Development of Organotypic Skin Disease Equivalents Mimicking Hallmarks of Atopic Dermatitis for Basic Research and Preclinical Drug Evaluation

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Dedicated to my beloved mother

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LIST OF ABBREVIATIONS

AD	atopic dermatitis
AMP	antimicrobial peptide
CD40L	CD40 ligand
CLDN-1	claudin-1
DC	dendritic cell
DHA	docosahexaenoic acid
ECM	extracellular matrix
EDC	epidermal differentiation complex
FFA	free fatty acids
FLG	filaggrin
GM-CSF	granulocyte-macrophage colony-stimulation factor
HUVEC	human umbilical vein cord endothelial cell
IFNγ	interferon gamma
IgE	immunoglobulin E
IL	interleukin
ILR	interleukin receptor
IVL	involucrin
JAK	Janus kinase
LC	Langerhans cell
LOR	loricrin
MDC	macrophage-derived chemokine
OX40L	OX40 ligand
PCA	pyrrolidone carboxylic acid
PPAR	peroxisome proliferator-activated receptor
RHE	reconstructed human epidermis
RHS	reconstructed full-thickness human skin
RXR	retinoid X receptor
S. aureus	Staphylococcus aureus
SC	stratum corneum
siRNA	small interfering RNA
STAT	signal transducers and activators of transcription
TARC	thymus- and activation-regulated chemokine
Th	T helper
TNFα	tumour necrosis factor alpha
T_{reg} cell	regulatory T cell
TSLP	thymic stromal lymphopoietin
TSLPR	thymic stromal lymphopoietin receptor
UCA	urocanic acid
UV	ultraviolet

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1. INTRODUCTION

The skin is the body's primary barrier against physical damages, microbial pathogens and allergens. It represents a unique environment in which immune cells interact with skin cells to maintain tissue homoeostasis and induce immune responses. However, dysregulated immune responses, as well as mutations in essential barrier proteins, can cause skin disorders such as atopic dermatitis (AD). AD is a chronic, relapsing skin disease with a multifaceted pathogenesis including inflammatory processes and a diminished barrier function. Its underlying causes are still not fully understood. Current therapeutic options mainly focus on controlling inflammation, rather than addressing the disease aetiology. Nevertheless, new therapeutic approaches are in continuous development. The use of organotypic skin equivalents in dermatological research and in the field of skin disease research in particular has contributed to a deeper understanding of the pathological mechanisms in AD, complementing the widely used animal models in drug development. Due to ethical issues and, most importantly, interspecies-related differences between animals and humans, the use of organotypic skin equivalents in dermatological research has become more and more important. Therefore, there is a pressing need to continuously improve and validate existing skin equivalents, as well as to develop new *in vitro* test systems to ensure the generation of reproducible and reliable data.

1.1. Atopic Dermatitis (AD)

1.1.1. Epidemiology

AD is a non-infectious, chronic inflammatory skin disease with recurrent acute episodes characterised by intense itch and eczematous lesions. The terms "atopic eczema" and "atopic dermatitis" are commonly used synonymously, although the term eczema is preferentially reserved for the acute manifestation of the disease. AD is still the most frequent chronic disease in children, with a prevalence between 10-30% in children, and 2-10% in adults (Silverberg and Hanifin, 2013; Weidinger and Novak, 2016). In Germany, 13% of all children suffer at least temporarily from AD. In around 60% of cases, the disease manifests during the first year of life (early-onset) and up to 85% experience an onset below the age of five (Bieber, 2008). The lifetime prevalence of AD has increased in industrialised countries over the past three decades, but now appears to have reached a plateau (Deckers *et al.*, 2012; Thomsen, 2014; Williams *et al.*, 2008). By contrast, its prevalence remains lower in many developing countries, though the number of reported cases is increasing. At least in part, the rise AD incidence also seems to be due to a growing attention to the disease pattern. The ISAAC (International Study of Asthma and Allergies in Childhood) phase three study included more than a million children from 97 countries (Odhiambo *et al.*, 2009). Worldwide, they found significant differences in the prevalence of AD in 6-7 year old schoolchildren, ranging from 0.9% (India) to 22.5% (Ecuador), as well as in 13-14 year old

children, from 0.2% (China) to 24.6% (Colombia). A recent study analysed the relationship between urbanisation and prevalence of asthma, rhino-conjunctivitis and eczema symptoms based on the ISAAC study (Pacheco-Gonzalez *et al.*, 2016). Interestingly, they found no association between urban and rural centres, suggesting other responsible factors like altitude, climate, proximity to different vegetation, and residency near the coast. The risk factors for AD vary from environmental and social-economic influences to genetic predisposition. The strongest factor for developing atopic conditions is a positive family history of atopy (i. e. affected parents and twins) (Apfelbacher *et al.*, 2011). Well-known environmental risk factors are a western diet, small family size, high level of education in the subject's household, living in regions with low exposure to ultraviolet (UV) radiation or low humidity, and treatment with broad-spectrum antibiotics during pregnancy and infancy. Otherwise, protective effects were reported for early child admission day care, endotoxin exposure, feeding with unpasteurised farm milk, and contact to dogs (Flohr and Mann, 2014; Kantor and Silverberg, 2017; Weidinger and Novak, 2016). Birth cohort studies suggested that 70% of children show a spontaneous remission before adolescence. An early and severe onset, family history of AD, and early allergen sensitisations are risk factors for a longer disease course (Bieber, 2008; Kim *et al.*, 2016). Around 50-75% of all paediatric patients with early-onset AD are sensitised to one or more allergens, such as food allergens, house dust mites, or pets. This form of AD has been termed as the immunoglobulin E (IgE)-associated, allergic or extrinsic type, while the absence of specific IgE for environmental allergens and food allergens is characteristic for the non-allergic or intrinsic type (Karimkhani *et al.*, 2015; Park *et al.*, 2006; Tokura, 2010). The disease can also start or relapse in adults (i.e. after 20 years of age), but those with late-onset AD are less often sensitised compared to children (Novak and Bieber, 2003). The group of patients with very late onset AD (age > 60 years) can be divided into those who had AD in the past with a longer period of remission and those who develop the disease very late in life. These patients show a relatively severe form of the disease with strong sensitisation (Tanei and Hasegawa, 2016).

Evidence indicates that AD is the cutaneous manifestation of a systemic insult that also gives rise to further atopic conditions. Indeed, AD is often the first step in the so-called "atopic march", which leads to asthma, rhino-conjunctivitis and/or allergic rhinitis in the majority of affected patients (Spergel and Paller, 2003). In the "Study of the Atopic March" (NCT00124709), approximately 11% of the included infants developed asthma within three years, 14-22% developed other atopic conditions, and the development of allergic rhinitis and food allergy correlated with AD severity (Schneider *et al.*, 2016). Accordingly, different cohort studies have revealed that only about 20-30% of children with AD develop respiratory symptoms, and less than 7% show development that resembled an atopic march, thus, the risk is lower than widely assumed (Belgrave *et al.*, 2014; van der Hulst *et al.*, 2007).

Nevertheless, early and effective treatment of AD could potentially interrupt atopic progression and decrease the risk of asthma. In addition, severe eczema in childhood and persistence into adult life are associated with considerable direct costs. For example, expenditures such as for non-prescription treatments and skin care could constitute up to 10% of the household annual income (Ricci *et al.*, 2006). Constant itching, which can lead to sleep disturbance, and the stigma associated with visible skin lesions can have a major impact on quality of life for the affected patient and its family (Holm *et al.*, 2016; Spergel and Paller, 2003). Considering the continuously increasing incidences of AD worldwide, inefficient treatment will affect both patients and health care systems.

1.1.2. Pathogenesis

AD is a multifactorial disorder and results from the complex interaction between defects in skin barrier function, immune abnormalities, environmental and infectious agents and represents a wide spectrum of disease from mild to severe forms. The clinical manifestations of AD vary with age, but four stages can be distinguished (**figure 1**). The first eczematous lesions (age < 2 years) usually arise on the cheeks and the scalp, while scratching causes crusted erosions. During childhood (age 2-12 years), eczema spreads to the flexural areas, the nape and the dorsum of feet and hands. Whether the disease persists in adolescence and adulthood (age 12-60 years), lichenified plaques affect the flexures, head, and neck as well as the hands (chronic hand dermatitis). AD in the elderly (age > 60 years) is a quite underestimated clinical phenotype with extensive eczematous lesions up to erythrodermic features with a strong pruritic component (Bieber, 2008; Bieber *et al.*, 2017). The hallmarks of skin lesions in AD do not differ from other eczemas such as contact eczema and have no essential impact on the diagnosis of AD. Histologic features of acute eczematous lesions are characterised by a red infiltrate with epidermal intercellular oedema (spongiosis) and a perivascular infiltrate of lymphocytes, monocyte-derived macrophages, dendritic cells (DCs), as well as few eosinophils in the dermis. Additionally, retention of the cell nuclei of keratinocytes leads to parakeratosis. In subacute and chronic lichenified lesions, the stratum corneum (SC) is hyperkeratotic, and the epidermis thickened (acanthotic), but with less lymphocytic infiltrates and only minimum spongiosis. Excoriations, papules, and nodules dominate the subacute and chronic form. In addition, non-lesional skin of AD patients contains a low concentration of perivascular T cell infiltrate suggesting minimal inflammation (Bieber, 2010; Thomsen, 2014).

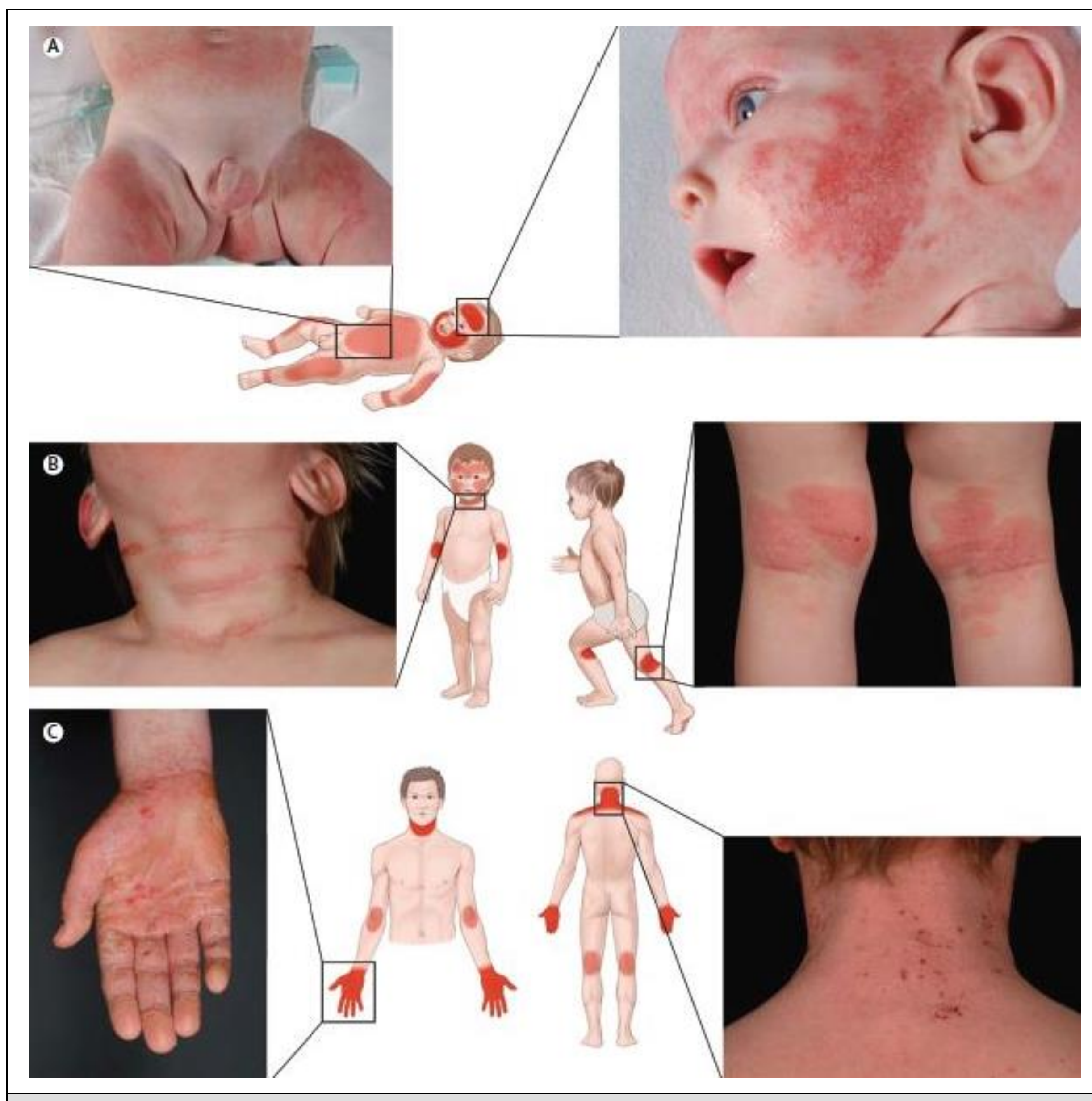

Figure 1. Common sites of eczema outbreaks and clinical feature of atopic dermatitis at different ages. (**A**) In infancy, lesions appear mainly on the cheek and the limbs. (**B**) During childhood, eczema affects the flexural areas, the nape and the dorsum of feet and hands (**C**) In adults, lichenified plaques involve flexures, head and neck, with additional chronic hand eczema. Reprinted by permission from Elsevier (Weidinger and Novak, 2016).

The interactions of environmental and genetic factors play an important role in the rise and development of AD. Consistent with current knowledge about the pathogenesis of AD, genes that are related to skin barrier abnormalities and immune dysregulation are jointly responsible for its aetiology. Until now, several possible AD-related loci have been identified through gene-mapping studies e.g. on chromosomes 3q21, 17q25, 20p and 1q21 (Ellinghaus *et al.*, 2013; Paternoster *et al.*, 2015). The latest includes most of the genes regulating the terminal differentiation and cornification of keratinocytes, the epidermal differentiation complex (EDC) (Cookson *et al.*, 2001). The EDC comprises approximately 60 different genes

encoding proteins involved in epidermal maturation such as filaggrin (FLG), involucrin (IVL), loricrin (LOR), the serine protease inhibitor Kazal type 5, as well as small proline-rich proteins and late cornified envelope proteins (Henry *et al.*, 2012). Among them, FLG (filament-aggregating protein) is a key player in epidermal differentiation and maintaining barrier function. In skin, it is expressed in the granular layer of the SC during terminal epidermal differentiation (Mischke *et al.*, 1996). *FLG* encodes a large (>400 kDa) insoluble polyprotein, profilaggrin, which is the major component of keratohyalin granules. Profilaggrin is proteolytically processed into 10-12 basic 37 kDa FLG monomers. Its degradation is initiated by an increasing intracellular Ca^{2+} concentration and is catalysed by protein phosphatases and serine proteases (Gan *et al.*, 1990; Presland *et al.*, 1992). The FLG monomers bind to keratin intermediate filaments which are cross-linked by transglutaminases to form a highly insoluble keratin matrix. This matrix serves as a template for the attachment of cornified envelope proteins and lipids that form the SC, contributing to its mechanical strength and integrity (Heimall and Spergel, 2012; Sandilands *et al.*, 2009). Subsequently, FLG is cleaved by e.g. caspase 14 into hygroscopic amino acids, including histidine, which are further metabolised to urocanic acid (UCA) and pyrrolidone carboxylic acid (PCA) (Hoste *et al.*, 2011). These acids are an important component of the skin's natural moisturising factor, a key contributor to maintenance of SC hydration and skin antimicrobial defence (Kezic *et al.*, 2008). Over the last decade, loss-of-function mutations in the *FLG* gene have been shown to cause lowered FLG protein expression, reduced NMF levels, and severe early-onset AD and ichthyosis vulgaris (a prevalent keratinisation disorder) with significant increases in total IgE levels (Palmer *et al.*, 2006; Sandilands *et al.*, 2006; Smith *et al.*, 2006; Weidinger *et al.*, 2006). In European populations, there is a carrier frequency of about 10% for at least one mutation in the *FLG* gene, but up to 60% of carriers will not develop atopic diseases (Irvine *et al.*, 2011). Overall, 80% of European AD patients with *FLG* mutations carry one of the two most prominent mutations, R501X and 2282del4 (Gruber *et al.*, 2007; Palmer *et al.*, 2006). However, null mutations are associated with about 50% of moderate to severe AD, but only about 15% of mild to moderate AD may be explained by *FLG* mutations (Brown and McLean, 2009). Functional similarities of other genes in the EDC may potentially alleviate the negative effect of *FLG* mutations (Heimall and Spergel, 2012). Nevertheless, it has been shown that especially patients with loss-of-function mutations in the *FLG* gene suffer from severe forms of AD with a chronic progression, whereas exposure to specific environmental allergens during early time-points of life might increase the risk for eczema (Fallon *et al.*, 2009; Schuttelaar *et al.*, 2009). Besides the possible impact of loss-of-function mutations in the *FLG* gene on skin barrier integrity, other known risk genes for AD playing a role in immune mechanisms, particularly in innate immune signalling, T cell activation, and T cell specification (Barnes, 2010; Guttman-Yassky *et al.*,

2009). For example, the locus on chromosome 5q31-33 has been suggested containing genes for the T helper (Th) type 2 cytokines interleukin (IL-)3, IL-4, IL-5, IL-13, and granulocyte macrophage colony stimulation factor (GM-CSF) (Forrest *et al.*, 1999). Text boxes in **figure 2** show the most plausible candidate genes associated with AD identified through genome-wide association studies.

An intact, healthy skin barrier is a fundamental first line of defence against various microbes, irritants, and allergens. Under healthy conditions, the protective function of the skin is mainly mediated by the SC which provides protection from various environmental stimuli, including allergens, irritants, physical changes, microbial infection, and also prevents transepidermal water loss. The SC consists of corneocytes (dead cornified cells) filled with keratin fibrils, surrounded by a cornified cell envelope of a densely cross-linked layer of proteins (**figure 2A**). An outer lipid layer, primarily made up of long chain ceramides, is esterified to the cornified envelope and forms the matrix for the intercellular lipid bilayers. Corneodesmosomes build up connections between corneocytes and must be degraded for desquamation (Walters and Roberts, 2002; Weidinger and Novak, 2016). The appearance of AD is a chronic, relapsing form of skin inflammation, a disturbance of epidermal-barrier function that leads to dry skin (xerosis), as well as sensitisation to food and environmental allergens. *FLG* deficiency has multiple consequences on epidermal organisation and function. The most direct result of a lack of FLG in AD is a decrease in SC hydration, resulting in increased transepidermal water loss (Kezic *et al.*, 2008). FLG breakdown products play an important role in SC acidification. Hence, a decreased formation of FLG metabolites due to *FLG* deficiency may result in an increased skin pH which is a hallmark of atopic skin (Eberlein-König *et al.*, 2000; Jungersted *et al.*, 2010; Schmid-Wendtner and Korting, 2006). Recently, in a *FLG*–deficient skin equivalent it has been shown, that although histidine-to-UCA/PCA pathway was decreased, the skin surface pH remained unaffected due to a compensatory up-regulation of NHE-1 (sodium/hydrogen antiporter) and $sPLA_2$ (secretory phospholipase A_2) (Vávrová *et al.*, 2014). These findings suggest additional key players involved in the acidification process beside FLG. However, the dysregulation of the skin acidification pathway has an impact on skin lipids as well. Elevated pH causes a decrease in acidic sphingomyelinase catalytic activity and degradation of β-glucocerebrosidase as well as acidic sphingomyelinase which are important enzymes responsible for ceramide synthesis (Hachem *et al.*, 2005). In patients with AD, a decrease in ceramides, which are crucial for the characteristic lipid bilayer organisation, contribute to an increase in skin permeability (Jungersted *et al.*, 2010). Additionally, tight junction proteins, which mediate keratinocyte adhesion in the stratum granulosum, are thought to contribute to this diffusion barrier. A down-regulation of claudin-1 (CLDN-1), which was observed in AD patients, may enhance the uptake of allergens by leading to impaired SC and tight junction

barriers (De Benedetto *et al.*, 2011). However, a compensatory up-regulation of tight junction proteins in a *FLG*-deficient skin equivalent has been demonstrated recently, that was then diminished after cytokine exposure (for details see **chapter 2.1.2.** and (Hönzke *et al.*, 2016b)). These results suggest that tight junction impairment in AD is secondary to inflammatory processes and is not directly linked to a lack in *FLG*.

These structural abnormalities of the epidermis lead to transepidermal water loss and increased penetration of allergens and microbes into the skin (**figure 2B**). The most infectious microbes involved in AD is *Staphylococcus aureus* (*S. aureus*), which colonises in approximately 90% of AD patients (Gong *et al.*, 2006). *S. aureus* contributes to disease aggravation and chronic manifestation through the production of proteases, stimulation of innate signalling pathways, the release of enterotoxins, and IgE-mediated sensitisation and direct induction of mast cell degranulation (Kobayashi *et al.*, 2015; Nakamura *et al.*, 2013). Increased colonisation of *S. aureus* is strongly linked to a raised severity of the disease. Furthermore, impaired innate immune responses also seem to contribute to increased bacterial and viral infections in patients with AD (Williams and Gallo, 2015).

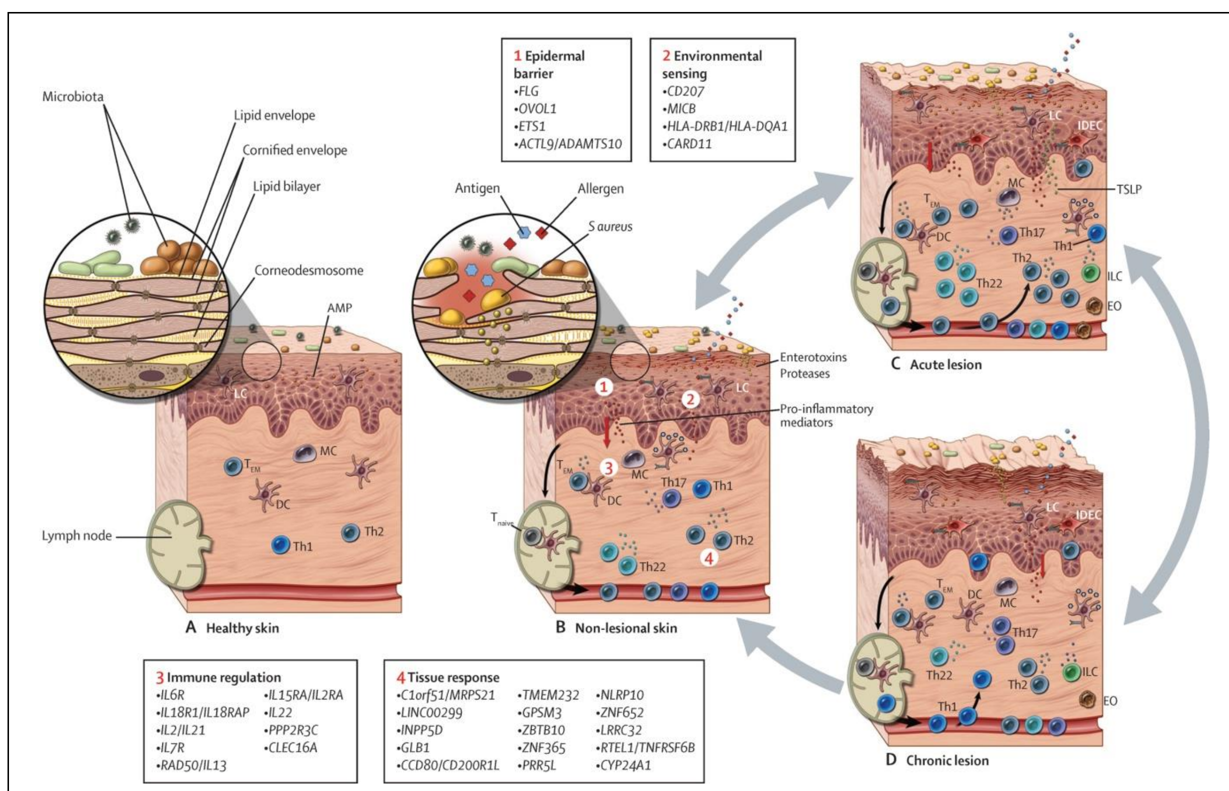

Figure 2. Mechanisms in the pathogenesis of AD. (**A**) Skin structure and composition of healthy skin. (**B**) Non-lesional skin of atopic dermatitis (AD) patients with epidermal barrier dysfunction and release of innate immune cytokines with subsequent signs of low-level inflammation. Text boxes show the most plausible candidate genes related to the development of AD. (**C-D**) Interplay between barrier dysfunctions and immunological dysregulations in the acute and chronic phases of AD. Figure reprinted by permission from Elsevier (Weidinger and Novak, 2016).

Innate and adaptive immunity have a major role in the development and maintenance of AD. This process involves complex interactions of T cells, antimicrobial peptides (AMPs) and keratinocytes. As components of innate immunity, AMPs, which e.g. include proteins from the β-defensin family, are crucial in the defence against microbial pathogens and in maintaining epidermal barrier homoeostasis. It was shown, that Th2 cytokines IL-4 and IL-13 inhibit skin production of AMPs, predisposing AD skin to *S. aureus* infections, which further contribute to skin inflammation and barrier defects (Howell *et al.*, 2006; Kisich *et al.*, 2008; Mu *et al.*, 2014). Uncontrolled activation of Th2 cells, which is a critical characteristic of the pathogenesis of AD, is crucial for inducing isotype class switching to IgE synthesis and therefore sensitisation (Czarnowicki *et al.*, 2014). The Th2 cytokines IL-4, IL-5 and IL-13, and IL-31, as well as the Th22 cytokine IL-22 are significantly increased in lesional and non-lesional skin in the acute phase of AD (**figure 2B-C**) (Bilsborough *et al.*, 2006; Neis *et al.*, 2006; Nograles *et al.*, 2009). Induction and development of Th2 responses are further mediated via IL-25, IL-33, and thymic stromal lymphopoietin (TSLP) (Paul and Zhu, 2010). Additionally, acute lesions are also associated with, to a lesser degree, IL-23/Th17 cell infiltration as well as elevated expression of S100-calcium binding proteins 7-9 (Guttman-Yassky *et al.*, 2008; Koga *et al.*, 2008; Nograles *et al.*, 2009). Further, Th2 cytokine-producing type 2 innate lymphoid cells have been found at increased levels in AD lesions and contribute to local inflammation (Salimi *et al.*, 2013). Besides continuous recirculation through the skin, antigen-primed T cells persist as resident effector memory cells in local skin pools and are therefore able to initialise rapid recall responses (Islam and Luster, 2012). In chronic lesions, the T cell infiltrate is further expanded but contains both Th2 and Th1 cells with fewer Th22 and Th17 cells than in acute lesions (**figure 2D**) (Gittler *et al.*, 2012). The switch from a Th2 to a more Th1 profile in the chronic phase of AD may be conditioned by a predominant type of DCs, the inflammatory dendritic epidermal cells that belong to the group of myeloid DCs (Novak and Bieber, 2005). The switch is characterised by the production of interferon gamma (IFNγ), IL-12, and GM-CSF, coinciding with the infiltration of macrophages and eosinophils (Yamanaka and Mizutani, 2011). Recruitment of T cells into the skin of AD patients, particularly by $CD4^+$ but also $CD8^+$ T cells, is conducted by a complex network of mediators that contribute to chronic inflammation (Bang *et al.*, 2001; Masopust and Schenkel, 2013). Inflammatory cells and keratinocytes in lesional skin express high levels of chemoattractants, including the keratinocyte-derived TSLP which is known to induce DCs to trigger Th2 inflammatory response (for details see **chapter 1.3. and 2.1.3.**) (Leyva-Castillo *et al.*, 2013). After the initial T cell trafficking into the skin, Th2 cell recruitment is significantly increased after T cell activation by mature DCs in the draining lymph node, where T cells also undergo rapid proliferation (Caux *et al.*, 2000). Recently, regulatory T (T_{reg}) cells ($CD4^+CD25^+$) have been found to play a role in allergy since they have the ability to suppress

the induction and proliferation of T cells which is also important in the prevention of autoimmune diseases (Beissert *et al.*, 2006; Verhagen *et al.*, 2006). It has been shown, that mutations of the nuclear factor *FOXP3* (forkhead box P3; scurfin) in these cells result in hyper IgE, food allergy and dermatitis and may thereby contribute to skin inflammation (Torgerson *et al.*, 2007). Concordantly, a recent study showed that children with low T_{reg} cell numbers at birth, due to prenatal tobacco smoke exposure, had a predisposition for AD during the first three years of life (Herberth *et al.*, 2014).

Whether skin barrier abnormalities cause immune dysregulation ('outside-inside' hypothesis) or immune dysregulation causes barrier changes ('inside-outside' hypothesis), it is important to distinguish that *FLG* mutations do not occur in all AD patients, and contrariwise, individuals with *FLG*-null alleles may not develop AD (Elias *et al.*, 2008). The pathophysiology of AD remains to be fully elucidated, but regardless of "outside-inside and inside-outside hypothesis", all patients show a combination of immune dysregulation and skin barrier dysfunction.

1.1.3. Diagnosis and Management

Diagnosis of AD depends primarily on the patient's and family's history as well as on the clinical phenotype according to morphology and distribution of the lesions at different stages (Bieber, 2010). As already mentioned, AD has a wide spectrum in terms of severity, ranging from a mild to a severe disease pattern which is best classified by using validated scoring systems (Schmitt *et al.*, 2015), such as the SCORAD (Score in Atopic Dermatitis) (European-Task-Force-on-Atopic-Dermatitis, 1993) or EASI (Eczema Area and Severity Index) (Housman *et al.*, 2002), based on revised Hanifin and Rajka diagnostic criteria (Hanifin and Rajka, 1980). Adult AD frequently shows atypical clinical and morphological features with related diagnostic challenges, because of missing validated diagnostic criteria (Napolitano *et al.*, 2016). Skin tests and investigation of allergen-specific serum IgE concentrations are useful in the search of provocation factors such as food or environmental allergens and helps to exclude other conditions like contact dermatitis or psoriasis (Darsow *et al.*, 1995; Eichenfield *et al.*, 2017). Nevertheless, analytical results should always be discussed in the context of the patient's history and skin tests. Many patients show high total or allergen-specific serum IgE concentrations, although their absence does not exclude an AD diagnosis. Especially in the set-up and evaluation of clinical trials, there is a need for reliable and measurable outcome parameters. Therefore, the utilisation and validation of appropriate biomarkers are of high interest. Not only in the area of clinical trials but also in the new field of personalised medicine, biomarkers will allow an optimised prevention and treatment with the available drugs to find the best benefit/risk ratio for the individual patient. Prominent candidates related to disease severity include TSLP, thymus- and activation-

regulated chemokine (TARC; CCL17), macrophage-derived chemokine (MDC; CCL22), cutaneous T-cell-attracting chemokine (CCL27), IL-31, IL-33, IL-22, IL-18, and IL-16. However, none of the mentioned candidate biomarkers have attained validation (Andersen *et al.*, 2016; Bieber *et al.*, 2017; Nygaard *et al.*, 2016; Ungar *et al.*, 2017). Screening for mutations in genes encoding for epidermal structural proteins, such as FLG, also represent an additional diagnosis tool to identify subjects with a high risk of suffering from AD, but the feasibility in the daily practice is very low.

Beside diagnostic evaluation, management of AD remains a clinical challenge where the primary goals are to improve skin barrier function, to prevent microbial colonisation and suppress inflammation. The therapeutic measures should always be adapted to the severity of the condition, while education of the patient or the parents of an affected child is highly important (Werfel *et al.*, 2016). The chronic and/or relapsing nature of AD causes difficulties to propose long-term management strategies. Most important is the daily skin care even in the absence of eczematous lesions, whereas dry skin is one main characteristic of AD. Bathing with hypoallergenic soaps or non-soap cleaners increases skin hydration while eliminating residual bacteria, crusting, and irritants, but transepidermal water loss can occur through evaporative losses after bathing. The use of moisturisers directly after bathing is crucial to maintain cutaneous hydration and improve skin barrier function (Cheong, 2009; Eichenfield *et al.*, 2017). Usually, emollients contain a moisturiser such as urea or glycerol, and should not contain possible allergens to reduce the risk of allergic sensitisation. While lessen the symptoms of AD like erythema and pruritus, their use also decreases the amount of prescription topical agents needed for suitable control of AD (Grimalt *et al.*, 2007). In patients with recurrent skin infections the guidelines recommend consideration of proactive antimicrobial bleach baths rather than the use of topical and systemic antibiotics (Eichenfield *et al.*, 2017). If AD is not controlled by moisturisers alone, the use of topical corticosteroids like dexamethasone or betamethasone dipropionate on acute lesions is recommended as first line therapy (Broeders *et al.*, 2016). They act on a variety of immune cells by binding to their cytoplasmic glucocorticoid receptor whereby they suppress the release of pro-inflammatory cytokines such as IL-4, IL-5 or IL-13 (Leung *et al.*, 2004). Most topical corticosteroids are proven to be safe and effective. However, side effects, such as skin atrophy, limit their long-term use, but are directly related to the potency ranking of the compound and the length of application (Callen *et al.*, 2007). Alternatively, a second class of anti-inflammatory topical therapy is available for short-term and intermittent treatment. Topical calcineurin inhibitors, like pimecrolimus or tacrolimus, inhibit the calcineurin-dependent T cell and mast cell activation, and prevent the production of pro-inflammatory cytokines and mediators of the inflammatory response in AD. They provide a good anti-inflammatory effect with only minor side effects (Chen *et al.*, 2010; Chia and Tey, 2015).

Combined therapy with emollients should be routine during the period of treatment with topical anti-inflammatory agents. AD patients usually benefit from natural sun exposure. Hence, children and adults with moderate to severe AD often require phototherapy with different spectra of UV light, like narrow-band UVB (Clayton *et al.*, 2007) and UVA1 (Rombold *et al.*, 2008), but skin cancer history and the use of photosensitising medications might limit its use. The treatment with photochemotherapy including psoralen and UVA (PUVA) is suggested for patients with severe extensive AD only (Sidbury *et al.*, 2014a). Systemic immunomodulating agents are an additional treatment option for the management of chronic and/or severe states of AD (Schmitt *et al.*, 2017). Cyclosporine A is an immunosuppressant that reduces Th1/Th2, Th17, Th22, T_{reg} cells, B cells, as well as DCs, and down-regulates transcription of cytokines like IL-2 (Malik and Guttman-Yassky, 2017). Several studies have demonstrated its efficacy in both children and adults with severe, refractory AD, although side effects like nephrotoxicity and hypertension limit its long-term use and make a close monitoring mandatory (Hernández-Martín *et al.*, 2017; Schmitt *et al.*, 2010; Sibbald *et al.*, 2015). Other second-line therapy options for severe flares are the off-label use of the immunosuppressants azathioprine (Meggitt *et al.*, 2006), methotrexate (Weatherhead *et al.*, 2007) and mycophenolate mofetil (Haeck *et al.*, 2011), as studies provide evidence for their efficacy in improving skin symptoms. Nevertheless, their application is limited due to insufficient data about optimal dosing, duration of therapy, and monitoring protocols. The use of systemic glucocorticoids, such as prednisolone, is restricted to a short-term treatment of severe, acute exacerbations due to several steroid-associated side effects including steroid rebound (Hengge *et al.*, 2006; Schmitt *et al.*, 2010).

Although many studies confirm the positive effects of conventional treatments, the satisfaction and compliance are usually very low in AD patients. There is a large need that exists for safer, more efficacious systemic therapeutics for moderate to severe AD. Due to the current progress in the understanding of the pathomechanisms of AD, there is the chance for the development of new strategies and therapies in its management (Furue *et al.*, 2017). Biologics are commonly used in dermatology, in particular for the treatment of psoriasis and autoimmune diseases, but so far only one of the available agents has been approved for AD therapy while others were tested in individual patients or small clinical trials only (Simon and Bieber, 2014). Omalizumab is an anti-IgE antibody which is approved for asthma therapy. Since AD patients usually display very high levels of IgE, the potential benefit of omalizumab was investigated in small patient groups. Although the IgE levels were significantly reduced, no significant effects on clinical improvement were detected (Heil *et al.*, 2010; Krathen and Hsu, 2005). Additionally, ligelizumab, a more potent IgE antagonist compared to omalizumab (Arm *et al.*, 2014), was tested in a phase II trial (NCT01552629), with no clinical benefits compared to placebo and cyclosporine A. However, owing to its

disease relevance, the role of IgE targeting should still be further investigated in clinical trials. Several other biologics are currently in ongoing clinical trials. These include tralokinumab/lebrikizumab (anti-IL-13) (Simpson *et al.*, 2016a; Wollenberg *et al.*, 2017), nemolizumab (anti-IL-31) (Nemoto *et al.*, 2016), and ustekinumab (anti-IL-23) (Weiss *et al.*, 2017), all of which have shown promise for therapeutic benefits in selected patients. Dupilumab, an interleukin 4 receptor-α (IL-4Rα) antagonist which thereby blocks the two key mediators of Th2 pathway IL-4 and IL-13, is highly efficient for controlling skin disease in patients with moderate to severe AD (Beck *et al.*, 2014; Hamilton *et al.*, 2014). It has shown excellent safety and efficacy in phase II trials, where it was well-tolerated with mostly transient side-effects, comparable with the placebo groups (Thaci *et al.*, 2016). Furthermore, the fast and robust clinical AD improvement was confirmed in two large phase III studies (Simpson *et al.*, 2016b). In March 2017, dupilumab (Dupixent®) received its first global approval in the USA followed by its EU registration in September 2017, where it is permitted for the treatment of adult patients with moderate to severe AD whose disease is not adequately controlled with topical therapies or when those therapies are not advisable (Boguniewicz, 2017; European-Medicines-Agency, 2017a). Additionally, other treatment options are under clinical evaluation like the efficiency of small molecule agents such as JAK inhibitors (tofacitinib) (Levy *et al.*, 2015), histamine H4 receptor antagonists (Murata *et al.*, 2015), and phosphodiesterase 4 inhibitors (apremilast) (Samrao *et al.*, 2012).

So far, no primary prevention strategy has been established. Most interventions focused on allergen avoidance or immunomodulation via dietary supplements, but there is no clear evidence for the effectiveness of measures such as elimination diets, omega-3 or omega-6 fatty acid supplementation, interventions with prebiotics or probiotics, and vitamin D or E supplementation (Sidbury *et al.*, 2014b). Nevertheless, several studies reported that a daily full-body application of moisturisers from birth reduced the incidence of AD in high-risk infants by 30-50% (Horimukai *et al.*, 2014; Simpson *et al.*, 2014). The prognosis for patients with AD is overall positive since most children outgrowing the condition by early adolescence. However, patients with severe courses and attendant atopic conditions, like asthma and allergic rhinitis, have a higher risk to experience poorer outcomes. Hence, early and disciplined management could improve the outcome and quality of life for patients living with AD.

1.2. Organotypic Skin Equivalents - Chances and Limitations

1.2.1. Organotypic Skin Equivalents in Basic and Preclinical Research

Successful launch of a commercial drug is generally comprised of two major steps: discovery and characterisation of a novel active substance in the preclinical phase and drug evaluation in terms of safety and efficacy in the clinical phase. In 2017, the EMA (European Medicines Agency) recommended the approval of 92 drugs with 35 new active substances (European-Medicines-Agency, 2017b). This followed years of intensive research. Recently, the cost of drug development was estimated at US$0.6-2.7 billion per successfully launched drug (DiMasi *et al.*, 2016; Prasad and Mailankody, 2017). Notably, only one in ten of these drugs entering phase I clinical trials was finally approved. Overall, animal models have been used in the preclinical drug discovery process to identify pathophysiological mechanisms, evaluate their mode of action, discover new biomarkers and drug targets, as well as toxicological effects (McGonigle and Ruggeri, 2014). The high failure rate of drugs evaluated in clinical trials strongly indicates the necessity for improved preclinical testing and animal alternative methods that are also in line with the 3Rs guidelines (Bergers *et al.*, 2016). According to the ethical principles of the '3R rule' for reduction, replacement and refinement of animal experiments, scientific efforts are based on limiting the suffering of animals, as well as the numbers used in experimental work or the development of suitable *in vitro* alternatives to animal testing (Russell and Burch, 1959). In 1986, the EU Directive 86/609/EEC on the protection of animals used for experimental purposes had already banned the use of animal experiments when scientifically approved alternatives exist (European-Commission, 1986). The EU Directive 2010/63/EU requires the evaluation and authorisation of all research projects and training activities involving the use of animals (European-Commission, 2010). In Germany, Directive 2010/63/EU is implemented into the revised Animal Welfare Act, which took effect on July 2013 (TierSchG, 2006). However, the implementation of the new EU Chemicals Regulation REACH (Registration, Evaluation and Authorization of Chemicals) in 2006 led at first to an increased need for animal experiments to achieve more precise data for the risk assessment of preliminary estimated 30,000 chemicals on the EU market to be safe for humans and the environment (European-Commission, 2006; Rovida and Hartung, 2009). By contrast, the EU Cosmetics Directive 1223/2009, which became mandatory in full by 2013, prohibits the testing of any cosmetic products or individual ingredients on animals (European-Commission, 2009). The regulatory measures for the reduction or replacement of animal experiments introduced by politics, science and industry are driving forward the efforts currently made to develop *in vitro* alternative methods for already established *in vivo* assays. A total of about 11.5 million animals were used in the 27 member states of the EU within 2011, according to a report of the European Commission from December 2013. This is a reduction of over half a million animals used in the EU from the number reported in 2008

(European-Commission, 2013). Nevertheless, an increase of animals used in research in general and a lack of efforts to implement the 3Rs, particularly reduction, is demonstrated as seen in the rising number of articles reporting animal use in four of the highest ranking journals - *Science*, *Cell*, *Nature* and *Nature Biotechnology* - between 1983 and 2007 (Ormandy *et al.*, 2009). Animal models can be of great value to the drug discovery and development process, but preclinical drug evaluation often lacks predictive power. Primary reasons for unreliable preclinical drug evaluation include interspecies-related differences between human drug targets and their animal orthologues, poor study design, and irreproducible tests, as they rarely consider randomisation or blinding (van Luijk *et al.*, 2014). Toxic effects in rodents and humans are poorly correlated, since the absence of toxicity in animals seems not to provide evidence to the absent toxicity in humans (Bailey *et al.*, 2014). Well known discrepancies in both innate and adaptive immunity, inflammatory responses, and morphological features e.g. in skin structure (Mestas and Hughes, 2004; Pasparakis *et al.*, 2014; Seok *et al.*, 2013) have initiated a lively debate over the prospects and limitations of murine models (Leist and Hartung, 2013; Osuchowski *et al.*, 2014; Warren *et al.*, 2015). The process of drug development requires a significant investment of time and financial resources, wherein failures at the last stage become a reason for fatal miscarriage in drug discovery. With this in mind, there is a real need for innovations in alternative technologies that can give reliable predictions of efficacy and toxicology of experimental compounds early in the drug discovery process.

Besides animal testing, for the evaluation of skin irritation, corrosion or absorption of chemical substances or dermal compounds, the use of human donor skin *ex vivo* is limited. Thus, alternatives are required. The development of valid human cell-based approaches for biomedical testing started with monolayer cultures of human cells or cell lines. However, due to the lack of cell-cell interactions and a sufficient skin barrier, monolayer cell cultures frequently overestimate potential toxic effects (Gibbs, 2009). Human-cell derived reconstructed human epidermis (RHE) and reconstructed full-thickness human skin (RHS) mimic the morphology, differentiation, and growth of native human skin *in vitro*, enabling crosstalk between different cell types as well as cell-matrix interactions. These organotypic constructs have become increasingly important as alternative approach to animal tests. For regulatory purposes and broader use, new *in vitro* toxicity tests must first undergo successful validation. RHE-based test procedures for *in vitro* evaluation of skin corrosion (OECD-431, 2014), assessment of skin irritation (OECD-439, 2015), and re-evaluation of phototoxicity of chemical substances (OECD-432, 2004) are already approved by the OECD (Organization for Economic Cooperation and Development). Concerning skin absorption testing, though the OECD has approved pig and mouse skin to test percutaneous drug absorption *in vitro* (OECD-428, 2004), additional work demonstrating the validity of reconstructed skin

equivalents for this work has also been conducted (Ackermann *et al.*, 2010; Schäfer-Korting *et al.*, 2008). Beside the increasing number of individually generated "in-house" skin equivalents within academia, there is a broad interest in industry, particularly within cosmetic companies, to develop and market artificial human skin constructs. Commercially available constructs such as the RHEs EpiDerm™ (Mattek Corp., USA), SkinEthic® and EPISKIN® (L`Oréal, France), or the RHSs EpiDerm-FT™ (Mattek Corp., USA) and Phenion® FT Skin Model (Henkel, Germany), have been the subject of several validation studies which finally resulted in the integration of these RHEs/RHSs in respective OECD guidelines as alternatives to animal testing (Kandárová *et al.*, 2006; Netzlaff *et al.*, 2005; Spielmann *et al.*, 2007). In addition to the aforementioned uses, skin equivalents are being applied to a wide variety of fields, including wound healing testing (for details see **chapter 2.2.1. and 2.2.2.**) (Koehler *et al.*, 2017a; Koehler *et al.*, 2017b; Küchler *et al.*, 2010) and as test model in which the influence of even single parameters in skin pathologies can be investigated without interference from factors such as the influence of endothelialisation of epidermal differentiation processes (for details see **chapter 2.2.3.**) (Groeber *et al.*, 2016; Khiao In *et al.*, 2015).

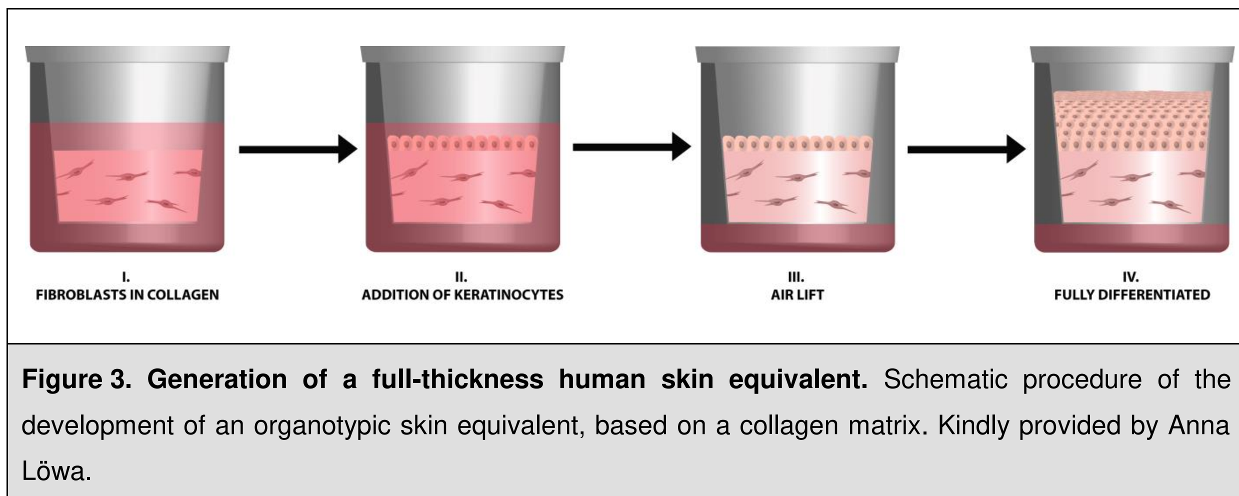

Figure 3. Generation of a full-thickness human skin equivalent. Schematic procedure of the development of an organotypic skin equivalent, based on a collagen matrix. Kindly provided by Anna Löwa.

Reconstructed skin equivalents consist of normal human-derived epidermal keratinocytes (NHK) and, in the case of RHS, normal human-derived dermal fibroblasts (NHDF). The dermal compartment of RHS may be generated by embedding fibroblasts in a matrix consisting of collagen (Carlson *et al.*, 2008), seeding them on artificial scaffolds (Sun *et al.*, 2007), using scaffold-free approaches via 3D bioprinting (Pourchet *et al.*, 2017) or human de-epidermised dermis (Prunieras *et al.*, 1983). Subsequently, keratinocytes are seeded on top of the dermal equivalent to form a multi-layered matrix. After proliferation, the cells are exposed to the air-liquid interface to induce differentiation (**figure 3**). *In vitro* generated RHS closely mimic their *in vivo* counterparts in terms of morphological structure (Ponec *et al.*, 2002), expressed differentiation markers (Netzlaff *et al.*, 2005), lipid

composition and organisation (Ponec *et al.*, 2000), and metabolic capacity (Grohmann *et al.*, 2017).

With the improvements achieved in recent years, reconstructed skin equivalents have become an essential tool not only as an alternative to animal testing, but also for investigative dermatology, especially for addressing skin homeostasis, skin repair, skin regeneration, and skin diseases. However, these skin equivalents can still be improved. The increased SC thickening seen in RHE and RHS over prolonged cultivation times limits the use of the constructs to a few weeks. This thickening is likely caused by the missing desquamation process and a lack of tissue renewable stem cells (Ponec, 2002). The well-known weaker barrier function of reconstructed skin equivalents as compared to human skin might influence the permeation and penetration profile of chemicals or pharmaceutical substances (Küchler *et al.*, 2013; Netzlaff *et al.*, 2007). It is thought deviations in lipid lamellar organisation plus differences in free fatty acid profiles and contents, as well as a not fully developed basement membrane absent of hemidesmosomes result in a lower barrier function in reconstructed skin equivalents (Ponec *et al.*, 2002; Thakoersing *et al.*, 2012). Several groups have attempted to overcome this problem by improving culture conditions through specific medium supplements or dynamic nutritional supply with additional variable mechanical shear stress using a bioreactor (Strüver *et al.*, 2017; Thakoersing *et al.*, 2015). Although the permeation of xenobiotics might be overestimated in skin equivalents using e.g. the Franz diffusion cell set-up, these are still in clear concordance with those obtained in human skin *ex vivo* (Schäfer-Korting *et al.*, 2008; Van Gele *et al.*, 2011). The absence of skin appendages like hair follicles and sweat glands, as well as adipose tissue and neuronal cells, might also contribute to altered results obtained in equivalent *in vitro* studies. Furthermore, the lack of blood flow in reconstructed skin equivalents – a key factor in cell nutrition, crosstalk, metabolism, and xenobiotic permeation, as well as a source of endogenous mechanical action – must also be considered for their research and clinical applications. Attempts to remedy this are however in motion, including the implementation of endothelial cells into the skin equivalents (Khiao In *et al.*, 2015), and the development of innovative fluidic devices like bioreactors (Strüver *et al.*, 2017) or skin-on-chip technologies (Wufuer *et al.*, 2016). Providing a fully vascularised dermal compartment would feature an optimal environment for immune cells, which are difficult to maintain within *in vitro* skin equivalents. For the assessment of skin irritation and sensitisation, having a functional immune system in skin equivalents including e.g. lymphocytes, monocytes and macrophages, remains a major goal. The implementation of immune cells into skin equivalents has been tried by a few groups (Bock *et al.*, 2018; Kosten *et al.*, 2015; van den Bogaard *et al.*, 2014), but so far standardised protocols are missing. However, such models require a high level of logistical organisation and man hours. A continuous supply of fresh blood donations is required to

derive primary immune cells, the correct cultivation of which is complicated by high donor variability. Therefore, it should not be the overall goal to mimic the *in vivo* situation exactly, but to establish reliable test systems that allow for the generation of reproducible and predictive data for its particular use.

Despite their limitations, the development and use of reconstructed skin equivalents has expanded enormously over the last decade, and they are already accepted as a reliable and useful *in vitro* research tool and as an alternative to animal testing for fundamental (patho)physiological investigations. Nevertheless, further progress is required, especially with regard to the necessary improvements to skin barrier function and culture period.

1.2.2. Organotypic Skin Disease Equivalents - Alternative Methods in Research on Atopic Dermatitis

Besides normal reconstructed skin, research efforts are also being made in the field of reconstructed skin disease models. Though skin diseases such as AD, ichthyosis vulgaris, psoriasis, and non-melanoma skin cancer are a major public health burden, their treatment options are limited and their underlying pathologies are not fully understood. Studies in humans are challenging to conduct and raise ethical issues. A number of mouse models for AD have been developed such as the Nc/Nga mouse (Jin *et al.*, 2009), however ethical concerns alongside limited clinical transferability of results derived from these have led to controversial discussions regarding their use (Ewald *et al.*, 2017; Seok *et al.*, 2013). Today, a variety of *in vitro* skin disease models based on human skin equivalents are available in addition to animal and human cell models. They enable not only the investigation of new dermal or systemic therapeutic options, but also give a deeper insight into the pathophysiology of the respective skin disease. These skin equivalents include models of wound healing (Küchler *et al.*, 2010; Rossi *et al.*, 2015; Xie *et al.*, 2010), ichthyosis vulgaris (Eckl *et al.*, 2011), non-melanoma skin cancer (Commandeur *et al.*, 2011; Martins *et al.*, 2009; Zoschke *et al.*, 2016) and AD (Küchler *et al.*, 2011; Mildner *et al.*, 2010; Pendaries *et al.*, 2014). There is a broad spectrum of *in vitro* human-based AD models generated from varied cell sources and construction protocols. These constructs consist either of NHKs, patient-derived keratinocytes, or immortalised human cell lines such as HaCaT, the latter of which do not reflect the intrinsic genetic variability found in constructs cultivated using primary keratinocytes (Seo *et al.*, 2012). Beside the use of patient-derived cells (van Drongelen *et al.*, 2015), AD like phenotypes can be induced in skin equivalents by supplementation of AD associated cytokines, such as IL-4 and IL-13 (Danso *et al.*, 2014), or by small interfering RNA (siRNA) mediated knock down of disease-related genes such as *FLG* (for more details see **chapter 1.1.2.**) (Mildner *et al.*, 2010). Although all the above approaches differ in the variables that determine the organotypic AD model, they collectively

provide little information of the underlying immune component in AD. Therefore, there is a need for more multifaceted skin equivalents that represent the whole complexity of AD including the loss of *FLG*, the reduction in skin barrier function, and characteristic immune responses, to bring us even closer to disease aetiology and consequently to new therapeutic options (for more details see **chapter 1.1.3.**).

Today, the use of skin disease equivalents offers a broad range of scientific applications. It is well-known that the molecular weight cut-off for effective permeation across skin is approximately 500 Da. However, an impaired skin barrier, a common feature of diseased skin like in AD, leads to a higher absorption of compounds that normally would not be able to overcome a healthy, intact skin barrier (Halling-Overgaard *et al.*, 2016; Kezic *et al.*, 2014). This offers the opportunity not only to deliver therapeutic compounds for a local or systemic effect past the SC such as glucocorticoids (Zöller *et al.*, 2008), but also to replace dysfunctional disease-related proteins like transglutaminase 1 via e.g. thermoresponsive nanogels (Gerecke *et al.*, 2017; Witting *et al.*, 2015) or liposomes (Aufenvenne *et al.*, 2013). Additionally, skin disease equivalents can serve as test matrix for the intra-epidermal and dermal delivery efficiency of drug-loaded nanocarrier systems such as CMS (core-multishell nanocarriers) (Edlich *et al.*, 2018; Hönzke *et al.*, 2016a). These systems can improve the therapeutic outcome of the treatment of chronic inflammatory skin diseases.

1.3. Thymic Stromal Lymphopoietin (TSLP)

1.3.1. The Biology of TSLP

Epithelial cell-derived TSLP was first identified in 1994 from the conditioned medium of the murine thymic stromal cell line Z210R.1, as a growth factor that supported B cell lymphopoiesis *in vitro* (Friend *et al.*, 1994). Subsequently, a cDNA clone encoding human TSLP was isolated by *in silico* methods (Quentmeier *et al.*, 2001; Reche *et al.*, 2001). TSLP is an IL-7-like, four-helix bundle cytokine with two *N*-glycosylation sites and six cysteine residues (Liu, 2009). It is mainly expressed by epithelial cells at barrier surfaces including the skin, the gastrointestinal tract and airways (Takai, 2012). Additionally, recent studies have shown, that other cell types such as fibroblasts, smooth muscle cells, mast cells, basophils, cancer and cancer-associated cells have the potential to produce TSLP as well (De Monte *et al.*, 2011; Sokol *et al.*, 2008; Soumelis *et al.*, 2002). The TSLP receptor (TSLPR) is a heterodimeric receptor complex consisting of the IL-7Rα and the TSLP-binding subunit (CRLF2). In comparison to its binding affinity alone, the TSLPR reveals only in combination with the IL-7Rα a high binding affinity to TSLP (Verstraete *et al.*, 2017; Ziegler and Liu, 2006). The functional TSLPR is expressed by multiple hematopoietic cell populations like T cells, B cells, natural killer T cells, monocytes, basophiles, eosinophils and DCs, as well as some non-hematopoietic cell types such as epithelial cells (Ziegler *et al.*, 2013). On binding its receptor complex, TSLP activates a variety of signal transduction pathways. Previous studies have shown that stimulation of the receptor complex by TSLP induces the phosphorylation and activation of Janus kinases (JAKs). Subsequently, activated JAKs regulate the activity of all known signal transducers and activators of transcription (STAT) factors except for STAT2 (Arima *et al.*, 2010; Rochman *et al.*, 2007; Wohlmann *et al.*, 2010). In addition, several other proteins such as ERK 1/2 (extracellular signal-regulated kinase 1/2) and ribosomal protein S6 kinase have also been shown to be activated on TSLP stimulation (Zhong *et al.*, 2014).

As reviewed above, TSLP exerts its function on a broad range of tissue and cell types. Initially, human TSLP was found to act preferentially on myeloid cells. It enhances the maturation and function of CD11c$^+$ DCs which results in high expression levels of MHC class II molecules CD40 and CD80 (Soumelis *et al.*, 2002). DCs directly activated by TSLP express OX40 ligand (OX40L; CD252) to prime naïve CD4$^+$ T cells to differentiate into pro-inflammatory Th2 cells. The activation is characterised by the release of high amounts of IL-4, IL-5, IL-13, and tumour necrosis factor alpha (TNFα), but not IL-10 and IFNγ (Ito *et al.*, 2005; Soumelis *et al.*, 2002). Further studies reveal that TSLP-mediated Th2 response in the lung and skin of wild type mice is significantly inhibited by neutralisation of OX40L *in vivo* (Seshasayee *et al.*, 2007). TSLP-activated DCs induce the proliferation of naïve CD4$^+$ T cells at higher levels than CD40 ligand (CD40L)- or lipopolysaccharide-activated DCs.

Interestingly, while last-mentioned activated DCs produce IL-12, TNFα and IL-1, TSLP activated DCs produce high levels of the Th2-attracting chemokines TARC/CCL17 and MDC/CCL22, but no IL-12 (Soumelis *et al.*, 2002). More importantly, the simultaneous stimulation of DCs with TSLP and CD40L results in the production of high levels of IL-12, so that naïve $CD4^+$ T cells polarised by TSLP/CD40L DCs produce both Th1 and Th2 cytokines (Watanabe *et al.*, 2005). Therefore, TSLP can promote the maintenance of a Th2-driven inflammation even in the presence of IL-12.

1.3.2. Differences of TSLP Biology between Mice and Humans

TSLP exhibits interspecies-related functions and may function differently in humans and mice. The mouse *TSLP* gene is located on chromosome 18, while the human *TSLP* gene is located on chromosome 5q22.1, next to the atopic cytokine cluster 5q31. The homology between mouse and human TSLP is only 43% and 35% for the TSLPR, with no cross-reactivity between the species (Quentmeier *et al.*, 2001; Sims *et al.*, 2000). Despite the poor amino acid sequence homology between murine and human TSLP and TSLPR, the fact that high binding of TSLP to its receptor in both species requires IL-7Rα suggests that human and mouse TSLP/TSLPR are orthologous (Pandey *et al.*, 2000; Park *et al.*, 2000). Although the role for TSLP/TSLPR in both species seems to be similar, the function of the IL-7Rα chain demonstrates interspecies-related differences. Mice deficient in the IL-7Rα subunit show diminished T and B cell development (Peschon *et al.*, 1994), while humans with severe combined immunodeficiency due to IL-7Rα mutations lack T cells but have normal B cell numbers (Puel *et al.*, 1998). In contrast to mouse TSLP which stimulates the growth and differentiation of pre-B cells and peripheral $CD4^+$ T cells (Al-Shami *et al.*, 2004; Chappaz *et al.*, 2007; Vosshenrich *et al.*, 2003), human TSLP acts mainly on myeloid DCs (Liu *et al.*, 2007; Soumelis *et al.*, 2002). Less clear is the exact role of TSLP in human B cell development. To date, there has only been one *in vitro* study, reporting the influence of TSLP and IL-7 in the proliferation and differentiation of human fetal B cell precursors (Scheeren *et al.*, 2010). A new *in vivo* study uses a chimeric mouse model that is able to produce TSLP to study the effect of human TSLP on human B cell development. The results from the latest study demonstrate that, despite the almost absent expression of the receptor, TSLP can support human B cell lymphopoiesis (Francis *et al.*, 2016). In mice, the effect of TSLP on DCs remains unclear. The first assumption, that TSLP does not have any biological effects on mouse DCs, was disproved by a study that showed that TSLP activates bone marrow-derived DCs and the release of TARC (Kashyap *et al.*, 2011). Most cytokine receptors, including the IL-7R, transfer signals to members of the STAT transcription family via JAK-dependent activation (Shuai and Liu, 2003). In humans, TSLPR has been shown to induce JAK2 tyrosine phosphorylation (Carpino *et al.*, 2004). In contrast, TSLPR in mice

failed to induce tyrosine phosphorylation of any of the four known JAKs (Levin *et al.*, 1999), revealing differences between mouse and human TSLP signalling.

Despite the fact, that several studies clearly depict similarities and/or differences in the biology of TSLP and its receptor complex, many others suffer from an imprecise separation of effects observed either in mice or human. There is a need both to order the existing knowledge about TSLP and to conduct further research into the impact of TSLP on human immunity in all its complexity.

1.3.3. TSLP in Disease

The normal function of TSLP is the maintenance of Th2-type homoeostasis at barrier surfaces (Ziegler and Artis, 2010). Physiological Th2 immune responses are thought to have a protective function in preventing the individual from parasitic helminths. It is assumed that TSLP, which is expressed by epithelial cells in the intestine, limits the production of non-protective Th1 cytokines and inflammation (Comeau and Ziegler, 2010). However, the variety of TSLP-responsive cell types demonstrates that TSLP may impact many different (patho)physiological pathways. Dysregulated TSLP expression can result, not only in the development of type 2 inflammatory responses followed by allergic disease, but also in infections, cancer and autoimmunity. Hence, there is evidence that TSLP is a master switch for allergic inflammation in AD and asthma (He and Geha, 2010). High TSLP expression was observed in skin lesions of acute and chronic AD patients but not in non-lesional skin and lesions of patients with contact dermatitis or cutaneous lupus erythematosus. TSLP was also found in keratinocytes of the apical layers of the epidermis, suggesting that its release is a characteristic of fully differentiated keratinocytes (Soumelis *et al.*, 2002). Interestingly, Langerhans cell (LC) migration and activation *in situ* is associated with high TSLP expression in atopic skin lesions, indicating that TSLP might contribute directly to the activation of LCs (Soumelis *et al.*, 2002). Subsequently, LCs migrate into the draining lymph nodes and prime allergen-specific Th2 responses (Ebner *et al.*, 2007). The crosstalk between keratinocytes and immune cells is also fundamental in the regulation of “itch” sensation. Keratinocyte-derived TSLP directly interacts with somatosensory nerves and increases Th2 cytokines such as IL-13 and IL-31, which stimulate neurons directly and up-regulate the release of pruritogens like histamine (Buddenkotte and Steinhoff, 2010; Mollanazar *et al.*, 2016; Wilson *et al.*, 2013). The resulting neurogenic inflammation promotes Th2 responses, keratinocyte proliferation, and epidermal thickening (Weidinger and Novak, 2016).

Several mouse models of induced TSLP expression in keratinocytes lead to allergic airway inflammation, suggesting that TSLP may be an important factor contributing to the progression from AD to allergic rhinitis and asthma (Demehri *et al.*, 2009; Zhang *et al.*, 2009; Ziegler *et al.*, 2013). While many of these models result in very high concentrations of

systemic TSLP not seen in AD patients, Han *et al.* observed, that intradermal application of TSLP triggers progression from AD to asthma in the absence of systemic TSLP (Han *et al.*, 2012). It is still not clear which role TSLP takes in the atopic march, but the dermal expression of TSLP is a plausible link between the large number of AD patients that develop asthma and other allergic disorders later in life.

As an overexpression of skin-derived TSLP leads to AD-like features, elevated expression of TSLP in bronchial epithelial cells has been associated with human asthma and correlates with disease severity (Ying *et al.*, 2008). While TSLP expression levels can differ in patients suffering from asthma, a study in asthmatic patients has revealed a direct correlation of Th2 cytokine and chemokine expression and inversely regulated lung function (Shikotra *et al.*, 2012). Furthermore, nasal epithelium biopsies taken from allergic rhinitis patients showed an increased TSLP expression associated with Th2 cytokine production and DC infiltration in nasal epithelial tissue (Kamekura *et al.*, 2009; Mou *et al.*, 2009). Additionally, the role for TSLP in human asthma has been supported by a variety of mouse models. TSLP expression was enhanced in mice lungs in an ovalbumin-driven model of airway inflammation. TSLPR-deficient mice failed to develop airway inflammation of allergic asthma (Zhou *et al.*, 2005). Furthermore, in an ovalbumin-induced mouse model of allergic rhinitis, inhibiting TSLP also prevented disease development (Miyata *et al.*, 2008). Mice overexpressing lung-specific TSLP developed a progressive asthma-like disease characterised by Th2-affected leukocyte airway infiltrates, increased serum IgE, airway hyperreactivity, and airway remodelling. A substantial reduction of Th2 cytokines, IL-4 and IL-13 prevented chronic lung inflammation, suggesting that TSLP-induced pathogenesis of asthma-like airway inflammation is mediated by intact Th2 responses (Zhou *et al.*, 2008).

Much progress has been made in the understanding of TSLP biology and its role in Th2-type inflammation. The challenge of managing allergic diseases including AD is to find a curative therapy that would prevent the recurrence of allergic episodes. Therefore, TSLP is an attractive target to consider for therapeutic intervention under both atopic and non-atopic conditions.

1.4. Aims

AD is by far the most frequent, chronic disease of children, but available treatment efficacy is still unsatisfactory. The unmet clinical need in AD therapy and the shortcomings of current preclinical drug development may both be aided by the establishment of predictive AD models, not yet introduced into preclinical drug evaluation. Due to its multifaceted pathogenesis including an immunological component, *in vitro* test systems have to fulfil this challenge to provide reliable data beside existing animal models. To get closer to this goal, this thesis aims for the further development of a skin disease equivalent mimicking hallmarks of atopic skin *in vitro*, allowing to study pathophysiological aspects as well as new drug candidates or therapeutic options for the treatment of AD. The implementation of immunocompetent cells into a skin equivalent emulating properties of atopic skin, lays the foundation for the development and testing of novel anti-inflammatory therapies, by meeting the requirements of a complex network like the immune system.

Advances in bioengineering of organotypic skin equivalents have led to the development of well-established test systems closely resembling native human skin *in vitro*. The possible fields of application range over studies in basic skin biology, metabolism, wound healing capacity, skin penetration, transdermal drug delivery and assessment of the efficacy and toxicity of compounds/drugs. As various animal models for the use in basic and preclinical research are described in the literature, problems such as limited availability and data reproducibility, interspecies-related problems and ethical issues still remain. To overcome these obstacles, this thesis further aims for the ongoing development of organotypic human-based skin equivalents, enlarging the manifold application purposes of these *in vitro* test systems.

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2. RESULTS

2.1. Development and Characterisation of Skin Disease Equivalents – One Step Closer to Mimic AD *In Vitro*

Due to the difficulties in the translation of results obtained with mouse models towards human test subjects, human-based organotypic skin equivalents that mimic AD as close as possible can be useful for biological and pharmacological studies. **Chapter 2.1.** describes the application and further development of a previously established *FLG*-deficient skin equivalent which has been used to gain a deeper insight into the pathomechanism of AD in terms of finding suitable therapies.

2.1.1. Stimulation of PPARα Normalises the Skin Lipid Ratio and Improves the Skin Barrier of Normal and Filaggrin-Deficient Reconstructed Skin

Current available therapeutic options for the treatment of *FLG*-associated skin diseases like AD are poorly targeting the underlying pathologic mechanisms, but rather address the arising symptoms. The enhancement or substitution of missing parts of the *FLG* pathway could contribute to the improvement of disease pattern (Otsuka *et al.*, 2014). Due to its receptor occurrence in epidermal keratinocytes, peroxisome proliferator-activated receptor (PPAR) agonists, which are fatty acid activated transcription factors that belong to the nuclear hormone receptor family, were identified to have a positive effect on *FLG* expression (Kömüves *et al.*, 2000; Schmuth *et al.*, 2014; Zhang *et al.*, 2012). To investigate the potential use in the therapy of *FLG*-related skin diseases like AD, a *FLG*-deficient skin equivalent was used to study the potential influence of PPARα and γ agonists on epidermal *FLG* level and barrier homoeostasis. After addition of the PPARα specific agonist WY14643, the PPARγ agonist ciglitazone, or the dual PPARα + γ agonist docosahexaenoic acid (DHA), the skin equivalents were characterised in terms of skin morphology, cornified envelope protein expression, skin lipid composition and barrier function. Overall, the supplementation with WY14643 led to an up-regulation in *FLG* mRNA and protein and normalised the disturbed free fatty acid profile which finally resulted in a significantly improved skin barrier function.

Hence, this study reveals the beneficial effects of PPARα agonists for the treatment of AD and the advantages of *in vitro* skin disease equivalents as alternatives in preclinical research (Wallmeyer *et al.*, 2015).

Publication

Title:	Stimulation of PPARα Normalizes the Skin Lipid Ratio and Improves the Skin Barrier of Normal and Filaggrin Deficient Reconstructed Skin
Authors:	**Leonie Wallmeyer**, Dominika Lehnen, Natascha Eger, Michaela Sochorová, Lukáš Opálka, Andrej Kováčik, Kateřina Vávrová, Sarah Hedtrich
Authorship:	First author
Year:	2015
Journal:	Journal of Dermatological Science
Source:	Wallmeyer L, Lehnen D, Eger N, Sochorová M, Opálka L, Kováčik A, Vávrová K, Hedtrich S. Stimulation of PPARalpha normalizes the skin lipid ratio and improves the skin barrier of normal and filaggrin deficient reconstructed skin. J Dermatol Sci. 2015, 80:102-10. https://doi.org/10.1016/j.jdermsci.2015.09.012

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Declaration of personal contribution:

- Planning, design and conducting of the experiments
- Generation of the skin equivalents
- Evaluation of the entire data set
  - morphological and functional characterisation
  - expression pattern analyses on mRNA and protein levels including quantitative real-time-PCR, immunohistochemistry, and Western blots
  - permeation studies
  - stratum corneum lipid analyses supported by Michaela Sochorová, Lukáš Opálka, Andrej Kováčik, and Kateřina Vávrová
- Interpretation of the entire data set in cooperation with the co-authors
- Drafting and revising the entire manuscript in agreement with Sarah Hedtrich

Contributions of the co-authors:

This publication was prepared by me under the supervision of Sarah Hedtrich. All co-authors participated considerably to the study design, evaluation of experimental results and review of the manuscript.

2.1.2. Influence of Th2 Cytokines on the Cornified Envelope, Tight Junction Proteins, and β-Defensins in Filaggrin-Deficient Skin Equivalents

The implementation of immune cells like T cells into organotypic skin equivalents is associated with great effort (Bergers *et al.*, 2016). However, AD is an inflammatory disease with a strong immunological component (Furue *et al.*, 2017). To study the influence of an inflammatory environment on skin cells and barrier homoeostasis independent from additional influencing factors, a simplified model of AD *in vitro* was developed. Therefore, the previously established *FLG*-deficient skin equivalent was supplemented with AD-specific Th2 cytokines IL-4 or/and IL-13 and characterised in terms of histology, barrier integrity and inflammatory response. The focus was placed on cornified envelope proteins, the skin surface pH, the regulation of tight junction proteins, and the expression of AMPs from the β-defensin family. It was revealed, that *FLG* deficiency alone led to an increase in TSLP and human β-defensin 2 and a compensatory up-regulation of skin barrier and tight junction proteins. These effects, except for enhanced TSLP expression, were diminished after cytokine supplementation which indicates that defects in skin homoeostasis are not primarily linked to *FLG* deficiency, but are rather secondarily induced by Th2 inflammation.

This publication clearly reveals the relevance for human-based *in vitro* test matrices like organotypic skin equivalents to investigate pathomechanisms independent from a complex system (Hönzke *et al.*, 2016). They enable the opportunity to simply modify parameters and subsequently, like in this study, gain deeper knowledge about the relationship between altered keratinocyte function due to *FLG* deficiency and additional inflammatory response through supplemented Th2 cytokines, as found in AD patients (Kuo *et al.*, 2013). Furthermore, this technique provides a useful tool for the development of new therapy strategies against skin diseases correlated with an overshooting Th2 response like AD.

Publication

Title: Influence of Th2 Cytokines on the Cornified Envelope, Tight Junction Proteins, and β-Defensins in Filaggrin-Deficient Skin Equivalents

Authors: Stefan Hönzke, **Leonie Wallmeyer**, Anja Ostrowski, Moritz Radbruch, Lars Mundhenk, Monika Schäfer-Korting, Sarah Hedtrich

Authorship: Co-author

Year: 2016

Journal: Journal of Investigative Dermatology

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Declaration of personal contribution:

- Conception, design and conducting of the experiments for
  - permeation studies
  - proportionately for the expression pattern analyses on mRNA and protein levels including quantitative real-time-PCR, immunohistochemistry, cytokine measurement by ELISA (enzyme-linked immunosorbent assay) and Western blots in cooperation with Stefan Hönzke
- Interpretation of the entire data set in cooperation with the co-authors
- Drafting of the skin permeation part of the manuscript
- Revising the entire manuscript in cooperation with the co-authors

2.1.3. TSLP is a Direct Trigger for T cell Migration in Filaggrin-Deficient Skin Equivalents

The implementation of immune cells into skin equivalents is still a challenge due to culture difficulties and donor cross reactions. However, to mimic AD skin *in vitro* the addition of active immune cells is indispensable. AD is a chronic inflammatory skin disease with a fundamental immune component (Werfel *et al.*, 2016). Therefore, to study pathomechanisms or new therapeutic options *in vitro*, the contribution of immune cells like T cells is of high interest (Engelhart *et al.*, 2005; Masopust and Schenkel, 2013). The relationships of skin cells, barrier impairment due to *FLG* deficiency and immune cells are still unknown. To minimise this gap, a well-characterised *FLG*-deficient skin equivalent with intrinsically high TSLP levels was used (Hönzke *et al.*, 2016) and subsequently supplemented with activated naïve $CD4^+$ T cells. Following successful model establishment, the skin equivalents were characterised in terms of cornified envelope and tight junction protein expression, skin surface pH, pro-inflammatory cytokine secretion, skin lipid composition and barrier function in the presence of the T cells. The T cell supplementation resulted in various morphological and molecular characteristics of AD. Additionally, an unknown interplay between *FLG* deficiency, TSLP and the migration of T cells were identified.

This publication provides a systematic investigation on the impact of naïve $CD4^+$ T cells on *FLG*-deficient skin equivalents without confounding factors (Wallmeyer *et al.*, 2017). The established immunocompetent skin equivalent lays the foundation for further studies on the interaction of skin and immune cells in a 3D environment and the development of anti-inflammatory treatment options for skin diseases like AD.

Publication

Title: TSLP is a Direct Trigger for T cell Migration in Filaggrin-Deficient Skin Equivalents

Authors: **Leonie Wallmeyer**, Kristina Dietert, Michaela Sochorová, Achim D. Gruber, Burkhard Kleuser, Kateřina Vávrová, Sarah Hedtrich

Authorship: First author

Year: 2017

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Declaration of personal contribution:

- Planning, design and conducting of the experiments
- Generation of the skin equivalents and isolation of naïve $CD4^+$ T cells
- Evaluation of the entire data set
  - morphological and functional characterisation
  - skin surface pH measurement
  - expression pattern analyses on mRNA and protein levels including quantitative real-time-PCR, immunohistochemistry, cytokine measurement by ELISA (enzyme-linked immunosorbent assay) and Western blots
  - migration assay and evaluation via flow cytometry
  - permeation studies
  - quantification of T cells supported by Kristina Dietert
  - SC lipid analyses supported by Michaela Sochorová and Kateřina Vávrová
- Interpretation of the entire data set in cooperation with the co-authors
- Drafting and revision of the manuscript together with Sarah Hedtrich

Contributions of other authors:

This publication was prepared by me under the supervision of Sarah Hedtrich. All co-authors participated considerably to the study design, evaluation of experimental results and review of the manuscript.

2.2. Applications for Human-Based Organotypic Skin Equivalents

As an alternative to animal testing *in vitro* human-based organotypic skin equivalents can be used for studying various aspects of skin biology. They exhibit many features of native human skin, such as similarities in morphology, expression of differentiation and proliferation markers as well as various SC barrier properties. Therefore, they provide a simple and reliable tool for the investigation and determination of (patho-)physiological processes and offer the opportunity for several scientific purposes. The following **chapter 2.2.** describes the possible applications of normal human-based organotypic skin equivalents and the further development including an increasing complexness of these systems due to the implementation of additional cell types.

2.2.1. pH Modulating Poly(Ethylene Glycol)/Alginate Hydrogel Dressings for the Treatment of Chronic Wounds

The treatment of chronic wounds is a challenge due to interruption in the healing process through continued inflammatory processes, persistent infections and inability of skin cells to respond to regenerative stimuli (Frykberg and Banks, 2015). They are a global health burden with an increasing number of cases because of an ageing population and rising incidences of diabetes and obesity (Fife and Carter, 2012). Chronic wounds are mainly characterised by elevated pH values which may have a strong impact on healing processes (Percival *et al.*, 2014). Therefore, to promote normal wound healing the application of pH-modulating wound dressings can be promising. To investigate a new possible treatment option for the management of chronic wounds, an acidic hydrogel dressing based on interpenetrating poly(ethylene glycol)/alginate networks was developed by the group of Professor Göpferich (University of Regensburg) and characterised in terms of swelling capacity, strength, base neutralising capacity and cell toxicity. The evaluation of the healing potential of this acidic wound dressing was performed using a 2D cell migration assay and a human-based 3D wound healing skin equivalent. When a pH-modulating alginate hydrogel was applied onto the wounded skin equivalent, the ingrowth of keratinocytes increased significantly as compared to the untreated control.

This study indicates the benefits of acidic wound dressings for wound healing and clearly demonstrates the advantages of skin equivalents in the development of new treatment options for an innovative wound management (Koehler *et al.*, 2017b).

Publication

Title: pH Modulating Poly(Ethylene Glycol)/Alginate Hydrogel Dressings for the Treatment of Chronic Wounds

Authors: Julia Koehler, **Leonie Wallmeyer**, Sarah Hedtrich, Achim M. Goepferich, Ferdinand P. Brandl

Authorship: Co-author

Year: 2017

Journal: Macromolecular Bioscience

Source: Koehler J, Wallmeyer L, Hedtrich S, Goepferich AM, Brandl FP. pH-Modulating Poly(ethylene glycol)/Alginate Hydrogel Dressings for the Treatment of Chronic Wounds. Macromol Biosci. 2017, 17.
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Declaration of personal contribution:

- Conception and design of the wound healing experiments with the skin equivalents
- Generation of the skin equivalents and wounding
- Interpretation of the corresponding data sets in cooperation with the co-authors
- Drafting of the skin equivalent parts of the manuscript in cooperation with Julia Köhler
- Revising the entire manuscript in cooperation with the co-authors

2.2.2. Alkaline Poly(Ethylene Glycol)-Based Hydrogels for a Potential Use as Bioactive Wound Dressings

Implementation of personalised topical therapeutics guided by molecular diagnosis e.g. investigation of the wound pH or a wound pH gradient, could significantly improve the outcome for patients suffering from chronic wounds and provides a directed and targeted approach to wound care (Dowd *et al.*, 2011). Therefore, based on the previous described study an additional approach was tested due to the recent discovery of an acidic pH of 6.5 at wound edges which leads to reduced cell proliferation and migration (Schreml *et al.*, 2014). Hence, alkaline interpenetrating polymer network hydrogels were developed by the group of Professor Göpferich (University of Regensburg). The alkaline hydrogels were analysed in terms of their wound healing capacity first and then characterised for their material properties, as described previously (Koehler *et al.*, 2017b). The developed hydrogels significantly increased the cell ingrowth in wounded human skin equivalents and showed up as suitable scaffolds for superficial cell attachment.

Here, for the first time, the positive influence of an alkaline wound management was shown, using a human-based organotypic skin equivalent (Koehler *et al.*, 2017a).

Publication

Title: Alkaline Poly(Ethylene Glycol)-Based Hydrogels for a Potential Use as Bioactive Wound Dressings

Authors: Julia Koehler, **Leonie Verheyen**, Sarah Hedtrich, Achim M. Goepferich, Ferdinand P. Brandl

Authorship: Co-author

Year: 2017

Journal: Journal of Biomedical Materials Research Part A

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- Conception and design of the wound healing experiments with the skin equivalents
- Generation of the skin equivalents and wounding
- Interpretation of the corresponding data sets in cooperation with the co-authors
- Drafting of the skin equivalent parts of the manuscript in cooperation with Julia Köhler
- Revising the entire manuscript in cooperation with the co-authors

2.2.3. The Effect of Endothelialisation on the Epidermal Differentiation in Human Three-Dimensional Skin Constructs – A Morphological Study

So far, investigating the crosstalk of single components of a complex system like human skin *in vivo* is a huge challenge. To simplify this issue the use of reconstructed skin equivalents offers the possibility to examine cell-cell interaction without confounding factors. Most skin equivalents consist of two types of skin-derived cells, particularly keratinocytes which form the epidermal equivalent and fibroblasts, embedded in a 3D biopolymer scaffold, presenting the dermal equivalent (Ali *et al.*, 2015). However, in order to mimic the morphology of human skin more closely, the inclusion of additional cell types such as endothelial cells is crucial. Current skin equivalents lack a functional vasculature, limiting clinical and research applications (Lei *et al.*, 2011; Montaño *et al.*, 2010). The goal here is to activate angiogenesis of endothelial cells in the dermal compartment, but little is known about the direct interplay of endothelial cells, fibroblasts and keratinocytes. To gain a deeper insight into the influence of endothelial cells on the epidermal development, a normal skin equivalent was used and co-cultured with human endothelial cells. The skin equivalents were analysed regarding structural differences by light microscopy and transmission electron microscopy, followed by quantitative, semi-quantitative as well as qualitative evaluation. It was shown that the endothelial cell layer influenced skin homeostasis by affecting keratinocyte proliferation and differentiation.

Here, the endothelialised skin equivalent enabled the analysis of the impact of single cell components within an interactive cell network and demonstrated the importance of cellular crosstalk on skin development (Khiao In *et al.*, 2015). Additionally, this endothelialised skin equivalent mimics physiological conditions of human skin more closely and may thus serve as an optimised substitute for animal models in research. However, the implementation of a full functional perfusable vasculature in an *in vitro* skin equivalent is still in the focus of research to reflect the physiological conditions of a full organ (Groeber *et al.*, 2016).

Publication

Title: The Effect of Endothelialization on the Epidermal Differentiation in Human Three-Dimensional Skin Constructs – A Morphological Study

Authors: Maneenooch Khiao-in, **Leonie Wallmeyer**, Sarah Hedtrich, Kenneth C. Richardson, Johanna Plendl, Sabine Kaessmeyer

Authorship: Co-author

Year: 2015

Journal: Clinical Hemorheology and Microcirculation

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Declaration of personal contribution:

- Generation of the endothelialised skin equivalents
- Preparation of the skin equivalents for histological analysis
- Interpretation of the entire data set in cooperation with the co-authors
- Drafting of the skin equivalent method parts of the manuscript
- Revising the entire manuscript in cooperation with the co-authors

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3. FINAL DISCUSSION AND PROSPECTS

3.1. Improvement of Skin Disease Equivalents Mimicking Hallmarks of Atopic Dermatitis and Their Scientific Use

AD is, by far, the most common chronic disease in children and a major public health burden. Even though it is not directly life-shortening, the impact it has on patient quality of life, and the correlation it has to allergic associated chronic diseases like asthma and food allergies. There is a pressing medical need to gain more knowledge about the aetiology of this disease in order to find strategies for adequate therapeutic management. Although much of our understanding about AD and its pathogenesis has been obtained using various mouse models (Jin *et al.*, 2009), their usage for studies that focus on the skin barrier and other epidermal features is limited by several factors, namely, differences in genetics, immunology, skin morphology, and skin barrier function (Leist and Hartung, 2013). These differences have often resulted in difficulties in the translation of the obtained results to humans (Warren *et al.*, 2015). Human reconstructed skin, which mimics many morphological and molecular characteristics of normal human skin, has been used to model various skin diseases such as psoriasis, ichtyosis vulgaris and AD (Eckl *et al.*, 2011; Jean *et al.*, 2009; Küchler *et al.*, 2011). Hence, human-based *in vitro* skin equivalents mimicking AD have the potential to reduce the gap between basic, preclinical and clinical research and results obtained so far underline their advantages.

3.1.1. Novel Approaches in Systemic Therapy of Atopic Dermatitis: Protective Role of PPAR Agonists on Skin Barrier Impairment

A previously established and characterised *FLG*-deficient skin equivalent mimicking hallmarks of AD *in vitro* was used as the basis for the performed studies (Küchler *et al.*, 2011). These skin equivalents suffer from impaired epidermal differentiation and increased susceptibility to skin irritating compounds. As most therapeutic approaches focus on the symptoms of AD only - e.g. inflammation, dry and itchy skin - the underlying causes are poorly addressed (Eichenfield *et al.*, 2017). The up-regulation of FLG expression could be a therapeutic option offering long-term beneficial effects to skin barrier homoeostasis. Peroxisome proliferator-activated receptors (PPARs), transcription factors activated by fatty acids and their derivatives belonging to the nuclear hormone receptor family, exhibit diverse functions that include stimulation of epidermal differentiation due to the regulation of profilaggrin expression (Sandilands *et al.*, 2009). The PPAR subfamily consists of the three isotypes: PPARα, PPARβ/δ and PPARγ, all of which have all been identified in keratinocytes (Kuenzli and Saurat, 2003). In inflamed skin PPAR expression is reduced, independent of the nature of inflammatory responses. Potentially, the down-regulation of PPARα could be involved in AD development or maintenance by affecting the lipid pathway (Dubrac and Schmuth, 2011). It has been shown that PPAR agonists reduce certain inflammatory

mediators in the skin, regulate epidermal barrier homeostasis and are involved in epidermal wound repair (Sertznig and Reichrath, 2011). Moreover, studies have revealed their beneficial effects on *FLG* expression in normal and *FLG*-deficient skin, making them interesting targets for AD therapy (Kömüves *et al.*, 2000; Schmuth *et al.*, 2014; Wallmeyer *et al.*, 2015; Zhang *et al.*, 2012). Here, for the first time, the increased expression of FLG at the protein and mRNA levels was shown in skin equivalents suffering from diminished *FLG* levels (**chapter 2.1.1.**). Significant up-regulations of FLG and the cornified envelope proteins IVL and LOR were seen after supplementation with the dual PPARα + γ agonist docosahexaenoic acid (DHA) and PPARα agonist WY14643 (**chapter 2.1.1., figure 1 and 3**). These results are concordant with a recent publication reporting a beneficial effect on FLG and LOR expression in a mouse model of AD after topical application of a PPARα agonist (Jung *et al.*, 2018). In a previous study, it was revealed that *FLG* deficiency had an impact on skin lipids with increased amounts of free fatty acids (FFA) that led to less ordered intercellular lipid lamellae as compared to normal skin equivalents (Vávrová *et al.*, 2014). DHA and WY14643 normalised the molar ratio of the main skin barrier lipids (FFA, ceramides and cholesterol) as well as the skin lipid profile in *FLG*-deficient skin equivalents (**chapter 2.1.1., figure 4 and 5**). Increased levels of shorter and unsaturated FFA in the SC were previously found in AD patients and their negative effects on the lipid order correlated negatively with transepidermal water loss and skin barrier function, suggesting a dysregulation of fatty acid biosynthesis in AD (Kezic *et al.*, 2014; van Smeden *et al.*, 2014). Finally, alone the supplementation with WY14643 significantly improved the skin barrier function in both normal and *FLG*-deficient skin equivalents (**chapter 2.1.1., figure 6**). This finding is well in line with a study, conducted on hairless mice that were treated topically with PPAR agonists (Man *et al.*, 2006). This treatment led to an improved barrier function due to a stimulation of lipid synthesis, increased lamellar body secretion, and post-secretory lipid processing. Regarding the failure of the PPARγ agonist ciglitazone, previous studies also demonstrated that PPARγ agonists are less effective than other liposensor activators in improving permeability barrier homeostasis (Chiba *et al.*, 2012; Mao-Qiang *et al.*, 2004). Since FLG expression is down-regulated in all AD patients irrespective of *FLG* status, an increase of FLG expression with PPARα modulators might be a therapeutic option for the treatment of *FLG*-associated skin diseases like AD, directly addressing the underlying pathology (Furue *et al.*, 2018; Zhang *et al.*, 2017). A small study in children demonstrated a beneficial effect of PPARα agonists in childhood AD, permitting a reduction in the doses of applied dermocorticoids and resulting in improved quality of life (De Belilovsky *et al.*, 2011). Combinatorial treatment of AD with topical glucocorticoids and a PPARα activator was even found to prevent the emergence of glucocorticoid-related epidermal side effects in a murine model of AD (Hatano *et al.*, 2011). Finally, our study clearly demonstrated the great potential

of PPAR agonists in the treatment of FLG associated skin diseases as well as the benefit of *FLG*-deficient skin equivalents in the evaluation of new treatment strategies for AD *in vitro.*

3.1.2. Th2 Cytokines Induce Atopic Dermatitis-Like Features in a Filaggrin-Deficient Skin Equivalent

Inflammatory skin diseases like AD are driven by a strong immunological component. In AD, keratinocytes, especially within eczematous lesions, exhibit a modified expression of pro-inflammatory cytokines, chemokines, and cell surface molecules (Homey *et al.*, 2006; Kuo *et al.*, 2013). The epidermal barrier is impaired in both lesional and non-lesional AD skin and characterised by the presence of Th1, Th2 and Th22 immune responses (Gittler *et al.*, 2012). Nevertheless, though the impact of inflammatory processes and FLG deficiency in AD pathogenesis is well-established, little is known about their direct interplay. To examine this relationship *in vitro*, the *FLG*-deficient skin equivalent was supplemented with Th2-specific cytokines IL-4 and IL-13 (**chapter 2.1.2.**). Subsequently, the direct role cytokines play in the development of pathophysiological changes occurring in AD was investigated, focusing on epidermal differentiation proteins, the regulation of tight junction proteins, and β-defensins. The up-regulation of IVL and occludin seen in *FLG*-deficient skin equivalents, as a possible feedback mechanism acting to compensate for the loss of *FLG*, was significantly disturbed after supplementation of IL-4 and IL-13 (**chapter 2.1.2., figure 2 and 3**). These findings are well in line with recent studies describing a negative affect of Th2 cytokines on skin lipid homoeostasis, skin barrier proteins, and skin barrier integrity (Danso *et al.*, 2014; Omori-Miyake *et al.*, 2014). The compensatory up-regulation of IVL and occludin in *FLG*-deficient skin equivalents might explain the lack of skin permeability differences between normal and *FLG*-deficient skin equivalents observed in skin permeability experiments. Further, a lack of *FLG* induced significant up-regulation of human β-defensin 2 expression compared to normal skin equivalents through an as yet unknown mechanism. Supplementation with IL-4/IL-13 significantly reduced human β-defensin 2 levels (**chapter 2.1.2., figure 5**), an important part of the cutaneous innate immune response. A decrease due to inflammatory cytokines enhances the risk of skin infections, a very common implication of AD (Clausen *et al.*, 2013; De Benedetto *et al.*, 2009). Besides loss-of-function mutations in the *FLG* gene, several other factors can reduce FLG expression such as environmental influences including skin irritation and mechanical damage, but also the skin cytokine milieu, as well as skin colonising microorganisms (Cabanillas and Novak, 2016). This might explain why *FLG* loss-of-function mutations are only present in 10-50% of AD patients, while skin barrier impairment, based on reduced expression of FLG is a general feature present in almost every AD patient (Howell *et al.*, 2009; Peng and Novak, 2015). Therefore, neutralising IL-4/IL-13 modulated inflammatory responses could be beneficial in

the treatment of AD. In March 2017, the US Food and Drug Association approved dupilumab (Dupixent®), a fully human monoclonal IgG4 antibody that binds to the shared alpha subunit of IL-4 receptor and thereby inhibits IL-4 and IL-13 signalling, for the treatment of adults with moderate-to-severe AD whose disease is inadequately controlled by topical therapies (Beck *et al.*, 2014; Eshtiaghi and Gooderham, 2018). This drug approval reflects the overlap between fundamental and pre-clinical/clinical research and demonstrates the benefit human-based *in vitro* systems could have in future drug development.

3.1.3. Immunocompetent Skin Disease Equivalents – New Opportunities in the Research of Inflammatory Skin Diseases

Differences in animal and human immunology are a large factor in the currently high failure rates of drugs in clinical testing phases (Seok *et al.*, 2013; Warren *et al.*, 2015). Therefore, alternatives to animal methods that integrate human immunology into *in vitro* skin disease equivalents are required (Bergers *et al.*, 2016). Additional work needs to be done to fully understand how innate immune signalling pathways mediate inflammatory effects and how the innate immune system is altered by the character of local T cell inflammation. *In vitro* models of inflammatory skin have previously been developed by supplementing the cell culture medium with disease associated cytokines as described above (Danso *et al.*, 2014; Hönzke *et al.*, 2016; Kamsteeg *et al.*, 2011). However, immuno-competent cells are missing, hence these models cannot fully reflect the complex interplay of the immune system. A few research groups have investigated the incorporation of different types of immune cells – e.g. T cells, LCs or DCs - into normal skin equivalents (Bock *et al.*, 2018; Uchino *et al.*, 2009; van den Bogaard *et al.*, 2014). Nevertheless, the aforementioned *in vitro* models lack complex components of diseased skin with a sufficient incorporation of immune cells. This will be mandatory for a human-based test system mimicking hallmarks of inflammatory skin diseases like AD. To close this gap and investigate the effects of T cells in *FLG*-deficient skin, an immunocompetent *FLG*-deficient skin equivalent that enables the migration of activated $CD4^+$ T cells into the dermis equivalent was developed (**chapter 2.1.3.**). As observed in the preliminary study (**chapter 2.1.2.**), the initial compensatory up-regulation of epidermal barrier and tight junction proteins in *FLG*-deficient skin equivalents was abolished after T cell exposure (**chapter 2.1.3., figure 2**). Compared to the results obtained after Th2 cytokine supplementation, the decrease of these important structural proteins was less dramatic, which suggests a more physiologically relevant set-up than established by overload with cytokines. Exposure to activated T cells induced an inflamed phenotype in the skin equivalents, displayed by the significantly enhanced levels of pro-inflammatory cytokines IL-6 and IL-8, and a significant increase in the surface pH of the skin equivalents (**chapter 2.1.3., figure 1A-C**). The findings are well in line with observations made in

patients suffering from AD, as elevated skin surface pH values are characteristic for atopic skin lesions (Panther and Jacob, 2015) favouring colonisation by pathogenic organisms, particularly *S. aureus* (Baurecht *et al.*, 2018). Interestingly, the development of an inflamed phenotype occured irrespective of the *FLG* level of the skin equivalents, concordant with a study that found no differences in the expression of AD-specific cytokines in patients with and without *FLG* mutations (Dajnoki *et al.*, 2016). Furthermore, $CD4^+$ T cell exposure to normal skin equivalents led to a disturbed skin lipid organisation, increased FFA content, and reduced ceramide levels in the SC (**chapter 2.1.3., figure 3**) in the same manner as previously seen for *FLG*-deficient skin equivalents (**chapter 2.1.1., figure 4 and 5** and (Vávrová *et al.*, 2014)). Inflammation has been revealed to be a key contributing factor to changes in the expression of lipid synthesising enzymes (e.g. for ceramides and FFAs). Th2 cytokines modify the mRNA and protein levels of these enzymes, which results in altered levels of the corresponding lipids and subsequent effects on the skin barrier in AD (Sawada *et al.*, 2012; Tawada *et al.*, 2014). Due to the effects on epidermal barrier proteins and skin lipids, the permeability of skin equivalents increased in the presence of activated T cells (**chapter 2.1.3., figure 1D**). The *FLG*-deficient skin equivalent is characterised by intrinsically high TSLP levels (**chapter 2.1.2., figure 4 and chapter 2.1.3., figure 5A and B**). This characteristic is well in line with results obtained in *FLG*-deficient epidermis equivalents (Lee *et al.*, 2011). TSLP is an epithelial cell-derived inflammatory IL-7-like cytokine with significantly elevated expression in the epidermis of lesional skin from patients with acute and chronic AD (Miazgowicz *et al.*, 2009; Nygaard *et al.*, 2016). TSLP is capable of activating DCs that subsequently prime human $CD4^+$ T cells into Th2 cytokine-producing cells (Soumelis *et al.*, 2002). Interestingly, T cell migration into the dermis equivalent was exclusively observed in *FLG*-deficient skin equivalents, even in the absence of DCs (**chapter 2.1.3., figure 4**), indicating a direct relationship between *FLG*-deficiency, TSLP and T cell migration (**chapter 2.1.3., figure 5**). To the best of the author's knowledge, this interaction has not yet been described in the literature. Furthermore, enhanced TSLP levels due to T cell exposure reduced the Th1/Th17-polarisation profile of activated $CD4^+$ T cells, while secretion of Th2 cytokine IL-13 and Th22 cytokine IL-22 was unaffected (**chapter 2.1.3., figure 6**). This finding suggests TSLP directly causes a shift from a Th1/Th17 to a more Th2/Th22 phenotype. In mice, Th2 cells were observed to inhibit the Th17 inflammation response (He *et al.*, 2009), while TSLP induced proliferation of effector Th2 cells but not Th1 nor Th17 cells (Kitajima *et al.*, 2011). Over the last decade, several studies attempted to uncover the direct effect of TSLP on T cells. It was shown, that activated human naïve $CD4^+$ T cells express low levels of TSLPR, while TSLP activates STAT5 and promotes the proliferation of activated $CD4^+$ T cells (Rochman *et al.*, 2007). A recent *in vivo* study revealed that $CD4^+$ T cells from AD patients exhibited enhanced

TSLPR expression with a correlation to the disease activity and that TSLP directly promote Th2 response, resulting in the production of higher amounts of IL-4 compared to normal subjects (Tatsuno *et al.*, 2015). This observation is well in line with results obtained in the here discussed study, as TSLPR expression on non-activated $CD4^+$ T cells increased significantly from 0.56% to 43.9% after activation (**chapter 2.1.3., figure S7**). Moreover, TSLPR expression was found on skin-associated T_{reg} cells mediating suppressive functions under pro-inflammatory conditions (Kashiwagi *et al.*, 2017). TSLP signalling in $CD4^+$ T cells is also essential for memory formation after Th2 sensitisation (Wang *et al.*, 2015) and it activates group 2 innate lymphoid cells, which are further important players in the pathogenesis of several inflammatory skin diseases (Kim *et al.*, 2013). Although the above studies all suggest that TSLP can directly induce $CD4^+$ T cell proliferation or Th2 differentiation, the level of TSLPR expression on activated $CD4^+$ T cells is extremely low when compared with that of myeloid DCs in the human system.

TSLP overproduction is triggered by skin barrier defects which reflect the severity of these impairments. This soluble cytokine can also reach systemic levels, sufficient to trigger the atopic march in AD patients e.g. mirrored by the development of asthma. If serum TSLP levels are elevated in AD patients, therapeutic interventions to counteract this could not only lower the burden of suffering from acute and chronic skin lesions, but also lower the incidence of asthma in later life (Demehri *et al.*, 2009; Pattarini and Soumelis, 2017). A phase I clinical trial assessing the safety of tezepelumab (formerly named MEDI9929 and previously AMG-157), a monoclonal antibody directed against circulating TSLP, in healthy and AD subjects [NCT00757042]. Subsequently, its safety and efficacy was assessed in a phase IIa study with 155 adults with AD [NCT02525094] (Brunner *et al.*, 2017; Nygaard *et al.*, 2017; Snast *et al.*, 2017; Tidwell and Fowler, 2018). Although little evidence regarding the treatment of AD via TSLP blockade is currently publically available, the data for the treatment of asthma patients published to date are promising (Corren *et al.*, 2017; Gauvreau *et al.*, 2014). Nuclear receptors like the retinoid X receptor (RXR), retinoic acid receptors, and PPARs are members of a superfamily of ligand-dependent transcription factors that regulate diverse aspects of reproduction, development, homeostasis, and immune responses by induction of appropriate genes and pathways (Francis *et al.*, 2003). These receptors have been suggested to be involved in the repression of TSLP expression in mouse keratinocytes (Li *et al.*, 2005). Thus, PPAR agonists could play an important role in the TSLP signalling pathway, which is a fundamental part of AD pathogenesis (**figure 4**). Additionally, RXR and PPAR agonists were able to reduce IL-1β-induced TSLP mRNA expression in a human bronchial epithelial cell line (Lee *et al.*, 2008). Oral application of the PPARγ agonist rosiglitazone to NC/Tnd mice, an AD mouse model, revealed a significant reduction in the severity of skin lesions and scratching behaviour. The expression of skin-derived TSLP and

maturation and migration of DCs were also markedly suppressed (Jung *et al.*, 2011). Even in an *in vivo* study in children dealing with the chronic autoimmune enteropathy celiac disease, a condition characterised by high intrinsic TSLP levels, PPARγ agonists were shown to act as an inhibitory regulator of TSLP-stimulated inflammatory processes (Sziksz *et al.*, 2014).

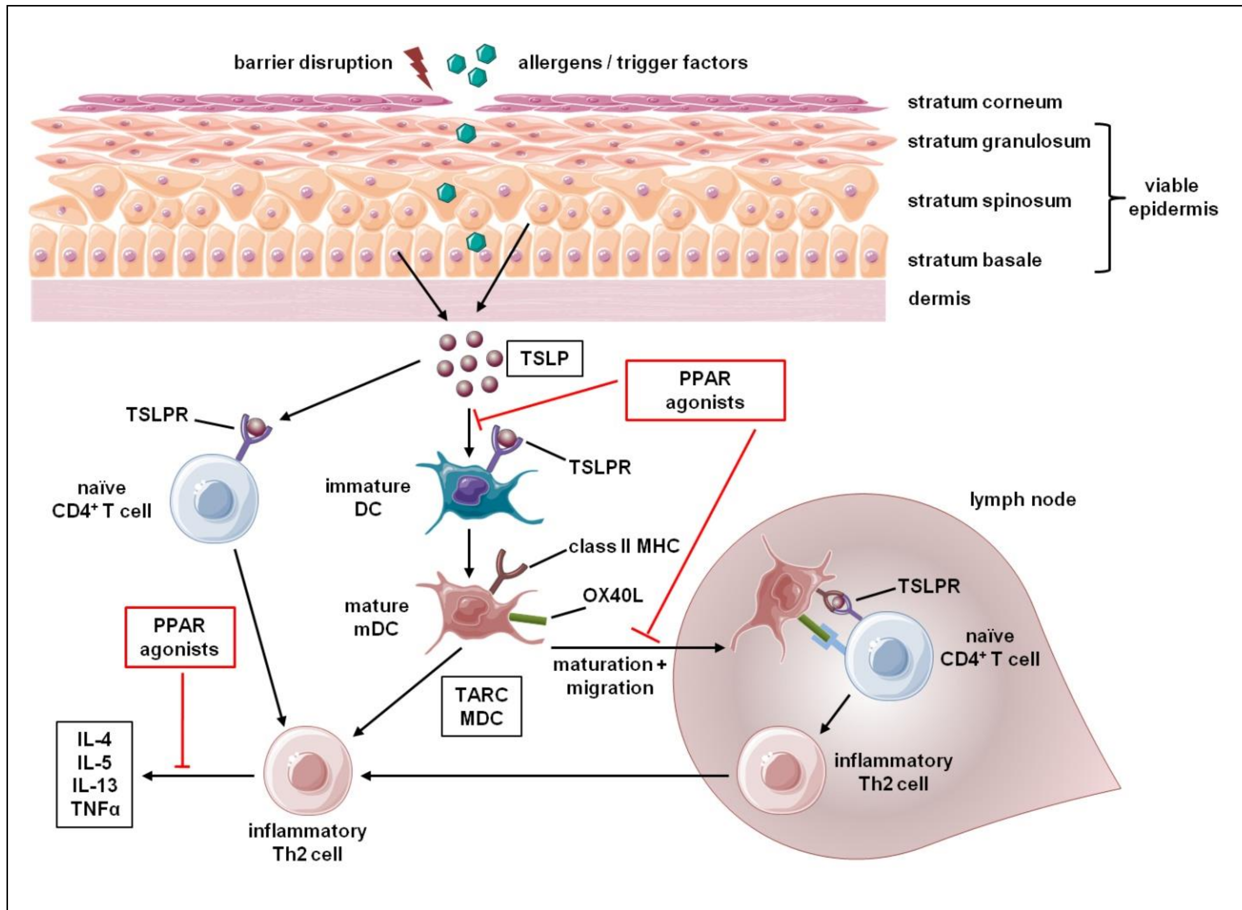

Figure 4. Pathophysiology of TSLP in allergic inflammation and the role of PPAR agonists. Invasion of allergens stimulates skin cells to produce thymic stromal lymphopoietin (TSLP). TSLP activates immature dendritic cells (DCs) which start producing T helper (Th) type 2 cell-attracting chemokines like thymus- and activation-regulated chemokine (TARC) and macrophage-derived chemokine (MDC). TSLP-activated myeloid DCs (mDCs) mature and migrate into the draining lymph nodes where they trigger the differentiation of allergen-specific naïve $CD4^+$ T cells into inflammatory Th2 cells via OX40 ligand (OX40L). Th2 cells then migrate back to the site of inflammation due to the local production of TARC and MDC. TSLP is also capable to directly stimulate the differentiation of naïve $CD4^+$ T cells into Th2 cells. Th2 cytokines interleukin (IL-)4, IL-5, IL-13, and tumour necrosis factor alpha (TNFα), produced by the Th2 cells, initiate allergic inflammation. Peroxisome proliferator-activated receptor (PPAR) activation via corresponding agonists might modulate the early atopic inflammation through inhibition of both, function and production of TSLP as well as the effects of inflammatory Th2 cytokines like IL-4. Graphic uses images from Servier Medical Art. Modified according to (Jung *et al.*, 2011; Jung *et al.*, 2018; Liu, 2006).

As shown in the previous studies, TSLP seems to be a promising target for the development of innovative treatment options for TSLP-associated inflammatory diseases, while PPAR agonists seem to provide an adequate therapeutic alternative for the treatment of atopic lesions, aiming for an improvement to the underlying aetiology. However, there is still no direct proof of the therapeutic value of anti-TSLP treatment in AD.

Finally, the development of a human-based immunocompetent *FLG*-deficient skin equivalent enabled not only the identification of a new mechanism of T cell stimulation, but also offers the possibility to investigate targets of various therapeutic treatments for skin diseases like AD. Furthermore, the development of an *in vitro* immunocompetent skin equivalent for the use as standardised test system could be of great value for the identification of skin sensitising substances. As after exposure to potentially sensitising substances a complex molecular communication can be achieved that enables the activation and migration of T cells according to the mechanisms of the adaptive immune response. So far, some RHE are already accepted by the ECVAM (European Centre for Validation of Alternative Methods) as an alternative *in vitro* method for the identification of the irritant and cytotoxic potential of chemicals. However, these are not a predictive system for sensitising substances (Spielmann *et al.*, 2007). Up to now, no stand-alone non-animal method can estimate skin sensitisation (Kleinstreuer *et al.*, 2018; Sauer *et al.*, 2016). The detection of IL-18 secretion, an important regulatory cytokine in the induction phase of allergic contact dermatitis by epidermal keratinocytes, the clinical manifestation of skin sensitisation, could be a promising biomarker for the identification of sensitising compounds (Gibbs *et al.*, 2013). As the clinical manifestation of skin sensitisation is a T cell-mediated delayed hypersensitivity reaction, the established immunocompetent skin equivalent containing $CD4^+$ T cells was tested for the evaluation of the sensitising potential of cosmetic UV filters (Benevenuto *et al.*, submitted). Conditioned by their IL-18 release, the skin equivalents were able to distinguish between the sensitizer and the irritant controls and were successfully used for chemicals with low solubility (data not shown), an ability lacking in the presently established *in vitro* models. This outcome is well in line with a recent study that used RHE to identify and classify skin sensitizers via their IL-18 release (Galbiati *et al.*, 2017). Thus, immunocompetent skin equivalents could contribute to the prediction of the sensitising potential of substances and subsequently reduce the use of animals. Nevertheless, for a safe application of substances in humans tested with this approach, the described method requires further improvement.

3.2. Organotypic Skin Equivalents in the Focus of Basic and Preclinical Research

For almost three decades the development of organotypic human skin equivalents has been in the focus of several research groups worldwide. Continuous refinements are based on better cell culture techniques and media supplements, as well as novel cell biology methods including genetic modification and engineering techniques (Zhang and Michniak-Kohn, 2012). Skin equivalents mimic key components of human skin and have been valuable in demonstrating the relevance of cell-cell interactions in skin homeostasis, as well as the role of a complex cellular microenvironments in the organisation of epidermal proliferation and differentiation processes (Ali *et al.*, 2015). Organotypic skin equivalents may be applied to a broad field of applications to address scientific and medical questions in dermatology and skin biology (Groeber *et al.*, 2011). Aiming for a reduction of animal tests in preclinical drug development and for an improvement to the rate of success in clinical drug testing, organotypic skin (disease) equivalents offer the possibility for the evaluation of local drug efficacy and safety.

3.2.1. Advantages of Skin Equivalent Wound Models for the Development of Novel Wound Dressings

Wound healing has been studied in various two-dimensional skin cell cultures. These cultures are supportive for studying keratinocyte proliferation and migration in response to wounding, but cannot be used to investigate cellular cross-talk during wound repair, as they fail to reflect the relevant complexity of the *in vivo* microenvironment and cell–cell interactions of native human skin (Knight and Przyborski, 2015; Safferling *et al.*, 2013). To study cutaneous wound healing, the comparability to the *in vivo* situation is crucial for the generation of reliable data, as cell migration and matrix generation are key elements of wound healing. The use of animal models in terms of analysing skin barrier properties and other epidermal features is limited, as few of these translate well to their human counterparts due to e.g. discrepancies in hair follicle distribution and numbers of epidermal cell layers (Nunan *et al.*, 2014; Pasparakis *et al.*, 2014). To overcome these obstacles, organotypic skin equivalents can be used for the evaluation of wound healing processes, inclusive of bacterial colonisation, and the assessment of new therapeutic approaches for the treatment of chronic wounds (Kirker and James, 2017; Küchler *et al.*, 2010; Rossi *et al.*, 2015; Wolf *et al.*, 2009; Xie *et al.*, 2010). Alginate-, chitosan- and collagen-containing hydrogels were shown to enhance cell migration and angiogenesis or suppress microbial infections (Jayakumar *et al.*, 2011; Moura *et al.*, 2014). Due to the predominantly elevated pH found in chronic wounds (Schneider *et al.*, 2007), pH-modulating hydrogels consisting of poly(ethylene glycol) diacrylate (PEGDA), acrylic acid (AA), and alginate were developed by the group of Professor Göpferich (University of Regensburg) to restore the physiological tissue

pH of 7.0-7.4 that supports the natural healing cascade (**chapter 2.2.1.**). To prove their wound healing effect, a human-based organotypic skin equivalent was wounded and, subsequently, treated with hydrogel cylinders from the most promising formulation (PEGDA/AA/alginate hydrogel with 0.25% AA content). When treated with the pH-modulating hydrogel, the ingrowth of keratinocytes increased by 164% as compared to the untreated control (**chapter 2.2.1., figure 7**). Observations made in human wounds demonstrated that the cells at edges of incisions started to migrate like an "epithelial tongue" within 48 h after wounding (Odland and Ross, 1968), well in line with the observations made with the injured skin equivalents (**chapter 2.2.1., figure 6**). The reason for this could be the breakthrough of the cycle of chronicity, enabling the wound to progress to healing (Kruse *et al.*, 2017). Additionally, it was shown in an animal model of skin wound healing that alginate is able to modulate the concentration of cytokines and growth factors in a manner found to promote fibroblast proliferation and migration (Lee *et al.*, 2009). These findings were also confirmed in a 2D cell migration assay using human dermal fibroblasts (**chapter 2.2.1., figure 5**).

Interestingly, recent findings identified an acidic environment (pH 6.5) at wound edges as a responsible factor for reduced cell proliferation and migration (Schreml *et al.*, 2014). The trend of the pH shift in chronic wounds is dependent on actual wound size, the state of chronicity and on both the type and amount of bacterial load (Sheybani and Shukla, 2017; Sirkka *et al.*, 2016). To deal with wounds with acidosis, alkaline poly(ethylene glycol)-based hydrogels were developed by the group of Professor Göpferich (University of Regensburg) and tested for the first time as alternative suitable wound dressings (**chapter 2.2.2.**). In one approach, the PEGDA hydrogel was combined with the alkaline precursor N-vinylimidazole (VI) that acts as a weak base (pKa = 7.0) and neutralises the surrounding medium whilst avoiding the formation of an alkaline environment (Horta and Pierola, 2009). In another, PEGDA gels were combined with the strong base 2-dimethylaminoethyl methacrylate (DMAEMA; pKa = 8.4), as the alkaline part of the primary network. Either alginate or modified chitosan (CEC) were used as secondary network. In the first instance, the wound healing capacities of the different hydrogel formulations under acidic conditions were assessed by human dermal fibroblasts in a 2D cell migration assay (**chapter 2.2.2., figure 2**) as well as by their potential positive effect on wounded human skin equivalents (**chapter 2.2.2., figure 3**). Histological analysis of the skin equivalents revealed enhanced cell migration of keratinocytes at wound edges. Here, cell ingrowth increased by 46.2% with PEGDA/VI/alginate with 3.5% VI and by 89.1% with PEGDA/DMAEMA/CEC with 2.0% DMAEMA, as compared to their respective hydrogels lacking an alkaline component. For the first time, this study clearly depicted the positive impact of alkaline wound dressings for the treatment of acidic wounds. The promising results of both studies demonstrated the great advantage of pH-modulating wound dressings as promising treatment option for

chronic wounds. The pH value in the context of wound healing is a dynamic factor (Schneider *et al.*, 2007). An improved understanding of the pH distribution in acute and chronic wounds could help further the development of diagnostic tools and therapeutic methods in wound care, where personalised treatment depending on wound pH could be taken into consideration (Xie *et al.*, 2010). This advantage was already proven based on the results of molecular diagnostics from a clinical study with more than 1000 patients, of which one half received personalised topical therapeutic gels. The average healing time and healing rate of the former group was reduced significantly and reliance on systemic antibiotics was nearly eliminated (Dowd *et al.*, 2011).

As shown above, studies using organotypic skin equivalents are likely to be of great benefit in the generation of new fundamental information regarding wound healing processes as well as the preclinical development and evaluation of novel treatment options. In particular, they are valuable models for studying the chronology of re-epithelialisation, cell proliferation, migration, differentiation and cell-cell crosstalk.

3.2.2. Endothelialised Skin Equivalents: a Model to Study Effects on Epidermal Differentiation

In human skin, epithelial cells form a structured and well-organised epithelium consisting of basal, spinous, granular, and cornified strata. Their development during embryogenesis, regeneration in wound healing, and in the maintenance of the epidermal homoeostasis depends on epithelial interactions with their underlying connective tissue, the dermis. The continuous progression of keratinocyte proliferation and terminal differentiation is mainly regulated by mesenchymal influences, resulting in a complex epidermal specific barrier system (Stark *et al.*, 2004; Watt, 2014). Fibroblasts secrete and organise the extracellular matrix (ECM), a network of proteins and proteoglycans that provide a microenvironment for cellular development, homeostasis, and regeneration. Additionally, ECM production and maintenance is an important feature of endothelial cell functionality crucial to the development of blood vessels, and endothelial cell signalling responses to this natural matrix *vice versa* (Anderson and Hinds, 2012; Rozario and DeSimone, 2010; Soucy and Romer, 2009). So far, the investigation of cellular growth, signalling processes, cell-cell interactions, and the effects of specific extracellular matrix components and soluble factors have relied on culture systems mainly developed by combining the two major cell types of human skin, keratinocytes and fibroblasts. However, to mimic the architecture and cellular environment of human skin more closely, endothelial cells have been implemented into the dermal part of full-thickness skin equivalents, which results in the alignment of endothelial cells to vessel-like structures (Lei *et al.*, 2011; Montaño *et al.*, 2010; Tonello *et al.*, 2005). Innovative techniques like bioprinting of artificial blood vessels are currently in testing, but

still struggle to accurately emulate normal physiological function (Hoch *et al.*, 2014). However, little is known about the direct effect of endothelialisation on epidermal structures and differentiation. Therefore, a previously established normal human-based organotypic skin equivalent (Küchler *et al.*, 2011) was co-cultured with human umbilical vein cord endothelial cells (HUVEC) (**chapter 2.2.3.**). Normal skin equivalents as well as skin equivalents containing a HUVEC layer were analysed in terms of their morphology and ultrastructure via light and electron microscopy, and compared to human skin *ex vivo*. The results revealed that skin equivalents with a HUVEC layer had significantly more mitotic cells in the stratum spinosum (11 vs. 5.5), more cell layers in the stratum granulosum (2.5 vs. 2) and higher numbers of keratohyalin granules (22.88 vs. 13.63 per 10 cells) and lamellar bodies compared to normal skin equivalents (**chapter 2.2.3., figure 1 and 7**). Compared to this mitosis rates in normal human epidermis examined *in vivo* and *in vitro*, the results obtained in the skin equivalents co-cultured with HUVECs were considerably higher (Marks and Nishikawa, 1973; Parakkal and Alexander, 1972). The abundance of keratohyalin granules and lamellar bodies found in the endothelialised constructs may be lead to improved skin barrier function due to the increased synthesis of keratin filaments, keratin filament associated proteins, and lipid matrix components (Norlén and Al-Amoudi, 2004; Salas *et al.*, 2016). This hypothesis should be investigated in future studies. Interestingly, the epidermal structure of skin equivalents cultured with HUVECs was unorganised, with gaps between the corneocytes and granulosum cells which were irregularly shaped in 50% of the samples (**chapter 2.2.3., figure 4B and D**). These are signs of impaired maturation such as hyperproliferation and subsequent disturbed epidermal layer formation. A cytokine potentially responsible for this is IL-6, which stimulates the proliferation of cultured human keratinocytes. It is expressed at high levels in pathologic skin lesion, such as in psoriasis, characterised by the loss of intercellular cohesion between keratinocytes (Grossman *et al.*, 1989; Tjabringa *et al.*, 2008). Overall, these skin equivalents showed features of excessive epidermal differentiation potentially conditioned by growth factors produced by HUVECs, fibroblasts and/or the keratinocytes themselves as a result of their cellular interactions in the co-culture. It is well known that endothelial cells produce cytokines that activate keratinocytes and stimulate their differentiation such as fibroblast growth factor, IL-1, IL-6, IL-8 and GM-CSF (Hegde and Behr, 2012; Suter *et al.*, 2009). Using a human-based organotypic skin equivalent supplemented with endothelial cells helped to gain a deeper insight into the effect of these cells on epidermal differentiation and their interactions with keratinocytes and fibroblasts within a three-dimensional network. Due to the better understanding of the influence of endothelial cells on keratinocyte differentiation, this study could be helpful for the development of fully vascularised skin equivalents.

3.3. Limitations of Currently Existing Organotypic Skin (Disease) Equivalents

In recent years, improved culture techniques and biotechnological enhancements have contributed to an increase in the reliability of *in vitro* test systems in the ever developing field of *in vitro* methods. Nevertheless, there are still several limitations in the use of human-based organotypic skin equivalents, particularly in the development of disease models (Ali *et al.*, 2015; Löwa *et al.*, 2018a). Some limitations have already been highlighted in **chapter 1.2.**. The use of primary cells in place of their respective cell lines has several drawbacks including a limited lifespan, large donor-to-donor variation, and limited availability. Primary cells like keratinocytes and fibroblasts derived from juvenile foreskin, used as standard cell source in full-skin equivalents, may not reflect physiological properties of aged or diseased skin. Furthermore, the lack of a full capillary network, which guarantees the adequate perfusion of nutrients and oxygen, has a negative influence on cell nutrition and metabolism. Cutaneous vasculature is crucial for several physiological and pathophysiological processes including wound healing, tumour angiogenesis, and the transdermal penetration of substances. Additionally, organotypic skin equivalents mimic only one organ and therefore cannot predict systemic drug effects. Furthermore, the emulation of a functional immune system in skin equivalents remains a major objective. Although mouse models have efficiently advanced our knowledge about basic immunological mechanisms, they cannot reflect all aspects addressing human skin immunology. Several studies have highlighted important differences between the innate and adaptive immunities between mice and humans, such as in Th1/Th2 differentiation, and T cell signalling pathway components, as well as chemokine and chemokine receptor expression (Mestas and Hughes, 2004; Warren *et al.*, 2015). Additionally, skin equivalents lack a neuronal system even though the density of neurons in the epidermis is increased in immune skin diseases such as psoriasis and AD. This could be, therefore, an interesting approach of investigation for future work (Botchkarev *et al.*, 2006; Harvima *et al.*, 2010). Although significant progress has been made in the research of inflammatory skin diseases, the initial cause for AD as well as its mechanisms of the progression is still ambiguous. This is likely the result of not only the complexity of the disease, but also the lack of sufficient disease models.

3.4. Conclusion

In conclusion, the overall aim of this thesis was the further development of a skin equivalent mimicking hallmarks of AD *in vitro*. It succeeded in incorporating immune cells into a *FLG*-deficient skin equivalent that will enable the future investigation of anti-inflammatory compounds, and which could reduce animal testing in basic and preclinical research. Additionally, the immunocompetent, *FLG*-deficient skin equivalent characterised by intrinsically high TSLP levels allowed the identification of a so far unknown mechanism of T cell migration. Furthermore, the *FLG*-deficient skin equivalent was successfully used to examine the beneficial effects of PPAR agonists on skin barrier homoeostasis, and demonstrated their therapeutic potential for the treatment of FLG associated skin diseases like AD. Finally, the studies included in this thesis contribute to a further development of existing organotypic human-based skin equivalents, in order to meet the requirements for the provision of reliable preclinical test systems - from cell biology to dermocosmetics, modelling diseases, drug development, pathophysiology and regenerative medicine. The applications and improvements, made on the basis of an “in-house” developed skin equivalent, are highlighted in **figure 5**. Despite their limitations, it was shown that the use of skin equivalents was highly beneficial to the acquisition of knowledge over physiological and pathophysiological processes in human skin - from wound healing to mimic AD *in vitro*.

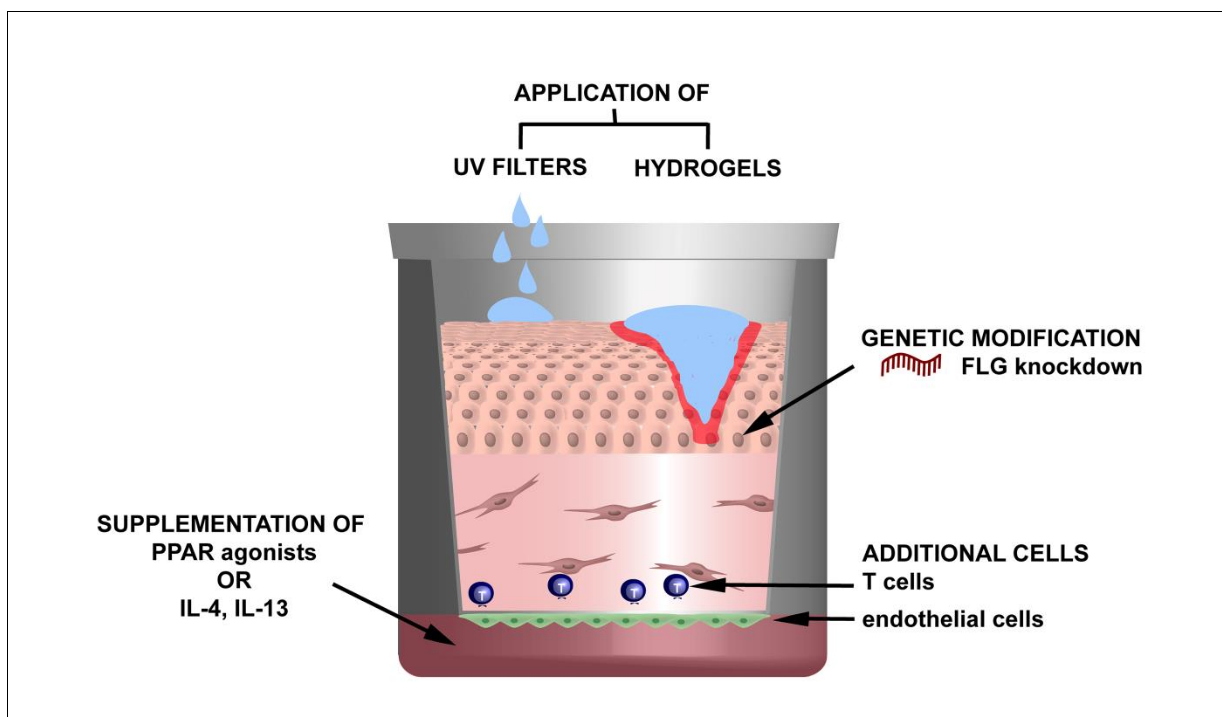

Figure 5. Skin equivalents in basic and preclinical research. Schematic summary of the applications and modifications of organotypic skin (disease) equivalents performed and discussed in the presented studies. Kindly provided by Anna Löwa.

3.5. Prospects

Although AD is the most common chronic disease in children, its therapeutic options are still limited. Mouse models, widely used in the preclinical development and testing of new drugs, suffer from poor transferability and not least, ethical concerns. Organotypic skin equivalents are already used for the assessment of skin absorption (OECD-428, 2004; Schäfer-Korting *et al.*, 2008) and for the evaluation of toxicological endpoints (OECD-439, 2015). Nevertheless, despite their great improvement in recent years, skin disease equivalents are still far from being implemented as a standardised test systems in drug development and an alternative to animal testing. Primary cells derived from AD patients closely emulate the pathophysiological characteristics of the condition, but the limited availability of sufficient numbers of these cells hampers their broad use in skin equivalents mimicking AD. Promising cell sources are induced pluripotent stem cells (Itoh *et al.*, 2013) and outer root sheath cells found adherent to plucked human hair that were recently used to generate full thickness, autologous skin models without the need to perform invasive skin biopsies (Löwa *et al.*, 2018b). This technique would also help to prevent rejection reactions possibly arising by the use of cells from different donors, particularly by combining skin and immune cells from diverse sources. Therefore, blood and hair samples could be collected from one patient to obtain different cell types from the same donor. Even more challenging is the establishment of an immunocompetent skin equivalent. Although this thesis describes the successful migration of activated T cells into a skin disease equivalent, immunology of inflammatory skin diseases like AD is much more complex and orchestrates a multitude of different immune cells. Therefore, the incorporation of various immune cell types like T cells, DCs and mast cells would further increase the validity of AD-specific immune reactions. Even though current skin equivalents lack a functional vasculature, which limits basic and preclinical research applications, much effort has been made to generate perfusable vascularised tissues. The dynamic cultivation of skin equivalents on a perfusion platform allows the continuous supply with oxygen and nutrients and also increases the skin barrier homoeostasis due to mechanical, physiological-like shear stress (Strüver *et al.*, 2017). This technique could also be used to achieve a physiological temperature gradient, existing between hypodermis (37 °C) and the skin surface (32 °C) (Viano *et al.*, 2017). Since enzyme activity and expression is known to be temperature dependent, a temperature gradient may be important for the proper function of enzymes involved in epidermal differentiation and lipid synthesis (Borowiec *et al.*, 2013). Therefore, the culture medium could be maintained at 37 °C, while setting the surrounding temperature to room temperature in order to mimic *in vivo* conditions. Furthermore, microfluidic human-on-a-chip approaches have gained increasing interest as they combine different tissues on one chip including systemic-like circulation (Skardal *et al.*, 2016; Wagner *et al.*, 2013). This would provide an optimal

environment for immune cells that are difficult to maintain in statically cultured skin equivalents. Nevertheless, the development of a fully vascularised skin equivalent with a perfusable vascular network, a very ambitious aim, would provide a more physiological environment and could increase the, to date, very limited life period of an organotypic skin equivalent. All the possible improvements discussed for skin equivalents could also be translated to other organ systems that are the target of inflammatory diseases, for instance lung tissue (Zscheppang *et al.*, 2018). The development of an immunocompetent lung equivalent could be helpful increasing the knowledge about allergic-associated conditions like asthma and evaluate new therapeutic strategies.

Overall, the generation of organotypic skin equivalents show high intra- and inter-batch variations, particularly between different laboratories. To reach the goal of implementing skin disease equivalents into new drug development for skin diseases like AD, methods need to be standardised in terms of cell sources, culture media as well as culture conditions.

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4. SUMMARY

4.1. Summary in English

Atopic dermatitis (AD) is a chronic inflammatory skin disease and a prominent health burden that markedly impact patient quality of life, affecting 2-10% of the adult population and 10-30% of children worldwide. Current therapeutic options tackle disease symptoms, but leave the disease's still poorly understood aetiology seemingly unaltered. Due to its relapsing nature and the associated risk of gaining further, potentially life-threatening, atopic disorders such as asthma and food-allergies, AD presents a notable unmet clinical need on the development of innovative therapeutic strategies. The use of human-based organotypic skin equivalents in basic research is constantly increasing. These, not only circumvent ethical concerns, they also overcome the difficulties of translating results from animal models to humans owing to differences in physiology and anatomy. Despite this however, skin equivalents are still not broadly used in preclinical drug development owing to certain physiological limitations and a lack of model standardisation. This thesis aimed to further develop a previously established skin equivalent in which filaggrin deficiencies, a major component of the known AD pathology, were induced.

Firstly, the filaggrin-deficient skin equivalent was used to investigate the therapeutic potential of peroxisome proliferator-activated receptor (PPAR) agonists. Supplementation with the PPARα agonist WY14643 led to an up-regulation of filaggrin, normalised free fatty acid distribution, and significantly improved skin barrier function, all of which indicate a probable positive impact upon filaggrin-associated skin diseases like AD.

Next, to study the influence of an inflammatory environment on skin cells and barrier homoeostasis, independent of additional factors, the filaggrin-deficient skin equivalent was supplemented with the AD-specific T helper (Th) type 2 cytokines interleukin (IL)-4 and IL-13, alone or in combination. The compensatory up-regulation of skin barrier and tight junction proteins seen following the induction of the filaggrin deficiency was diminished upon cytokine supplementation. This indicates that defects in the equivalent's skin homoeostasis are not primarily linked to filaggrin deficiency but are, rather, secondarily induced by Th2 inflammation. As AD is a chronic inflammatory skin disease with a fundamental immune component, the contribution of immune cells like T cells is of high interest. Thus, the filaggrin-deficient skin equivalents were supplemented with activated $CD4^+$ T cells applied underneath the dermal layer. This resulted in an inflammatory phenotype, and led to the discovery of an unknown link between thymic stromal lymphopoietin (TSLP), a pro-inflammatory cytokine found at intrinsically high levels in the filaggrin-deficient skin equivalent, and the migration of $CD4^+$ T cells in filaggrin-deficient skin. The immunological component instated by either supplementation of the AD-specific Th2 cytokines, or by the implementation of activated $CD4^+$ T cells contributed to the approximation of the *in vivo* situation.

In addition, this thesis revealed several possible applications for human-based skin equivalents and how these can be improved by the implementation of additional cell types. Normal organotypic skin equivalents were used to test the wound healing capacity of different hydrogels modulating wound pH in an attempt to find a new approach for the treatment of chronic wounds characterised by shifted pH values. After application, wounded skin equivalents showed a marked recovery as measured by keratinocyte migration and wound closure. Current *in vitro* skin equivalents lack of functional vasculature, limiting their preclinical and basic research applications. To gain deeper insight into the crosstalk of keratinocytes and endothelial cells, a normal organotypic skin equivalent was complemented with endothelial cells, resulting in altered keratinocyte proliferation and differentiation, demonstrating the key roles cellular crosstalk plays during skin development.

In conclusion, organotypic skin disease equivalent mimicking crucial hallmarks of AD *in vitro* were developed, and which will enable precise basic research and preclinical drug evaluation for this still poorly understood disease. Moreover, this thesis contributed to the furthered progression of current organotypic skin equivalents towards meeting the requirements of a reliable preclinical test system applicable from cell biology to dermocosmetics, disease modelling, drug development, pathophysiological investigation and regenerative medicine.

4.2. Summary in German (Zusammenfassung)

Die atopische Dermatitis (AD) ist eine chronisch-entzündliche Hauterkrankung und stellt eine erhebliche, gesundheitliche Belastung dar, die die Lebensqualität der Patienten massiv beeinträchtigt. Sie betrifft 2-10% der erwachsenen Bevölkerung und 10-30% der Kinder weltweit. Momentan verfügbare therapeutische Optionen richten sich gegen die Krankheitssymptome, lassen aber die noch immer wenig verstandene Krankheitsursache außen vor. Aufgrund der hohen Rezidivgefahr und des Risikos, im weiteren Verlauf der Krankheit zusätzliche, potenziell lebensbedrohliche atopische Erkrankungen wie Asthma oder schwerwiegende Nahrungsmittelallergien zu entwickeln, besteht ein großer klinischer Bedarf in der Entwicklung innovativer, therapeutischer Strategien. Die Nutzung humaner Hautmodelle in der Grundlagenforschung steigt kontinuierlich an. Sie bieten nicht nur eine Alternative gegenüber Tierversuchen, die ethisch umstritten sind, sondern haben auch den Vorteil, die durch Unterschiede in der Physiologie und Anatomie von Tier und Mensch bedingte schlechtere Übertragbarkeit von in Tierversuchen generierten Daten zu überwinden. Dennoch finden Hautmodelle aufgrund mangelnder Modellstandardisierung und einiger physiologischer Limitierungen nach wie vor keine breite Anwendung in der präklinischen Arzneimittelentwicklung. Ziel dieser Arbeit war es, ein bereits etabliertes Filaggrin-defizientes Hautmodell weiter zu entwickeln, das die wesentlichen Merkmale der AD-Pathogenese noch realistischer abbildet.

Zuerst wurde das Filaggrin-defiziente Hautmodell verwendet, um den positiven Effekt von Peroxisomen-Proliferator-aktivierten Rezeptor (PPAR)-Agonisten auf die Verbesserung der Hautbarriere zu untersuchen. Die Zugabe des PPARα-Agonisten WY14643 führte zu einer Hochregulierung von Filaggrin, einer normalisierten Verteilung der freien Fettsäuren und einer signifikant verbesserten Hautbarrierefunktion. Diese Ergebnisse deuten auf eine mögliche positive Auswirkung auf Filaggrin-assoziierte Hautkrankheiten wie der AD hin.

Um den Einfluss einer entzündlichen Umgebung auf die Hautzellen und die Barrierehomöostase unabhängig von weiteren Einflussfaktoren zu untersuchen, wurde das *FLG*-defiziente Hautmodell mit für den AD-spezifischen T-Helfer (Th)-Typ-2-Zytokinen Interleukin (IL)-4 und IL-13 versetzt, jeweils allein oder in Kombination. Die kompensatorische Hochregulation von Hautbarriere- und Tight Junction-Proteinen, die nach der Induktion des Filaggrinmangels beobachtet wurde, war nach Zytokinexposition vermindert. Dies deutet darauf hin, dass Defekte in der Hauthomöostase des Modells nicht in erster Linie mit dem Filaggrinmangel in Zusammenhang stehen, sondern sekundär durch eine Entzündungsreaktion ausgelöst werden. Da die AD eine chronisch-entzündliche Hauterkrankung mit einer grundlegenden immunologischen Komponente ist, ist der Einfluss von Immunzellen wie T-Zellen von großem Interesse. Daher wurden die Filaggrin-defizienten Hautmodelle im weiteren Verlauf mit aktivierten $CD4^+$-T-Zellen versetzt, die unterhalb der

dermalen Schicht eingebracht wurden. Dies führte zu einem entzündlichen Phänotyp innerhalb des Modells und zur Entdeckung einer unbekannten Verbindung zwischen Thymus stromal lymphopoietin (TSLP), einem proinflammatorischen Zytokin, welches in hohen Konzentrationen in dem Filaggrin-defizienten Hautmodell vorliegt, und der Migration von $CD4^+$-T-Zellen in Filaggrin-defizienter Haut. Die immunologische Komponente, die entweder durch den Zusatz AD-spezifischer Th2-Zytokine oder durch die Implementierung aktivierter $CD4^+$-T-Zellen eingebracht wurde, trug zur Annäherung der *in vivo* Situation in atopischer Haut bei.

Darüber hinaus wurden in dieser Arbeit verschiedene Anwendungsmöglichkeiten humaner Hautmodelle und deren Optimierung durch die Implementierung weiterer Zelltypen aufgezeigt. Humane Hautmodelle wurden verwendet, um die Wundheilungskapazität verschiedener pH-modulierender Hydrogele zu testen und um einen neuen Ansatz für die Behandlung chronischer Wunden zu entwickeln, welche durch veränderte pH-Werte gekennzeichnet sind. Nach der Applikation der jeweiligen Hydrogele zeigten die verwundeten Hautmodelle eine Verbesserung der Wundheilung, gemessen anhand des Wundverschlusses durch die Migration von Keratinozyten. Gegenwärtig verwendeten *in vitro* Hautmodellen fehlt ein funktionelles Gefäßsystem, was präklinische und grundlegende Forschungsanwendungen einschränkt. Um einen genaueren Einblick in die Zellkommunikation zwischen Keratinozyten und Endothelzellen zu erhalten, wurde ein humanes Hautmodell mit Endothelzellen versetzt, was zu einer veränderten Keratinozytenproliferation und -differenzierung führte. Dies demonstriert die Bedeutung von zellulärem Austausch für die Hautentwicklung.

Zusammenfassend wurde ein humanbasiertes Hautkrankheitsmodell entwickelt, das grundlegende Merkmale atopischer Haut *in vitro* nachahmt und somit zielgerichtete Grundlagenforschung und präklinische Arzneimittelentwicklung für diese noch wenig verstandene Krankheit ermöglichen wird. Darüber hinaus trug diese Arbeit zur Weiterentwicklung humaner Hautmodelle bei, um den Anforderungen an die Nutzung zuverlässiger präklinischer Testsysteme gerecht zu werden – für die Anwendung in der Zellbiologie und Pathophysiologie, der Modellierung von Krankheiten, über der Testung von Kosmetika, der Arzneimittelentwicklung und in der regenerativen Medizin.

5. STATEMENT OF AUTHORSHIP (SELBSTSTÄNDIGKEITSERKLÄRUNG)

Hiermit versichere ich, Leonie Verheyen, die vorliegende Arbeit selbstständig verfasst zu haben. Alle verwendeten Hilfsmittel und Hilfen habe ich angegeben. Die Arbeit wurde weder in einem früheren Promotionsverfahren angenommen noch als ungenügend beurteilt.

Berlin, 31.05.2018

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Leonie Verheyen

6. CURRICULUM VITAE

Due to data protection reasons, the CV has been removed.