred, incubated for 20 min at RT and centrifuged for 10 min (at 13.000 rpm; 4 °C). Supernatant is discarded.
4. 800 µL of 70% ethanol are added, samples stirred briefly and centrifuged for 5 min (at 13.000 rpm, 4 °C). Supernatant is discarded.
5. RNA pellets are dried for 20 minutes in the Eppendorf concentrator 5301.
6. 60 µL of nuclease free water are added to each sample, samples are stirred and RNA activated for 10 min in a water bath at 65 °C (BIOSAN Dry Block Heating Thermostat). Samples are stirred briefly and cooled down on ice.
7. Concentration and purity of RNA samples are determined with NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Hungary). Samples were kept at -80 °C until further analysis.

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

In our studies, the tow-step TaqMan® qRT-PCR was performed in order to quantify the mRNA expression level of mouse genes involved in retinoid metabolism, skin homeostasis and immune response.

In the reverse transcription step, 750 ng of total RNA were reverse transcribed into complementary DNA (cDNA) using the High Capacity cDNA Reverse Transcription Kit (Life Technologies) according to the manufacturer's instructions (Table 1) and the indicated thermal protocol (Table 2).

Table 1. Master mix protocol for reverse transcription (per one reaction).

Reagent	**Volume (µL)**
10x RT Buffer	3.0
25x dNTP Mix (0.1M)	1.5
10x RT Random Primers	3.0
MultiScribe Reverse Transcriptase Enzyme (50 U/µL)	1.5
Nuclease free water	17.5
RNA (750 ng)	5.0
Total (one reaction)	**31.5**

Table 2. Thermal conditions for reverse transcription reactions.

Time (min)	**Temperature (°C)**
10	25
120	37
5	72
∞	4

In the second step, the cDNA samples were amplified by the enzyme *Taq* DNA polymerase and their quantity measured to determine the mRNA expression level. qRT-PCR measurements were performed in triplicates using pre-designed TaqMan® Gene Expression Assays (ROX-MGB assays) or FAM-TAMRA assays as well as TaqMan® reagents. The master mix protocols differed according to the respective assay used as presented in Table 3. TaqMan® Low Density Array (TLDA) cards were used for the OVA mouse model with duplicate determinations using TaqMan® Gene Expression Master Mix (all Applied Biosystems).

Table 3. Master mix protocol for FAM-TAMRA assays or TaqMan® ROX-MGB assays (per one reaction).

	Volume (µL)	
Reagent	*FAM-TAMRA*	*ROX-MGB*
NFW	2.1	1.9
10x Taq buffer	1.0	1.0
25 mM $MgCl_2$	1.2	1.2
2.5 mM dNTP	0.5	0.5
Taq DNA polymerase (5 U/µl)	0.06	0.06
100 µM m reverse primer	0.04	-
100 µM m forward primer	0.04	-
20 µM probe	0.06	-
50x ROX	-	0.2
20x oligomix	-	0.15
cDNA (diluted 1:5)	5.0	5.0
Total (one reaction)	**10.0**	**10.01**

All gene expression analyses were conducted on an ABI Prism 7900 using the following thermal protocols (Table 4).

Table 4. Thermal conditions for real time-PCR per one cycle (for a total of 40 cycles).

Time (sec)	**Temperature (°C)**
qRT-PCR	
60	94
12	94
30	60
TLDA	
120	50
600	94.5
30	97
60	59.7

Tables 5 and 6 present gene names, symbols and assay IDs (Table 5) or primer and probe sequences (Table 6), respectively, of all genes investigated within this study.

Table 5. Gene names, symbols and assay IDs of ROX-MGB assays.

Gene name	Symbol	Assay ID
Alcohol dehydrogenase 7	Adh7	Mm00507750_m1*
Aldehyde dehydrogenase 1A1	Aldh1a1	Mm00657317_m1*
Aldehyde dehydrogenase 1A2	Aldh1a2	Mm00501306_m1*
Aldehyde dehydrogenase 1A3	Aldh1a3	Mm00474049_m1*
Alkaline ceramidase 1	Acer1	Mm00460332_m1*
ATP-binding cassette A12	Abca12	Mm00613683_m1
Beta-carotene oxygenase 2	Bco2	Mm00460051_m1*
Cellular retinoic acid binding protein 1	Crabp1	Mm00442776_m1
Cellular retinoic acid binding protein 2	Crabp2	Mm00801691_m1*
Cellular retinol binding protein 1	Rbp1	Mm00441119_m1*
Chemokine (CC motif) ligand 11 / eotaxin-1	Ccl11	Mm00441238_m1*
Chemokine (CC motif) ligand 17 / Tarc	Ccl17	Mm00516136_m1*
Chemokine (CC motif) ligand 22 / Mdc	Ccl22	Mm00436439_m1*
Chemokine (CC motif) ligand 24 / eotaxin-2	Ccl24	Mm00444701_m1*
Chemokine (CC motif) receptor 3	Ccr3	Mm01216172_m1
Cyclophilin A[1]	Cyclo	Mm02342430_g1
Cytochrome P450 26A1	Cyp26a1	Mm00514486_m1*
Cytochrome P450 26B1	Cyp26b1	Mm00558507_m1*
Cytochrome P450 2S1	Cyp2s1	Mm00512037_m1*
Diacylgylcerol O-acyltransferase	Dgat	Mm00442776_m1
Fatty acid binding protein 5	Fabp5	Mm00783731_s1*
Filaggrin	Flg	Mm01716522_m1*
Glucocerebrosidase	Gba	Mm00484700_m1
Heparin-binding EGF-like growth factor	Hbegf	Mm00439307_m1*
Interferon γ	Ifng	Mm00801778_m1
Interleukin 4	Il4	Mm00445259_m1*
Interleukin 10	Il10	Mm00439614_m1*
Interleukin 12A	Il12a	Mm00434165_m1*
Involucrin	Ivl	Mm00515219_s1*
Kallikrein-related peptidase 5	Klk5	Mm01203811_m1

Gene name	Symbol	Assay ID
Kallikrein-related peptidase 7	Klk7	Mm00496274_m1*
Keratin 4	Krt4	Mm00492996_g1
Keratin 6B	Krt6b	Mm00834035_gH*
Keratin 16	Krt16	Mm00492979_g1*
Keratin 17	Krt17	Mm00495207_m1*
Lecithin-retinol acyltransferase	Lrat	Mm00469972_m1*
Loricrin	Lor	Mm01219285_m1*
Matrix metalloproteinase 9	Mmp9	Mm00442991_m1*
Nuclear receptor family 4, group A, number 1	Nr4a1/Nurr77	Mm01300401_m1*
Peroxisome proliferator-activated receptor delta	Ppard	Mm00803184_m1*
Retinoic acid receptor α	Rara	Mm00436264_m1*
Retinoic acid receptor β	Rarb	Mm01319677_m1*
Retinoic acid receptor γ	Rarg	Mm00441083_m1
Retinoic acid receptor responder 2	Rarres2	Mm00503579_m1*
Retinoid X receptor α	Rxra	Mm00441182_m1
Retinol binding protein 4	Rbp4	Mm00803266_m1*
Retinol dehydrogenase 10	Rdh10	Mm00467150_m1*
Retinol dehydrogenase 16	Rdh16	Mm01625764_s1
Serine palmitoyltransferase	SptIc2	Mm00448871_m1
Serine peptidase inhibitor, Kazal-type 5	Spink5	Mm00511522_m1*
Short chain dehydrogenase/reductase 16C5	Sdr16c5	Mm00725380_m1*
S100 calcium binding protein A7A / psoriasin	S100a7a	Mm01218201_m1*
Transglutaminase 1	Tgm1	Mm00498375_m1*
Transglutaminase 2	Tgm2	Mm00436987_m1*
UDP-glucose ceramide glucosyltransferase	Ugcg	Mm00495925_m1
3-Hydroxy-3-methylglutaryl-CoA synthase 2	Hmgcs2	Mm00550050_m1*

[1] Gene expression analysis using TLDA

Table 6. Gene name, symbol and sequences of forward and reverse primers as well as probe of the used FAM-TAMRA assay.

Gene name	Symbol	Sequence	
Cyclophilin A[1]	Cyclo	forward	77+ 5´-CGATGACGAGCCCTTGG-3´
		reverse	142- 5´-TCTGCTGTCTTTGGAACTTTGTC-3´
		probe	5´-TCTGCTGTCTTTGGAACTTTGTC-3´

[1] Gene expression analysis using qRT-PCR

Relative quantification of mRNA expression was achieved using the comparative C_T method and values were normalized to cyclophilin A mRNA. Gene expression values below detection limit were assumed to be zero for the purpose of statistical analysis. The Sequence Detector Software version 2.1 was used for data analysis. qRT-PCR analyses of n=5-6 mice per treatment group were performed by J. Gericke with support from Éva Papp (technical assistant).

Histological analysis

Skin biopsies were taken from similar shaved, dorsal body sites and kept at -80 °C until analysis. Frozen specimens were sectioned (four µm) and stained with hematoxylin and eosin (H&E) for histological analysis by Dr. D. Töröcsik (retinoid model; n=3). For the OVA model, skin samples were fixed overnight with 4% paraformaldehyde at 4 °C and embedded in paraffin. Five µm sections were stained with H&E or Giemsa (n=8) at the Medical University of Innsbruck, Austria. Mast cells were quantified under a light microscope using 20x lenses and a calibrated grid (six fields per sample) while epidermal thickness was measured using x10 lenses (five measurements per mouse). Evaluation was performed by J. Gericke.

Immunohistochemical analysis

Frozen five µm skin sections (n=5 mice per treatment group) were fixed in acetone, blocked with mouse seroblock FcR block (AbD Serotec) or 10% goat serum (NGS; Vector Laboratories) and incubated with FITC rat anti-mouse CD3 molecular complex (17A2); biotin rat anti-mouse CD8a (53-6.7); purified hamster anti-mouse CD11c (HL3; all BD Biosciences - Pharmingen); or purified rat anti-mouse CD4 (GK1.5; BioLegend). Antibody binding was detected using biotinylated goat anti-rat Ig for anti-CD4 (Amersham Biosciences UK limited) and biotin mouse anti-hamster IgG cocktail for anti-CD11c (BD Biosciences - Pharmingen),

followed by incubation with Alexa Fluor 594-linked streptavidin (Invitrogen) for anti-CD4, anti-CD11c, and anti-CD8. Skin sections stained for CD11c were counterstained with FITC-linked rat anti-mouse I-A/I-E (BD Biosciences - Pharmingen) to identify MHC class II-positive cells. Staining of paraffin-embedded skin sections with rabbit Fabp5 polyclonal antibody (1:50; ProteinTech) was performed following the manufacturer's directions using antigen retrieval buffer (0.1 M sodium citrate, 0.1 M citric acid) and blocking with 5% donkey serum. Biotinylated donkey anti-rabbit Ig (Amersham Biosciences UK limited) and Alexa Fluor 594-linked Streptavidin were applied for detection of antibody binding. Nuclei were visualized with DAPI and all sections were mounted with Vectashield Mounting Medium (Vector Laboratories). $CD3^+$, $CD4^+$, $CD8^+$, MHC-class II^+ and $CD11c^+$ cells were counted under an Olympus BX60 epifluorescence microscope using 40x objective lenses and a calibrated grid (six fields per section). All immunohistochemical stainings and evaluation were conducted by J. Gericke.

Total IgE levels in serum

Sera of OVA-sensitized mice (n=8 per treatment group) were collected at day 70 and kept at -80 °C until analysis. Plasma IgE concentration was measured using the mouse ELISA kit from BD-Pharmingen by J. Gericke.

Determination of Fabp5 protein in skin

Fabp5 protein levels were determined in protein lysates from whole mouse skin (n=2 per group) by J. Gericke. Skin samples were lysed in RIPA lysis buffer in the presence of protease inhibitor (Pierce). Lysates were separated by 4-12% SDS-PAGE and then transferred to a nitrocellulose membrane (Invitrogen). The membrane was incubated in blocking solution (Pierce) for 30-60 min at RT. Subsequently, the membrane was incubated with the Fabp5 antibody (1:500, ProteinTech) diluted in blocking buffer overnight at 4 °C. Endogenous proteins were detected with Alexa A680-conjugated anti-rabbit secondary antibody (1:10.000, Invitrogen). Blots were scanned with a LI-COR Biosciences analyzer. Anti-beta-actin (Sigma) was used as loading control.

Cytokine levels in serum

Levels of TSLP, IL-4 and IL-12 (p70) were determined in serum (n=4 mice per group) using Quantikine Mouse Immunoassays (R&D Systems) by Dr. J. Mihály. Assay sensitivity was 2.63 pg/mL for TSLP, <2 pg/mL for IL-4, and <2.5 pg/mL for IL-12.

High performance liquid chromatography mass spectrometry – mass spectrometry (HPLC MS-MS) analysis

Concentrations of ATRA and retinol were determined in mouse skin samples (n≥5 mice or n=3 mice for the retinoid or OVA model, respectively) by our previously described HPLC MS-MS method by Dr. R. Rühl (Rühl, 2006). The HPLC system consisted of a Waters 2695XE separation module (Waters), a diode-array detector (model 996, Waters) and an MS-MS detector (Micromass Quattro Ultima Pt, Waters). In summary, 100 mg of skin biopsy (if samples were below 100 mg water was added up to the used standard weight: 100 mg) were diluted with a threefold volume of isopropanol, tissues were minced by scissors, vortexed for 10 sec, put in an ultra sonic bath for 5 min, shaken for 6 min and centrifuged at 13.000 rpm in a Heraeus BIOFUGE Fresco at 4 °C. After centrifugation, the supernatants were dried in an Eppendorf concentrator 5301 (Eppendorf) at 30 °C. The dried extracts were resuspended with 60 µL of methanol, vortexed, shaken, diluted with 40 µL of 60 mM aqueous ammonium acetate solution and transferred into the autosampler for subsequent analysis.

Statistical Analysis

Data are indicated as mean ± standard error of mean (SEM). Satistical analysis of qRT-PCR data was performed using one-way ANOVA followed by Dunett's post-test (retinoid model) or followed by Tukey correction (OVA model). Significance of HPLC MS-MS results was determined using Student's *t*-test. Differences were considered significant at $p<0.05$. All statistical analyses were performed by J. Gericke.

4 RESULTS

4.1 Topical treatment of mice with various retinoid receptor agonists and antagonists

In the first part of the study mice were treated topically with the endogenous RAR agonist ATRA or with synthetic agonists specific for RARα, RARγ, or RXR for a period of 14 days. Further groups of mice were treated with retinoid receptor specific antagonists for (pan) RAR, RARα, RARγ, or pan RXR according to the same protocol.

4.1.1 Retinoid signaling in the skin after topical treatment with various retinoid receptor agonists or antagonists

Initially, we investigated the expression pattern of genes involved in retinoid metabolism, retinoid transport, and retinoid signaling such as retinoid receptors and target genes in murine skin upon treatment with various retinoid receptor selective agonists or antagonists. By means of qRT-PCR the mRNA expression levels were determined and compared to those obtained for the vehicle control (acetone) treated mice. Data is indicated as fold change of gene expression and presented as mean per treatment group (n≥5 mice) ± SEM of triplicate measurements per data point. Significant changes (p<0.05) are indicated with asterisks.

4.1.1.1 RARα and RARγ differentially regulate retinoid-mediated signaling in mouse skin

Since both, RARα and RARγ are expressed in skin (Fisher *et al.*, 1994) we were interested in the effect of topically applied RAR subtype-selective agonists on retinoid metabolism. Interestingly, we found that treatment with the synthetic RARα agonist down-regulated the expression of all investigated genes with a role in retinoid metabolism that is RA synthesis, retinoid receptors, and target genes (Table 7, Figure 10).

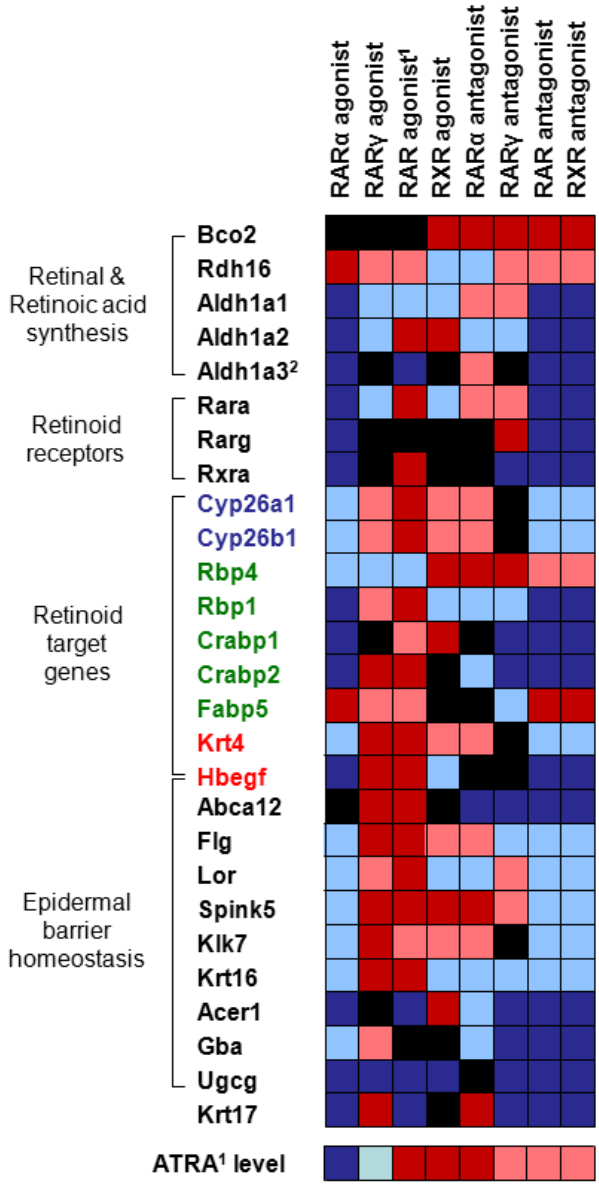

Figure 10. Heat map displaying fold change of gene expression in mouse skin (n≥5 per group) after treatment with retinoid receptor specific agonists and antagonists compared to control mice (acetone). Genes are differentiated according to roles in retinoid metabolism or epidermal homeostasis. Retinoid target genes are further distinguished by specific function, i.e. retinoic acid metabolism (blue), retinoid transport (green), and genes unrelated to retinoid signaling (red). Color codes: dark red – significantly up-regulated; light red – non-significantly up-regulated; black – not regulated (±20%); light blue – non-significantly down-regulated; dark blue – significantly down-regulated. A *p*-value <0.05 was considered significant. [1]all-*trans* retinoic acid; [2]also relevant as retinoid target gene.

Only mRNA levels of the lipid transporter Fabp5 and an enzyme involved in retinal synthesis (Rdh16) were significantly increased by the agonist. In contrast, the synthetic agonist for RARγ and the natural RAR agonist ATRA induced the expression of nearly all retinoid target genes in the skin of mice, e.g. Cyp26a1, Cyp26b1 (both degradation enzymes), Rbp1, Crabp1, Hbegf, and Krt4 as a marker for retinoid activity (Table 7, Figure 10). We were also interested in the effect of a synthetic RXR agonist on skin retinoid metabolism. Topical application of this agonist induced the expression of some retinoid target genes (Cyp26a1, Cyp26b1, Rbp4, Crabp1, Krt4), while the treatment did not affect or slightly decrease the expression of other targets (Crabp2, Fabp5, Rbp1, Hbegf). Moreover, repetitive treatment with the RARγ-selective agonist showed no significant effect on retinal and RA synthesis enzymes and retinoid receptor gene expression in the skin. In contrast, the endogenous RAR ligand ATRA and the RXR agonist markedly increased mRNA levels of Aldh1a2 and ATRA further induced Rara and Rxra gene expression, while it decreased Aldh1a3 expression in the skin (Table 7, Figure 10).

Table 7. Fold change of retinoid metabolism-related gene expression in the skin of mice after two weeks topical treatment with retinoid receptor specific agonists.

		Agonists (Fold change)			
Gene name	**Symbol**	**RARα[1]**	**RARγ[2]**	**ATRA[3]**	**RXR[4]**
Retinal synthesis					
Beta-carotene oxygenase 2	Bco2	UDL	UDL	UDL	3305 ± 192***
Short chain dehydrogenase / reductase 16C5	Sdr16c5	UDL	0.6 ± 0.1	9.4 ± 1.3***	0.9 ± 0.1
Retinol dehydrogenase 10	Rdh10	0 ± 0***	1.5 ± 0.1***	0.7 ± 0.1*	0.6 ± 0.1**
Retinol dehydrogenase 16	Rdh16	493 ± 128***	1.3 ± 0.3	2.5 ± 0.3	0.5 ± 0.1
Alcohol dehydrogenase 7	Adh7	0.2 ± 0.1	1.8 ± 0.3	2.8 ± 0.5***	2 ± 0.1*
Retinoic acid synthesis					
Aldehyde dehydrogenase 1A1	Aldh1a1	UDL**	0.7 ± 0.1	0.6 ± 0.1	0.3 ± 0.1
Aldehyde dehydrogenase 1A2	Aldh1a2	0 ± 0*	0.7 ± 0.2	3.4 ± 0.5***	2.4 ± 0.3**
Aldehyde dehydrogenase 1A3[5]	Aldh1a3	UDL***	1.0 ± 0	0 ± 0***	0.9 ± 0.2
Retinoid receptors					
Retinoic acid receptor α	Rara	UDL*	0.3 ± 0.1	5.8 ± 0.4***	0.7 ± 0.1
Retinoic acid receptor β[5]	Rarb	UDL***	1.1 ± 0.2	1.4 ± 0.1*	0.6 ± 0.1*
Retinoic acid receptor γ	Rarg	UDL***	1.1 ± 0.1	0.9 ± 0.3	0.9 ± 0.1
Retinoid X receptor α	Rxra	0 ± 0***	0.9 ± 0.1	1.4 ± 0.1***	1 ± 0.1
Retinoid target genes					
Retinoic acid degradation					
Cytochrome P450 26A1	Cyp26a1	UDL	19 ± 5.4	1410 ± 161***	1.5 ± 0.3
Cytochrome P450 26B1	Cyp26b1	0 ± 0	13 ± 0.5	299 ± 54***	1.6 ± 0.2
Cytochrome P450 2S1	Cyp2s1	UDL**	0.9 ± 0.1	0.6 ± 0.1	0.9 ± 0.1
Retinoid transport proteins					
Retinol binding protein 4	Rbp4	UDL	UDL	UDL	2816 ± 244***
Cellular retinol binding protein 1	Rbp1	0 ± 0***	1.4 ± 0.2	2.8 ± 0.2***	0.7 ± 0.1
Cellular retinoic acid binding protein 1	Crabp1	0 ± 0**	1 ± 0.2	1.7 ± 0.3	1.8 ± 0.2*
Cellular retinoic acid binding protein 2	Crabp2	0 ± 0***	1.5 ± 0.2*	2 ± 0.2***	0.8 ± 0
Fatty acid binding protein 5	Fabp5	10 ± 0.8***	1.9 ± 0.4	2.2 ± 0.1	1.2 ± 0.1
Retinol esterification					
Lecithin-retinol acyltransferase	Lrat	0 ± 0***	2.3 ± 0.2***	2 ± 0.1***	1 ± 0.1
Diacylgylcerol O-acyltransferase	Dgat	UDL***	1.5 ± 0.2***	0.4 ± 0.1***	0.2 ± 0***
Further retinoid target genes not involved in retinoid signaling					
Keratin 4	Krt4	UDL	6470 ± 646***	3167 ± 679***	7.7 ± 3
Retinoic acid receptor responder 2	Rarres2	UDL	1.8 ± 0.2	1.3 ± 0.3	1.6 ± 02
Heparin-binding EGF-like growth factor[6]	Hbegf	0 ± 0*	2.7 ± 0.6***	2 ± 0.2*	0.3 ± 0.2

[1] BMS753; [2] BMS961; [3] all-*trans* retinoic acid; [4] LG268; [5]retinoid target genes; [6] target genes not involved in retinoid signaling; [7] gene also relevant in epidermal homeostasis; UDL, under detection limit

Fold change data are expressed as mean ± SEM and were determined in skin specimens of topically treated mice (n≥5) by qRT-PCR. *p<0.05, **p<0.01, ***p<0.001, vs. control (acetone).

4.1.1.2 RAR and RXR antagonists decrease the expression of genes involved in retinoid signaling in mouse skin

Topical application of antagonists for RARα or RARγ showed a more homogeneous pattern and resulted in non-significantly reduced or unaltered expression of several genes involved in retinoid signaling in skin. However, some genes seemed to be slightly induced by both antagonists, such as Bco2, Rbp4, Aldh1a1 which is responsible for RA synthesis, Rara, Rarg, and some target genes like Cyp26a1, Cyp26b1, and Krt4 (Figure 10, Table 8). In contrast, antagonists for RAR and RXR decreased the expression of nearly all of these genes below detection limit. Only mRNA levels of Bco2, Rdh16, Rbp4, and Fabp5 were found to be elevated by the antagonists. Most surprisingly, this expression pattern strongly resembled to that which we observed in mice treated with the RARα agonist (Figure 10, Table 8).

Table 8. Fold change of retinoid metabolism-related gene expression in the skin of mice after two weeks topical treatment with retinoid receptor specific antagonists.

		Antagonists (Fold change)			
Gene name	**Symbol**	**RARα[1]**	**RARγ[2]**	**RAR[3]**	**RXR[4]**
Retinal synthesis					
Beta-carotene oxygenase 2	Bco2	443 ± 84***	124 ± 78	90 ± 41	117 ± 14
Short chain dehydrogenase / reductase 16C5	Sdr16c5	0.7 ± 0	0.3 ± 0	UDL	UDL
Retinol dehydrogenase 10	Rdh10	0.8 ± 0.1	0.1 ± 0***	0 ± 0***	0.1 ± 0***
Retinol dehydrogenase 16	Rdh16	0 ± 0	3.5 ± 0.7	6.5 ± 1.2	1.3 ± 0.5
Alcohol dehydrogenase 7	Adh7	0 ± 0*	1 ± 0.1	0 ± 0**	0 ± 0*
Retinoic acid synthesis					
Aldehyde dehydrogenase 1A1	Aldh1a1	1.3 ± 0.3	1.5 ± 0.3	UDL**	UDL**
Aldehyde dehydrogenase 1A2	Aldh1a2	0.5 ± 0.1	0.2 ± 0	0.1 ± 0.1*	0 ± 0*
Aldehyde dehydrogenase 1A3[5]	Aldh1a3	1.3 ± 0.2	1 ± 0.2	0 ± 0***	0 ± 0***
Retinoid receptors					
Retinoic acid receptor α	Rara	1.5 ± 0.3	1.3 ± 0.1	UDL*	UDL*
Retinoic acid receptor β[5]	Rarb	0.6 ± 0	0.7 ± 0.1	UDL***	UDL***
Retinoic acid receptor γ	Rarg	0.8 ± 0.1	1.7 ± 0.2**	UDL***	UDL***
Retinoid X receptor α	Rxra	0.9 ± 0.1	0.8 ± 0.1*	0 ± 0***	0 ± 0***
Retinoid target genes					
Retinoic acid degradation					
Cytochrome P450 26A1	Cyp26a1	1.6 ± 0.6	0.9 ± 0.3	UDL	UDL
Cytochrome P450 26B1	Cyp26b1	1.3 ± 0.2	1.1 ± 0.2	0 ± 0	0 ± 0
Cytochrome P450 2S1	Cyp2s1	0.9 ± 0.1	1.6 ± 0.1*	UDL***	0 ± 0**
Retinoid transport proteins					
Retinol binding protein 4	Rbp4	448 ± 18***	1710 ± 505***	12 ± 12	30 ± 30
Cellular retinol binding protein 1	Rbp1	0.6 ± 0.1	0.7 ± 0	0 ± 0***	0 ± 0***
Cellular retinoic acid binding protein 1	Crabp1	1.1 ± 0.3	UDL***	0 ± 0**	0.1 ± 0**
Cellular retinoic acid binding protein	Crabp2	0.7 ± 0.1	0.5 ± 0**	UDL***	0 ± 0***
Fatty acid binding protein 5	Fabp5	0.9 ± 0.1	0.2 ± 0	13 ± 0.6***	8 ± 1***
Retinol esterification					
Lecithin-retinol acyltransferase	Lrat	1.2 ± 0.1	0.6 ± 0.1	0 ± 0***	0 ± 0***
Diacylgylcerol O-acyltransferase	Dgat	0.2 ± 0***	0.1 ± 0***	UDL***	0 ± 0***
Further retinoid target genes not involved in retinoid signaling					
Keratin 4	Krt4	154 ± 37	UDL	UDL	UDL
Retinoic acid receptor responder 2	Rarres2	3.4 ± 0.6***	1 ± 0.3	UDL	0 ± 0
Heparin-binding EGF-like growth factor[6]	Hbegf	0.8 ± 0.1	0.8 ± 0.1	UDL	UDL*

[1] BMS614; [2] UVI2041; [3] BMS493; [4] UVI3003; [5] retinoid target genes; [6] target genes not involved in retinoid signaling; [7] gene also relevant in epidermal homeostasis; UDL, under detection limit

Fold change data are expressed as mean ± SEM and were determined in skin specimens of topically treated mice (n≥5) by qRT-PCR. *p<0.05, **p<0.01, ***p<0.001, vs. control (acetone).

4.1.2 ATRA levels in the skin of topically treated mice

Gene expression analysis indicated alterations of retinoid metabolism (RA synthesis and degradation enzymes) in murine skin in response to various retinoid receptor specific agonists or antagonists. Therefore, we were interested whether the level of ATRA in the skin of mice was also affected by the various treatments.

4.1.2.1 An RXR agonist and RARα antagonist increase ATRA levels in skin via induced synthesis

ATRA levels in the skin were determined via HPLC MS-MS and were found to be differentially affected depending on the applied receptor selective agonist or antagonist (Figure 10, Table 9).

Table 9. ATRA concentration (ng/g) in murine skin after two weeks topical treatment with retinoid receptor selective agonists or antagonists.

		Agonists				**Antagonists**			
	acetone	**RARα[1]**	**RARγ[2]**	**RAR[3]**	**RXR[4]**	**RARα[5]**	**RARγ[6]**	**RAR[7]**	**RXR[8]**
ATRA	2.5 ± 0.4	1.1 ± 0.4*	1.8 ± 1.1	5001 ± 1127**	25 ± 9.4*	14 ± 4.2*	4.3 ± 1.7	5.6 ± 1.6	4.6 ± 2

[1] BMS753; [2] BMS961; [3] all-*trans* retinoic acid; [4] LG268; [5] BMS614; [6] UVI2041; [7] BMS493; [8] UVI3003

Concentrations were determined in skin specimens of topically treated mice (n≥5) by HPLC MS-MS and were calculated as ng/g skin. Data are indicated as mean ± SEM. *p<0.05, **p<0.005, vs. control (acetone).

Concentrations of ATRA were significantly decreased in the skin of mice treated with the synthetic RARα agonist and non-significantly by the RARγ agonist. Furthermore, treatments with antagonists for RARγ, RARs, or RXRs resulted in elevated ATRA concentrations, while only the RARα antagonist induced a significant increase. As expected, we found ATRA levels markedly elevated upon treatment with this RAR agonist itself (highest level among all groups). Noticeably, however, was the pronounced elevation of ATRA in mouse skin after application of the synthetic RXR agonist which is in accordance with a significantly elevated gene expression of the RA synthesis enzyme Aldh1a2.

4.1.3 Impact of topical retinoid receptor agonists and antagonists on skin and immune homeostasis

RAR-RXR signaling pathways are well known to be involved in the modulation of immune responses (Rühl, 2007; Rühl *et al.*, 2004; Rühl *et al.*, 2007) as well as skin physiology (Roos *et al.*, 1998). Furthermore, we recently found disturbed retinoid signaling in skin of AD patients, indicating the involvement of retinoid signaling in the disease's pathogenesis (Mihály *et al.*, 2011). Therefore, we aimed to investigate the impact of topically applied retinoid receptor specific agonists and antagonists on murine skin and immune homeostasis.

4.1.3.1 RAR-RXR signaling pathways induce epidermal hyperproliferation

After two weeks topical treatment of mice with various retinoid receptor selective agonists or antagonists, obvious signs of dryness (scales) could be observed in some groups compared to control mice. Representative images of the treated skin area at day 14 (end of treatment) are shown in Figure 11.

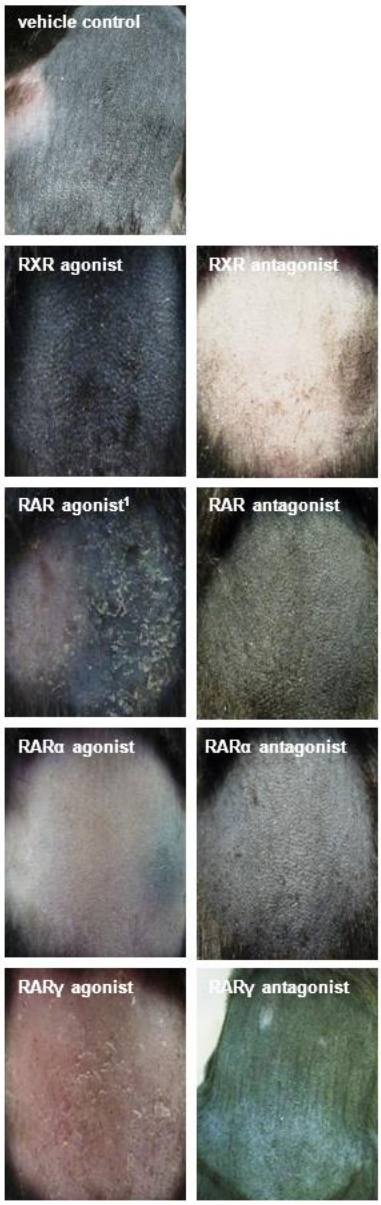

Figure 11. Representative photographs of dorsal skin areas from mice topically treated with vehicle control (acetone), or various retinoid receptor selective agonists or antagonists for 14 days. Note the scaly skin of mice treated with the synthetic RXR agonist or the synthetic RARγ agonist, and appearing most pronounced in the RAR agonist (ATRA) treated group. [1]all-*trans* retinoic acid / ATRA

Control animals were treated with acetone (vehicle) and their skin appeared normal without scales at the end of two weeks. Similar observations were made in the group treated with the RARα agonist showing only a very few scattered white scales on the back skin. In contrast, application of synthetic agonists for RXR or RARγ and the natural RAR ligand ATRA resulted in visibly dry and scaly skin. Compared to rather mild effects induced by the RXR agonist we could detect small scales already after the third treatment with the synthetic RARγ agonist. During the following days, number and size of scales increased and the skin

appeared red and slightly shiny compared to control mice (Figure 11). Application of ATRA (same amount as the synthetic RARγ agonist) showed the strongest effects resulting in apparently very dry skin with big white scales already shortly after initiating the treatment (not shown). Skin of these mice also seemed shiny compared to controls. Skin regions treated with receptor antagonists appeared mostly normal at day 14. A few small scales could be observed only after application of the RARα and RXR antagonists. In order to verify these macroscopic impressions we also performed histological analysis (Figure 12).

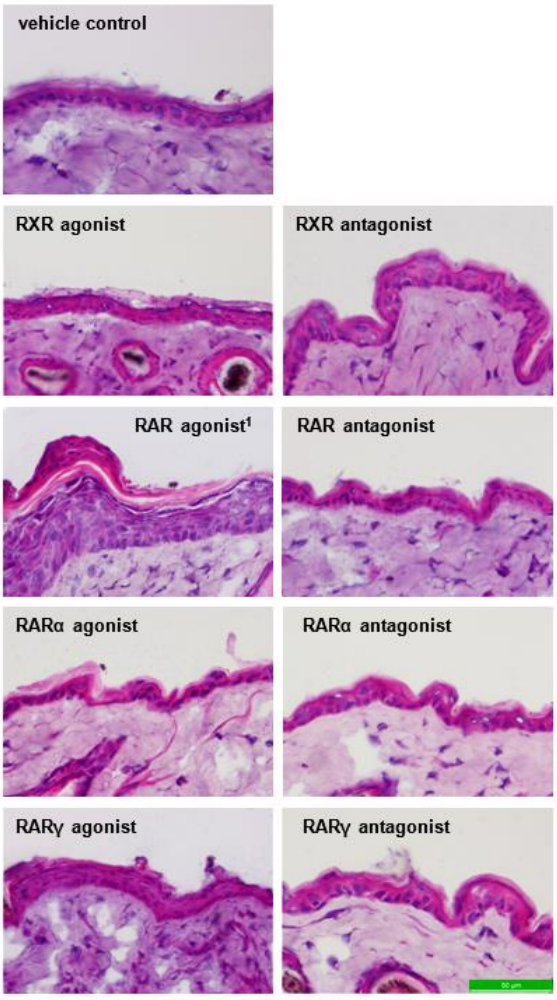

Figure 12. Representative photographs of H&E stained skin sections from mice topically treated with vehicle control (acetone), or various retinoid receptor selective agonists or antagonists for 14 days. Note the epidermal thickness of mice treated with synthetic agonists for RXR or RARγ, and appearing most pronounced in the RAR agonist (ATRA) treated group. Epidermal thickness seemed comparable to vehicle control in mice treated with RARα agonist, RXR antagonist, RAR antagonist, and selective antagonists of RARα or RARγ. Original magnification (×20) was digitally magnified. [1]all-trans retinoic acid / ATRA

In accordance, epidermal thickness seemed comparable to control mice in all treatment groups except for mice treated with the synthetic RXR agonist, RARγ agonist, or ATRA. Epidermal thickness was markedly increased in all three groups (1.73 fold, 2.46 fold, 5.98 fold increase, respectively) but appeared stronger in mice treated with the RARγ agonist and was most pronounced in ATRA-treated mice. Additionally, the epidermal surface seemed notably scaly after application of the synthetic RARγ agonist and ATRA (Figure 12).

4.1.3.2 RAR-RXR signaling pathways modify epidermal barrier homeostasis

We next investigated the expression of various genes with significant functions in epidermal barrier homeostasis upon treatment with retinoid receptor agonists and antagonists. As shown in Table 10 and Figure 10, application of the synthetic RARγ agonist and ATRA both induced genes involved in skin barrier function (Abca12, Flg, Lor, Spink5, Krt16, Hbegf). On the other hand, mRNA levels of genes implicated in ceramide metabolism (Acer1, Gba, Ugcg) or cholesterol synthesis (Hmgcs2) were mainly decreased or unaffected by the treatment. Compared to RARγ ligand application, expression of these genes was markedly down-regulated (in several cases below detection limit) when mice were treated with the synthetic RARα agonist (Table 10, Figure 10). Noticeably, the same expression profile was observed after application of RAR or RXR antagonists. Treatment with the RXR agonist and RARα- and RARγ-specific antagonists resulted in inconsistent gene expression patterns with an increase of some genes (Spink5, Flg, Klk7) and decrease of other genes (Abca12, Krt16, Ugcg) involved in epidermal barrier function (Table 10, Figure 10). Krt6b expression was below the limit of detection in all groups (not shown).

Table 10. Fold change of mRNA expression of genes involved in epidermal barrier homeostasis and chemotaxis in murine skin after two weeks of topical treatment with retinoid receptor specific agonists or antagonists.

		Agonists (Fold change)				**Antagonists** (Fold change)			
Gene name	**Symbol**	**RARα[1]**	**RARγ[2]**	**ATRA[3]**	**RXR[4]**	**RARα[5]**	**RARγ[6]**	**RAR[7]**	**RXR[8]**
Epidermal barrier homeostasis									
ATP-binding cassette A12	Abca12	1 ± 0.1	1.6 ± 0.2***	1.5 ± 0.1***	1 ± 0.1	0.6 ± 0.1*	0.5 ± 0.1**	0 ± 0***	0 ± 0***
Filaggrin	Flg	0.2 ± 0.1	11.4 ± 1.3**	32 ± 4.4***	4.1 ± 0.1	1.4 ± 0.1	2.2 ± 0.8	0 ± 0	0 ± 0
Involucrin	Ivl	0.9 ± 0.1	1.6 ± 0.1***	1.3 ± 0.1	0.9 ± 0.1	1 ± 0.1	1.2 ± 0.1	1.3 ± 0.1	0.8 ± 0.2
Loricrin	Lor	0 ± 0	1.8 ± 0.3	7.3 ± 0.8***	0.6 ± 0.1	0.7 ± 0.2	1.3 ± 0.3	0 ± 0	0 ± 0
Transglutaminase 1	Tgm1	UDL***	0.7 ± 0.2	2.5 ± 0.2***	0.1 ± 0***	0.4 ± 0.1**	UDL***	UDL***	UDL***
Serine peptidase inhibitor, Kazal-type 5	Spink5	0 ± 0	5.1 ± 1***	2.8 ± 0.4*	2.8 ± 0.3*	5.1 ± 0.6***	2.3 ± 0.3	0 ± 0	0 ± 0
Kallikrein-related peptidase 5	Klk5	UDL	4.9 ± 1.5***	2.8 ± 0.4	1 ± 0.1	2.4 ± 0.5	3.4 ± 0.7*	UDL	UDL
Kallikrein-related peptidase 7	Klk7	0 ± 0	5.6 ± 1.8***	2.7 ± 0.3	3.2 ± 0.6	2.1 ± 0.3	1.2 ± 0.1	0 ± 0	0 ± 0
Matrix metalloproteinase 9	Mmp9	0.4 ± 0	2.7 ± 0.5	2 ± 1.1	0.8 ± 0.1	4.7 ± 0.8***	UDL	0.3 ± 0.1	0.9 ± 0.3
S100 calcium binding protein A7A / psoriasin	S100a7a	0.2 ± 0.1	1.4 ± 0.1	3.4 ± 0.6***	0.8 ± 0.1	0 ± 0*	UDL**	UDL*	0 ± 0*
Keratin 16	Krt16	0 ± 0	3.8 ± 0.4***	2.8 ± 0.9*	0.6 ± 0.1	0.5 ± 0.1	0 ± 0	0 ± 0	0 ± 0
Heparin-binding EGF-like growth factor[9]	Hbegf	0 ± 0*	2.7 ± 0.6***	2 ± 0.2*	0.3 ± 0.2	0.8 ± 0.1	0.8 ± 0.1	UDL*	UDL*
3-Hydroxy-3-methylglutaryl-CoA synthase 2	Hmgcs2	0 ± 0***	0.5 ± 0.1*	0.1 ± 0***	1.3 ± 0.3	0.6 ± 0.1	UDL***	0 ± 0***	0.1 ± 0***
UDP-glucose ceramide glucosyltransferase	Ugcg	0.1 ± 0***	0.4 ± 0.1***	0.1 ± 0***	0.2 ± 0***	0.9 ± 0.1	UDL***	0 ± 0***	0 ± 0***
Glucocerebrosidase	Gba	0.4 ± 0.2	1.4 ± 0.3	0.9 ± 0.2	0.9 ± 0.1	0.5 ± 0.2	UDL***	UDL***	0 ± 0***
Alkaline ceramidase 1	Acer1	UDL*	1.2 ± 0.1	0 ± 0***	1.5 ± 0.2*	0.7 ± 0.1	0.1 ± 0***	UDL***	0.2 ± 0***
Immune response									
Chemokine (CC motif) ligand 11 / eotaxin-1	Ccl11	UDL***	0.6 ± 0.1*	0.6 ± 0.1*	0.5 ± 0.1*	2 ± 0.2***	UDL***	UDL***	UDL***
Chemokine (CC motif) ligand 24 / eotaxin-2	Ccl24	UDL***	0.1 ± 0.1***	0 ± 0***	0.5 ± 0.1*	1.9 ± 0.2***	UDL***	UDL***	UDL***
Chemokine (CC motif) ligand 17 / Tarc	Ccl17	2 ± 0.6	2.7 ± 0.4	9.6 ± 1.5***	0.6 ± 0.1	0.2 ± 0.1	13.9 ± 2.8***	UDL	0.1 ± 0.1
Chemokine (CC motif) ligand 22 / Mdc	Ccl22	UDL	4.3 ± 1***	1.9 ± 0.3	0.8 ± 0.1	0.5 ± 0.2	UDL	UDL	UDL
Keratin 17	Krt17	0 ± 0***	1.5 ± 0.1***	0.2 ± 0***	1 ± 0.1	1.3 ± 0.1*	0 ± 0***	0 ± 0***	0 ± 0***

[1] BMS753; [2] BMS961; [3] all-*trans* retinoic acid; [4] LG268; [5] BMS614; [6] UVI2041; [7] BMS493; [8] UVI3003; [9] gene also relevant as retinoid target gene; UDL, under detection limit

Fold change data are expressed as mean ± SEM and were determined in skin specimens of topically treated mice (n≥5) by qRT-PCR. *p<0.05, **p<0.01, ***p<0.001, vs. control (acetone).

4.1.3.3 RAR-RXR signaling pathways modify skin based immune responses

Retinoid-mediated signaling is known to play an important role in the immune system and a dysregulated retinoid metabolism was found in skin of AD patients (Mihály *et al.*, 2011). Therefore, we investigated whether topical application of receptor selective retinoids is sufficient to alter the expression of genes implicated in the immune response in skin, such as the Th2 cell attracting chemokines Ccl11 (eotaxin-1), Ccl17 (Tarc), Ccl22 (Mdc), Ccl24 (eotaxin-2), the chemokine receptor Ccr3, and the inflammatory marker Krt17 (Table 10). The synthetic RXR activator exerted only a slight effect on their gene expression in skin, while levels of chemokines and Krt17 were markedly decreased in response to the RARα agonist (except for Ccl17). Once more, this result strongly resembled to those found after application of RAR or RXR antagonists. Topical treatment with the synthetic RARγ agonist and ATRA as well as the RARγ antagonist decreased mRNA levels of Ccl11 and Ccl24 but induced Ccl17 and partly Ccl22, while it was the opposite in mouse skin treated with the RARα antagonist. Moreover, the chemokine receptor Ccr3 was below detection level regardless of which agonist or antagonist was applied (not shown). Expression of Krt17 was increased only in response to the RARγ agonist or RARα antagonist while it was decreased or unaltered in all other groups (Table 10).

4.2 Mouse model of allergen-induced dermatitis

Following determination of the contribution of retinoid receptor specific signaling to skin barrier homeostasis and immune response in the skin, in the second part of the study we were interested in the regulation of retinoid metabolism and retinoid-mediated signaling in the skin of mice under cutaneous disease conditions. Therefore, we applied a mouse model of allergen-induced dermatitis (Dahten *et al.*, 2008; Weise *et al.*, 2011) using combined systemic and topical OVA treatment. Further, we investigated the effect of systemic OVA sensitization without additional topical application on retinoid metabolism and signaling in murine skin.

4.2.1 Induction of allergen-induced dermatitis by systemic and systemic plus topical OVA application

Several parameters involved in immune response pathways in the skin as wells as skin homeostasis, such as immune cell infiltration, total IgE levels, and gene expression were

investigated in response to OVA treatment in order to verify the induction of an allergen-induced dermatitis. mRNA expression analysis was performed using qRT-PCR and TLDA and results are indicated as fold change of gene expression versus control mice (PBS-treated). Data are presented as mean per treatment group (n=6 mice) ± SEM of triplicate (qRT-PCR) or duplicate (TLDA) measurements per data point. Significant changes ($p<0.05$) are indicated with asterisks.

4.2.1.1 Systemic sensitization with OVA triggers mild allergen-induced dermatitis when compared to additional topical OVA applications

BALB/c mice were systemically sensitized with OVA in addition or not to topical sensitization onto shaved back skin (Figure 8 and 9) and compared with PBS-injected mice (controls). Sensitization with OVA induced mild but statistically significant focal hyperplasia with a two-fold (OVA i.p.; $p<0.001$ vs. PBS i.p.) or three-fold (OVA i.p.+e.c.; $p<0.001$ vs. PBS i.p.; $p<0.001$ vs. OVA i.p.) increase in epidermal thickness, respectively. Histological analysis further revealed scaly skin in both OVA-sensitized groups (Figure 13).

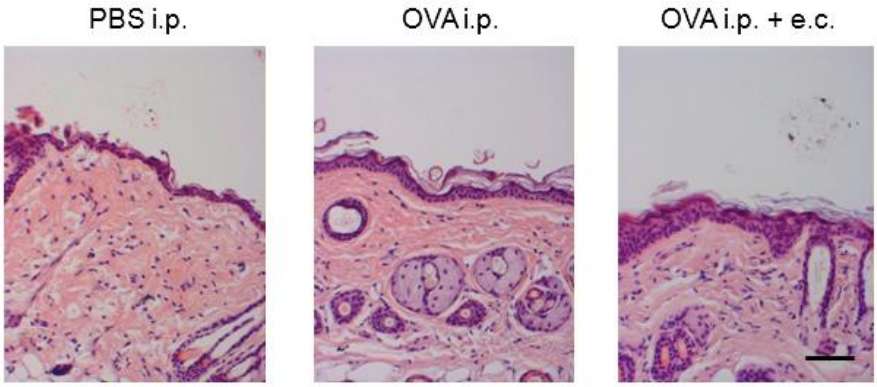

Figure 13. H&E staining of five-micrometer skin sections obtained from PBS or OVA treated dorsal skin sites (n=8 per group). Images were taken at x10 magnification (scale bar = 50 µm).

Interestingly, repeated systemic sensitization with OVA did not alter total serum IgE levels compared to control mice (Figure 14). However, combined systemic and topical sensitization leading to allergen-induced dermatitis resulted in significantly increased total serum IgE (21.696 ± 1.655 ng/ml) when compared to controls and systemically sensitized mice (Figure 14).

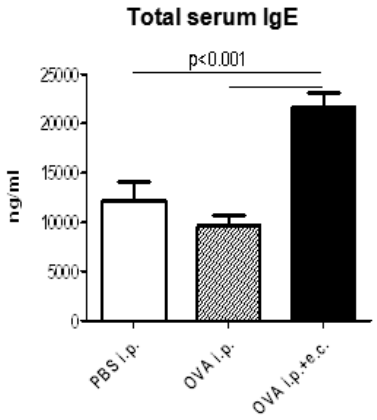

Figure 14. Total IgE levels were determined in the serum of mice treated systemically with or without additional topical sensitization with OVA. Data are presented as mean values ± SEM of three independent measurements with triplicate determination of n=8 mice/group.

Furthermore, inflammatory cells infiltrating the skin were characterized by Giemsa staining and immunohistochemical methods. Numbers of mast cells were significantly elevated in the dermis of both OVA-sensitized groups, while the increase in macrophages, dermal dendritic cells, and CD4+ lymphocytes reached statistical significance only in allergen-induced dermatitis (Table 11). Sensitization with OVA induced elevated numbers of CD3+ lymphocytes in dermis and epidermis (data not shown). Only few CD8+ lymphocytes could be detected in skin sections of OVA-sensitized mice (data not shown). Eosinophils could only be found in the dermis of OVA-sensitized mice and predominantly in allergen-induced dermatitis (Table 11). In the epidermis, several Langerhans cells exhibiting activated morphology could be observed in OVA-sensitized mice (data not shown). Altogether, numbers of mast cells, macrophages and dermal dendritic cells were significantly higher only in allergen-induced dermatitis (Table 11).

Table 11. Systemic sensitization with ovalbumin (OVA) induced mild allergic dermatitis when compared to additional topical OVA applications.

	Sensitization		
Analysis	**PBS i.p.**	**OVA i.p.**	**OVA i.p.+e.c.**
***Inflammatory cell infiltrate in skin*[1]**			
Mast cells[2]	7 ± 1	27 ± 2***	45 ± 3$^{***\#\#\#}$
Eosinophils[2]	0 ± 0	5 ± 1*	9 ± 2***
Macrophages (MHC II^{+}CD11c^{-})[3]	307 ± 16	369 ± 21	474 ± 14$^{***\#\#}$
Dermal dendritic cells (MHC II^{+}CD11c^{+})[3]	40 ± 3	59 ± 4	90 ± 10$^{***\#}$
CD3^{+} lymphocytes[3]	164 ± 13	240 ± 15**	285 ± 10***
CD4^{+} lymphocytes[3]	4 ± 1	11 ± 2	14 ± 2**
***Cytokine serum levels (pg/ml)*[4]**			
Th1-type			
Interleukin 12 p70 (IL-12)	7.4 ± 3.9	6.5 ± 4	8.4 ± 5.6
Th2-type			
Interleukin 4 (IL-4)	73 ± 22	179 ± 24*	198 ± 15**
Thymic stromal lymphopoietin (Tslp)	3.5 ± 0.5	3.7 ± 1.2	3.4 ± 0.4

e.c., epicutaneous; i.p., intraperitoneal; OVA, ovalbumin; PBS, phosphate-buffered saline; Th, T-helper cell.

[1] Positively stained cells were counted in six fields per section of n=5 (IHC-staining) or n=8 animals (Giemsa-staining), respectively, and expressed as cells per mm². [2] Determined in Giemsa-stained skin sections. [3] Determined in IHC-stained skin sections. [4] Cytokine levels in sera were determined as triplicate measurements of n=4 mice per group using Quantikine mouse immunoassay kits. Cell numbers are indicated as mean ± SEM, cytokine levels are indicated as pg/ml.. *p<0.05, **p<0.01, ***p<0.001, vs. PBS i.p.; $^{\#}p$<0.05, $^{\#\#}p$<0.01, and $^{\#\#\#}p$<0.001, vs. OVA i.p.

4.2.1.2 Systemic sensitization with OVA triggers IL-4 serum levels

Levels of the cytokines IL-4, TSLP and IL-12 (IL-12p70) were determined in the sera of OVA-sensitized and control mice. IL-4 but not TSLP was significantly increased in both OVA-treated groups (Table 11, Figure 15). Serum levels of IL-12, a Th1-type cytokine, remained unaltered in all groups (Table 11).

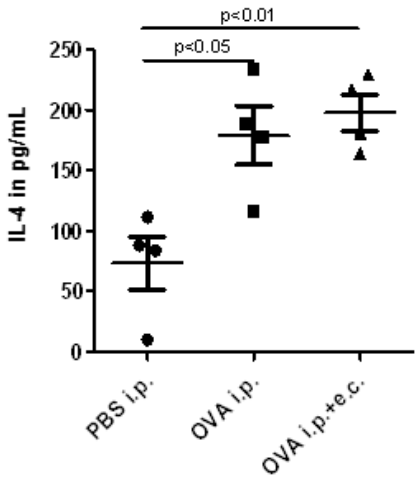

Figure 15. IL-4 serum levels after systemic with or without additional topical OVA sensitization (n=4 mice per group).

4.2.1.3 Expression of Th1- and Th2-type immune response genes is only altered in allergen-induced dermatitis

Expression of genes relevant for Th1- or Th2-type immune responses was analyzed in the skin of mice by qRT-PCR and TLDA. As shown in Table 12, gene expression levels of the inflammation-associated Krt17 were moderately increased in the skin of OVA-sensitized mice. In contrast, expression of Th1 related genes including interferon γ (Ifng) and interleukin 12A (Il12a) and of Th2 associated genes including interleukin 4 (Il4), interleukin 10 (Il10), Ccl11, and its corresponding receptor Ccr3 was significantly elevated only in the skin of mice with allergen-induced dermatitis (Table 12). Altogether, these results (Tables 11 and 12) show that only combined systemic and topical sensitizations with OVA fully recapitulate allergen-induced dermatitis symptoms, which are also found in chronic AD (Grewe *et al.*, 1998; Horikawa *et al.*, 2002; Leung, 1995; Leung and Bieber, 2003; Werfel *et al.*, 1996).

Table 12. Systemic and topical OVA sensitization results in inflammation.

		Fold change	
Gene name	**Symbol**	**OVA i.p.**	**OVA i.p.+e.c.**
Immune response			
Keratin 17	Krt17	1.6 ± 0.2*	1.7 ± 0.2**
Th1-type			
Interleukin 12A	Il12a	1639 ± 719	2613 ± 740*
Interferon γ	Ifng	11.1 ± 5.4	22.1 ± 2.8**
Th2-type			
Interleukin 4	Il4	11.7 ± 4.5	92.7 ± 30.9$^{**\#}$
Interleukin 10	Il10	12.4 ± 5.5	32.7 ± 13.1*
Chemokine (CC motif) ligand 11 / eotaxin-1	Ccl11	0.2 ± 0.2	13.8 ± 5.1$^{*\#}$
Chemokine (CC motif) receptor 3	Ccr3	63.7 ± 34.4	1763 ± 534$^{***\#\#}$

e.c., epicutaneous; i.p., intraperitoneal; OVA, ovalbumin; Th, T-helper cell.

Fold change data are expressed as mean ± SEM and were determined in skin specimens of sensitized mice (n=6) by TLDA or qRT-PCR. *p<0.05, **p<0.01, ***p<0.001, vs. control (PBS i.p.); $^{\#}p$<0.05, $^{\#\#}p$<0.01, and $^{\#\#\#}p$<0.001, vs. OVA i.p.

4.2.1.4 Systemic sensitization with OVA is sufficient to modify the expression of genes involved in epidermal barrier homeostasis

mRNA levels of several genes involved in epidermal barrier formation and maintenance, such as keratinocyte differentiation markers Ivl and Lor, matrix metalloproteinase 9 (Mmp9), and Spink5 were significantly decreased in the skin of OVA-treated mice (Table 13). Notably, expression levels of all tested genes, except for psoriasin (S100a7a), were lower in the OVA-treated mice. Indeed, psoriasin expression was induced in mice treated with OVA (Table 13).

In contrast, genes related to the epidermal lipid components were mainly up-regulated in mouse skin after OVA challenges. In fact, mRNA expression levels of Hmgcs2, involved in cholesterol synthesis, of serine palmitoyltransferase 2 (Sptlc2), Ugcg, both catalyzing the synthesis of ceramides and glycosyl-ceramides, and of alkaline ceramidase 1 (Acer1), which is responsible for ceramide degradation, were significantly elevated in the skin of mice with allergen-induced dermatitis and/or solely systemic OVA sensitization (Table 13). Noticeably, only expression of Abca12, which is responsible for lipid loading into lamellar bodies, was decreased in OVA-treated groups (Table 13).

Table 13. Systemic and topical OVA sensitizations disturb epidermal barrier homeostasis.

Gene name	Symbol	Fold change	
		OVA i.p.	OVA i.p.+e.c.
Epidermal barrier homeostasis			
ATP-binding cassette A12	Abca12	$0.3 \pm 0^{***}$	$0.6 \pm 0.1^{***\#\#}$
Involucrin	Ivl	$0.6 \pm 0.1^{*}$	$0.4 \pm 0^{***}$
Loricrin	Lor	$0.5 \pm 0^{*}$	$0.8 \pm 0.1^{\#}$
Serine peptidase inhibitor, Kazal-type 5	Spink5	$0.2 \pm 0^{***}$	$0.4 \pm 0^{***}$
Matrix metalloproteinase 9	Mmp9	$0.1 \pm 0^{***}$	$0.6 \pm 0.1^{\#\#}$
S100 calcium binding protein A7A / psoriasin	S100a7a	$2.6 \pm 0.3^{**}$	$2.1 \pm 0.3^{*}$
3-Hydroxy-3-methylglutaryl-CoA synthase 2	Hmgcs2	0.4 ± 0.1	$4.4 \pm 1.1^{**\#\#}$
Serine palmitoyltransferase long chain base subunit 2	Sptlc2	1.2 ± 0.3	$33.8 \pm 10.6^{**\#\#}$
UDP-glucose ceramide glucosyltransferase	Ugcg	$196 \pm 16.9^{***}$	$161 \pm 14.4^{***}$
Alkaline ceramidase 1	Acer1	2.2 ± 0.6	$3.9 \pm 0.3^{***\#}$

e.c., epicutaneous; i.p., intraperitoneal; OVA, ovalbumin.

Fold change data are expressed as mean ± SEM and were determined in skin specimens of sensitized mice (n=6) by TLDA or qRT-PCR. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, vs. control (PBS i.p.); $^{\#}p<0.05$, $^{\#\#}p<0.01$, and $^{\#\#\#}p<0.001$, vs. OVA i.p.

Taken together, our results suggest that systemic sensitization with OVA is sufficient to decrease the expression of differentiation markers and to increase the expression of proteins involved in the cutaneous lipid metabolism. This, in turn, might lead to an impaired epidermal barrier function.

4.2.2 Retinoid metabolism and signaling in allergen-induced dermatitis

While retinoid-mediated signaling in skin is involved in several physiological processes, little is known about RAR-mediated signaling in inflammatory skin diseases (Mihály *et al.*, 2011). By means of our mouse model we aimed to investigate whether repeated systemic and combined systemic and topical sensitizations with OVA are able to induce changes in retinoid metabolism and retinoid-mediated signaling on the gene expression level in the skin.

4.2.2.1 ATRA levels are increased in allergen-induced dermatitis

Interestingly, we found significantly elevated concentrations of ATRA only in the skin of mice with allergen-induced dermatitis. Furthermore, retinol levels in mouse skin remained unchanged in all treatment groups (Table 14).

Table 14. Systemic and topical OVA sensitizations result in increased ATRA levels in skin.

Retinoid concentrations in ng/g	PBS i.p.	OVA i.p.	OVA i.p.+e.c.
ATRA	0.5 ± 0.2	0.8 ± 0.2	1.4 ± 0.2*
Retinol	100 ± 14	126 ± 36	136 ± 13

e.c., epicutaneous; i.p., intraperitoneal; OVA, ovalbumin; PBS, phosphate-buffered saline.

Concentrations are expressed as ng/mg with mean ± SEM calculated and were determined by HPLC MS-MS method in skin specimens of control mice and OVA-sensitized mice (n=3 per group). *p<0.05 vs. PBS i.p.

4.2.2.2 Retinoid metabolism is increased in allergen-induced dermatitis

After sensitization, the expression of Sdr16c5, responsible for the oxidation of retinol to retinal, was induced compared to controls while expression of Rdh10 remained unchanged (Table 15). In contrast, expression of enzymes responsible for the conversion of retinal to the bioactive retinoid ATRA (aldehyde dehydrogenases; Aldh1a1, 1a2 and 1a3) was significantly increased only in allergen-induced dermatitis (Table 15) and corresponding to ATRA levels in the skin. Effects of ATRA are mediated by different retinoid receptors in the skin. In parallel to the determined ATRA skin content, elevated mRNA levels of RARγ and RXRα, both the most abundant retinoid receptors in skin, were evidenced only in allergen-induced dermatitis (Table 15).

Table 15. Systemic and topical OVA sensitizations induce RA synthesis and dysregulate retinoid-mediated signaling in the skin of mice.

		Fold change	
Gene name	**Symbol**	**OVA i.p.**	**OVA i.p.+e.c.**
Retinal synthesis			
Short chain dehydrogenase/reductase 16C5	Sdr16c5	$1.7 \pm 0.2^{*}$	$1.8 \pm 0.2^{*}$
Retinol dehydrogenase 10	Rdh10	1.1 ± 0.1	1.3 ± 0.1
Retinoic acid synthesis			
Aldehyde dehydrogenase 1A1	Aldh1a1	1.8 ± 0.2	$2.4 \pm 0.4^{**}$
Aldehyde dehydrogenase 1A2	Aldh1a2	0.5 ± 0	$3.9 \pm 1.3^{*\#}$
Aldehyde dehydrogenase 1A3[1]	Aldh1a3	$4.8 \pm 0.4^{**}$	$4.0 \pm 0.8^{**}$
Retinoid receptors			
Retinoic acid receptor α	Rara	0.8 ± 0.1	1.0 ± 0.1
Retinoic acid receptor β[1]	Rarb	0.8 ± 0.1	0.9 ± 0.1
Retinoic acid receptor γ	Rarg	0.8 ± 0.1	$1.3 \pm 0.2^{\#}$
Retinoid X receptor α	Rxra	0.7 ± 0.1	$1.6 \pm 0.2^{*\#\#\#}$
RAR target genes involved in retinoid signaling			
Retinoic acid degradation			
Cytochrome P450 26A1	Cyp26a1	2.1 ± 0.7	$7.9 \pm 2.2^{**\#}$
Cytochrome P450 26B1	Cyp26b1	0.6 ± 0.1	$1.9 \pm 0.2^{*\#\#}$
Retinoid transport proteins			
Cellular retinol binding protein 1	Rbp1	$3.5 \pm 0.2^{***}$	$3.0 \pm 0.2^{***}$
Cellular retinoic acid binding protein 2	Crabp2	1.3 ± 0.1	1.4 ± 0.1
Retinol esterification			
Lecithin-retinol acyltransferase	Lrat	2.4 ± 0.3	2.5 ± 0.7
Further RAR target genes not involved in retinoid signaling			
Keratin 4	Krt4	0.6 ± 0.2	$0.3 \pm 0^{*}$
Retinoic acid receptor responder 2	Rarres2	$0.5 \pm 0.1^{*}$	0.6 ± 0.1
Transglutaminase 2	Tgm2	0.9 ± 0.1	0.7 ± 0.1

e.c., epicutaneous; i.p., intraperitoneal; OVA, ovalbumin. [1] RAR target genes

Fold change data are expressed as mean ± SEM and were determined in skin specimens of sensitized mice (n=6) by TLDA. *p<0.05, **p<0.01, ***p<0.001, vs. control (PBS i.p.); $^{\#}p$<0.05, $^{\#\#}p$<0.01, and $^{\#\#\#}p$<0.001, vs. OVA i.p.

4.2.2.3 Retinoid-mediated signaling is increased in allergen-induced dermatitis

To further investigate the induction of retinoid-mediated signaling in sensitized skin, we assessed the expression of RAR target genes. Accordingly, we found that expression of genes encoding RA degradation enzymes, cytochrome P450 26a1 (eight-fold induction), and Cyp26b1 (two-fold increase) was increased in allergen-induced dermatitis (Table 15). Expression of proteins involved in retinoid transport (Rbp1, Crabp2) and metabolism (Lrat) was similarly increased in the skin of mice treated with OVA, regardless of further topical sensitization with OVA. In contrast, expression of RAR target genes not involved in retinoid signaling (Krt4, Rarres2, Tgm2) was not significantly altered (Table 15).

Notably, the ratio of Fabp5 vs. Crabp2 gene expression, both delivering ATRA to their respective cognate NR, was significantly increased in allergen-induced dermatitis (Figure 16).

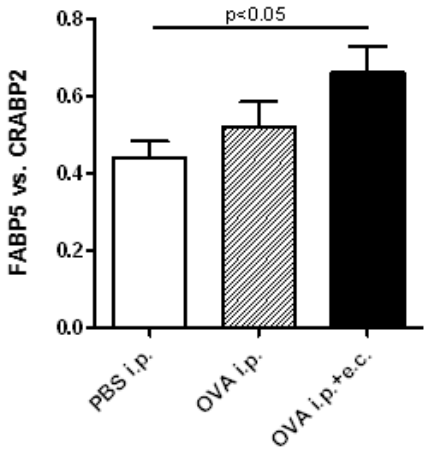

Figure 16. Ratio of Fabp5 vs. Crabp2 expression in the skin of OVA-treated mice (n=6 per group) compared to control mice (PBS i.p.). Data are presented as mean values ± SEM.

4.2.3 PPARδ-mediated signaling in allergen-induced dermatitis

4.2.3.1 Gene targets involved in PPARδ pathways in the skin are mainly up-regulated in allergen-induced dermatitis

Systemic or systemic plus topical sensitization of mice with OVA led to reduced Ppard gene expression compared to controls and this decrease was somewhat more pronounced in mice systemically sensitized only. In contrast, mRNA expression of Fabp5, the fatty acid binding protein 5 which delivers ligands to PPARδ, was increased after sensitization with OVA (Table 16).

Table 16. Systemic and topical OVA sensitizations induce PPARδ target gene expression in skin.

		Fold change	
Gene name	**Symbol**	**OVA i.p.**	**OVA i.p.+e.c.**
PPARδ signaling			
Peroxisome proliferator-activated receptor δ	Ppard	$0.4 \pm 0.0^{**}$	$0.6 \pm 0.0^{*}$
Fatty acid-binding protein 5	Fabp5	1.7 ± 0.3	$2.2 \pm 0.2^{**}$
ATP-binding cassette A12	Abca12	$0.3 \pm 0.0^{***}$	$0.6 \pm 0.1^{***\#\#}$
Keratin 6B	Krt6b	5.8 ± 0.6	$632 \pm 177^{**\#\#}$
Keratin 16	Krt16	0.5 ± 0.0	$5.7 \pm 1.2^{***\#\#\#}$
Heparin-binding EGF-like growth factor	Hbegf	1.1 ± 0.1	$2.1 \pm 0.3^{*\#}$
3-Hydroxy-3-methylglutaryl-CoA synthase 2	Hmgcs2	0.4 ± 0.1	$4.4 \pm 1.1^{**\#\#}$

e.c., epicutaneous; i.p., intraperitoneal; OVA, ovalbumin.

Fold change data are expressed as mean ± SEM and were determined in skin specimens of sensitized mice (n=6) by TLDA or qRT-PCR. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, vs. control (PBS i.p.); $^{\#}p<0.05$, $^{\#\#}p<0.01$, and $^{\#\#\#}p<0.001$, vs. OVA i.p.

Moreover, Krt6b, Krt16, Hbegf, and Hmgcs2, all of which known to be induced upon PPARδ activation and involved in epidermal barrier homeostasis (Calleja *et al.*, 2006; Romanowska *et al.*, 2008; Romanowska *et al.*, 2010) showed significantly elevated gene expression levels in skin after systemic and topical sensitization. Only the PPARδ target gene Abca12 (Jiang *et al.*, 2009) which is responsible for epidermal barrier formation and maintenance showed decreased mRNA levels in both OVA treatment groups (Table 16). Altogether, our results suggest an induction of gene targets which are involved in PPARδ

signaling pathways, most noticeably Fabp5, in murine skin in response to systemic and topical OVA sensitization.

4.2.3.2 Systemic sensitization with OVA increases Fabp5 protein levels

Because Fabp5 gene expression was induced in the skin after repeated systemic OVA sensitization (Table 16) we also assessed levels of Fabp5 protein in the skin of mice in our experimental conditions. Levels of Fabp5 protein as measured by Western Blots, increased in the skin of mice sensitized with OVA compared to controls. However, highest Fabp5 protein levels were detected in whole skin of mice systemically treated with OVA (Figure 17).

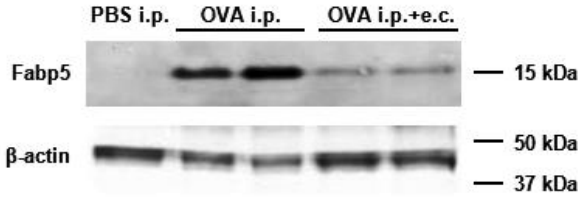

Figure 17. Fabp5 protein levels in the skin of mice with allergen-induced dermatitis. 150 µg proteins were loaded per lane and beta-actin was used as control for even protein loading. Representative image of 3 independent experiments

In order to determine the localization of Fabp5 across the skin, we performed immunohistochemical analysis. We found intense staining for Fabp5 in the thickened epidermis and around hair follicles of mice treated with OVA (Figure 18). Thus, systemic sensitization with OVA is sufficient to increase levels of the PPARδ ligand-binding protein Fabp5 in the skin of mice.

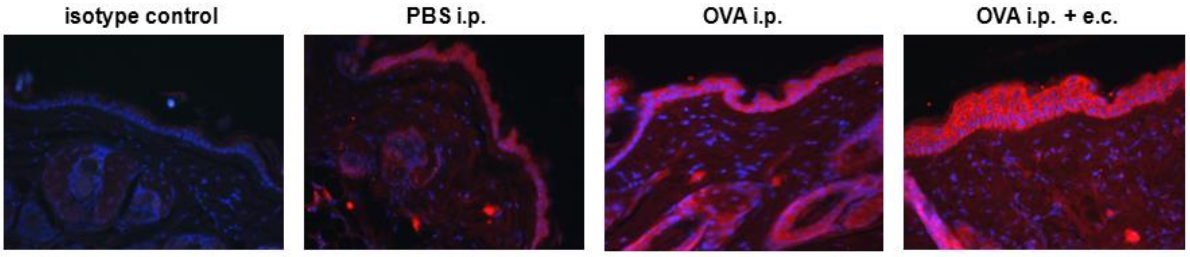

Figure 18. Immunohistochemical analysis of Fabp5 protein expression in five-micrometer back skin sections of OVA-sensitized mice. Representative images for n=3 mice per group.

5 DISCUSSION

5.1 Retinoid function in the skin

Retinoids, such as ATRA are important modulators of epithelial surface maintenance (Blomhoff, 1994) and immune competence (Rühl, 2006; Schuster *et al.*, 2008; Stephensen, 2001). Through their role in the regulation of several processes of skin cell proliferation, differentiation, apoptosis, epidermal barrier function, and immune regulation (Elias, 1987; Elias *et al.*, 1981), they are in particular essential for the physiology of the skin (Roos *et al.*, 1998). Retinoids mediate their functions mainly via gene expression regulation through NRs such as RARs and RXRs. Both these receptor families can be found in the skin, though with varying subtype distribution (Fisher *et al.*, 1994). Up to now it is unknown whether all retinoid receptor subtypes present in the skin contribute equally to retinoid metabolism and signaling and, thereby, to skin physiology or whether differences may exist. Notably, alterations of retinoid metabolism and signaling were already found in the skin of patients with various skin diseases, such as psoriasis (Saurat, 1999), ichthyosis (Mevorah *et al.*, 1996), and AD (Mihály *et al.*, 2011). However, it is still unclear whether these alterations are the trigger or if they are consequence of these skin diseases.

In order to determine the effect of selective retinoid-mediated signaling in the skin on retinoid metabolism, epidermal barrier homeostasis, and immune regulation mice were treated topically with various retinoid receptor specific agonists or antagonists. The main finding is the strong difference between the positive retinoid-mediated signaling via RARγ pathways in contrast to the negative retinoid-mediated signaling via RARα. Furthermore, using an OVA-induced allergic dermatitis mouse model, the present work additionally demonstrates that the immune response in allergen-induced dermatitis is associated with increased retinoid signaling and RA concentrations in murine skin. Moreover, signaling via PPARδ-mediated pathways, mostly through Fabp5 up-regulation, is mainly enhanced in allergen-induced dermatitis. Thus, retinoid-mediated signaling is involved in the pathogenesis and/or maintenance of allergic dermatitis and possibly further atopic skin diseases such as AD but the exact pathway has yet to be determined.

5.1.1 Retinoid signaling in the skin is oppositely regulated by RARα and RARγ

Topical treatment with retinoid receptor specific agonists affected the expression of all genes involved in retinoid-mediated signaling in the skin (retinoid metabolism, transport, target genes) in general oppositely to receptor antagonists. Likewise, target genes were mainly induced after treatment with ATRA or the synthetic RARγ agonist (Figure 10 and Table 7), as previously reported (Balmer and Blomhoff, 2002; Fisher and Voorhees, 1996; Lee *et al.*, 2009; Virtanen *et al.*, 2000). Moreover, both agonists induced very similar gene expression patterns and given the fact that RARγ is the predominant RAR subtype in the skin (Fisher *et al.*, 1994) it is indicated that ATRA mediates its activity in skin through RARγ rather than the RARα subtype (Chapellier *et al.*, 2002; Fisher *et al.*, 1994; Goyette *et al.*, 2000).

Most interesting however, was a consequent down-regulation of gene expression by the synthetic RARα agonist which is in line with reduced ATRA levels in mouse skin, possibly due to decreased ATRA synthesis via Aldh enzymes (Table 7). Only Fabp5 and Rdh16 expression was increased in response to the agonist. This expression pattern strongly resembled to that in response to RAR or RXR antagonists while both antagonists further seemed to induce Bco2 and Rbp4 expression (Figure 10, Tables 7 and 8). The proteins encoded by those genes are implicated in retinoid metabolism and transport (Chai *et al.*, 1997; Goodman, 1984; Kiefer *et al.*, 2001; Schug *et al.*, 2007). Thus, it seems plausible that ATRA or retinoid derivatives different from ATRA, like oxo-retinoids or still unknown endogenous RAR ligands, could be generated upon retinoid receptor antagonism and shuttled to NRs different from RARs, as it was already proposed for Fabp5-mediated ATRA-induced PPARδ activation (Schug *et al.*, 2007; Shaw *et al.*, 2003; Tan *et al.*, 2001).

5.1.2 Retinoid-mediated signaling is induced in allergen-induced dermatitis

Corresponding to topical RARγ agonist treatment, several RAR target genes as well as genes involved in RA synthesis, degradation, transport, and esterification were induced in allergen-induced dermatitis indicating the involvement of this NR in the skin disease. In contrast, the expression of RAR targets which are not implicated in retinoid signaling or which are rather related to epidermal differentiation (such as Krt4, Rarres2, Tgm2) remained unaltered or reduced. These data indicate that potentially increased ATRA synthesis via Aldh1a enzymes and elevated ATRA levels in mouse skin, as observed in allergen-induced

dermatitis, might not result in an overall increase of RAR-mediated signaling. In fact, these data further suggest the involvement of additional RA-mediated signaling pathways in murine skin. However, OVA treatment might also affect the level of several other lipids and NR agonists different from ATRA which might impact on gene expression.

5.1.3 Alternative retinoid-mediated signaling pathways in the skin

The involvement of other NRs than RARs in retinoid-mediated signaling is not unlikely as also NR4A1/NUR77 and RXR were shown to form heterodimers which respond to RXR activators *in vivo* and *in vitro* (Perlmann and Jansson, 1995). Such heterodimers might participate in retinoid signaling especially when RARs are antagonized. Moreover, Volakakis *et al.* (2009) demonstrated that NR4A1/NUR77 can induce the expression of Fabp5 in HEK293 cells which potentially enhances RA-mediated PPARδ signaling. Indeed, it has previously been shown that ATRA can activate PPARδ when the ratio of the lipid transporters Fabp5 vs. Crabp2 is high within cells such as keratinocytes (Schug *et al.*, 2007; Shaw *et al.*, 2003). Interestingly, we found Nr4a1/Nur77 and Ppard expression in the skin of NR ligand-treated mice significantly decreased or below detection limit in response to those ligands which markedly induced Fabp5 expression, namely the RARα agonist, RAR and RXR antagonists (Tables 7, 8 and 17). This may be indicative of (late) negative feedback regulations on the gene expression level in response to induced Fabp5 expression.

Whether Fabp5-mediated PPARδ signaling and/or a novel, as yet undetermined retinoid(s) might mediate such an alternative retinoid pathway in the skin is currently unknown. In fact, mRNA levels of ATRA-synthesizing enzymes (Aldhs) following RAR and RXR antagonist application were not in accord with elevated ATRA levels in the skin of those mice. This suggests that ATRA synthesis upon antagonist treatment may be mediated by other enzymes such as Bco2, Rdh16, Rbp4, and/or other pathways, from precursors present in the skin and/or via transporter-mediated pathways delivering retinoids to the skin (Goodman, 1984).

Table 17. Fold change of mRNA expression of nuclear hormone receptors NR4A1/NURR77 and PPARδ in the skin of mice after two weeks topical treatment with retinoid receptor specific agonists or antagonists.

		Agonists (Fold change)				**Antagonists** (Fold change)			
Gene name	**Symbol**	**RARα[1]**	**RARγ[2]**	**ATRA[3]**	**RXR[4]**	**RARα[5]**	**RARγ[6]**	**RAR[7]**	**RXR[8]**
Nuclear receptor family 4, group A, number 1	Nr4a1/Nurr77	0.1 ± 0.04[#]	0.9 ± 0.1	0.7 ± 0.1	0.4 ± 0.04	0.007 ± 0.001[#]	1.2 ± 0.1	UDL	UDL
Peroxisome proliferator-activated receptor delta	Ppard	0.03 ± 0.01[#]	1.1 ± 0.1	0.9 ± 0.1	0.8 ± 0.04[*]	1 ± 0.1	UDL	0.0009 ± 0.0009[#]	0.04 ± 0.01[#]

[1] BMS753; [2] BMS961; [3] all-*trans* retinoic acid; [4] LG268; [5] BMS614; [6] UVI2041; [7] BMS493; [8] UVI3003

UDL, under detection limit

Fold change data are expressed as mean ± SEM (n≥5) and were determined in skin specimens of topically treated mice by qRT-PCR. **p*<0.05, #p<0.001, versus control (acetone).

5.1.3.1 ATRA-induced PPARδ-mediated signaling in allergen-induced dermatitis

Moreover, the increased Fabp5 vs. Crabp2 ratio in the skin of mice with allergen-induced dermatitis further suggested favored ATRA signaling through PPARδ under disease conditions (Figure 16). This signaling pathway may significantly contribute to the specific gene expression patterns observed in this mouse model (Figure 19). Indeed, PPARδ signaling and several of its target genes were previously found increased in psoriasis and lesional AD skin (Romanowska *et al.*, 2008; Romanowska *et al.*, 2010; Westergaard *et al.*, 2003) and Romanowska *et al.* (2010) further demonstrated the induction of an inflammatory skin disease similar to human psoriasis in PPARδ-overexpressing mice. Interestingly, in our mouse model of allergen-induced dermatitis we observed an increased expression of several target genes involved in PPARδ signaling pathways in the skin. This further indicates the involvement of PPARδ signaling pathways in allergen-induced dermatitis.

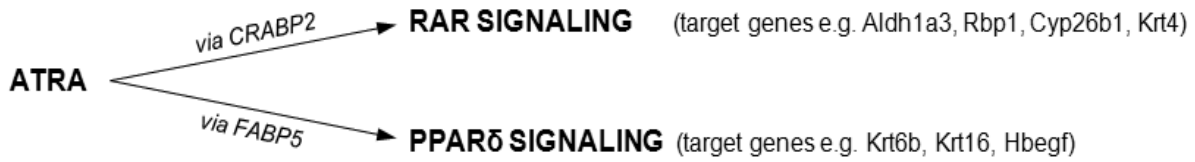

Figure 19. ATRA-induced nuclear receptor-mediated signaling pathways depending on the predominant cellular transport protein.

Interestingly, we determined highest Fabp5 protein levels in the skin of mice treated systemically with OVA (Figure 17). In contrast, immunohistochemical analysis showed particularly intense staining in the epidermis and around hair follicles of mice with allergen-induced dermatitis (Figure 18). In the literature, Fabp5 protein is described to be predominantly present in epidermis (Ogawa *et al.*, 2011), sebaceous glands and hair follicles (Collins and Watt, 2008), and in subcutaneous adipocytes (Zhou *et al.*, 2010) and might explain our observations in whole skin biopsies.

5.1.4 Retinoid receptor subtypes have distinct roles in mouse skin

Furthermore, our observations on retinoid receptor-selective signaling in murine skin indicate different roles of RXR-, RARα-, and RARγ-mediated signaling pathways in the skin

(Figure 20). These data further suggest that induction of RARα signaling might result in the suppression of RARγ-mediated pathways in the skin of mice. Considering the induced RARα gene expression after topical ATRA treatment, this appears to be an efficient physiological switch to different retinoid-mediated signaling pathways.

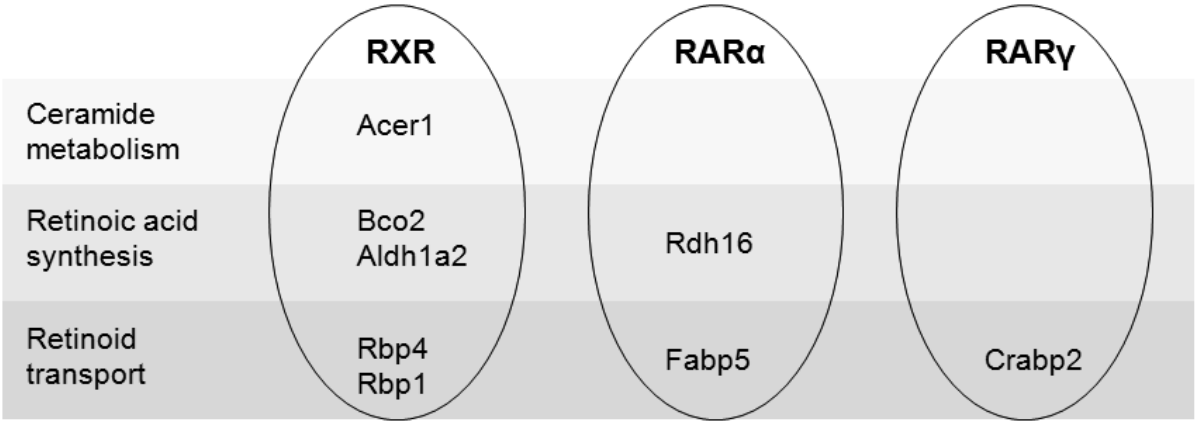

Figure 20. Retinoid receptor selective induction of genes with specific roles in retinoid signaling or epidermal barrier homeostasis in skin of mice treated topically with selective receptor agonists.

So far, it is unknown how RARα mediates its suppressive action on RARγ signaling. High RARα expression was found in inflammatory cells infiltrating the skin in several dermatoses (Haider *et al.*, 2008), however, in normal skin its expression level is fairly low compared to RARγ (Fisher *et al.*, 1994). Thus it seems unlikely that a competition between both receptors for RXRα as heterodimer partner could be the explanation. Instead, RARα apparently regulates the expression of different sets of genes, possibly also in different skin cell types than does RARγ and might also induce the transcription of co-repressor molecules upon activation.

5.1.5 RXR-mediated epidermal hyperproliferation

Another well established effect of RAR-activation in the skin is epidermal hyperproliferation (Chapellier *et al.*, 2002; Cheepala *et al.*, 2007; Fisher and Voorhees, 1996). This was found induced by topically applied ATRA and the synthetic RARγ agonist (Figure 12) and also in the allergen-induced dermatitis model (Figure 13). Furthermore, epidermal hyperproliferation was supported by an induced expression of regulators of desquamation such as Spink5, Klk5, and Klk7 (Brattsand *et al.*, 2005; Caubet *et al.*, 2004; Roelandt *et al.*,

2009) in RAR agonist-treated skin. Moreover, elevated mRNA levels of Hbegf and Krt16, which have already been related previously to induced keratinocyte proliferation (Chapellier *et al.*, 2002; Lee *et al.*, 2009; Weiss *et al.*, 1984; Yoshimura *et al.*, 2003), further confirmed the data (Table 10, Figure 10).

Surprising however, was the induction of epidermal proliferation by the synthetic RXR agonist since no such observation was reported in a previous study using another synthetic RXR agonist (Chapellier *et al.*, 2002). Retinoid effects in skin are most likely mediated by RARγ-RXR heterodimers while their transcriptional activity is dependent on the RAR-activating ligand (Chapellier *et al.*, 2002; Feng *et al.*, 1997). Upon treatment with the RXR agonist we observed increased Aldh1a2 gene expression and elevated ATRA levels in the skin (Table 7 and 9). This indicates an induced ATRA synthesis which might account for the mild epidermal hyperproliferation, most probably mediated by the RAR partner. However, another RXR heterodimer partner, PPARδ, was previously found to be implicated in the regulation of keratinocyte hyperproliferation (Michalik *et al.*, 2001; Romanowska *et al.*, 2008; Romanowska *et al.*, 2010). Compared to RAR-RXR, this heterodimer is permissive which means the ligand of PPAR or RXR, respectively, is sufficient to activate transcription of respective target genes (Tan *et al.*, 2005). This might suggest alternative pathways to be involved in RXR agonist-induced hyperproliferation.

5.1.6 Retinoid-mediated signaling is one piece of the epidermal barrier puzzle

Additionally, retinoid receptor ligand application affected various other processes in the skin as indicated by altered expression levels of genes involved in epidermal barrier homeostasis, such as Abca12, Flg, and Lor (Akiyama, 2011; Proksch *et al.*, 2008), and of genes with roles in lipid barrier formation and ceramide metabolism, e.g. Hmgcs2, Ugcg, Gba, Acer1 (Harris *et al.*, 1997; Holleran *et al.*, 2006; Jennemann *et al.*, 2007; Mao *et al.*, 2003). Consistently, such retinoid-mediated effects have already been reported by Lee *et al.* (2009) in epidermal keratinocytes. These results suggest that retinoid-mediated signaling is required for normal barrier homeostasis and that retinoid-induced dysregulation may be a predisposing factor for dermatological diseases such as allergen-induced dermatitis. Thereby, both antagonism and induction of RAR- and/or RXR-mediated signaling in skin appear to disturb barrier homeostasis (Attar *et al.*, 1997; Fullerton and Serup, 1997; Li *et al.*, 2001; Li *et al.*, 2000; Stücker *et al.*, 2002). Interesting in this regard is our observation from another study

that only barrier disruption by repeated tape stripping (without further sensitization) alters retinoid metabolism in the skin of mice. This was indicated by reduced expression of Adh and Rdh enzymes while RA synthesis enzymes (Aldh1a isoforms) as well as most of the retinoid target genes were induced (unpublished data). These data further support a strong link between epidermal barrier homeostasis and retinoid metabolism and signaling.

Furthermore, also allergen-induced dermatitis led to altered expression of genes responsible for epidermal barrier formation and/or maintenance. However, this pattern was rather different from that observed after NR ligand application (e.g. Abca12, Lor, Ivl mainly down-regulated in allergic dermatitis but mainly up-regulated by topical ATRA and RARγ agonist). This data indicates the involvement of additional, non RARγ/RXR-mediated signaling pathways in epidermal barrier disturbance under disease conditions.

5.1.7 Allergen-induced immune response in the skin is associated with increased RA signaling

It is well established that retinoids play important roles in the immune system (Rühl, 2007; Stephensen, 2001), especially in Th2-type cell differentiation (Dawson *et al.*, 2008; Iwata *et al.*, 2003; Stephensen *et al.*, 2002). Interestingly, the expression of various chemokines which are preferentially attracting Th2-type lymphocytes during inflammatory processes (Bonecchi *et al.*, 1998; Imai *et al.*, 1999; Sallusto *et al.*, 1997) and which are known to be altered in human AD skin (Gros *et al.*, 2009; Owczarek *et al.*, 2010) and in an AD mouse model (Wang *et al.*, 2007) was differently altered by the applied retinoid receptor ligands (Table 10). However, undetectable mRNA levels of the corresponding receptor Ccr3 which is expressed by infiltrating immune competent cells such as eosinophils (Dulkys *et al.*, 2001; Menzies-Gow *et al.*, 2002; Sabroe *et al.*, 1999) suggests the absence of inflammatory cells in the skin upon retinoid treatments.

The opposite was true for the applied mouse model of allergen-induced dermatitis where high numbers of infiltrating dermal macrophages, dendritic cells, and mast cells were found in the skin (Table 11) as well as enhanced expression of Ccl11 and Ccr3 as previously reported (Wang *et al.*, 2007). Moreover, a mixed Th1- and Th2-type immune response was present in those mice (Table 11 and 12), indicating that high RA levels in the skin might directly impact on systemic and local immune responses (Rühl, 2007; Rühl *et al.*, 2007; Schuster *et al.*, 2008;

Stephensen, 2001; Stephensen *et al.*, 2007). In contrast, mice systemically treated with OVA exhibited only a partial phenotype with lower inflammatory infiltrates and cytokine expression in the skin. Interestingly, the highest levels of immune response-related gene expression, inflammatory cell infiltrates, and serum cytokines correlated with increased expression of RA synthesizing enzymes and ATRA levels in inflamed skin.

Increased levels of ATRA in the skin of OVA-sensitized mice (Table 14) might reflect the induced expression of Aldh1a enzymes (Table 15) responsible for elevated ATRA synthesis in murine skin. Besides resident skin cells, infiltrating immune cells might be a source of ATRA in sensitized skin. For example, human basophils which have been shown to infiltrate AD skin (Ito *et al.*, 2011) were found to express Aldh1a2 enzyme and to produce RA upon activation with IL-3 in an *ex vivo* model (Spiegl *et al.*, 2008). Unfortunately, identification of specific cell types producing RA in inflamed skin is currently not feasible due to problems in acquiring sufficiently large numbers of highly purified cells from the skin.

Thus our results indicate that topical retinoids can modify potential immune responses at least in part by altering the chemokine expression of resident skin cells and that the outcome of immune alterations seems to differ depending on the RAR subtype activated. Moreover, our data suggest that only overt allergen-induced dermatitis leads to increased ATRA concentration and altered RA signaling in the skin of mice.

5.1.8 Inside-out pathogenesis in allergic skin disease

Notably, one further major outcome of the present work is to demonstrate that systemic OVA sensitization of mice per se is sufficient to induce partial skin immune responses and an impairment of expression of key genes involved in skin homeostasis and barrier function (Table 11, 12 and 13, Figure 15). Previous studies and reviews reported an "outside-inside-outside" pathogenic mechanism of AD (Cork *et al.*, 2009; Elias *et al.*, 2008; Marsella and Samuelson, 2009). In contrast, our data support an "inside-out" mechanism initiated by immune system activation without preceding epidermal barrier impairment. Result of an increasing immune response is epidermal barrier disruption which facilitates the penetration of allergens and significantly contributes to the development of overt skin inflammation. Moreover, the OVA-induced alterations in the skin are accompanied by partially altered retinoid signaling (Table 15), strongly suggesting a causative relationship.

5.1.9 Retinoids act on various pathways in mouse skin with implication for allergic skin disease

In summary, this study lets us emphasize that there must be yet unidentified alternative retinoid signaling pathways or a broader range of endogenous retinoids present in the skin for selective RARα, RARγ, or RXR activation (Figure 21). Furthermore, also NR independent pathways might be involved in retinoid function in the skin. Though 13-*cis* RA has, in contrast to ATRA, little or no ability to bind to Crabp2 or RARs it is the most effective retinoid used in acne therapy (Layton, 2009). 13-*cis* RA is converted intracellularly into various RA derivatives such as 9-*cis* RA, ATRA, or oxo-retinoids (Layton, 2009) which may all contribute in some extent to the effects seen during acne therapy. However, only the 13-cis RA isomer is able to induce apoptosis of sebocytes (independent of RAR) resulting in the strong reduction of sebum secretion during acne therapy with this retinoid (Nelson *et al.*, 2011; Nelson *et al.*, 2006; Zouboulis, 2006) and making it more effective than ATRA.

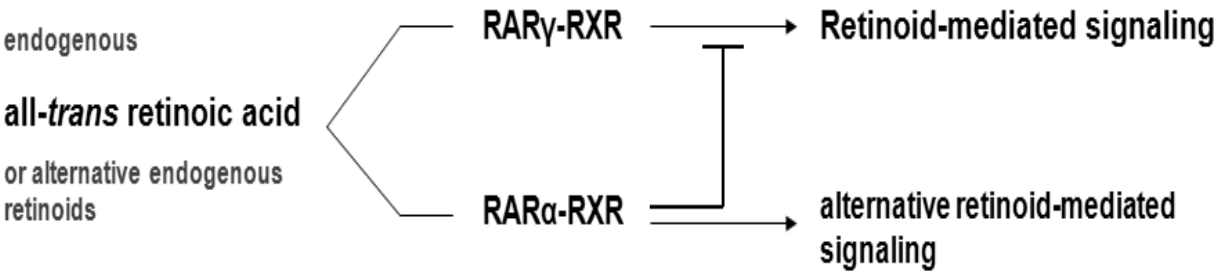

Figure 21. Proposed outcome of selective signaling via RARα-RXR or RARγ-RXR in the skin of mice induced by endogenous retinoids such as all-*trans* retinoic acid (ATRA).

Moreover, our data indicate that unbalanced retinoid signaling in the skin mediated by RARα, RARγ, and/or RXR signaling pathways as well as potential unknown pathways, affect epidermal barrier homeostasis and skin-based immune responses in mice. This retinoid dysregulation may play a central role in various skin diseases as it is also indicated in our mouse model of allergen-induced dermatitis which is associated with increased retinoid signaling and elevated ATRA levels in the skin. Because expression of genes involved in all aspects of RA metabolism is increased, whereas expression of RAR target genes involved in other pathways such as epidermal differentiation remains largely unchanged, allergen-induced

dermatitis might additionally redirect intracellular retinoid flux and metabolism. It is worth mentioning that high VA supplementation diet (600 mg retinol/kg for four weeks) is able to induce retinoid target gene expression in mouse skin as compared to normal VA diet (Mihály *et al.*, 2012). Therefore, even diet might influence epidermal barrier homeostasis and skin-based immune response and thereby might be of importance in skin diseases. Moreover, PPARδ gene targets are mainly induced indicating that RAR-mediated signaling and certain pathways/molecules involved in PPARδ signaling are altered in allergic dermatitis skin. Furthermore, systemic sensitization with an allergen is sufficient to modify the expression of genes central to epidermal homeostasis and immune response suggesting an "inside-out" effect in allergic skin disease pathogenesis possibly by increasing allergen penetration through the skin in response to immune system activation. Whether disturbed retinoid metabolism and retinoid-mediated signaling are symptoms or potential initiators of atopic sensitization still remains to be elucidated.

5.2 The role of mouse models for human allergic skin disorders

Mouse models of allergic skin diseases are of great importance to understand the pathomechanism of these disorders and to target possible therapies. However, the pathology of skin inflammation induced in mice and present in patients is not entirely the same. Therefore, data obtained within animal studies such as our retinoid and allergen-induced dermatitis mouse models provide indispensable scientific knowledge but demand further verification using human samples such as skin explants as well as human studies with allergic skin diseases before the results can be translated into treatment strategies in the clinic.

6 SUMMARY

Endogenous retinoids like all-*trans* retinoic acid (ATRA) play important roles in skin physiology and immune-modulatory events in the skin via nuclear hormone receptor-mediated signaling through RARs and/or RXRs. Moreover, it has been shown recently that ATRA can activate another nuclear receptor involved in skin homeostasis, namely PPARδ, depending on specific transport proteins: Fabp5 initiates PPARδ signaling whereas Crabp2 promotes signaling via RAR. Notably, alterations in retinoid metabolism, signaling, and concentrations have been found in various skin diseases such as atopic dermatitis. Thereby, it remains unclear whether these changes are symptoms or the trigger of such diseases.

The aim of this study was to determine how topically applied agonists or antagonists selective for RARs or RXRs and how the induction of an allergic immune response by systemic or combined systemic and topical treatment with ovalbumin influence retinoid metabolism and signaling in mouse skin. Of further interest were nuclear receptor ligand treatment effects on epidermal barrier homeostasis and skin-based immune regulation relevant for skin disorders such as atopic dermatitis, as well as their correlation to the allergen-induced dermatitis mouse model.

Our data indicate that RARα and RARγ subtypes possess different roles in mouse skin and may be of relevance for the auto-regulation of endogenous retinoid signaling in the skin. Moreover, dysregulated retinoid signaling mediated by RXR, RARα and/or RARγ as well as potential unidentified pathways may promote skin-based inflammation and disturbance of epidermal barrier properties. This is further supported by elevated ATRA levels and mainly increased signaling mediated by RAR or PPARδ in allergen-induced dermatitis skin. Furthermore, systemic sensitization with an allergen is sufficient to modify the expression of genes central to epidermal homeostasis suggesting an “inside-out” effect of allergen in allergic skin disease pathogenesis possibly by increasing allergen penetration through the skin. In summary, disturbed retinoid metabolism and retinoid-mediated signaling in the skin may contribute to the development and/or maintenance of allergic skin diseases. Whether these alterations are symptoms or potential initiators of atopic sensitization still remains to be elucidated.

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7.2 Publication list prepared by the Kenézy Life Sciences Library

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List of publications related to the dissertation

1. **Gericke, J.**, Ittensohn, J., Mihály, J., Dubrac, S., Rühl, R.: Allergen-induced dermatitis causes alterations in cutaneous retinoid-mediated signaling in mice.
PLoS One. 8 (8), e71244, 2013.
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2. **Gericke, J.**, Ittensohn, J., Mihály, J., Álvarez, S., Álvarez, R., Törőcsik, D., de Lera, Á.R., Rühl, R.: Regulation of Retinoid-Mediated Signaling Involved in Skin Homeostasis by RAR and RXR Agonists/Antagonists in Mouse Skin.
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List of other publications

3. Mihály, J., **Gericke, J.**, Törőcsik, D., Gáspár, K., Szegedi, A., Rühl, R.: Reduced lipoxygenase and cyclooxygenase mediated signaling in PBMC of atopic dermatitis patients.
Prostaglandins Other Lipid Mediat. Article in press, 2013.
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H-4032 Debrecen, Egyetem tér 1. e-mail: publikaciok@lib.unideb.hu

4. Mihály, J., **Gericke, J.**, Aydemir, G., Weiss, K., Carlsen, H., Blomhoff, R., Garcia, J., Ralph, R.: Reduced retinoid signaling in the skin after systemic retinoid-X receptor ligand treatment in mice with potential relevance for skin disorders.
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5. **Gericke, J.**, Aydemir, G., Ulbricht, G., Rühl, R.: Statistical calculation of the transition of vitamin A and beta-carotene ingestion in the former West and East German regions between 1986 and 1993.
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8 KEYWORDS

Allergen-induced dermatitis, all-*trans* retinoic acid, atopic dermatitis, fatty acid binding protein 5, gene expression, nuclear hormone receptor, peroxisome proliferator-activated receptor δ, retinoic acid receptor, retinoids, retinoid X receptor, skin

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