IN VITRO SKIN DISEASE EQUIVALENTS AND THEIR APPLICATIONS IN BASIC DERMATOLOGICAL RESEARCH

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...to those who I care for

and those who care for me

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ABBREVIATIONS

2-D	two-dimensional
3-D	three-dimensional
AD	atopic dermatitis
AMP	antimicrobial peptide
APC	antigen presenting cell
ARCI	autosomal-recessive congenital ichthyosis
βCD	β-cyclodextrin
CD4	cluster of differentiation 4
CE	cornified envelope
CMS	core-multishell
COL1A1	collagen type 1 alpha 1
COL3A1	collagen type 3 alpha 1
DC	dendritic cell
DED	de-epidermized dermis
dPG	dendritic polyglycerol
DXM	dexamethasone
ECM	extracellular matrix
ELISA	enzyme-linked immunosorbent assay
EPR	electron paramagnetic resonance
ERK	extracellular signal-regulated kinase
EU	European union
FBS	fetal bovine serum
FLG	filaggrin
GC	glucocorticoid
GM-CSF	granulocyte macrophage colony-stimulating factor
GR	glucocorticoid receptor
hBD	human β-defensin
HSE	human (full thickness) skin equivalent
ICAM1	intercellular adhesion molecule 1
IgE	immunoglobuline E
IL	interleukin
IL-4Rα	IL-4 receptor alpha chain
iPSC	induced pluripotent stem cell
IVL	involucrin
LC	Langerhans cell
LOR	loricrin
mPEG	monomethoxypoly(ethylene glycol)
NMF	natural moisturizing factor
OECD	Organization for Economic Co-Operation and Development
OCLN	occludin
PCL	polycaprolactone
PI	propidiumiodid
pNIPAM	poly(N-isopropylacrylamide)
PBS	phosphate buffered saline
RHE	reconstructed human epidermis
RT-PCR	real-time polymerase chain reaction
SB	stratum basale
SC	stratum corneum
SCCP	scientific committee on consumer products
SEM	standard error of the mean
SG	stratum granulosum
SS	stratum spinosum

STAT	signal transducers and activators of transcription	tNG	thermoresponsive nanogel
		TJ	tight junction
TCI	topical calcineurin inhibitors	TNF	tumor necrosis factor
Th	T helper	TSLP	thymic stromal lymphopoetin
TIMP	tissue inhibitors of metalloproteinases	UV	ultraviolet

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[1]) M.G. and G.Y. contributed equally to this work.
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DIRECTORY

1

INTRODUCTION

1.1 TISSUE ENGINEERING OF THE SKIN

Tissue engineering was defined in the 1980´s as the application of principles and methods of engineering and life sciences. Both scientific areas were merged to address scientific goals like understanding the fundamental structure-function relationship in normal or pathological human tissue and to drive clinical research for the development of biologic substitutes which can restore, maintain and improve tissue function (Patil et al., 2006). Tissue engineering has been studied and applied for various organs (Griffith and Naughton, 2002), but for the organ, which is the most efficient barrier against external influences such as UV radiation, microbial agents and mechanical disturbances, the last decades of research were a success story: the SKIN.

Critics argue that the use of appropriate animal models or immortalized cell lines revealed essential information's for the understanding the pathogenesis of skin disorders e.g. atopic dermatitis (Jin et al., 2009) and their treatment assessment with new therapeutics (Abdel-Mottaleb et al., 2014). Indeed, there is no doubt that these animal models and cell culture techniques have helped to make major contributions to the field, but the scientific community is increasingly recognizing that these models and their outcomes suffer from serious limitations. Potential therapeutics were deemed to be safe and effective in animals, but failed in humans due to e.g. non-rigorous study design (Perrin, 2014).

The organ skin is serving as an impressive example: Human and mouse skin share many features, but recent gene analysis identified significant differences between the two species (Gerber et al., 2014). While a subset of skin related genes are only present in one or the other genome, many shared genes also exhibited profound variation of expression between the species. This affects in particular genes associated with skin morphogenesis and growth and various immune-associated genes, which are only represented in the mouse genome (Gerber et al., 2014). These findings contribute to the disagreement between poor (Seok et al., 2013) and great correlation (Takao and Miyakawa, 2015) of murine and human genomic response to inflammation and initiated a vibrant discussion about the capabilities and limitations of murine models (Dirnagl, 2014; Warren et al., 2015). Additionally, ethical concerns are arising regarding the sacrificed animals used for efficacy and safety testing of pharmaceutical, chemical or cosmetic compounds. The reduction of animal use is demanded by the general public, as well as by the relevant authorities. Therefore, the European legislation banned animal tests for the evaluation of cosmetic products and their ingredients by regulation EC No 1223/2009, which became mandatory in full by 2013 (Buzek and Ask, 2009). Tissue engineering of skin has been started in the 1970´s and owing to this important EU regulation

the impact of reconstructed human epidermis (RHE) or human full-thickness skin equivalents (HSE) in replacing animal-based tests increases. These organotypic skin equivalents led to apparent changes in the preclinical safety evaluation of topical applied substances, as seen by the animal replacement in the skin corrosion (OECD 431, 2014) or skin irritation test (OECD 439, 2013) by RHEs. Further they emerged as valuable tools for pharmaceutical requests. They can be utilized as complex human-based organ-like test systems for basic biology or disease research (Groeber et al., 2011; Ali et al., 2015) and are accessible for efficacy evaluation of novel drug delivery technologies (Flaten et al., 2015; Planz et al., 2016).

ENGINEERING DERMIS

The dermal layer is structurally divided in two layers. The upper layer, the stratum papillare, is composed of a thin arrangement of collagen fibers. It contains blood vessels and is so responsible for the adequate nourishment and waste removal of the epidermis. The following layer, the stratum reticulare is composed of a tight connective tissue and collagen fibers parallel to the surface of the skin. The dermal layer mostly consists of extracellular matrix (ECM), which presents the structuring element. It consists mainly of collagen and elastin as well as extrafibrillar matrix, composed of glycosaminoglycans or proteoglycans. Fibroblasts, the main cellular component of the dermis, provide a constant secretion of these matrix components (Bouwstra et al., 2003). The matrix is primarily responsible for the skin´s mechanical strength and hosts a variety of skin appendages and important cells like macrophages and mast cells, which are responsible for the immune response (Naves et al., 2016).

3-D culture One approach to mimic a dermis in skin equivalents was developed using acellular human de-epidermized dermis (DED). Using abdominal skin from human donors DED is prepared by the removal of the epidermis, while preserving the basal membrane followed by dermal sterilization (Tjabringa et al., 2008). Among the fact, that the composition of the DED is not clearly defined and has differences among the donors, a major disadvantage is the absence of fibroblasts. This is especially important considering the fact, that the presence of these cells stimulates proliferation of the keratinocytes, improves epidermal morphology and enhances the formation of certain proteins (El-Ghalbzouri et al., 2002; Marionnet et al., 2006; Wong et al., 2007). To prevent this obstacles, the dermal layer can be produced by using the "self assembly method" (Michel et al., 1999). This concept is based on the capacity of fibroblasts to create their own extracellular matrix *in vitro*, which makes it possible to obtain cell sheets which are easy to handle (Jean et al., 2009). Another procedure to obtain

adequate and stable 3-D structure can be achieved by seeding the fibroblasts in a scaffold. While the cells proliferate, differentiate and produce ECM, the scaffold can be degraded by hydrolysis or enzymatic reactions. In this thesis, another very practicable alternative to construct *in vitro* HSEs was used by seeding the keratinocytes on top of a self-prepared extracellular matrix (Figure 1).

In this process – firstly introduced by Bell and colleagues (Bell et al., 1983) – the fibroblasts were embedded into a matrix of collagen. The matrix approach is widely accepted and is also used in various commercially available full-thickness skin equivalents, which are pretty common in clinical and laboratory applications (Groeber et al., 2011; Kamel et al., 2013).

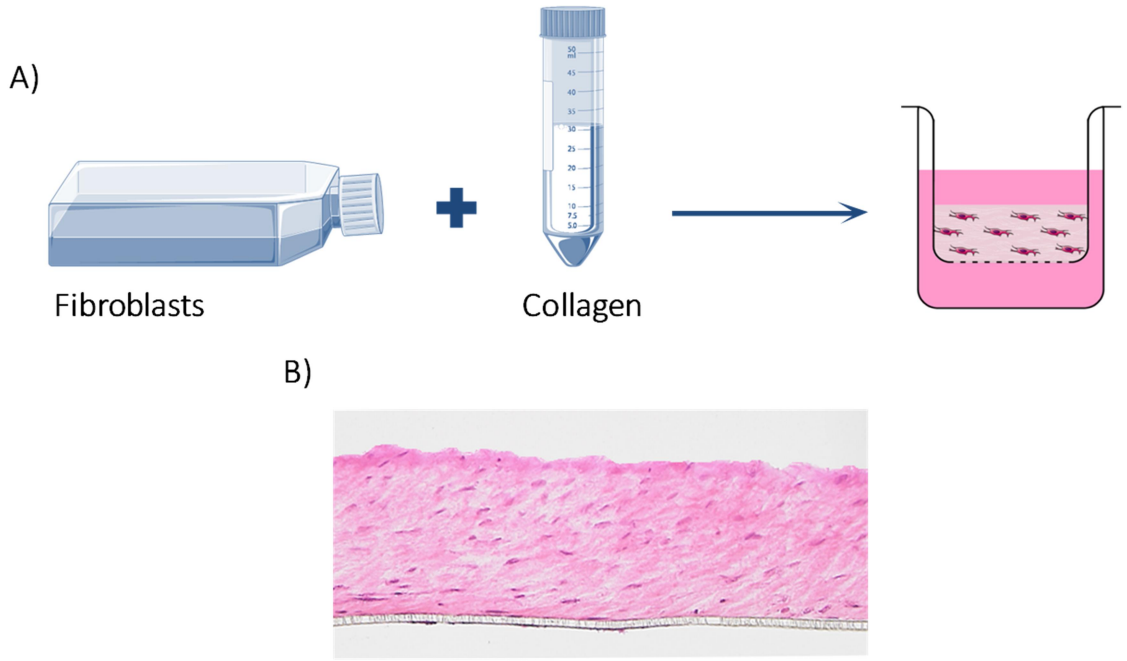

Figure 1. Engineering dermis. A) Schematic procedure outlines the generation of a collagen matrix-based dermis. B) Histological picture of the *in vitro* dermal layer on a polymer membrane (Hematoxylin and Eosin (H&E) staining; original magnification 200x). Scheme uses images from Servier Medical Art.

ENGINEERING EPIDERMIS

The top layer of the skin, called the epidermis, contains no blood vessels and is consequently supplied with nutrients from the blood capillaries in the upper dermis. The epidermis comprises a very small amount of ECM and keratinocytes are the predominant cell types found in the epidermis with up to 95-97%. A wide variety of different cell types is embedded in this matrix, including cells of the immune system responsible for antigen uptake, such as Langerhans cells; melanocytes, which are cells responsible for pigmentation; adnexal cells, which form hair glands and nails and sensory cells for pressure, known as Merkel cells

(Kanitakis, 2002). From bottom to top, the keratinocytes can be differentiated into four different layers: stratum basale (SB), stratum spinosum (SS), stratum granulosum (SG) and the stratum corneum (SC) (Figure 2). The journey of the keratinocytes – a very dynamic process – starts in the basal layer containing the stem cells of the skin. During stratification, stem cells undergo an asymmetric cell division, where different cell fate determinants are segregated unequally between the two daughter cells. This process allows the development of suprabasal cells that terminally differentiate and establish the skin barrier (Blanpain and Fuchs, 2009).

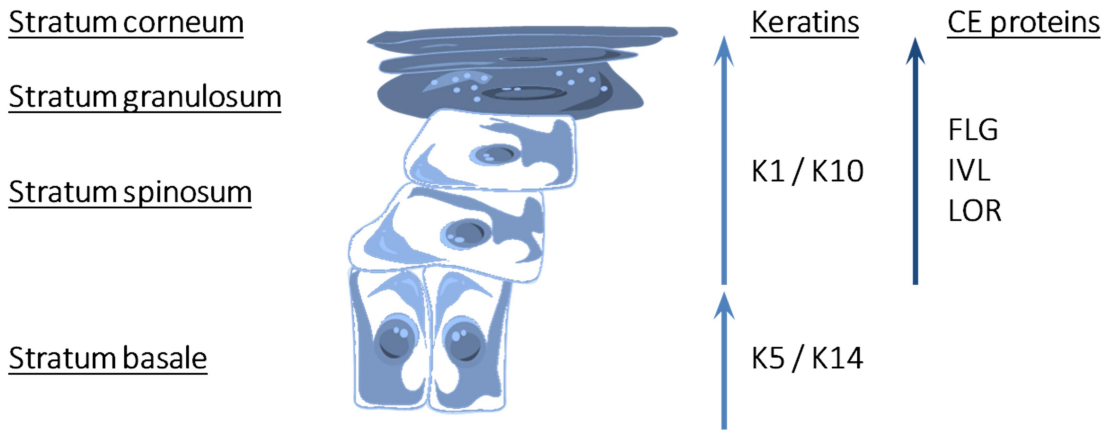

Figure 2. Schematic overview of epidermis structure. During differentiation, the keratinocyte show the expression of several keratins (e.g. K1, K5, K10, K14) and cornified envelope (CE) proteins in specific layers. At the stratum granulosum/stratum corneum interface, lamellar bodies containing lipid precursors are extruded. Scheme uses images from Servier Medical Art. (FLG = filaggrin, IVL = involucrin, LOR = loricrin).

During the differentiation process, which results in an enormous change in the function of the keratinocytes, they start to express several early and late differentiation markers. In the stratum spinosum the keratinocytes start to produce lipid-enriched lamellar bodies. In the upper spinous layers the keratinocytes become flatter and move into the direction of the stratum granulosum. Once in the stratum granulosum, the keratinocytes accumulate keratohyalin granules, which contain a number of barrier proteins, such as profilaggrin, loricrin (LOR) and involucrin (IVL) (Steven et al., 1990; Ishida-Yamamoto et al., 1993). At the interface of the stratum granulosum/stratum corneum the keratinocytes initiate the terminal differentiation process. The keratin filaments aggregate into a keratin matrix after interaction with the filaggrin subunit of profilaggrin. Additionally, enzymes will start to degrade the cell components, such as the nucleus and cell organelles. Furthermore, desmosomes, which link the keratinocytes together, are transformed into corneodesmosomes and a cornified envelope is formed around the plasma membrane. The cornified envelope is composed of several structural proteins like IVL, LOR and the small proline-rich proteins, which are cross-linked by transglutaminases (Candi et al., 2005). The lipid and enzymatic content of the

lamellar bodies is excreted via exocytosis at the stratum granulosum/stratum corneum interface and a major change in lipid composition occurs (Wertz, 2000; Feingold, 2009). All these changes in the keratinocytes and the secretion of the lipids into the intercellular regions consequently lead to the formation of the stratum corneum. In the end, this layer consists of dead flattened and nuclei absent cells, referred to as corneocytes, embedded in a continuous hydrophobic lipid matrix or envelope. This fundamental process leads to the development of a barrier against excessive water evaporation and protection against penetration of exogenous substances (Candi et al., 2005).

3-D culture Engineered epidermis provides an opportunity to study specific skin conditions and disorders of interest under controlled conditions. Mirroring the complexity of questions and issues that are able to address there is a relatively large number of different *in vitro* skin equivalents. The simplest approach is to build reconstructed human epidermis (RHE). These equivalents are composed only of an epidermal layer and were produced by seeding keratinocytes on the surface of a polycarbonate or cellulose acetate membrane forming a multi-layered matrix. Although they have the big disadvantage that they contain no fibroblasts, they have their scientific justification in particular for commercially available RHEs, which were used for skin corrosion and skin irritating testing (Groeber et al., 2011; OECD 431, 2014; OECD 439, 2013).

One of the first approaches of 3-D full-thickness human skin equivalents (HSE) were developed in the 1970´s. Freeman *et al.* cultured human epidermal cells, which were finally seeded on decellularized pig dermis (Freeman et al., 1976). To obtain a better differentiation of the keratinocytes this scientific concept was improved by culturing the keratinocytes in the above described human de-epidermized dermis (DED) (Tjabringa et al., 2008). Thereby, the procedure of engineering and the process behind the development of the epidermis is the same no matter if the dermis equivalent is self assembled (Jean et al., 2009) or based on the scaffold/matrix approaches as using in our experiments (Figure 3). The keratinocytes were seeded on the dermal compartment and following an immersion period – during the cells settled – the keratinocyte/dermis complex is placed at the air-liquid interface, thereby inducing keratinocyte differentiation and the formation of a stratified epidermis (Prunieras et al., 1983).

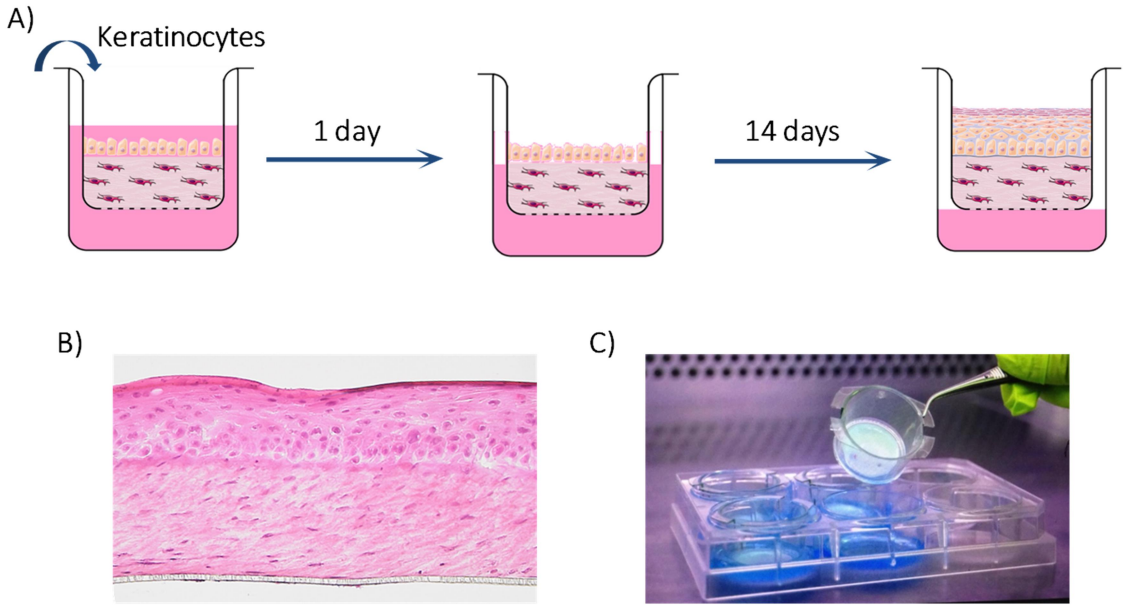

Figure 3. Engineering of a full-thickness human skin equivalent. A) Schematic procedure outlines the development of an *in vitro* skin equivalent after adding keratinocytes. B) Histological image of the equivalent clearly demonstrating the differentiation of the keratinocytes into a stratified multilayered epidermis (Hematoxylin and Eosin (H&E) staining; original magnification 200x). C) Representative picture of the skin equivalent in the trans-well system. Scheme uses images from Servier Medical Art.

1.2 ATOPIC DERMATITIS

Atopy describes a hereditary tendency to be hypersensitive to certain allergens and is immunologically defined by an inherited tendency to produce immunoglobuline E (IgE) antibodies against these allergens. Dermatitis presents a compound word, deriving from the Greek "derma" which means *skin* and "itis", which means *inflammation*. Together these words are describing a relapsing skin disease with a chronic, highly pruritic and inflammatory phenotype. Historically atopic dermatitis (AD) was described as disease solely affecting the skin but from today's perspective, AD is recognized as systemic disease with various disease associations (Darlenski et al., 2014). The disease results in significant morbidity and adversely affects quality of life (McKenna and Doward, 2008). The prevalence of AD has increased over the past 30 years and is currently estimated for children with 10 - 20% and 1 - 3% for adults in the developed countries (Nutten, 2015). The disease often starts in early infancy, given the fact that approximately half of the patients develop symptoms within their first year of life. One of four patients remains to have eczema into adulthood or experience a relapse of symptoms after symptom-free years (Williams, 2005; Bieber, 2008).

In the acute and subacute form the clinical manifestations of the eczema is characterized by active red infiltrate with oedema, vesicles, oozing and crusting. The diagnostic criteria can build upon characteristics starting with the essential presence and distribution of itchy eczema (e.g. hands and feet (Figure 4A)) in combination with associated features of the patient (e.g. atopy, onset age, comorbidities etc.) (Hanifin et al., 2004). However, many AD patients have a general tendency to develop dry skin, due to an extensive water loss trough the epidermis and the skin is pale because of increased tension in the dermal capillaries (Williams, 2005; Bieber, 2008).

Histological characteristics in acute lesions of AD patients mainly involve the epidermis. The suprabasal layers typically exhibit intercellular oedema when compared to normal tissue. The principal reason for this spongioses is the alteration in cohesion between the keratinocytes, resulting in enlarged intercellular spaces. Inflammatory infiltrate from lymphocytes can also be noticed in the epidermal layer. The differentiation of the keratinocytes is dramatically disturbed, clear visible by the retention of the nuclei as the cells ascending into the stratum corneum, known as parakeratosis (Figure 4B). Further epidermal hyperplasia corresponds to a thickening of the spinous layers and a high number of IgE bearing Langerhans cells are present. The lesional dermis is characterized by infiltration of T cells, macrophages, eosinophils together with elevated numbers of mast cells (Mu et al., 2014).

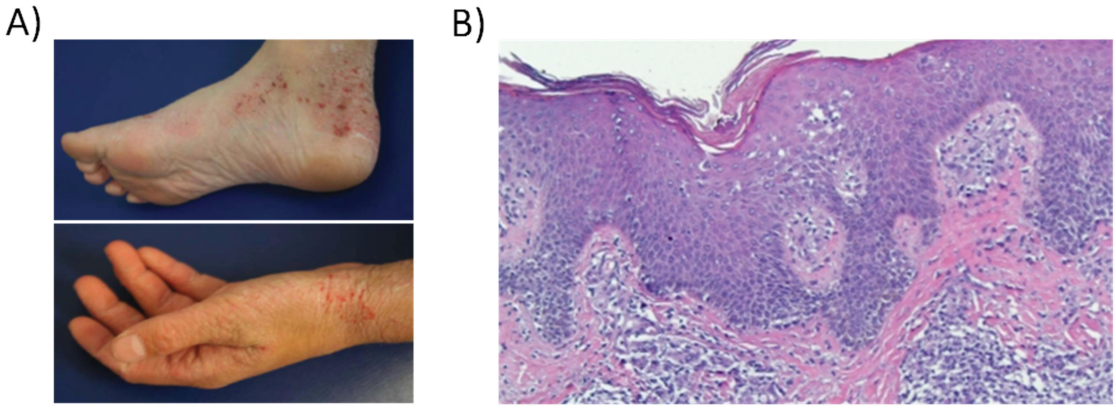

Figure 4. Clinical presentation and histopathology of atopic dermatitis. A) Visible lesions on the hand and feet. B) Typical disturbed differentiation of keratinocytes (spongiose, hyperplasia) in the epidermal layer of the skin (Hematoxylin and Eosin (H&E) staining; original magnification x200). (A, Adapted and reprinted by permission from BioMed Central using the terms of the Creative Commons Attribution License CC BY 2.0, Atopic dermatitis, copyright 2011, (Watson and Kapur, 2011)) (B, Reprinted by permission from dermpedia.org using the terms of the Creative Commons Attribution License CC BY-NC-ND 3.0, dermpedia textbook chapter: Atopic dermatitis, author: Stephen Lyle, copyright 2010).

PATHOPHYSIOLOGY

The pathogenesis of AD has been attributed to a complex interaction of the environment and host susceptibility. This supported the hypothesis, that AD can present a cutaneous symptom of a systemic disorder that also give rise to other atopic conditions. It is known, that the disease is often the initial step on the "atopic march", which leads to asthma, allergic rhinitis or both (Zheng et al., 2011). Besides the mentioned factors, there are also other triggers of AD, which play an equally important role. These are a disturbed skin barrier caused by epidermal/dermal alterations (Cork et al., 2009), dysregulations in the immune response (Levin et al., 2013) and changes of the biological innate defense system (Kopfnagel et al., 2013; Williams and Gallo, 2015), each of them being potentially relevant for the induction of other alterations, creating a vicious circle responsible for the acute lesions. Much too often, each component has been studied independently. However, recent findings have suggested that they interact in a highly complex manner in the development of that severe disease (Boguniewicz and Leung, 2011).

Skin barrier An intact, healthy skin barrier is a critical first line of defense against external influences and xenobiotics. Conditioned from significant barrier disruption in the epidermal layer, AD patients have an increased susceptibility to allergic sensitization and higher rates of microbial colonization and infections (Zöllner et al., 2000; Boguniewicz and Leung, 2010). In

up to half of the AD patients – depending on the evaluated population (Kim and Leung, 2012) – the described barrier disruption is directly correlated to a loss-of-function mutation in a gene, which encodes the crucial epidermal barrier protein filaggrin (FLG). These mutations results either in a reduction or complete absence of filaggrin and its degradation products and are considered as the strongest known risk factor for developing AD (Palmer et al., 2006; Weidinger et al., 2006). FLG deficiency leads to an insufficient aggregation of the keratin cytoskeleton and drives an incomplete formation of the cornified envelope. It is hypothesized that this results in decreased stratum corneum hydration, increased transepidermal water loss and reduced natural moisturizing factor (NMF) (Brown and McLean, 2012). The increased enzyme activity could lead to further barrier breakdown and precipitate Th2 inflammation (Elias, 2010). These findings verified the “outside-inside” hypothesis that the barrier abnormality can function as the initial trigger for the disease activity. The opposite is described by the "inside-outside" hypothesis, where epidermal permeability and infections are assumed to be downstream consequences of an immunologic abnormality (Howell et al., 2009). This is of special interest, when considering that half of the AD patients do not suffer from *FLG* mutations (Kim and Leung, 2012) and reduced NMF levels were found independent from the *FLG* genotype (Kezic et al., 2011). The current most probable explanation is the combination of both hypotheses ("outside-inside-outside") (Figure 5).

In addition to the cornified envelope in the stratum corneum, tight junctions (TJ) that are located on opposing membranes in keratinocytes form a second barrier in the stratum granulosum. TJs are complexes of adhesive and scaffolding proteins that control the paracellular passage of water, ions, and solutes (Niessen, 2007). In AD patients it was shown that the reduced expression of claudin-1 – a major transmembrane protein in epidermal TJ – inversely correlates with levels of circulating eosinophils and serum IgE (De Benedetto et al., 2011). Furthermore, decreased expression of occludin and zonula occludens-1 have also been detected in the epidermis of filaggrin-deficient patients, which might be one factor contributing to the skin barrier impairment in these subjects (Gruber et al., 2011). The role of TJs in AD is still unknown and although these findings indicate an impaired TJ barrier, the reasons for TJ dysfunctions as well as their linkage to *FLG* abnormality and immunological dysregulations remains to be fully elucidated.

Immune response When compared to normal skin, acute lesions of AD patients are infiltrated of considerably high number of interleukin (IL)-producing $CD4^+$ lymphocytes that differentiate to a T helper (Th) cells phenotype. The infiltrated cell population is divided into a significantly greater number of IL-4, IL-5 and IL-13 expressing cells – described as Th2 cells – compared to a few interferon-γ or IL-12 (Th1 cells) and antigen-presenting cells (APCs) like Langerhans

cells, dendritic cells (DCs) and macrophages to a lesser extent (Oyoshi et al., 2009). The predominant Th2 phenotype in the acute phase is widely accepted as a characteristic of the pathogenesis. Evidence suggests that the two sister cytokines IL-4 and IL-13 are potent mediators and further key drivers of the inflammation in AD (Leung et al., 2004; Ong and Leung, 2006; Bieber, 2008; Boguniewicz and Leung, 2010) (Figure 5). Transgenic mice overexpressing IL-4 developed skin lesions spontaneously which exhibits clinical diagnostic criteria for human AD (Chan et al., 2001). Selective expression of IL-13 causes skin inflammation characterized by immune cell infiltration and significant skin remodeling (Zheng et al., 2009). In AD these cytokines are growth factors for other immune cells (e.g. B cells), initiators of isotype class switching to IgE and are reported to increase eosinophil recruitment to the eczematous skin. Additionally IL-4 itself presents a differentiation factor polarizing naive $CD4^+$ to the Th2 phenotype (Gandhi et al., 2017). Both cytokines have a strong effect on the skin barrier. In keratinocytes, presence of IL-4/IL-13 induce a dramatically downstream regulation of the cornified envelope proteins LOR, IVL (Kim et al., 2008) and in particular FLG (Howell et al., 2009).

Beside T cells, another major source of Th2 cytokines are mast cells (Horsmanheimo et al., 1994). The number of these bone marrow-derived cells is increased in AD lesions, especially in areas of lymphocytic infiltration in the papillary dermis. Their IgE-sensitized degranulation directly induce and activate Th2 cells, DCs and eosinophils and is correlated with the severity of AD (Zhao et al., 2006). In addition, thymic stromal lymphopoetin (TSLP) in AD patients can activate mast cells to produce more Th2 cytokines (Nagarkar et al., 2012). TSLP, a cytokine from the IL-7 family, constitute a critical connection link between the barrier defects in AD and the Th2 inflammation. Increased expression in keratinocytes is induced by enhanced allergen penetration, after mechanical stimulation e.g. scratching (Oyoshi et al., 2010), or by Th2 related cytokines (Bogiatzi et al., 2007). Additionally, TSLP expression is induced by abovementioned barrier influencing *FLG* mutations (Lee et al., 2011; Moniaga et al., 2013). TSLP is considered to play a major role in AD pathogenesis. TSLP activates dendritic cells, which induce naive T cell proliferation to Th2 phenotype, aggravating the inflammatory response and the production of IL-4, IL-13 and TNFα (Wu et al., 2010; Ziegler, 2010). It converts epidermal Langerhans cells into APCs that induce proallergic T cells (Ebner et al., 2007) and is able to trigger direct T cell migration into the epidermal layer (Wallmeyer et al., 2017). Thus, TSLP plays an important role in the Th2 skewing, which is an integral part of AD (Figure 5). At last it has to be mentioned, that TSLP itself is able to induce a downregulatory effect of filaggrin expression in keratinocytes mediated by STAT3- or ERK-dependent pathways (Kim et al., 2015).

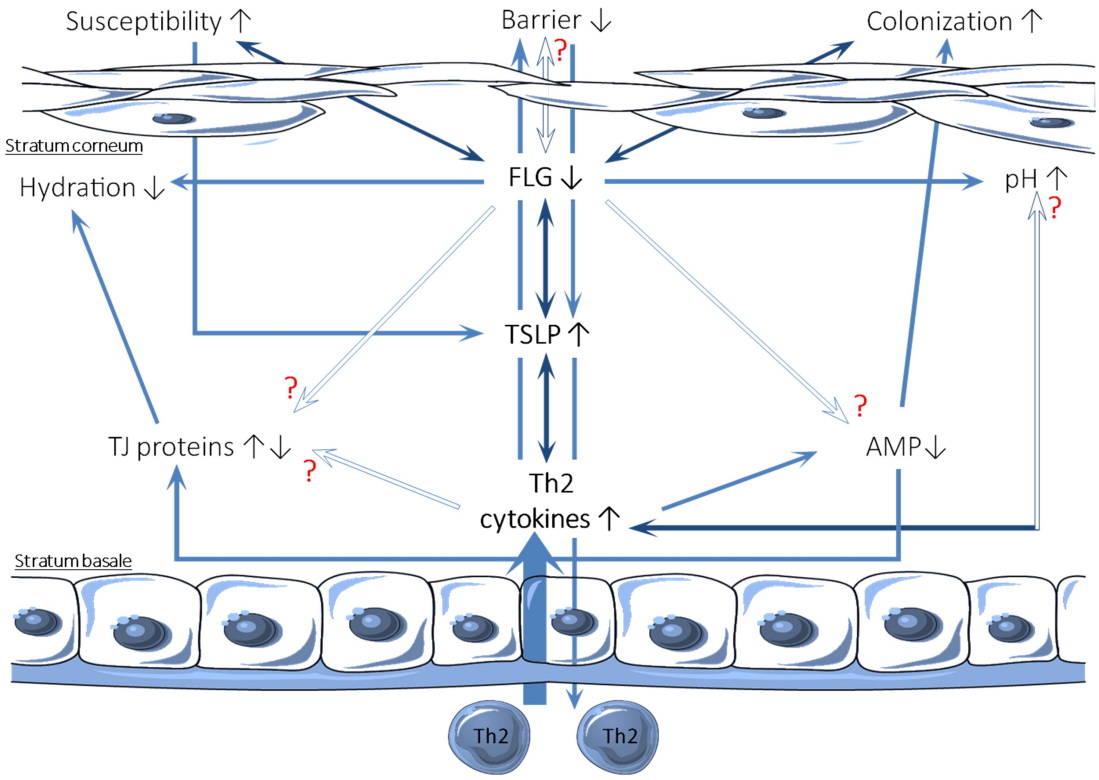

Figure 5. Essential pathogenesis characteristics of atopic dermatitis. Outside-inside initial provocation of AD can lead to an "outside-inside-outside" vicious cycle. Although several triggers and outcomes are known, there are still not investigated connections. Scheme uses images from Servier Medical Art. (AMP = antimicrobial peptide, FLG = filaggrin, TJ = tight junction, TSLP = thymic stromal lymphopoetin, Th2 = T helper cell type 2).

However, it should be noted, that AD is a biphasic inflammatory disease. Although the Th2-biased immune response is closely linked to the acute phase, there is a switch towards a Th1 predominant paradigm in its chronic phase (Grewe et al., 1998), which may be attributable to the corresponding predominate type of DCs (Novak and Bieber, 2005). Continuously other immune cells, like Th17 and Th22 cells had been found to be increased in AD. This newly discovered subpopulations are different from Th1 and Th2 cells and their associated expressed cytokines (IL-17 or IL-22) also induce immune cell recruitment, skin inflammation and changes in the epidermal barrier (Mu et al., 2014).

Defense system A clinical manifestation of AD patients is a dysbiosis in their skin microbial flora, in particular an increased colonization with *Staphylococcus aureus,* observed for the first time in the 1970´s (Leyden et al., 1974). The prevalence of the increased colonization in AD lesions varies, but the capacity to culture *S. aureus* from the patients was reported to be approximately 80-100% and is strongly correlated to increased severity of the disease (Gong et al., 2006). Interestingly, recent investigations revealed that apart from an overall increase

of colonization, the total number of different bacteria types is also decreased (microbial diversity) in the involved skin (Kong et al., 2012). A potential basic explanation for the dysbiosis is the dramatically changed environment for bacterial growth in AD skin compared to normal skin. Defective functions in the physical skin barrier results in an increase in serine protease activity and skin surface pH that favors *S. aureus* growth (Rippke et al., 2004). This explanation can be further supported by immunologic factors influencing the skin microbial composition. As components of the innate immune and defense system, antimicrobial peptides (AMPs) are a crucial factor for preventing pathogenic microbes infecting the skin and for maintaining epidermal barrier effectiveness. The AMPs are divided into cathelicidins, psoriasin, ribonuclease 7 and the important human β-defensin family including hBD-1, 2 and 3. These amphiphatic, positive charged structures are mainly synthesized in the stratum granulosum and have a broad spectrum of antimicrobial activity (Nakatsuji and Gallo, 2012). The role of AMPs in atopic skin is complex and has been the subject of many discussions (Kopfnagel et al., 2013). In AD patients, reduced levels of the peptides – especially hBDs – further enhance the risk of skin infections (De Benedetto et al., 2009). Interestingly, higher levels of hBD-2 and hBD-3 have been detected in AD skin relative to healthy controls. However, compared with psoriasis, hBD levels are dramatically diminished in AD, likely due to down-regulatory effects of the Th2 cytokines found in IL-4 and IL-13 treated keratinocytes (Nomura et al., 2003; de Jongh et al., 2005; Albanesi et al., 2007) (Figure 5). In fact, the relationship of skin barrier dysfunction, immune abnormalities and increased susceptibility to microbial infections remains to be fully elucidated which is also seen in the gap, that the direct relationship between FLG deficiency and AMP expression has not been investigated to date.

IN VITRO INVESTIGATIONS OF ATOPIC DERMATITIS

To understand the fundamental mechanisms leading to the development of the AD, especially the interaction between the various trigger facts, extensive studies in the future are necessary. Therefore, different mice models – developing AD symptoms after specific procedures (Jin et al., 2009) – have been generated to investigate the development of AD and to examine potentially protecting or resolving treatments and drugs. There is no dispute that human and mouse skin exhibit many similarities. Nevertheless, beside the fundamental difference that mice are not able to spontaneously develop AD, further variations limit the translation of disease conditions from experimental mice to humans. These are for instance a higher number of hair follicles and a lower number of epidermal layers and, of particular interest in terms of investigating drug delivery approaches. Further problems are shortened

skin tissue renewal time (10 days vs. 28 days), lacks of sweat glands, different T cell populations (Wagner et al., 2010; Pasparakis et al., 2014) and, as mentioned at the beginning, important gene expression variations (Gerber et al., 2014). In fact, using *in vitro* skin equivalents will not heal all these differences in an instant, but several approaches regarding AD have been published in the last decade focusing on either immune dysfunctions or epidermal barrier alterations. The results showed, that the use of appropriate skin equivalents can support preclinical research to overcome these issues.

2-D cultures Although monocultures of single cell types like immune, epithelial or connective tissue cells do not display complex structures and constitute the simplest *in vitro* approaches, they have still their significance. They are used for investigations of basic questions regarding the biology of the specific cell types and cultures. They are efficient at providing results with potential significance and have the advantage of simple handling and analytical probing.
Epidermal differentiation of keratinocytes can be mimicked *in vitro* through control of Ca^{2+} concentration or cell number (Poumay and Pittelkow, 1995). To study the effect of the Th2 cytokines either HaCaT cells (Omori-Miyake et al., 2014) or primary human keratinocytes (Howell et al., 2009) have been incubated with IL-4/IL-13 to identify downstream mechanisms affecting proteins that are crucial for the skin barrier and structure, e.g. keratins, FLG or IVL. Experiments on keratinocyte monolayers showed that silencing of *FLG* expression induced alterations in the amounts of synthesized cornified envelope related proteins and results in release of Th2 cytokines (Dang et al., 2015). In contrast to this "disease-inducing" approach, keratinocytes obtained from AD patients were cultured and investigated. Compared to cells from healthy donors, these patient cells exhibited an altered chemokine production inside the cell, e.g. granulocyte macrophage colony-stimulating factor (GM-CSF) (Pastore et al., 1997). In addition, further cytokine treatment led to the production of other chemokines, revealing an increased sensitivity of these cells against external trigger facts (Giustizieri et al., 2001).
Although keratinocytes cultured as immersed monolayers are simple and useful research tools, it has to be considered that these 2-D cultures do not stratify and produce an efficient barrier. Hence, the development of epidermal *in vitro* equivalents that produce a functional barrier has become crucial to screen treatments targeting the skin barrier or the inflammation.

3-D cultures With the help of RHE/HSE approaches the scientific insights from the 2-D culture regarding the negative influence of the Th2 cytokine milieu were successfully transferred in 3-D equivalents. Firstly performed in 2011, Kamsteeg and colleagues found morphological and molecular characteristics of AD in a skin equivalent (Kamsteeg et al., 2011). Here, distinct

epidermal changes were found after incubation with IL-4 and IL-13. In various skin equivalents, independent from the additional treatment of IL-25 (De Vuyst et al., 2016), IL-31 (Danso et al., 2014) or IL-21 (Bernard et al., 2012) spongiosis and hyperkeratosis were found in combination with decreased expression of epidermal proteins like FLG and LOR or increased TSLP secretion. Additionally, alteration in barrier function and SC lipid compositions were observed. A much more complex approach to study the effect of the inflammatory aspect on skin characteristics used HSEs made from HaCat cells (Engelhart et al., 2005) or primary keratinocytes (van den Bogaard et al., 2014) following the exposure to T cells. In concordance to 2-D culture, also the use of cells from AD patients and healthy controls were recently considered to study e.g. keratinocyte-fibroblast interaction (Berroth et al., 2013).

Regarding keratinocyte differentiation, the negative effect of primary FLG deficiency (e.g. due to mutations) were also proven by the use of 3-D skin equivalents. Sufficient downregulation of FLG expression by using siRNA has been reported first by Mildner and colleagues and resulted in increased permeability, hypogranulosis and enhanced susceptibility against external factors (Mildner et al., 2010). Reports from our group confirmed these conclusions with additional findings on disturbed keratinocyte differentiation and SC development (Küchler et al., 2011; Wallmeyer et al., 2015), altered acidification pathways and impaired lipid profile (Vávrová et al., 2014). Similar findings were obtained by silencing *FLG* using shRNA interference, which results also in disturbed epidermal differentiation (e.g. hypogranulosis) and increased barrier permeability, together with reduced number of epidermal layers, cornified layer thickness, and decreased levels of NMF (Pendaries et al., 2014). Interestingly, epidermal equivalents were also used to investigate the connection between FLG deficiency and increased bacterial colonization (van Drongelen et al., 2014). For studying the effects of primary FLG deficiency also skin equivalents with the use of AD patient cells were developed and showed similar characteristics such as impaired lipid metabolism (Blunder et al., 2017).

Current research on 3-D *in vitro* AD cultures were able to evaluate the predicted pathological effects of inflammation or skin barrier alterations and success is mirrored in the clinical hallmarks which were found in these skin equivalents. In contrast, it also supports the controversial discussion on the role of single effects – in particular FLG deficiency – on the pathogenesis. After *FLG* silencing in HSEs no alterations were reported neither regarding epidermal morphology or lipid composition nor epidermal permeability (van Drongelen et al., 2013). These supported either the findings that even individuals with *FLG*-null allele may not develop AD (O'Regan et al., 2008) but also endorsed AD as a multifactorial disease, where individual aspects should be investigated altogether (Kabashima, 2013). Due to this fact, it was more astonishing, that at the beginning of this thesis in 2013/2014 there was no *in vitro*

HSE available which combined the primary FLG deficiency and the inflammatory Th2 aspect. Thus, one of our research aims was to establish an HSE approach, which implies the effects of both AD hallmarks to gain an insight in their direct interaction.

TREATMENT OF ATOPIC DERMATITIS

AD is not curable and many patients will experience a chronic course in later life. Guidelines from expert associations (e.g. The German Society of Dermatology) suggests a stepwise regimen for the treatment in terms of restoring epidermal barrier defects and reducing skin inflammation when necessary (Werfel et al., 2016).

The first aim relates primarily to prevention. The avoidance of specific and unspecific irritants, allergens and trigger factors such as particular food or clothing is crucial and known to postively influence the severity of the disease. This aim is best addressed by trying to reduce the dryness of skin with a topical basic therapy, which only contains skin moisturizing cream or emollients as well as regular bathing. Intensive use of emollients increases the hydration of the epidermis, mainly by reducing evaporation. Moisturizers have a more complex mode of action as they act by restoring the structural lipid components of the SC, which can correct the increased permeability (Thomsen, 2014). The maintenance of epidermal hydration can improve the management of AD by reducing the need for further topical drug containing therapies (Lebwohl et al., 2013). Interestingly, the prophylactic use of emollients at the neonatal period is associated with a trend towards reduced incidence of AD at later ages (Lowe et al., 2017). For moderate to severe AD the application of topical glucocorticoids (GCs) is the first line of pharmacological therapy (Figure 6). These agents effectively control atopic flares through their anti-inflammatory, anti-proliferative, and immunosuppressive actions. The sufficient effect is based on their binding to the glucocorticoid receptor (GR). After activation the receptor works as a nuclear transcription factor and is able to induce/inhibit gene transcription and is known to inhibit the activity of other immune-modulating transcription factors (Ratman et al., 2013). Due to the massive involvement in the regulation of genes, the main disadvantages of topical GCs are their side effects. In the same time, where the anti-inflammatory effect occurs, GCs downregulate the expression of ECM proteins (e.g. collagens) as well as matrix metalloproteinase inhibiting enzymes (e.g. TIMP-1 or TIMP-2). These are major compartments of the connective tissue and their provoked decrease by long term treatment results in skin atrophy (Schoepe et al., 2006). Besides these facts, when used properly the risk of side effects is very small and the fear should not inhibit the compliance since insufficient use can cause worsening of the eczema (Thomsen, 2014).

Approved for the second line treatment are topical calcineurin inhibitors (TCIs). But, given the fact of high costs and the consideration of their not fully known long-term safety profile (e.g. rare cases of skin malignancy and lymphoma have been reported) long-term use should be avoided. TCIs are reserved for adults and infants with persistent disease that would require continuous topical corticosteroid treatment or for patients severely affected in sensitive skin areas (e.g., around the eyes or genitals), where systemic absorption and the risk of skin atrophy are of particular concern. Systemic immunosuppressive treatments with GCs are reserved for acute treatment of severe flare-ups with therapy duration of 3 days. Other systemic drugs like cyclosporin A are strictly approved only for very severe, persistent forms of the disease. Adjuvant therapy options (e.g. phototherapy), symptomatic treatments with antihistamine and interventions of antimicrobial infections – especially occurring during topical GC treatment – with oral or topically administered agents can be given during all stages of the disease (Werfel et al., 2016).

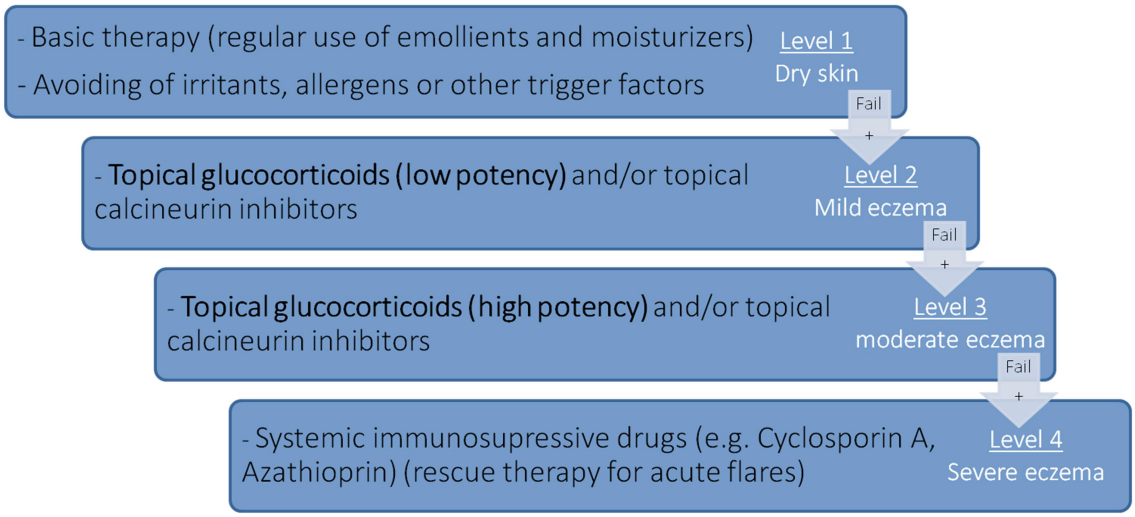

Figure 6. Stepwise algorithm for the treatment of atopic dermatitis. Adapted from the guidelines from The German Society of Dermatology (Werfel et al., 2016).

1.3 TOPICAL DRUG DELIVERY

For many skin diseases including atopic dermatitis, topical treatment is preferred to systemic drug application. Higher drug doses at the target site and reduced systemic adverse effects can be achieved by topical administration compared to parenteral or oral drug administration. Furthermore, cutaneous drug uptake prevents extensive metabolism in the liver before the drug gets to the target. Three major routes of cutaneous drug uptake are possible after topical application: the intercellular route, the transcellular route, and the transappendageal route (*via* hair follicles and sweat glands). Despite these permeation routes and the large surface area, the skin provides an efficient barrier to drugs owing to the lipids and keratin in the SC, tight junctions in the SG and various metabolizing enzymes in the viable epidermis. Hence, most therapeutic compounds demonstrate a poor penetration capacity into the human skin.

Therefore, enormous efforts have been made to develop intelligent transdermal drug delivery systems in particular with focus on increasing therapeutic activity and minimizing undesirable side effects (Prausnitz and Langer, 2008; Rizwan et al., 2009). This include for example improvements of conventional carrier systems like liquids, semi-solid (e.g. ointments and creams) or solid systems (e.g. patches) (Daniels and Knie, 2007). Elsewhere, smart strategies to overcome the skin barrier were developed such as microneedles (Bariya et al., 2012; Witting et al., 2015a) and especially nanoparticulate carrier systems, which have gained notable attention in the last decade (Prow et al., 2011; Goyal et al., 2016).

Considering the fact, that not every drug delivery system – physical or chemical based – is suitable to apply in patients with inflammatory skin disease, various nanoformulations were developed. But, the question rises why only one dermal application on a nano-based formulation is currently in a clinical trial (Ragelle et al., 2017). On the one hand this problem is based on reported limited particle instability or problematic safety profiles (Duncan and Gaspar, 2011), which requires the development of new chemistry strategies. On the other hand preclinical results are often overinterpreted and the translation into clinical trials presents a tough challenge (van der Meel et al., 2017).

NANOCARRIERS FOR TOPICAL DRUG DELIVERY

The definition of nanocarriers designated by the American National Nanotechnology Initiative has been adopted by national institutes for regulatory purposes as particles with all dimensions between 1 nm and 100 nm (Lövestam et al., 2010) but extended in research up to 1000 nm (Jain, 2017). An innumerable amout of different nanocarrier types have been

developed as transdermal drug delivery systems. The theory and practical aspects of the nanocarriers-mediated delivery especially for the treatment of skin diseases have been covered in various referenced texts (Korting and Schäfer-Korting, 2010; Gupta et al., 2012; Raphael et al., 2015). The developed approaches increase drug solubilization in the formulation, partitioning within the skin layers and permeation through the skin. Improvements in drug stability, therapeutic potential and drug safety are achieved by packaging the drug in targeted carriers that localize drug action to the site of disease. Nanocarrier accumulation in the upper skin layers or formation of semiocclusive films of their components increases formulation retention time and prolongs drug release. Additionally, nanocarriers have been shown to aggregate within hair follicles and skin furrows providing a potential depot effect.

Dendritic approaches Among the various polymeric architectures, dendritic (tree-like) polymers have received a substantial scientific focus for their highly branched, multifunctional, and well-defined structures. In a dendritic molecule, the modular arrangement of branches confers a two-fold structural parameter which is critical for drug delivery applications. With increasing orders of branching, the molecule takes on a 3-D spherical shape due to symmetrical congestion of the branching units, thereby yielding supramolecular void spaces. Furthermore, multiple surface functional groups which render the molecule amenable to a wide range of chemical modifications (Menjoge et al., 2010). These two features have particularly made dendritic polymers suitable for delivering bioactive guests. Thereby, dendritic scaffolds have found many applications for designing nanoscale drug delivery carriers where the active compounds are homogeneously distributed within the nanocarrier (Quadir and Haag, 2012).

As one of these promising architectures dendritic core-multishell (CMS) nanocarriers were introduced in 2007 as liposome-analog amphiphilic carrier with superior stability compared to liposomes (Radowski et al., 2007). The initial approach was based on hyperbranched poly(ethylenimine) core architectures which were replaced by dendritic polyglycerol due to its lower cytotoxicity (Frey and Haag, 2002; Calderon et al., 2010). The dendritic core is conjugated by an amide linker with a surrounding lipophilic inner shell, composed of octadecanoicacid (C18), followed by a hydrophilic outer shell based on monomethoxypoly(ethylene glycol) also known as mPEG (Figure 7). The final result of the synthesis are globular nanocarriers with a molecular weight of 70,000 g/mol and a diameter of 7-16 nm (Boreham et al., 2014), but these unimers are able to build aggregates up to 80 nm depending on the concentration and the encapsulated guest molecule (Fleige et al., 2012). In terms of drug delivery, these CMS nanocarriers are of special interest due to the

fact, that they are able to encapsulate a wide range of guest compounds (Radowski et al., 2007) and are able to transport them to polar and non-polar environments. As a logical consequence, penetration enhancement of lipophilic and hydrophilic substances was shown in viable skin layers after topical CMS nanocarrier application (Küchler et al., 2009a; Küchler et al., 2009b) and has been confirmed in various following approaches. These include the penetration of spin labels for electron paramagnetic resonance (EPR) spectroscopy (Haag et al., 2011), peptides (Do et al., 2014) or fatty acids into skin appendages (Lohan et al., 2016).

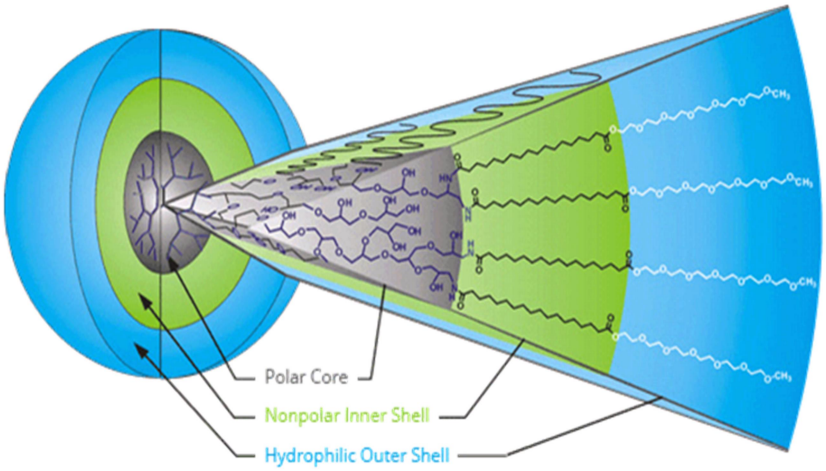

Figure 7. Core-multishell (CMS) architecture. Hyperbranched dendritic polyglycerol and connected inner and outer shell (Reprinted by permission from John Wiley and Sons, Radowski et al., copyright 2007, (Radowski et al., 2007)).

The same question arises for another promising polymeric architecture using dendritic polyglycerol named thermoresponsive nanogels (tNGs). In these carriers the multifunctional surface of dendritic polyglycerol (dPG) enabled its function as a macro-crosslinker between linear thermoresponsive units e.g. thermoresponsive polyglycerol (tPG) or poly(N-isopropylacrylamide) (pNIPAM). This results in 3-D scaffolds of polymeric building blocks forming particles due to chemical bonds and/or physical interaction (Kabanov and Vinogradov, 2009). Their behavior to absorb large amounts of water in their polymeric network in aqueous media enables them also to encapsulate biologically active molecules, providing a protective environment from degradation or rapid clearance (Billiet et al., 2012; Molina et al., 2014). They are attractive carriers for the topical delivery of drugs due to their unique ability: while they are swollen with water below their cloud point temperature (Tcp), the polymers of the tNGs undergo a reversible transition from highly hydrophilic to hydrophobic state when exposed to greater temperatures, resulting in gel shrinking and the expulsion of water (Figure 8). A broad range of therapeutic agents may be encapsulated within tNG and then released from these in a temperature sensitive manner e.g. by using the

thermal gradient of the skin (32 °C – 37 °C) as trigger (Asadian-Birjand et al., 2012; Bergueiro and Calderón, 2015; Sahle et al., 2017).

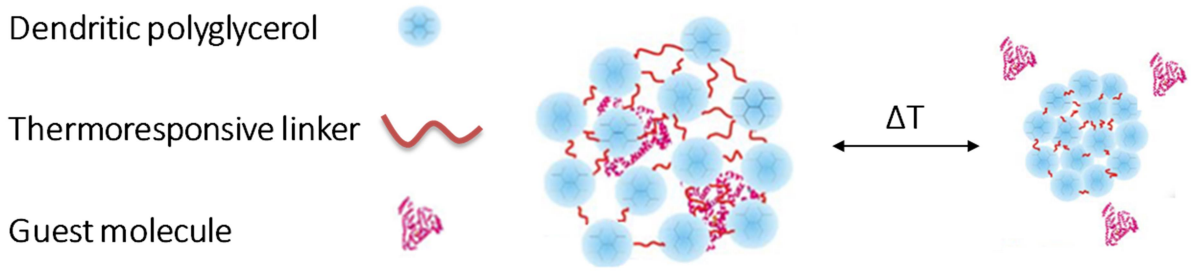

Figure 8. Thermoresponsive nanogels. Schematic picture of structure and mode of drug release (Adapted and Reprinted by permission from Elsevier, Witting et al., copyright 2015, (Witting et al., 2015b)). (ΔT = temperature gradient).

TOPICAL DRUG DELIVERY EVALUATION *IN VITRO*

Preclinical studies are necessary to evaluate new drugs or pharmaceutical approaches for skin diseases. For the evaluation, drug absorption and corresponding pharmacological effects have to be identified, to permit a reliable forecast for their medical possibilities. But, to fully utilize the capability of the transdermal route and the new agents in a reliable and ethical framework, the characterization of their potential on validated human *in vitro* test skin systems is necessary.

Drug absorption In general, excised human skin is the ideal model for *in vitro* skin absorptions experiments and is clearly acknowledged as the gold standard (Küchler et al., 2013; Flaten et al., 2015; Planz et al., 2016), although the follicular route is neglected in this model. Generally, breast or abdominal skin should be used, obtained from breast reductions or abdominoplastic surgeries (Küchler et al., 2013). The excised skin has the advantages of retaining their barrier properties, valid storage procedure at -20 °C as well as a very good and validated *in vitro/in vivo* correlation (Schäfer-Korting et al., 2006; Schäfer-Korting et al., 2008). Occasionally, the *in vitro* approach with excised skin can be even more sensitive than the *in vivo* setting when comparing different drug delivery systems (Franz et al., 2009).

Most of the absorption studies are performed using the static-typed or flow-through Franz-diffusion cell method. This consists of a donor and a receptor chamber, where the excised full-thickness skin is placed in between, that the stratum corneum facing the donor compartment, where examined formulations are applied. The high significance of this approach is based on the performance under validate conditions (temperature, receptor medium, stirring conditions etc. (OECD, 2004; Ng et al., 2010)) and the various feasible drug

quantification methods, which can be conducted with the skin sample e.g. spectroscopic or chromatographic methods (Flaten et al., 2015)

Pharmacological efficacy Aiming for highly predictive non-clinical testing, a variety of human cell-based *in vitro* skin systems have been developed and should be preferentially used whenever possible. Although some systems for skin disorders with special conditions are available, efficacy testing of new therapeutics on skin disease equivalents is not widely investigated and only a few approaches are described. In general, it can be stated that a main obstacle in this field is the generation of valid and adequate readout parameters.

For example, transglutaminase-1 deficient skin equivalents were developed, mimicking the main characteristic of autosomal-recessive congenital ichthyosis (ARCI) (Witting et al., 2015b), a disease with disturbed keratinocyte differentiation and altered skin barrier function. An efficacy testing of topical enzyme replacement therapy for ARCI with the help of nanogels, resulting in repaired skin barrier, is currently under investigation in patient-derived skin equivalents (Plank et al., in preparation). Another successful *in vitro* approach was described for non-melanoma skin cancer (Zoschke et al., 2016). Accordingly carcinoma cells were incorporated into the epidermal layer and skin equivalents were characterized after cultivation. Furthermore their suitability for efficacy testing of cytostatic agents was shown, by reducing the proliferation of the cells within the tissue. A similar issue was addressed from Ziegler and colleagues with their UVB-radiated skin equivalents (Zöller et al., 2008). For the topical applied compounds, anti-inflammatory as well as typical side effects were shown in the skin equivalents and this even depending on the strength of the compound. There are also *in vitro* approaches for skin infections. Reconstructed skin infected with the Herpes Simplex Virus was used to study the influence of antiviral agents such as topical applied Aciclovir on the progress of the infection (Andrei et al., 2005).

For psoriasis, several *in vitro* skin equivalents are described in literature (Soboleva et al., 2014) and their usefulness was successfully intensively reviewed (Desmet et al., 2017) and described by studying the effects of topical therapeutic drugs e.g. calcipotriol (Rüffer, 2011). In skin equivalents treated with TNFα, one major cytokine for skin inflammation, efficacy of topic glucocorticoids was shown by reducing pro-inflammatory cytokines (Weindl et al., 2011).

For AD the situation is a bit different. Although quite a few 3-D *in vitro* approaches have been published over the last decades, there is no publication that investigated the skin equivalent in respect to their ability to respond to known topical drugs, excepting an approach for eczematous dermatitis (Engelhart et al., 2005). Empirical knowledge about evaluation of systemic drug application on FLG deficient skin equivalents were recently obtained by our

group (Wallmeyer et al., 2015). Hence, skin equivalents – emulating characteristics of AD *in vitro* – were established in this thesis and investigated related to their suitability for biology und pharmaceutical research. The disease was selected as an example due to published studies on normal reconstructed skin, the high proportion of animals used for AD research, and the unmet clinical need in treating the disease.

1.4 AIM OF THE THESIS

This thesis aims to lay the foundations for the formation of relevant and reliable skin disease equivalents and to establish their applications in basic dermatological research. The equivalents were designed to emulate clinical characteristics of atopic skin *in vitro* by combining crucial pathogenic hallmarks. Their characterization was intended to reveal new insights into the pathogenesis of atopic dermatitis as well as to identify possible readout parameters for pharmacological investigation of therapeutic agents. Additionally, this thesis set out to evaluate new delivery approaches such as nanocarriers, intended to increase the efficacy of existing topical treatment options in atopic skin. In particular, their ability to increase the amount of the delivered drug into relevant skin layers and the corresponding pharmacodynamic response were assessed. Hence, the use of disease equivalents emulating characteristics of atopic skin intended to verify the efficacy of the most promising delivery approaches by using suitable parameters and techniques in an appropriate human-based *in vitro* system.

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2

DEVELOPMENT AND CHARACTERIZATION OF SKIN EQUIVALENTS EMULATING CHARACTERISTICS OF ATOPIC SKIN *IN VITRO*

2.1 VALUE FOR THE THESIS

Besides normal reconstructed skin, more and more research efforts are made in the field of reconstructed skin disease models. Since the translational capacity of mouse experiments for inflammatory skin disease research is limited (Seok et al., 2013; Gerber et al., 2014) and the availability of diseased skin is finite, the development of skin equivalents mimicking inflammatory skin diseases is inevitable. Several approaches to mimic atopic dermatitis were describe by implementing either FLG deficiency or Th2 driven inflammation into *in vitro* approaches and to investigate their influence on the pathogenesis (De Vuyst et al., 2017). However, due to the singular consideration the interdependencies between the genetic predisposition and the altered cytokine milieu is not fully understood and crucial connections as well as important factors of the disease pathogenesis are still unknown (Figure 5, Chapter 1.3).

Hence, the following publication investigates the influence of the Th2-driven inflammation together with the FLG deficiency on relevant AD hallmarks and aims to unravel significant interdependencies between both parameters (Hönzke et al., 2016a).

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2.2 PUBLICATION

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Title and authors

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

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Personal contribution

Development and realization of skin equivalent construction, histological investigations inclusive of immunostaining, protein quantification by Western blot, gene analysis using real-time polymerase chain reaction (RT-PCR), cytokine measurement by enzyme-linked immunosorbent assay (ELISA) and skin surface pH measurements with subsequent data evaluation. Manuscript design and preparation under the supervision of Prof. Dr. Sarah Hedtrich.

Co-author´s contribution

All co-authors contributed to the study design, evaluation of the experiments and manuscript revision. Leonie Verheyen (neé Wallmeyer) performed skin absorption experiments.

3

DRUG ABSORPTION EVALUATION USING *EX VIVO* HUMAN SKIN

3.1 VALUE FOR THE THESIS

Many different nanocarriers based on various chemistries were developed for the topical treatment of inflammatory skin diseases (Gupta et al., 2012), but only a few approaches overcome the obstacle of clinical trials and the market. This might be due to reported limited particle stability or problematic safety profiles even for widely used approaches like lipid-based nanocarriers (Winter et al., 2016) and might be due to over predicted results based on animal research (Perrin, 2014). For drug absorption studies, the use of tissue engineered skin equivalents is also limited due to lower barrier function and corresponding higher permeability compared to human skin. Hence, human skin *ex vivo* presents the best approach to evaluate the efficacy of topical nanocarriers in terms of drug absorption depending on their chemical composition.

The following publications provide an overview about techniques that we used with our cooperation partners for the evaluation of drug absorption. Therefore, various new designed and synthesized nanocarriers were loaded with the fluorescent dye Nile Red (Adeli et al., 2015; Du et al., 2016; Stefani et al., 2016; Müller et al., 2017) or with the glucocorticoid dexamethasone (Döge et al., 2016; Balzus et al., 2017), then applied on excised human skin and ability for dermal drug delivery was monitored. For dexamethasone extraction and quantification from human skin a specific protocol was developed (Chapter 3.2).

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3.2 SOP "DEXAMETHASONE EXTRACTION AND QUANTIFICATION FROM HUMAN SKIN *EX VIVO*"

Protocol: Dexamethasone extraction and quantification from human skin *ex vivo*	Freie Universität Berlin	
Author: Stefan Hönzke	**Valid From** 13.11.2015	**Page** 1 of 2

Materials

Extraction:

- Waterbath
- Leica CM 1510 S cryotome (Leica Biosystems, Nussloch, Germany)
- Vacuum concentrator e.g. SpeedVac® (Thermofisher)
- Screw glass vials for vacuum concentrator (12 ml) (VWR, 231751459)
- Freezer
- Forceps
- Eppendorf tubes (2ml)
- Sodiumfluorid solution [120 mg/ml] (NaF, Sigma, S7920)
- Ethyl acetate (Sigma, 270989)
- Methanol (VWR, 1.06012.1001)

Quantification:

- High-Performance-Liquid-Chromatography Device with UV-Detection
- Acetonitrile for HPLC (VWR, 1.14291.1000)
- Ultrasonic bath
- Prednisolone solution [100 µg/ml] (Prednisolone, Sigma, P6004)
- Column (VWR, 1.50170.7127)
  - Lichrocart RP 18 (Fa. Merck)
  - Particle size: 5 µm
  - Pore size: 100 Å
  - Length: 125 mm
  - Diameter: 4 mm

Working Procedure

Heat separation:

1. Preheat waterbath to 60°C approximately one hour bevor starting the experiment and place a small beaker with clean water into the waterbath
2. Transfer skin sample in the beaker and incubate for 1 minute at 60°C
3. Separate epidermis from dermis using forceps
4. Transfer separated epidermis into 2 ml tubes within 500µl NaF solution and freeze dermis in a straight position. Store at -20°C
5. Cut 8 slices of 50µm thickness from dermis in horizontal position using a freeze-microtom and pool slices in 500µl NaF solution. Store at -20°C

Extraction:

6. Following the addition of 20 µl internal standard into each sample perform 5 freeze-thaw cycles to disrupt membranes before extraction
7. Ad 500 µl ethyl acetate to the sample, mix them and transfer ethyl acetate into glass vials for vacuum rotation. Use of Tissue lyser for mixing simplified the process and improved extraction process.
8. Repeat extraction 2 times and exsiccate the combined extracts by vacuum rotation
9. Dissolve residues in 500µl Methanol, centrifuge and exsiccate again for cleaning samples
10. Dissolve residues in 100µl of acetonitrile and inject an appropriate amount in the HPLC system

Quantification:

11. Transfer sample into the appropriate sample glass vial (volume ca. 100 µl) and prepare HPLC system with the eluent (acetonitrile/water: 30%/70%)
12. Inject 20 µl of the sample and into the HPLC system and monitor the chromatographic process for 10 min at a wavelength of 254 nm (Flowrate 0.5 ml/min)
13. CAUTION: Prepare for each sample batch a standard curve of the internal standard for calculation of its concentration within each sample.

Buffers and Solutions

1. **Sodiumfluorid solution [120 mg/ml]**

   NaF (Sigma, S7920) 6 g
   Water ad 50 ml

   -Filter solution bevor use

2. **Prednisolone [100 µg/ml]**

   Prednisolone (Sigma, P6004) 100 µg
   Acetonitrile ad 100 ml

3. **Eluent: Acetonitrile/water**

   Acetonitril (VWR, 1.14291.1000) 300 ml
   Water ad 1000 ml

- Degas eluent for 30 min by using a ultrasonic bath

3.3 PUBLICATIONS

Results of the chapter were published 2017

European Journal of Pharmaceutics and Biopharmaceutics, Volume 116, Pages 31-37
https://doi.org/10.1016/j.ejpb.2016.12.013

Title and authors

Rhamnolipids form Drug-loaded Nanoparticles for Dermal Drug Delivery

Felix Müller [1], **Stefan Hönzke [1]**, Wulf-Ole Luthardt [1], Ee Lin Wong [1], Michael Unbehauen [2], Jörg Bauer [1], Rainer Haag [2], Sarah Hedtrich [1], Eckart Rühl [2], Jörg Rademann [1]

[1] Institute for Pharmacy, Pharmacology and Toxicology, Freie Universität Berlin, Germany

[2] Institute of Chemistry and Biochemistry, Freie Universität Berlin, Germany

Personal contribution

Drug delivery experiments using excised human skin with subsequent data evaluation. Support of HPLC measurements and involvement in manuscript preparation.

Co-author´s contribution

Felix Müller synthesized rhamnolipids, designed and conducted the remaining experiments together with Wulf-Ole Luthardt. Felix Müller prepared the manuscript under supervision of Prof. Dr. Rademann. All co-authors contributed to the study design, evaluation of the experiments and manuscript revision.

Results of the chapter were published 2015

RSC Advances, Volume 115, Issue 20, Pages 14958-14966
https://doi.org/10.1039/C4RA14619K

Title and authors

Synthesis of Multiarm Star Copolymers based on Polyglycerol Cores with Polylactide Arms and their Application as Nanocarriers

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http://pubs.rsc.org/en/content/articlelanding/2015/ra/c4ra14619k#!divAbstract

Personal contribution

Drug delivery experiments using excised human skin with subsequent data evaluation.

Co-author´s contribution

Fang Du synthesized the nanocarriers and conducted the remaining experiments together with the co-authors. Prof. Dr. Mohsen Adeli and Prof. Dr. Rainer Haag designed the experiments and prepared the manuscript.

Results of the chapter were published 2016

Polymer, Volume 96, Pages 156-166
https://doi.org/10.1016/j.polymer.2016.04.074

Title and authors

Hyperbranched Glycerol-based Core-Amphiphilic Branched Shell Nanotransporters for Dermal Drug Delivery

Stefano Stefani [3], **Stefan Hönzke [2]**, Jose Luis Cuellar Camacho [1], Falko Neumann [1], Ashok K. Prasad [4], Sarah Hedtrich [2], Rainer Haag [1], Paul Servin [3]

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Personal contribution

Drug delivery experiments using excised human skin with subsequent data evaluation. Support of HPLC measurements and involvement in manuscript preparation.

Co-author´s contribution

Stefano Stefani synthesized the nanocarriers, designed and conducted the remaining experiments, and prepared the manuscript under the supervision of Dr. Paul Servin and Prof. Dr. Rainer Haag. Falko Neumann performed toxicity experiments. All co-authors contributed to the study design, evaluation of the experiments and manuscript revision.

Results of the chapter were published 2016

Journal of Controlled Release, Volume 242, Pages 42-49
https://doi.org/10.1016/j.jconrel.2016.06.048

Title and authors

Development of Biodegradable Hyperbranched Core-multishell Nanocarriers for Efficient Topical Drug Delivery

Fang Du [1], **Stefan Hönzke [2]**, Falko Neumann [1,3], Juliane Keilitz [1], Wei Chan [1], Nan Ma [1,3], Sarah Hedtrich [2], Rainer Haag [1]

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Supplemental material for this publication is provided online:
https://www.sciencedirect.com/science/article/pii/S0168365916304254?via%3Dihub

Personal contribution

Drug delivery experiments using excised human skin with subsequent data evaluation. Significant support of HPLC measurements and involvement in manuscript preparation.

Co-author´s contribution

Fang Du synthesized nanocarriers, designed and conducted the remaining experiments and prepared the manuscript under the supervision of Prof. Dr. Rainer Haag. Falko Neumann performed toxicity experiments. Co-authors contributed to the study design, evaluation of the experiments and manuscript revision.

Results of the chapter were published 2016

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Title and authors

Ethyl Cellulose Nanocarriers and Nanocrystals Differentially Deliver Dexamethasone into Intact, Tape-stripped or Sodium Lauryl Sulfate-exposed *ex vivo* Human Skin - Assessment by Intradermal Microdialysis and Extraction From the Different Skin Layers

Nadine Döge [1,2], **Stefan Hönzke [2]**, Fabian Schumacher [3,4], Benjamin Balzus [5], Miriam Colombo [5], Sabrina Hadam [1], Fiorenza Rancan [1], Ulrike Blume-Peytavi [1], Monika Schäfer-Korting [2], Anke Schindler [6], Eckart Rühl [6], Per Stahl Skov [7,8], Martin K. Church [8], Sarah Hedtrich [2], Burkhard Kleuser [3], Roland Bodmeier [5], Annika Vogt [1]

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Personal contribution

Drug delivery experiments using excised human skin with subsequent data evaluation. A protocol to extract and quantify dexamethasone in skin was established (Chapter 3.2) and successfully transferred to the Clinical Research Center for Hair and Skin Science at the department of Dermatology and Allergy within the Charité, Berlin. Support of HPLC measurements and involvement in manuscript preparation.

Co-author´s contribution

Nadine Döge designed and conducted the remaining experiments and prepared the manuscript under the supervision of Dr. Annika Vogt. Benjamin Balzus and Miriam Colombo synthesized and characterized nanocarriers. All co-authors contributed to the study design, evaluation of the experiments and manuscript revision.

Results of the chapter were published 2017

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https://doi.org/10.1016/j.ejpb.2017.02.001

Title and authors

Formulation and *ex vivo* Evaluation of Polymeric Nanoparticles for Controlled Delivery of Corticosteroids to the Skin and the Corneal Epithelium

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[4] Department of Molecular Biology, University of Duisburg-Essen, Germany

Personal contribution

Drug delivery experiments using excised human skin with subsequent dexamethasone extraction and data evaluation and involved in manuscript preparation.

Co-author´s contribution

Benjamin Balzus designed the remaining experiments, synthesized nanocarriers and prepared the manuscript together with Dr. Fitsum Feleke Sahle under the supervision of Prof. Dr. Roland Bodmeier. Toxicity experiments were performed by Dr. Christian Gerecke. Dexamethasone amounts within the skin samples were quantified by Dr. Fabian Schumacher. All co-authors contributed to the study design, evaluation of the experiments and manuscript revision.

4

PHARMACOLOGICAL EVALUATION OF NANOCARRIERS USING SKIN EQUIVALENTS

PART 1: THERMORESPONSIVE NANOGELS

4.1 VALUE FOR THE THESIS

Highly hydrophilic, thermoresponsive nanogels (tNG) were shown to be attractive potential systems for the topical delivery of biomolecules, which can be encapsulated within the three-dimensional polymeric scaffold of the tNGs (Witting et al., 2015). They have the advantage to be highly biocompatible for epidermal skin cells (Gerecke et al., 2017) as well as for immune cells (Edlich et al., 2017). The question whether the delivered proteins maintain their pharmacological activity after overcoming the skin barrier is still an open question.
Concerning hydrophobic drugs, tNG suffer from low encapsulation capacities due to their hydrophilic chemistry and subsequently inefficient delivery of these substances. So their application for the standard atopic dermatitis treatment in particular glucocorticoids, might seem paradoxical at first sight. However intelligent modifications of the polymeric scaffold seem to be the most innovative solution to solve this issue and might result in delivery changes (Giulbudagian et al., 2016). Nevertheless, the efficacy of this approach is not proved yet.

The following publications investigate tNGs loaded either with dexamethasone (Giulbudagian et al., 2018b) or the anti-inflammatory protein Etanercept (Giulbudagian et al., 2018a) and demonstrate the usefulness of the developed *in vitro* skin disease equivalents (Chapter 2) for the pharmacological evaluation of the delivered agent.

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WITTING, M., MOLINA, M., OBST, K., PLANK, R., ECKL, K. M., HENNIES, H. C., . . . HEDTRICH, S. 2015. Thermosensitive dendritic polyglycerol-based nanogels for cutaneous delivery of biomacromolecules. *Nanomedicine*;11(5):1179-87.

4.2 PUBLICATIONS

Results of the chapter were published 2018

Nanoscale, Volume 10, Issue 1, Pages 469-479
https://doi.org/10.1039/C7NR04480A

Title and authors

Enhanced Topical Delivery of Dexamethasone by β-Cyclodextrin Decorated Thermoresponsive Nanogels

Michael Giulbudagian [1], **Stefan Hönzke [2]**, Julián Bergueiro [1], Dogus Işık [1], Fabian Schumacher [3,6], Siavash Saeidpour [4], Silke B. Lohan [5], Martina C. Meinke [5], Christian Teutloff [4], Monika Schäfer-Korting [2], Guy Yealland [2], Burkhard Kleuser [3], Sarah Hedtrich [2], and Marcelo Calderón [1]

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Personal contribution

Drug delivery experiments using excised human skin with subsequent data evaluation. The established protocol from Chapter 3.2 was used for dexamethasone extraction and quantification. Development and realization of skin equivalent construction, histological investigations and protein quantification by Western blot with subsequent data evaluation to monitor the anti-inflammatory effects of dexamethasone. Significant involvement in manuscript design and preparation.

Co-author´s contribution

Michael Giulbudagian synthesized the nanogels, designed and conducted the remaining experiments, and prepared the manuscript under the supervision of Prof. Dr. Marcelo Calderón. Electron paramagnetic resonance (EPR) spectroscopy was conducted by Dr. Silke Lohan and Dr. Siavash Saeidpour. Dexamethasone amounts within the skin samples were quantified by Dr. Fabian Schumacher. All co-authors contributed to the study design, evaluation of the experiments and manuscript revision.

Results of the chapter were published 2018

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https://doi.org/10.7150/thno.21668

Title and authors

Breaking the Barrier – Potent Anti-Inflammatory Activity following Efficient Topical Delivery of Etanercept using Thermoresponsive Nanogels

Michael Giulbudagian [1, †], Guy Yealland [2, †], **Stefan Hönzke [2]**, Alexander Edlich [3], Birte Geisendörfer [3], Burkhard Kleuser [3], Sarah Hedtrich [2 *], Marcelo Calderón [1]

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[†] These authors contributed equally to this work

Supplemental material for this publication is provided online:
http://www.thno.org/v08p0450.htm#SM0

Personal contribution

Development and realization of skin equivalent construction and protein quantification by Western blot with subsequent data evaluation. Involvement in manuscript preparation.

Co-author´s contribution

Michael Giulbudagian synthesized the nanogels, designed and conducted the remaining experiments together with Dr. Guy Yealland. Both prepared the manuscript under the supervision of Prof. Dr. Sarah Hedtrich and Prof. Dr. Marcelo Calderón. Immunotoxicity assays were conducted by Alexander Edlich and Birte Geisendörfer. All co-authors contributed to the study design, evaluation of experiments and manuscript revision.

5

PHARMACOLOGICAL EVALUATION OF NANOCARRIERS USING SKIN EQUIVALENTS

PART 2: CORE-MULTISHELL NANOCARRIERS

5.1 VALUE FOR THE THESIS

In the last decade dendritic core-multishell (CMS) nanocarriers were introduced as one of the most promising approaches, for the dermal delivery of several drugs (Chapter 1.3). They are able to encapsulate a wide range of guest compounds and are able to transport them to polar and non-polar environments (Quadir et al., 2008). The loading of lipophilic or hydrophilic substances onto CMS nanocarriers significantly increases their delivery to viable skin layers (Küchler et al., 2009a; Küchler et al., 2009b; Haag et al., 2011) and skin appendages (Lohan et al., 2016). Nevertheless, based on the limited availability of suitable *in vitro* approaches for skin, there is no study described that investigates the pharmacological efficacy of the drug that was delivered by CMS nanocarrier. Based on our developed *in vitro* equivalents emulating characteristics of atopic skin and the very promising results of preceding dermal drug absorption investigations, the implementation of studies using anti-inflammatory agents for skin disease treatment were logical next steps.

The following publication and the complemental data (Chapter 5.3) provide an evaluation and description of properties of newly developed ester-based CMS nanocarriers for dermal drug delivery. They were developed to address the weak point of conventional amide-based CMS nanocarriers: the amide linker between the polyglycerol core and inner alkyl shell that on degradation, generates highly toxic polyglycerol amines (Khandare et al., 2010; Hellmund et al., 2015). First, their abilities were investigated in terms of drug delivery efficacy for dexamethasone, biodegradation and biocompatibility by using 2-D cell culture models and *ex vivo* human skin as introduced in Chapter 3 (Hönzke et al., 2016b). The most efficient CMS nanocarriers were then applied onto *in vitro* equivalents mimicking atopic skin to evaluate the therapeutic efficacy of the delivered glucocorticoid (Hönzke et al., 2016b and Chapter 5.3).

To ensure consistency the complemental data are initially introduced and discussed for themself in Chapter 5.3 and were integrated afterwards in the overall concept in the general discussion (Chapter 6) similarly to the published data.

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5.2 PUBLICATION

Results of the chapter were published 2016

Journal of Controlled Release, Volume 242, Pages 50-63
https://doi.org/10.1016/j.jconrel.2016.06.030

Title and authors

Tailored Dendritic Core-multishell Nanocarriers for Efficient Dermal Drug Delivery: A Systematic top-down Approach from Synthesis to Preclinical Testing

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Supplemental material for this publication is provided online:
https://www.sciencedirect.com/science/article/pii/S0168365916304072?via%3Dihub

Personal contribution

Nanocarrier characterization in terms of size and drug loading efficiency. Realization of drug delivery experiments using excised human skin with subsequent data evaluation. The established protocol from Chapter 3.2 was used for dexamethasone extraction and quantification. Establishment of an approach to investigate the biodegradation of nanocarriers by skin enzymes together with Anja Elpelt and in close collaboration with Dendropharm GmbH. Development and realization of skin equivalent construction, histological investigations and protein quantification by Western blot with subsequent data evaluation. Manuscript design and preparation under the supervision of Prof. Dr. Sarah Hedtrich.

Co-author´s contribution

CMS Nanocarriers were synthesized by Dr. Emanuel Fleige and Dr. Florian Paulus from Dendropharm GmbH. Alkaline single-cell gel electrophoresis (COMET assay) and determination of reactive oxygen species (ROS) were performed by Dr. Christian Gerecke. Cell viability assays were conducted by Anja Elpelt. Nan Zhang performed red blood cell assay and bovine corneal opacity and permeability (BCOP) test. All co-authors contributed to the study design, evaluation of the experiments and manuscript revision.

5.3 COMPLEMENTAL DATA

INTRODUCTION

Animal models, especially mouse models, are still the gold standard for investigation of patho-physiological mechanisms in skin diseases and exploration of new treatment options (Kabashima and Nomura, 2017). Although the value of mouse models is undoubted, evidence is accumulating that interspecies-related differences present major problems for the correct interpretation of preclinical data, a point believed at least in part to underlie the failure of approximately 80% of drugs undergoing clinical trials (Seok et al., 2013; Perrin, 2014). Potential reasons include anatomical and physiological species-related differences of animal models, as well as incomplete model characterization (Perrin, 2014). For instance, there is only a 30% overlap between the skin-associated genes present in human and mouse skin, a point suggestive of major differences in skin physiology even before differences in gene expression are considered (Gerber et al., 2014). Indeed, there is a particularly poor representation of human genes associated with skin morphogenesis, growth and immune-responses in mice (Gerber et al., 2014), the latter of which needs particular attention when investigating inflammatory skin diseases such as atopic dermatitis (AD).

To overcome these obstacles, interest in human-based models has grown, resulting in the development of several organotypic skin equivalents for dermatological research. These models have emerged as potential tools to simulate physiologically active skin in health and disease (Danso et al., 2014; Mathes et al., 2014; Abd et al., 2016; De Vuyst et al., 2016; Naves et al., 2016; Bernard et al., 2017). However, there is still very little known regarding their suitability for assessing drug-related effects *in vitro.* Most importantly, valid and reproducible read-out parameters need to be identified.

Hence in the present study, we report on the utility of skin equivalents emulating characteristics of atopic skin for pharmacological studies *in vitro*. The disease phenotype was established by knockdown of the filaggrin gene (*FLG)* in keratinocytes and supplementing their subsequent skin equivalents with the Th2 cytokines IL-4 and IL-13 (*FLG*-IL4/IL13) as previously report (Hönzke et al., 2016a). Filaggrin deficiencies are a major risk factor for the manifestation of AD (Palmer et al., 2006), which is further characterized by an acute Th2-dominated inflammatory phase. The *FLG- IL4/IL13* skin equivalents were then used as a model to study the anti-inflammatory efficacies of topical dexamethasone (DXM) formulations. Although topical glucocorticoids are still the first line therapy for the treatment of AD (Lebwohl et al., 2013), their continuous usage is limited by long-term side effects such as skin thinning (Korting et al., 2002; Schoepe et al., 2006). Novel nanoparticulate formulations that

facilitate targeted drug delivery to the viable epidermis may improve the benefit-to-risk ratio associated with their use, as has been demonstrated with lipid nanocarriers (Fesq et al., 2003; Schlupp et al., 2011). Polymeric nanocarriers such as topically applied dendritic core-multishell (CMS) nanocarriers have been shown to greatly improve the intraepidermal delivery of DXM when compared to a conventional cream: Dexamethason LAW® (Hönzke et al., 2016b). However, whether this ultimately correlates with improved anti-inflammatory efficacy and decreased off-target effects is still unknown.

MATERIALS AND METHODS

All solutions for histology, formaldehyde solution 4%, Tween 20 and bovine serum albumin (BSA) were obtained from Carl Roth, Karlsruhe, Germany. The secondary antibodies IgG DyLight 488 and IgG DyLight 594 as well as 4',6-diamidin-2-phenylindol (DAPI) antifading mounting medium were bought from Dianova, Hamburg, Germany. Alexa Fluor 488 conjugated anti-human IgG (H+L) was from Invitrogen, Darmstadt, Germany. GRa/b antibody was from Santa Cruz Biotechnology, Heidelberg, Germany. Keratinocyte growth media (KGM) was obtained from Lonza, Basel, Switzerland. Primary antibodies against human filaggrin, involucrin, TSLP and Lamin B1 were purchased from Abcam (Cambridge, United Kingdom). The primary human β-actin antibody was purchased from Sigma Aldrich, Taufkirchen, Germany as was the Dulbecco's Modified Eagle Medium (DMEM) for fibroblast cultivation. The primary human antibodies for glucocorticoid receptor (D6H2L) and glycerinaldehyd-3-phosphat-dehydrogenase (GAPDH) were purchased from Cell Signaling, Frankfurt/Main, Germany. All primers for RT-PCR were provided by TibMolbiol, Berlin, Germany. Core mulitshell nanocarriers (10-E-15-350) were synthesized and loaded with dexamethasone as previously described (Hönzke et al., 2016b). Drug free cream vehicle (ingredients: glycerol, Kolliphor CS A, Kollicream IPM, propylene glycol, methyl-4-hydroxybenzoate, propyl-4-hydroxybenzoate, sorbic acid, sodium edetate, purified water) was kindly provided by Riemser Pharma, Greifswald, Germany.

Dexamethasone Formulations

Two dexamethasone-containing formulations were compared: A commercially available DXM cream (Dexamethason LAW® 0.05% DXM) and novel dendritic core-multishell (CMS) nanocarriers (3.6% DXM loading). The nanocarriers are composed of a hydrophilic hyperbranched polyglycerol core, a lipophilic inner shell composed of alkyl chains and a hydrophilic outer shell of methoxy poly(ethylene glycol). This composition allows efficient DXM encapsulation and superior intraepidermal delivery (Hönzke et al., 2016b). The CMS polymer concentration within the aqueous carrier solution was set at 5 mg ml^{-1}.

Each formulation and drug-free vehicle controls were applied at a concentration of 2 µg cm^{-2} DXM at day 11 and day 13 of tissue cultivation resulting in a total dosage of 4 µg cm^{-2} DXM. To investigate whether the CMS nanocarriers themselves do overcome the outermost layer, stratum corneum, of the skin equivalents, the CMS were tagged by the fluorescent dye indocarbocyanine (ICC) and applied onto the skin equivalents with the same amounts as used in the therapeutic treatment.

Generation of Inflammatory Skin equivalents and Topical Treatment with DXM-Loaded Cream and Nanocarriers

Normal (*FLG*+) and filaggrin-deficient (*FLG*-) skin equivalents were generated according to previously published procedures (Hönzke et al., 2016a; Wallmeyer et al., 2017). Primary human keratinocytes and fibroblasts were isolated from juvenile foreskin, acquired from circumcision surgeries (with permission). To induce gene knockdown, keratinocytes were transfected (HiPerFect®; Qiagen, Hilden, Germany) with *FLG*-specific siRNA (Sequence: CAGCUCCAGACAAUCAGGCACUCAU; NM_002016, Invitrogen, Darmstadt, Germany). A mixture of primary human fibroblasts, fetal bovine serum (FBS) Superior (Merck Millipore, Darmstadt, Germany) and bovine collagen I (PureCol; Advanced BioMatrix, San Diego, USA) was brought to neutral pH and poured into 3-D cell culture well inserts with a growth area of 3.2 cm^2 (BD Biosciences, Heidelberg, Germany). After 2 h at 37 °C, defined cell culture medium was added and the system transferred to an incubator with 5% CO_2 and 95% humidity. After 2 h, primary human keratinocytes (with or without filaggrin knockdown) were added on top of the collagen matrix. After 24 h, the skin equivalents were lifted to the air-liquid interface and a differentiation medium was added. The equivalents were cultivated for 14 days with media change every second day. Starting at day 10, the culture media was supplemented with IL-4 (15 ng ml^{-1}) and IL-13 (15 ng ml^{-1}). At day 11 and day 13, 2 µg cm^{-2} DXM incorporated in cream LAW® or loaded onto CMS nanocarriers as well as their respective drug free vehicles were applied on the surface of skin equivalents. For histological analysis, skin equivalents were immediately frozen, and cut into 8 µm vertical slices using a Leica CM 1510 S cryotome (Leica Biosystems, Nussloch, Germany). Skin sections were fixed with 4% formaldehyde, embedded in 4',6-diamidin-2-phenylindol (DAPI) antifading mounting medium and then analyzed with a fluorescence microscope (BZ-8000, objectives 20x/0.75, zoom 10x, Plan-Apo, DIC N2, Keyence, Neu-Isenburg, Germany).

Western Blot

The epidermis was gently removed from the equivalents and proteins were extracted with radioimmunoprecipitation assay (RIPA) buffer. Total protein concentrations were determined

using the Pierce® BCA Protein Assay Kit (Thermo Fisher Scientific, Schwerte, Germany). Subsequently, samples (30 µg protein) were boiled in standard SDS-PAGE sample buffer and separated by 10% SDS polyacrylamide gel electrophoresis (Bio-Rad, Munich, Germany). Gels were blotted onto nitrocellulose membranes (Bio-Rad, Munich, Germany). After blocking with 5% skimmed-milk powder for 1 h at 37 °C, membranes were incubated with primary antibodies at 4 °C overnight (rabbit polyclonal to filaggrin 1:1,000, rabbit polyclonal to involucrin 1:1,000, rabbit polyclonal to TSLP 1:1,000, mouse monoclonal to β-actin 1:10,000). Blots were washed and incubated with anti-rabbit horseradish-peroxidase-conjugated secondary antibody (dilution 1:1,000, Cell Signaling, Frankfurt/Main, Germany) for 1 h. Afterwards, blots were developed with SignalFire™ ECL reagent (Cell Signaling, Frankfurt/Main, Germany) and visualized by a PXi/PXi Touch gel imaging system (Syngene, Cambridge, UK). Protein expression was semi-quantified by densitometry using ImageJ version 1.46r (National Institutes of Health, Bethesda, USA) and normalized to β-actin levels. Potential off-target effects on respective protein expression levels due to siRNA transfection were excluded previously using skin equivalents generated with siRNA negative control (Hönzke et al., 2016a).

Subcellular Fractionation

After 6 hours treatment with the drug free and drug loaded formulations the epidermis was gently removed from the equivalents. For separation of viable keratinocytes from the stratum corneum, epidermis was gently shaken in 1 ml trypsin/EDTA solution (0.05%; Invitrogen, Darmstadt, Germany) at 37 °C for 5 min. After addition of 5 ml ice-cold DMEM with serum the obtained cell suspension was filtered through a 100 µm cell strainer (BD Biosciences, Heidelberg, Germany) and washed twice with ice-cold PBS. Subsequently, protein fractions from the cytoplasmic and nuclear compartments were isolated using the Subcellular Protein Fractionation Kit for Cultured Cells (Thermo Fisher Scientific, Frankfurt, Germany) according to the manufacturer's instructions. Protein expression was determined using Western blot. Here, 15 µg protein per sample was loaded and membrane was incubated with the corresponding primary antibodies (dilution 1:1000).

Cell Nuclei Isolation from Skin Equivalents and Flow Cytometry Analyses

To analyze glucocorticoid receptor translocation into the nuclei, the epidermis was gently removed and keratinocytes were separated as described in 2.4. Subsequently, the fractions of intact nuclei were isolated using the cytoplasmic extraction buffer (CEB) and membranous extraction buffer (MEB) from the Subcellular Protein Fractionation Kit for Cultured Cells (Thermo Fisher Scientific, Frankfurt, Germany). The resulting suspension of nuclei were fixed

using paraformaldehyde (1.5%) for 10 min, centrifuged and then permeabilized 30 min with cold (-20 °C) methanol. After resuspending in staining buffer, nuclei were incubated with a glucocorticoid receptor antibody (D6H2L) (dilution 1:100, Cell Signaling) for 1 h. Suspensions were further incubated with an AlexaFluor488-conjugated secondary antibody (dilution 1:500, Abcam) for 30 min. Fixation and staining of isolated nuclei were performed according a published protocol (Rosner et al., 2013). A total of 10^4 events were counted and examined using a Cytoflex flow cytometer (Beckman Coultman, Indianapolis, USA).

Quantitative Real-Time PCR

To assess glucocorticoid-related effects in fibroblasts, the dermal compartment of the skin equivalents was frozen and then milled for 30 s at 25 Hz using a TissueLyzer (Qiagen, Hilden, Germany). Subsequently, RNA was isolated using the innuPREP RNA Mini kit (Analytik Jena, Jena, Germany) according to the manufacturer's instructions. For cDNA synthesis, the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Munich, Germany) and RT-PCR was performed using SYBR Green I Masterplus (Bio-Rad Laboratories). GAPDH served as housekeeping gene. Primer sequences are listed in the table 1.

Table 1. Primer sequences

gene	primer sense 5'-3'	primer antisense 5'-3'
GAPDH	CTCTCTGCTCCTCCTGTTCGAC	TGAGCGATGTGGCTCGGCT
COL1A1	CCTCAAGGGCTCCAACGAG	TCAATCACTGTCTTGCCCCA
COL3A1	GATCAGGCCAGTGGAAATGT	GTGTGTTTCGTGCAACCATC
FN	GGTGACACTTATGAGCGTCCTAAAA	AACATGTAACCACCAGTCTCATGTG
TIMP1	GCCCAGAGAGACACCAGAGA	GAGGTCGGAATTGCAGAAG
TIMP2	AGATGCACATCACCCTCTGT	TTCTCTGTGACCCAGTCCAT

Immunofluorescence

Skin sections were fixed with 4% formaldehyde, washed with 0.01M PBS, permeabilized with 1% TritonX-100 and blocked with 5% normal goat serum (1:20 in PBS). The sections were incubated overnight at 4 °C with glucocorticoid receptor antibody (1:50 in 0.01M PBS, 0.0025% BSA, 0.025% Tween 20). Subsequently, the sections were incubated for an additional 1 h at room temperature with the secondary antibody (1:2000 in PBS, 0.0025% BSA, 0.025% Tween 20). Nuclear counterstaining was performed using DAPI. Finally, sections were mounted in Mowiol mounting medium (Sigma Aldrich, Taufkirchen, Germany), and analyzed

using LSM 780 or LSM5 Pascal confocal microscopes (objectives 63x, zoom: 1,9; Plan-Neofluor/oil, NA 1.3, Plan-Apochromat/oil, NA 1.4; Zeiss, Oberkochen, Germany).

Enzyme Linked Immunosorbent Assay (ELISA)

The epidermis from the skin equivalents was lysed in radioimmunoprecipitation assay (RIPA) buffer and protein concentrations were determined using the Pierce® BCA Protein Assay Kit (Thermo Fisher Scientific). Subsequently, hBD-2 levels in skin equivalents were quantified using the BD-2 OmnikineTM ELISA Kits (AssayBioTech, Sunnyvale, USA) according to the manufacturer's instructions. Release from epidermal derived cytokines (IL-1β, IL-6, IL-8, IL-10, TNFα) were quantified in the differentiation medium of the skin equivalents using ELISA-Ready Set Go kits (eBioscience, Hatfield, United Kingdom).

Statistical Analysis

The unpaired two-tailed student's *t*-test was used for direct comparisons of two independent groups and was performed using GraphPad Prism 6.0 (GraphPad Software Inc., La Jolla, California, USA). Asterisks indicate statistical significance over untreated FLG-$_{IL\text{-}4/IL\text{-}13}$ skin equivalents; $p \leq 0.05$ was considered statistical significant. Data from at least three independent experiments are presented as means ± standard error of the mean (SEM).

RESULTS

Dose response studies were performed on *FLG* knockdown skin equivalents to determine a clinically-realistic anti-inflammatory DXM concentration (Figure S1). 4 µg cm^{-2} was found to effectively decrease the expression of the pro-inflammatory keratinocyte cytokine thymic stromal lymphopoietin (TSLP) (Surjit et al., 2011). Topical application of DXM formulated in CMS nanocarriers, but not the commercially available cream, significantly decreased TSLP levels in the inflammatory skin equivalents (*FLG* knockdown plus IL-4 and IL-13 supplementation; FLG-$_{IL\text{-}4/IL\text{-}13}$) (Figure 1A). Similarly, CMS nanocarrier DXM significantly increased expression of the skin barrier protein involucrin (IVL) (Figure 1B). Though less pronounced than the CMS nanocarriers, treatment with the cream also produced enhanced IVL expression whether in combination with DXM or not. Fluorescence microscopy confirmed the CMS nanocarriers themselves did not overcome the stratum corneum of the skin equivalents (Figure 2).

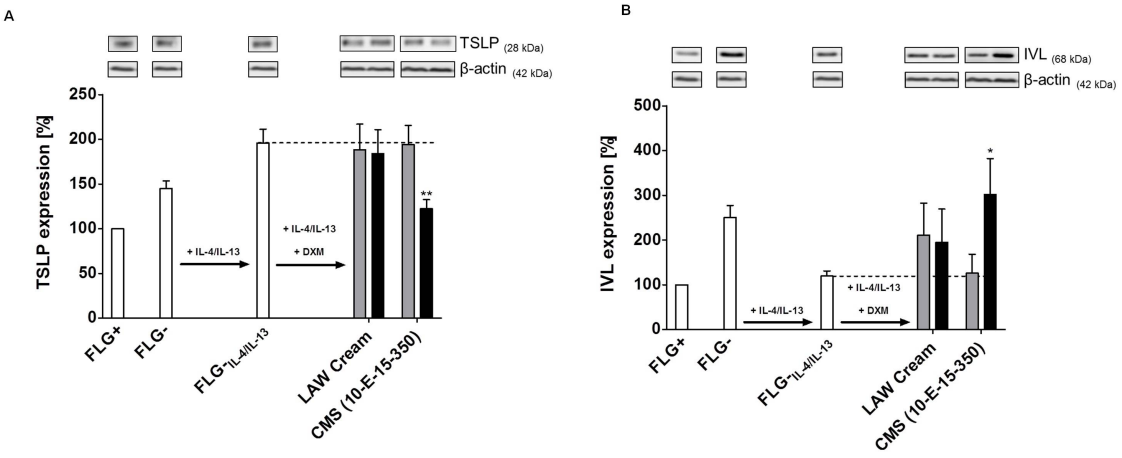

Figure 1. Anti-inflammatory efficacy in skin equivalents quantified from protein levels in epidermal lysates. A, B) Western blots and relative protein expression as semi-quantified by densitometry of TSLP (A) and IVL (B) levels in untreated normal (FLG+) and filaggrin knockdown (*FLG*-) (untreated and IL-4/IL-13 supplemented) skin equivalents. At day 11 and day 13, the skin equivalents were topically treated with Dexamethason LAW® cream or DXM-loaded CMS nanocarriers (black bars) and their corresponding drug-free vehicle controls (grey bars). Statistical differences were calculated by student's t–test compared to FLG-$_{IL-4/IL-13}$ (** = $p \leq 0.01$, * = $p \leq 0.05$) or compared to FLG+ (TSLP) or FLG- (IVL) (+ = $p \leq 0.05$, Mean ± SEM.; n = 5). (CMS = core multi-shell nanocarrier, FLG = filaggrin, IVL = involucrin, TSLP = thymic stromal lymphopoetin).

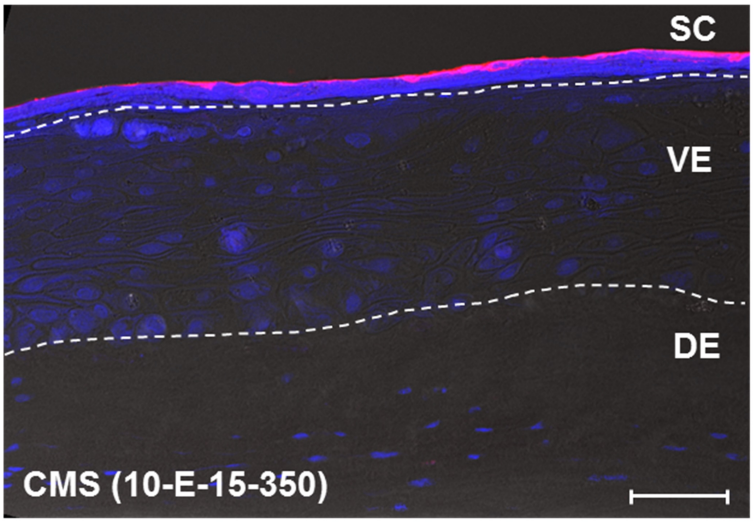

Figure 2. Core multi-shell nanocarriers do not overcome the stratum corneum. Representative fluorescence imaging of fluorescently labeled CMS nanocarriers (red) in FLG-$_{IL4/IL-13}$ skin equivalents. The skin equivalent sections were counterstained with 4',6-Diamidin-2-phenylindol (DAPI) (blue). Dashed lines indicate the stratum corneum (SC), viable epidermis (VE), dermis (DE). Scale bar = 50 µm. (CMS = core multi-shell nanocarrier).

To directly study the therapeutic mechanism of DXM, the translocation of glucocorticoid receptors (GR) to the cell nuclei in topically treated skin equivalents was investigated. Fluorescence microscopy of equivalent cyrosections and flow cytometry on isolated nuclei from the disaggregated epidermal compartment revealed enhanced GR translocation in DXM treated equivalents (Figure 3A-D). CMS nanocarrier DXM treatment resulted in GR translocations to the equivalent's dermal compartment (Figure 3D) not seen in the other equivalents. GR translocation was significantly increased in nanocarrier-treated equivalents relative to vehicle control (34.4 % ± 16.9 SD) (Figure 3E). A less pronounced increase was also seen the in DXM cream-treated equivalents (22.1 % increase ± 18.9 SD) (Figure 3E). Western blots (Figure 4) confirmed faster GR translocation into keratinocyte nuclei following application of the nanocarriers, accompanied by a corresponding decrease of cytoplasmic GR expression. Notably, drug free vehicle controls had no effect on this.

Further therapeutic readouts of DXM efficacy in the skin equivalents were trialed, but which failed to show a clear correlation to DXM's known therapeutic effects. The previously identified down-regulation of ß-defensin-2 in the inflamed skin equivalents was unaffected (Hönzke et al., 2016a) by DXM application (Figure S2). Application of the CMS nanocarrier or cream vehicle only controls resulted in decreased expression of extracellular matrix (ECM) proteins in the dermal compartment, a known side-effect of long term glucocorticoid treatment in humans (Figure S3). With regard to secreted pro-inflammatory cytokines, no changes to the IL-6 and -8 levels of the inflamed equivalents were seen, and IL-1β, IL-10 and TNFα were not excreted at levels detectable by ELISA, precluding their use as a therapeutic readout of DXM (Figure S4).

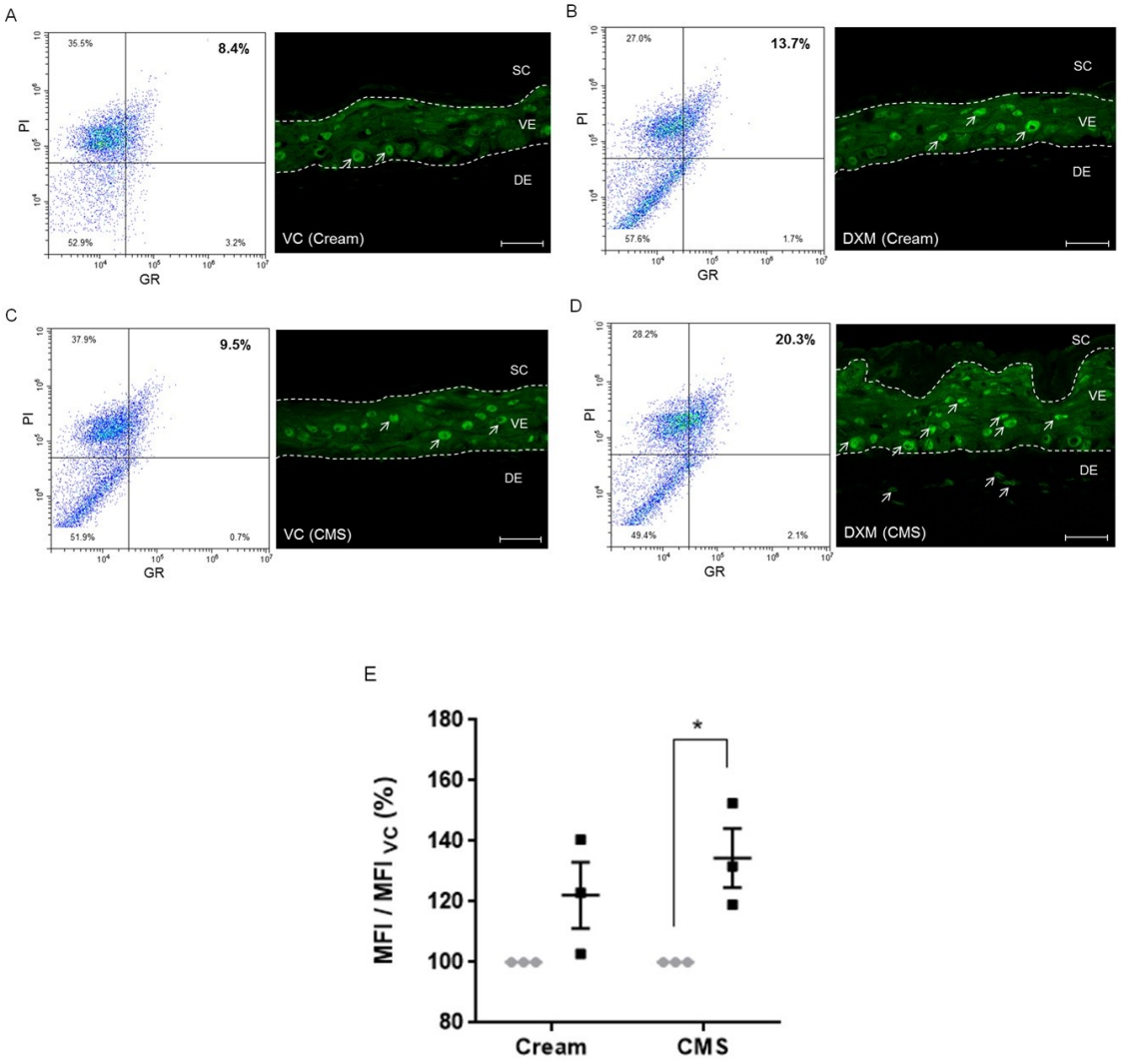

Figure 3. Dexamethasone efficacy in skin equivalents can be quantified by glucocorticoid receptor translocation. A-D) Representative dot plots of glucocorticoid receptor (GR) staining from isolated nuclei, as determined by flow cytometric analysis, and corresponding fluorescence microscopy images of stained GR (green) in FLG-$_{IL\text{-}4/IL\text{-}13}$ skin equivalents. Skin equivalents were topically treated with Dexamethason LAW® cream (C) or DXM-loaded CMS nanocarriers for 6 hours (D) and thier corresponding vehicle controls (VC) (A, B). Dashed lines indicate the stratum corneum (SC), viable epidermis (VE), dermis (DE). E) Median fluorescent intensities of GR staining in the nuclei of DXM treated skin equivalents, as determined by flow cytometric analysis, expressed as a percentage of the corresponding vehicle only controls (statistical differences between vehicle and DXM treatments were calculated by unpaired two-tailed student's t-test, * = $p \leq 0.05$, Mean ± SD.; n = 3). (CMS = core multi-shell nanocarrier, GR = glucocorticoid receptor, MFI = mean fluorescence intensitiy, PI = propidiumiodid, VC = vehicle control).

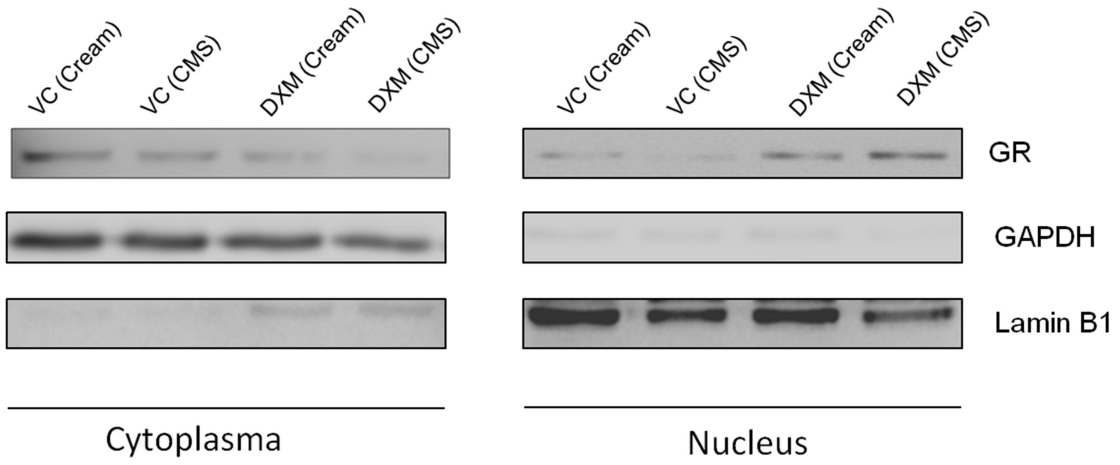

Figure 4. Glucocorticoid receptor translocation can be quantified by protein detection in the isolated nuclei of dissagregated equivalents. Glucocorticoid receptor (GR) protein levels in the nuclei or cytoplasm of keratinocytes dissagregated from FLG-$_{IL\text{-}4/IL\text{-}13}$ skin equivalents was determined by Western blot. Skin equivalents were topically treated with Dexamethason LAW® cream cream or DXM-loaded CMS and corresponding vehicle controls (VC) for 6 hours. Purity of the fractions were confirmed using antibodies against lamin B1 (only in nuclear fractions) and glycerinaldehyd-3-phosphat-dehydrogenase (GAPDH) (only in cytoplasmic fractions). (CMS = core multi-shell nanocarrier, VC = vehicle control).

DISCUSSION

Although animal studies remain the gold standard in preclinical testing, some controversy has arisen over their predictive power for human conditions and the frequency with which their results lack concordance to them. Moreover, awareness is increasing that many animal models are often poorly characterized (Perrin, 2014) and that their suitability for the study of drug-related effects are often unknown (Pischon et al., 2017). Consequently, interest in alternative human-based model systems has grown thanks to their relative ease of use, lower costs, and absence of interspecies-related differences. In dermatological research, skin equivalents have emerged as potential alternatives for the study of e.g. atopic dermatitis (Hönzke et al., 2016a) and skin tumors (Zoschke et al., 2016; Massaro et al., 2017). Although tremendous advances have been made, very few data are presently available on the actual use of skin equivalents in determining drug-related effects *in vitro*. Here, we report on the applicability of an inflammatory skin disease equivalent as a test-system to assess the anti-inflammatory effects of topically applied DXM, for which several valid read-out parameters were identified and used to compare the efficacies of two formulations.

TSLP was selected as a read-out parameter since glucocorticoids are known to directly suppress its production in keratinocytes by binding to negative glucocorticoid response

elements (Surjit et al., 2011). TSLP is also a key player in the inflammatory pathways of AD (Ziegler, 2010) with direct and indirect roles in T cell recruitment (Soumelis et al., 2002; Wallmeyer et al., 2017). It is upregulated in AD patients (Mu et al., 2014) and correspondingly in the FLG-$_{IL4/IL13}$ skin equivalents (Fig. 1A) (Hönzke et al., 2016a). Topical application of DXM significantly decreased TSLP levels in the inflammatory skin equivalents as compared to untreated controls (Figure 1A). Interestingly, DXM-loaded CMS nanocarriers induced greater TSLP reductions than the commercially available DXM cream, in line with the superior intra-epidermal DXM delivery by CMS nanocarriers (Hönzke et al., 2016b). Indeed, no clear anti-inflammatory effect was demonstrated by the cream. It should be noted that the CMS nanocarriers themselves did not overcome the stratum corneum of the skin equivalents (Figure 2), suggesting that their effects did in fact result from increased DXM skin absorption.

In addition to reduced TSLP expression, increased expression of the skin barrier protein involucrin (IVL) was observed, an indication of improvements to skin barrier function. IVL is an important marker of keratinocyte differentiation and part of the cornified envelope, a structure crucial to the skin's role as a physical barrier. Treatment with DXM-loaded CMS nanocarriers mitigated the downregulatory effect of the Th2 cytokines, resulting in protein expression levels similar to those *FLG*- skin equivalents without cytokine exposure (Figure 1B). For the cream, similar increases in IVL levels with both the DXM-containing formulation and its vehicle only control, indicating the cream alone had a pronounced impact on the skin equivalents. Notably, the cream induced increases in IVL were markedly lower than those induced by DXM-loaded CMS nanocarriers.

To identify further parameters to quantify the anti-inflammatory effects of DXM *in vitro*, its direct mechanism of action – the translocation of glucocorticoid receptors (GR) into cell nuclei – was investigated. GR immunostaining and flow cytometry on isolated nuclei demonstrated faster GR translocation in DXM-loaded CMS nanocarrier treated equivalents (Figure 3A-D). GR translocation was significantly increased in nanocarrier-treated equivalents over their vehicle controls (Figure 3E). A similar, though less pronounced trend was seen in cream-treated equivalents (Figure 3C). The high standard deviations seen in these results likely derive from inter-donor variation in the primary skin cells used to generate the skin equivalents, a point that must be considered in the interpretation of all skin-equivalent studies. Interestingly, fluorescence images also showed GR translocation within dermal fibroblasts of these but not other equivalents (Figure 3D), which may be again attributed to improved drug delivery by the CMS nanocarriers. Western blots confirmed the faster GR translocation into epidermal keratinocyte nuclei following application of the nanocarriers accompanied by a corresponding decrease of cytoplasmic GR expression (Figure 4). Notably, drug free vehicle controls had no effect on this.

Assessing the pharmacological efficacy of DXM in the inflammatory skin equivalents was repeatedly hampered by distinct vehicle effects. This was most pronounced for the cream, which contains high amounts of glycerol and the penetration enhancer propylene glycol, both known to influence epidermal proliferation and differentiation (Short et al., 2007). Occlusive effects of the cream may also play a role. Unexpectedly, topical application of either drug free vehicle resulted in reduced mRNA levels of extracellular matrix (ECM) proteins in the dermal compartment (Figure S3). ECM proteins such as collagen type 1 alpha 1 (COL1A1), collagen type 3 alpha 1 (COL3A1), fibronectin and matrix metalloproteinase inhibiting enzymes (e.g. TIMP-1) are important components of dermal connective tissue and are known to be down-regulated following long-term treatment with GCs, resulting in skin thinning (Schoepe et al., 2006). Unfortunately, this side effect could not be investigated in this skin equivalent owing to the vehicle only effects. This is in line with a study using commercially available full-thickness skin equivalents (Weindl et al., 2011). Additionally, the duration over which the skin equivalents were treated with DXM was likely too short to observe skin thinning in the dermal compartment since glucocorticoid-induced skin atrophy appears mostly after prolonged treatment.

Further, though unsuccessful attempts to identify valid read-out parameters of DXM's anti-inflammatory effects in skin-disease equivalents were made. The expression of antimicrobial peptides such as hBD-2 appeared interesting since they are down-regulated in inflammatory conditions (Hönzke et al., 2016a). However, the expression of hBD-2 was unaffected by topical application of the DXM formulations (Figure S2). Quantifications of IL-1β, IL-10, TNFα could not be used as a read-out parameter since they were not excreted from the equivalents at detectable levels. Quantification of IL-6 and IL-8 were also unusable as their levels did not differ between normal and diseased equivalents (Figure S4). Notably, previous reports have documented significantly decreased IL-6 and IL-8 release after topical application of DXM to skin equivalents containing $CD45RO^{+}$ T cells (Engelhart et al., 2005) or TNFα stimulated equivalents (Weindl et al., 2011). Another study reported distinct anti-inflammatory effects and the induction of atrophy following glucocorticoid treatment in UVB-irradiated skin equivalents (Zöller et al., 2008). Here, again, IL-6 and -8 releases were used as surrogate parameters to assess the anti-inflammatory effect of the glucocorticoids *in vitro.* In contrast to our study and that published by Weindl and colleagues, Engelhart et al. and Zöller et al. applied the glucocorticoids both topically and into the cell culture media of the skin equivalents, perhaps resulting in stronger drug related effects than seen here (Engelhart et el., 2005; Zöller et el., 2008; Weindl et al., 2011). However, this approach does not truly emulate the topical application of glucocorticoids used in the clinic. Moreover, the significance of both studies is limited, since no drug free vehicle controls were included.

The inclusion of drug-free vehicles as controls is imperative not only for *in vitro* models. For instance, the imiquimod-mouse, a broadly used *in vivo* model of psoriasis (van der Fits et al., 2009), recently failed to reproduce the anti-inflammatory effects of Tacrolimus, a drug already approved for topical treatment of psoriasis in humans (Pischon et al., 2017). They also identified pronounced "anti-inflammatory" effects induced by the drug-free vehicles, a fact that may have previously resulted in the over-interpretation of studies where correctly selected vehicle only controls were not included.

In summary, when using skin equivalents for the assessment of pharmacological and formulation-dependent effects *in vitro,* it is essential that their limitations are observed. The identification and validation of suitable read-out parameters direct to the test substances mode of action is essential, as is the use of correct drug-free vehicle controls since their absence may tremendously bias the interpretation of results. This, however, is true for all preclinical models and human trials. With this in mind, human-based skin equivalents hold potential for use in preclinical testing and may become powerful tools to overcome distinct shortages of animal models as well as the issues surrounding inter-species related differences to humans. Nonetheless, human-based tissue models remain relatively simple compared to the *in vivo* tissues and will require further refinement to more accurately model the conditions found *in vivo*. For skin equivalents, this might involve the incorporation of immune cells, something that would greatly broaden their significance as preclinical test systems, especially for inflammatory diseases.

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Supplementary Figures

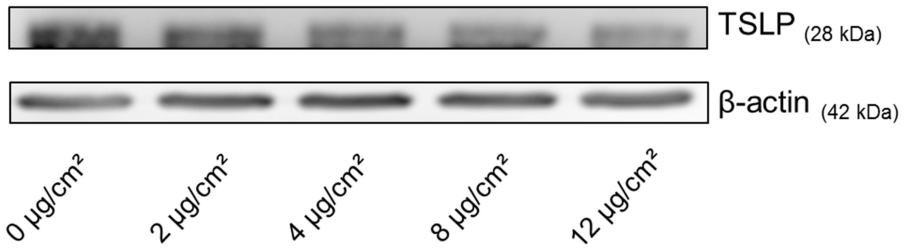

Figure S1. Representative Western blot of TSLP expression in inflammatory filaggrin knockdown skin equivalents treated with DXM concentrations [µg/cm²] in ascending order. β-actin served as loading control.

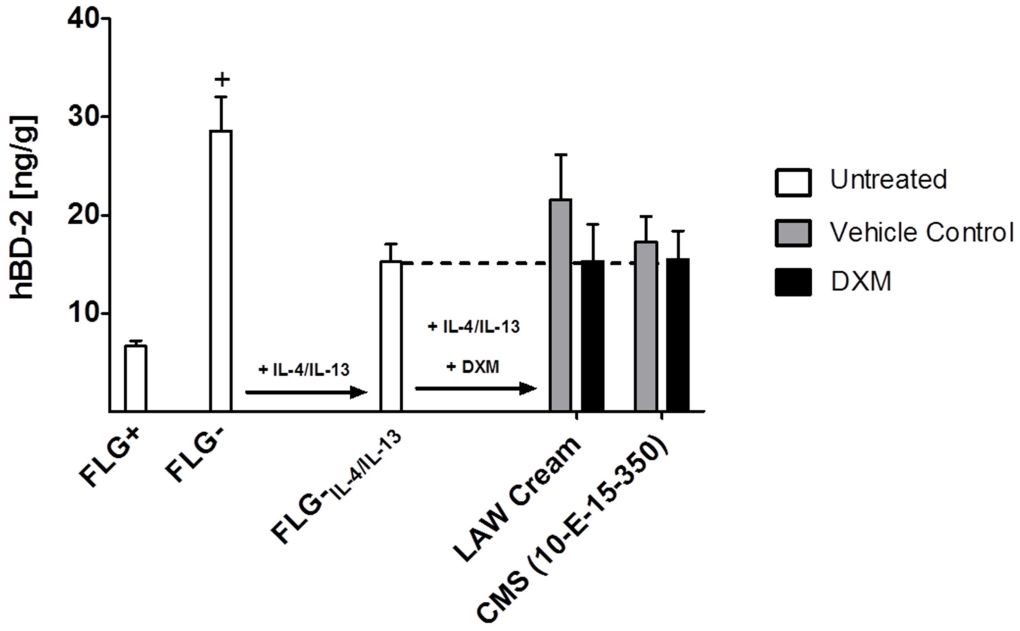

Figure S2. Human ß-defensin-2 (hBD-2) levels of untreated normal (*FLG+*) and filaggrin knockdown (*FLG-*) (no interleukin exposure and IL-4/IL-13 supplemented) skin equivalents, quantified by ELISA (calculated per [g] protein). Statistical differences were calculated by Student's t–test compared to *FLG+* (+ = $p \leq 0.05$, mean ± SEM; n = 5).

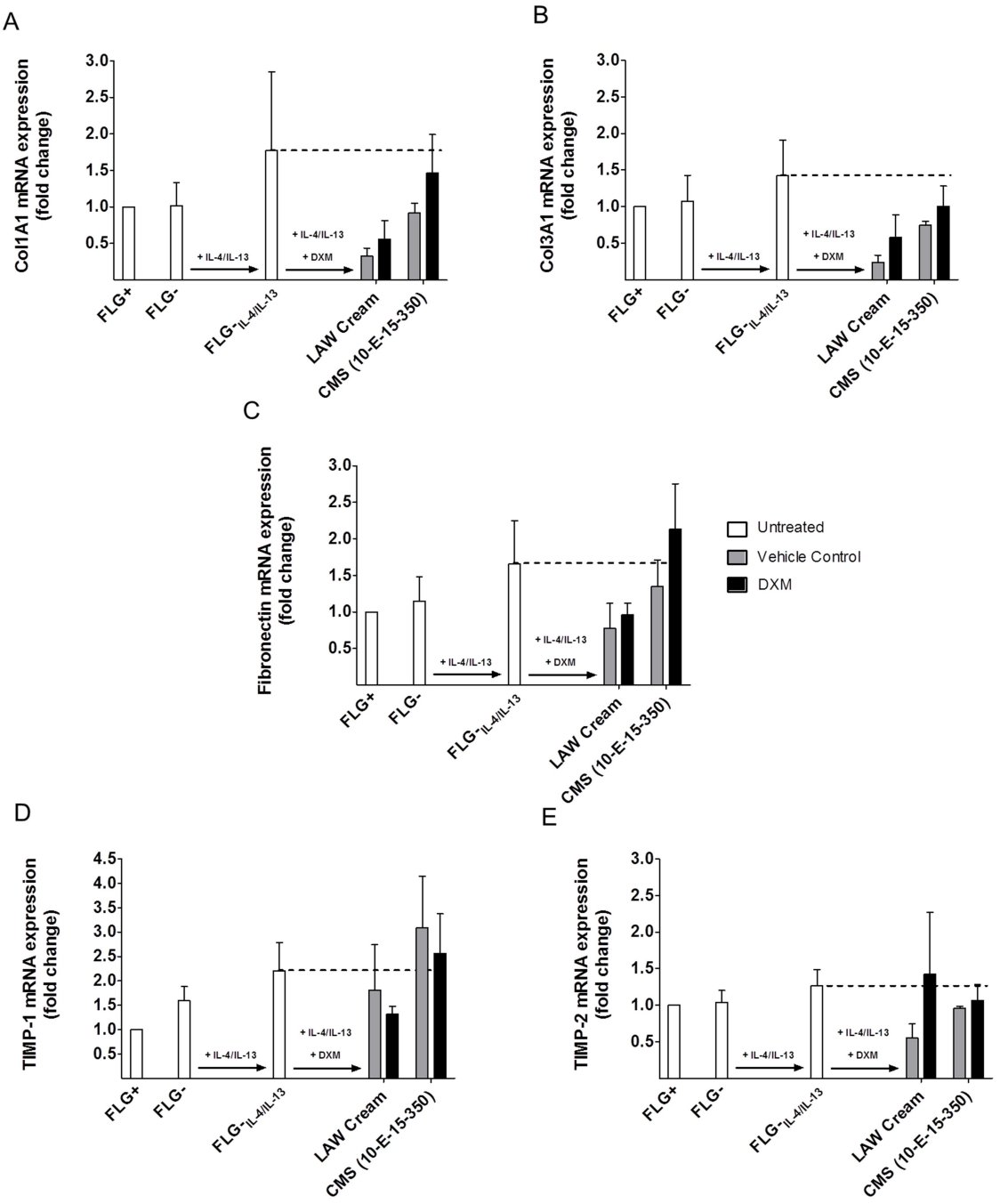

Figure S3. Relative mRNA expression of (A) collagen type 1 alpha 1 (COL1A1), (B) collagen type 3 alpha 1 (COL3A1), (C) fibronectin, (D) tissue inhibitors of metalloproteinases (TIMP-1) and (E) TIMP-2 in untreated normal (*FLG+*) and filaggrin knockdown (*FLG-*) (untreated and IL-4/IL-13 supplemented) skin equivalents. Mean ± SEM, n = 5.

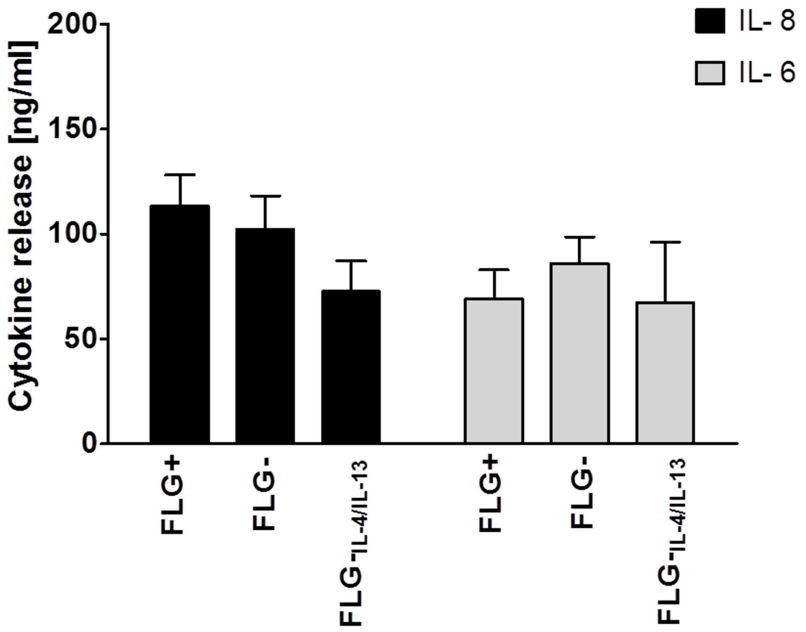

Figure S4. Expression of the pro-inflammatory cytokines IL-8 and IL-6 in culture medium of untreated normal (*FLG+*) and filaggrin knockdown (*FLG-*) (untreated and IL-4/IL-13 supplemented) skin equivalents. Mean ± SEM, n = 5

6

DISCUSSION AND PROSPECTS

6.1 DISCUSSION

Atopic dermatitis, the most common inflammatory skin disease of early childhood, presents a major public health problem with continuously rising incidences worldwide. To date, treatment options for atopic dermatitis, especially for severe progressive forms, are limited and the pathogenesis of the disease is not completely understood. The development of new drugs has been slow and reliant on the use of animal testing, the disadvantages of which, particularly with regard to human skin, have already been addressed. A deficit in therapeutic alternatives and the subsequently unmet clinical need in AD research are undisputed. The development of skin equivalents emulating specific disease characteristics might be able to address the knowledge gaps within our understanding of the disease pathogenesis and the current shortcomings in preclinical drug assessments.

In the frame of this thesis, an *in vitro* skin disease equivalent emulating characteristics of atopic dermatitis was continued developed based on a human organotypic skin equivalent (Eckl et al., 2011; Küchler et al., 2011). To capture the relevant complexity of the *in vivo* microenvironment, we transferred the atopic inflammation into a full-thickness human skin equivalent (HSE). The implementation of fibroblasts into the system was a conscious decision. Their presence in a dermal layer is crucial for an adequate production of fundamental skin structure proteins like collagens or laminin-5 leading to an improved development of the epidermal layer (Marionnet et al., 2006). This is particularly important for the barrier function of the equivalents, an especially important point when using the skin equivalents for drug delivery evaluation (Chapter 4 and Chapter 5). Further, the fibroblasts were crucial to evaluate drug-related effects in the dermal layer (Chapter 5.3, Figure 3 and Figure S3).

To establish an inflammatory skin disease equivalent emulating characteristics of AD, FLG deficiency and inflammation were combined in a HSE for the first time (Chapter 2, (Hönzke et al., 2016a)). The implementation of the two hallmarks of the disease, results in similar pathological alterations compared to the situation in patients (Figure 9). First, the Th2 cytokines IL-4/IL-13 led to major histological changes recognized by spongioses, parakeratosis and epidermal thickening, all features of AD (Foster et al., 2011; Watson and Kapur, 2011). Second, an increase of the surface pH in the diseased skin equivalents was identical to the increase in affected patient skin (Seidenari and Giusti, 1995). It might be possible to speculate that the cytokines disturb the recently discovered feedback mechanism, where the proton pump NHE-1 and the secretory phospholipase A_2 are upregulated (Vávrová et al., 2014), ultimately increasing skin surface pH.

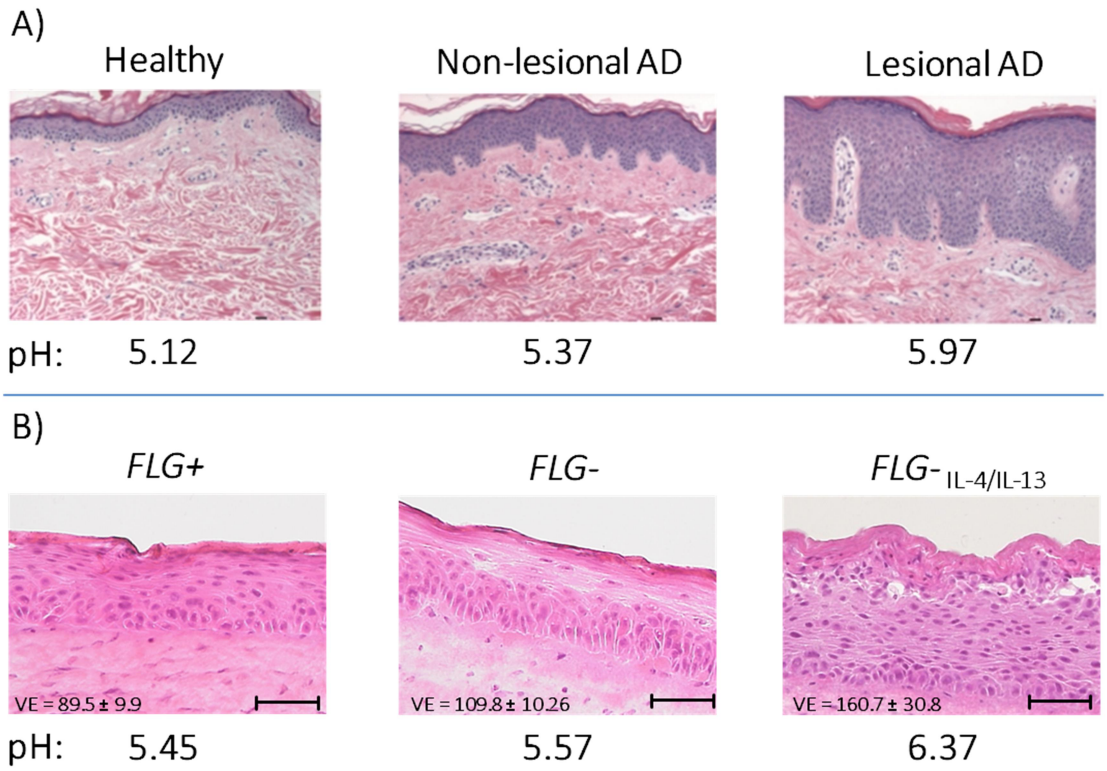

Figure 9. Pathological alterations. Changes in histology and surface pH of human skin (A) and the respective skin disease equivalents (B) (VE = viable epidermis, scale bar = 100 µm). (Human skin histology was reprinted with permission from PLOS ONE using the terms of the Creative Commons Attribution License CC BY 4.0, copyright 2011, (Foster et al., 2011). pH values were reprinted with permission from medicaljournals.se using the terms of the Creative Commons Attribution License CC BY-NC 4.0, copyright 1995, (Seidenari and Giusti, 1995). Data were shown and reprinted with permission from Elsevier (Hönzke et al., 2016a)). (AD = atopic dermatitis, FLG = filaggrin).

Additionally, the FLG-$_{IL-4/IL-13}$ equivalents revealed pathological protein patterns. As expected, expression of FLG was negatively influenced due to gene knockdown and cytokine treatment. The influence of FLG deficiency on TSLP expression, which was postulated in animal models (Moniaga et al., 2013), has now been verified in our system (Chapter 2, (Hönzke et al., 2016a) , Figure 4). Expression of this key cytokine was considerably increased, consistent with studies in humans (Sano et al., 2013).

Interestingly, with this approach new pathways that might play a role in AD pathogenesis were identified: a great impact of FLG deficiency on AMP expression was observed, resulting in higher hBD-2 levels (Chapter 2, (Hönzke et al., 2016a), Figure 5). Up to here only TNFα induced IL-1/IL-6 expression (served as positive control) or toll-like receptor-2 stimulation were identified as endogenous trigger factors (Liu et al., 2002) but not owing to a lack of FLG. Since hBDs stimulate the release of pro-inflammatory cytokines, these results might explain

the more severe AD phenotype in patients with *FLG* mutations (McAleer and Irvine, 2013). Interestingly, the hBD up-regulation was hampered by the Th2 inflammation and presents a potential reason for the lower AMP levels seen in AD patients compared to other skin diseases that results in higher infection rates with microbes (Kopfnagel et al., 2013). Compensatory regulation of IVL and occludin (OCLN), both relevant for skin barrier function, were also found in the FLG deficient skin equivalents (Chapter 2, (Hönzke et al., 2016a), Figure 2 and 3). Such compensatory up-regulation involving crucial skin barrier components have previously been reported only in filaggrin (Presland et al., 2000), loricrin (Koch et al., 2000) or claudin-1 (Furuse et al., 2002) deficient mice. Concordant to hBD-2, these pathways were dramatically disturbed when simultaneous inflammation occurred. This might explain the differences in skin barrier function between healthy and non-lesional skin and the big increase in AD lesions (Polańska et al., 2013). Permeability experiments of the skin equivalents support this hypothesis (Chapter 2, (Hönzke et al., 2016a), Figure S6).

Related to open issues in AD research (Chapter 1, Figure 5), FLG deficient skin equivalents were used to identify new pathological pathways and showed higher sensitivity to the detrimental effects of IL-4/IL-13 than skin equivalents with normal FLG expression. The results indicate that defects in the epidermal barrier, skin permeability, and cutaneous innate immune response are not primarily linked to FLG deficiency but are rather secondarily induced by Th2 inflammation. This conclusion was recently proven with a similar experimental approach in HSE by infiltration of $CD4^+$ T cells (Wallmeyer et al., 2017) and with Th2 cytokine stimulated epidermal equivalents derived from patient cells (Niehues et al., 2017). IL-4 and IL-13 seem to be important cytokines to the induction of an AD-like phenotype. Given the pleiotropic effects, preclinical and clinical evidence of their activity in AD has been widely reported (Oyoshi et al., 2009; Brandt and Sivaprasad, 2011; Mu et al., 2014). The overlapping function of IL-4 and IL-13 are due to receptor expression patterns. Both cytokines signal through two heterodimeric receptors with a shared receptor moiety, the IL-4 receptor alpha chain (IL-4Rα) (Gandhi et al., 2017). The clinical relevance of this pathway was recently demonstrated through the development of the monoclonal antibody Dupilumab (Dupixent®, SANOFI) – set to enter the German market in December 2017. The specific binding to IL-4Rα blocks both signaling pathways and has resulted in consistent and unprecedented therapeutic efficacy in AD disease end points, across several clinical studies, as monotherapy or as add-on to topical glucocorticoids (Beck et al., 2014; Simpson et al., 2016).

Although these findings are notable, they have to be valued regarding their demonstration in skin equivalents. The presence of only keratinocytes and fibroblasts in the skin equivalent do not represent the complexity within the skin. Micro-environmental factors including dynamic crosstalk between the epithelium and adjacent connective tissue, cells or skin appendages

like hair follicles and sebaceous glands are known to be a key regulator of epidermal morphogenesis and homeostasis. In particular the presence of immune cells like DCs and LCs and a functional vasculature might have significant influence on the results. Further differences in e.g. lipid composition and organization and the lacking of desquamation process led to lower barrier properties compared to human skin (Schäfer-Korting et al., 2008). It needs to be emphasized that the detected biological processes and pathways are significant but they have to be critically assessed in future investigations and have to be verified in experiments with AD patients.

Beside the development of new therapeutic strategies, another approach represents the improvement of existing therapies. As recommended by expert associations, topical application of GCs is still the first line therapy for moderate and severe AD (Lebwohl et al., 2013; Werfel et al., 2016). Based on their potency, GCs are hierarchically grouped into different classes and the dexamethasone (DXM) is one of the most used agents. Incorporated into cream, it is used both for treating acute flares of AD and for maintenance therapy (Abanta Pharma, Fachinformation Dexamethason Creme LAW® 2017). Topical administrations lead to local side effects including rosacea, skin infections and, most importantly, irreversible skin atrophy (Schoepe et al., 2006). Hence, research has focused on strategies to optimize the potency of corticosteroids. Enhancing topical bioavailability will reduce dose and frequency of drug administration (Senyigit and Ozer, 2012) and higher uptake rates will reduce the inter- or intraindividual variation of the available amount (Korting and Schäfer-Korting, 2010). To this end, various nanoparticulate structures have been proposed as potential drug carrier systems that could improve the retention of encapsulated corticosteroids within the epidermal layers of human skin (Gupta et al., 2012; Zhang et al., 2013).

To investigate, how the various nanocarriers alter the absorption of topical applied drugs the Franz cell approach combined with excised human skin – obtained from plastic surgery – presents the gold standard and was the method of choice. Drug absorption was not investigated in skin equivalents owing to their lower barrier properties compared to normal human skin (Schäfer-Korting et al., 2008). Due to that shortcoming, drug absorption is overestimated in skin equivalents (Planz et al., 2016). Beside the drug delivery efficacy, an efficient drug loading and decent biocompatibility are the most important properties to grade newly developed nanocarriers for their use in therapeutic strategies (Figure 10).

Core-shell nanocarriers showed increased delivery of the fluorescent dye Nile red compared to the cream formulation dependent on the length of the polycaprolactone (PCL) shell. Longer shells resulted in greater particle hydrophilicity that led to increased dye delivery (Chapter 3, (Adeli et al., 2015), Figure 11). Further, PCL was used to develop biodegradable

nanocarriers that showed distinct Nile red delivery (Chapter 3, (Du et al., 2016), Figure 5) but the hydrophilic environment probably restricted the loading efficacy for DXM. Core-shell nanocarriers with hydrophobic alkyl chains in the core outperform the cream in terms of dye delivery efficacy (Chapter 3, (Stefani et al., 2016), Figure 8) but the DXM loading remained low and the nanocarriers were toxic towards keratinocytes. For micelles based on rhamnolipids, biocompatibility and drug loading were acceptable, but the hydrophobicity within the micelles in combination with a passive mode of drug release resulted in limited dye delivery (Chapter 3, (Müller et al., 2017), Figure 4). However, the informative value for those delivery studies was limited by the use of the dye. Although Nile red and DXM has similar chemical properties (e.g. molecular size, logP value), the semi-quantitative analyses of fluorescence might overinterpret the efficacy (White and Errington, 2005).

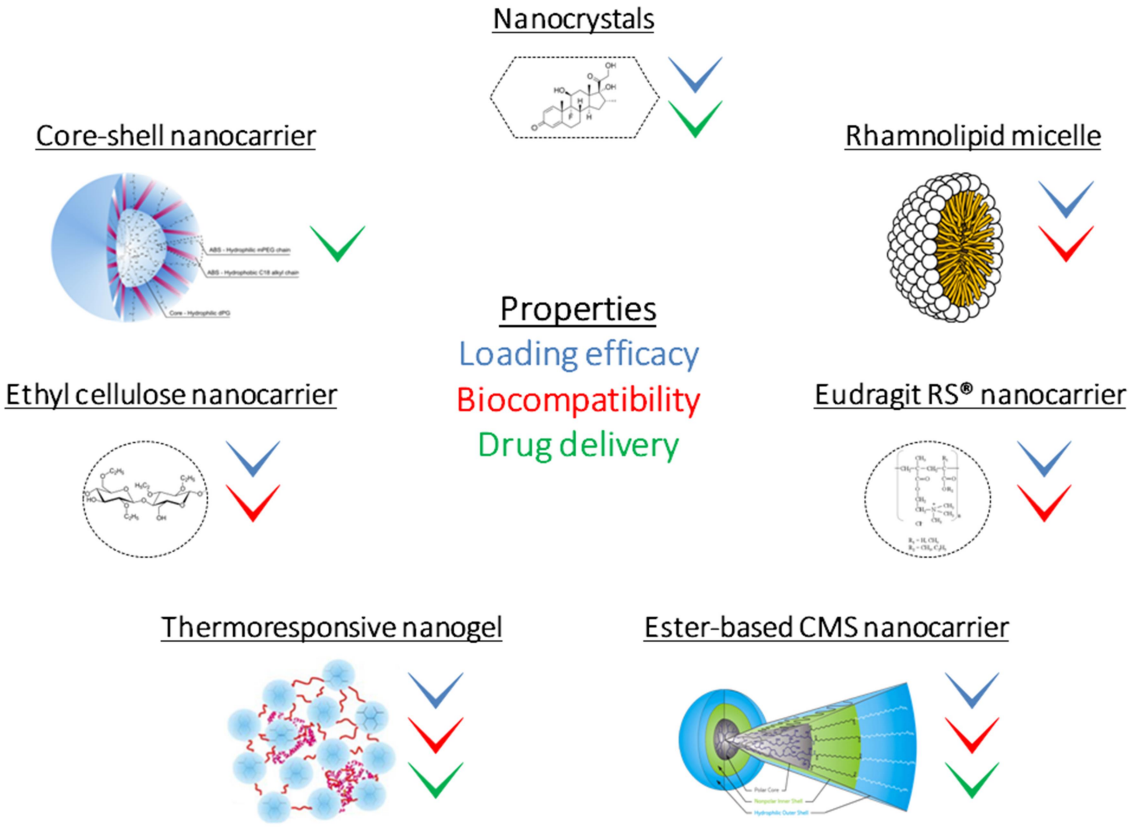

Figure 10. Investigated nanocarriers and their important properties for topical application. In strong cooperation, the new developed nanocarriers were investigated and their properties in terms of drug loading (blue), biocompatibility (red) and in particular drug delivery efficacy (green) were assessed using *in vitro* approaches. Presented ticks in corresponding color indicate the presence of necessary properties for evaluation on skin disease equivalents. (Reprinted by permission from Elsevier, Witting et al./Stefani et al, copyright 2015/2016, (Witting et al., 2015; Stefani et al., 2016)) and by permission from John Wiley and Sons, Radowski et al., copyright 2007, (Radowski et al., 2007)).

Those nanocarriers that demonstrated efficient DXM loading (Figure 10) were applied onto excised human skin and the delivered amounts of DXM were quantified using a reliable and validated protocol (Chapter 3.2). The elected method – human excised skin on Franz cell approach – has been identified as the only quantification approach of topical applied GCs with documented *in vitro*/*in vivo* correlation (Franz et al., 2009). Hence, nanocrystals significantly increased the delivered drug amount into the epidermis (Chapter 3, (Döge et al., 2016), Figure 2). These induced further cytokine release after topical application, revealing their irritative potential. Nanocarriers made from ethyl cellulose or Eudragit RS® failed to deliver DXM beyond the SC into deeper layers (Chapter 3, (Balzus et al., 2017), Figure 5). Their size (70-120 nm) exceeded the threshold of 20 nm of maximal particle size for skin absorption (SCCP, 2007) which might be the limiting factor in this approach. These particles might be relevant candidates for drug delivery *via* hair follicles (Lademann et al., 2015), but excised human skin neglect the follicular pathway and therefore the results should be supported by appropriate *in vitro* models for follicular penetration (Lademann et al., 2009).

Thermoresponsive nanogels (tNG) decorated with β-cyclodextrin (βCD) showed distinct higher DXM delivery efficacy into the skin compared to commercially available cream (Chapter 4, (Giulbudagian et al., 2018b), Figure 6). Interestingly, undecorated tNG failed too. Thus, the polymer surface modification with an penetration enhancer, which already showed an ability to deliver DXM (Loftsson and Duchêne, 2007), was successfully able to encapsulate hydrophobic compounds in a hydrophilic environment. The tNG exhibit also a controllable loading of DXM and excellent biocompatibility towards skin cells (Gerecke et al., 2017). The penetration enhancing effects of the tNG correlated with their ability to reorganize the SC. Ultrastructural analyses of tNG treated skin biopsies revealed clear disordering and/or swelling within the comprising structural components of the SC and apparent dilations of the spaces between these possibly formed transient pathways along which molecules may diffuse (Giulbudagian et al., 2016).

A strong interaction with the SC is also described for the second promising delivery approach within the thesis: CMS nanocarriers. These carriers accumulate within the SC lipid lamellae (Yamamoto et al., 2016), interact with the hydrophobic environment (Alnasif et al., 2014) and, owing to temperatures ≥ 31 °C, loaded cargos move towards the outermost shell of the CMS nanocarriers favoring the drug release (Boreham et al., 2014). Furthermore, the mPEG shell showed a retraction in lipophilic environments (e.g. SC lipids) alongside a simultaneous expansion of the inner shell (Rabe et al., 2014) that may also facilitate drug release. Consequently, CMS nanocarriers delivered 3-fold more DXM into the epidermis of the skin than the cream formulation (Chapter 5, (Hönzke et al., 2016b), Figure 6). Similar recovery rates for DXM applied with nanocarriers, were only achieved with polycaprolactone particles

(Beber et al., 2014) with the help on an infinite dose approach, which does not reflect the clinical use. To decrease toxicological potential, an ester bond was placed between core and inner shell. The high biocompatibility of the CMS (10-E-15-350) *in vitro* and *in vivo* supports this concept. Additionally, biodegradation of CMS nanocarriers by skin-derived enzymes was facilitated and has been shown for the first time (Chapter 5, (Hönzke et al., 2016b), Table 3) using a protocol especially designed for that purpose.

With the help of *in vitro* approaches, we were able to investigate various characteristics (size, hydrophilicity, surface modification, chemical composition) of topical nanocarrier systems and their influence on drug loading or biocompatibility. In particular, the combination with the *ex vivo* human skin approach was extremely useful in identifying tNG and CMS nanocarriers as the most effective of the tested carriers to increase drug bioavailability within the skin (Figure 10) and to decide whether it would be appropriate to evaluate the systems on the skin disease equivalents.

After topical application of drug loaded nanocarriers, specific requirements must be fulfilled for a stronger pharmacological effect compared the conventional approach e.g. cream treatment. These conditions can be described as increased drug delivery, faster activation of the corresponding receptor and a sustainable pharmacodynamic response (Gupta et al., 2012; Zhang et al., 2013), though the main challenge is to visualize these condition *in vitro*.

Human excised skin as an *in vitro* approach presents a short-term culture model and therefore the viability of the skin is only stable within 48 h after excision (Döge et al., 2016), a limiting factor for investigation of sustainable pharmacodynamic effects. These systems largely rely on molecular read-outs that, unfortunately, require longer time periods for effective and relevant regulation due to the latency of receptor mediated effects on protein formation (Döge et al., 2016). Hence, the use of skin disease equivalents may help to overcome these limitations.

The skin disease equivalents have been used to investigate the ability of tNG to deliver high molecular weight proteins, such as Etanercept, into HSEs (Figure 11A). The delivery of the anti-inflammatory protein was verified by demonstration of concordant downregulation of TNFα itself (present the specific target of Etanercept) as well as Intercellular adhesion molecule 1 (ICAM1) (Chapter 4, (Giulbudagian et al., 2018a), Figure 8), which was secondarily induced by TNFα. The reduction constitutes a relevant read-out parameter, due to the fact that these proteins are the main triggers for the activation and migration of immune cells in the skin (Heath and Carbone, 2013) aggravating dramatically the phenotype of skin diseases like AD.

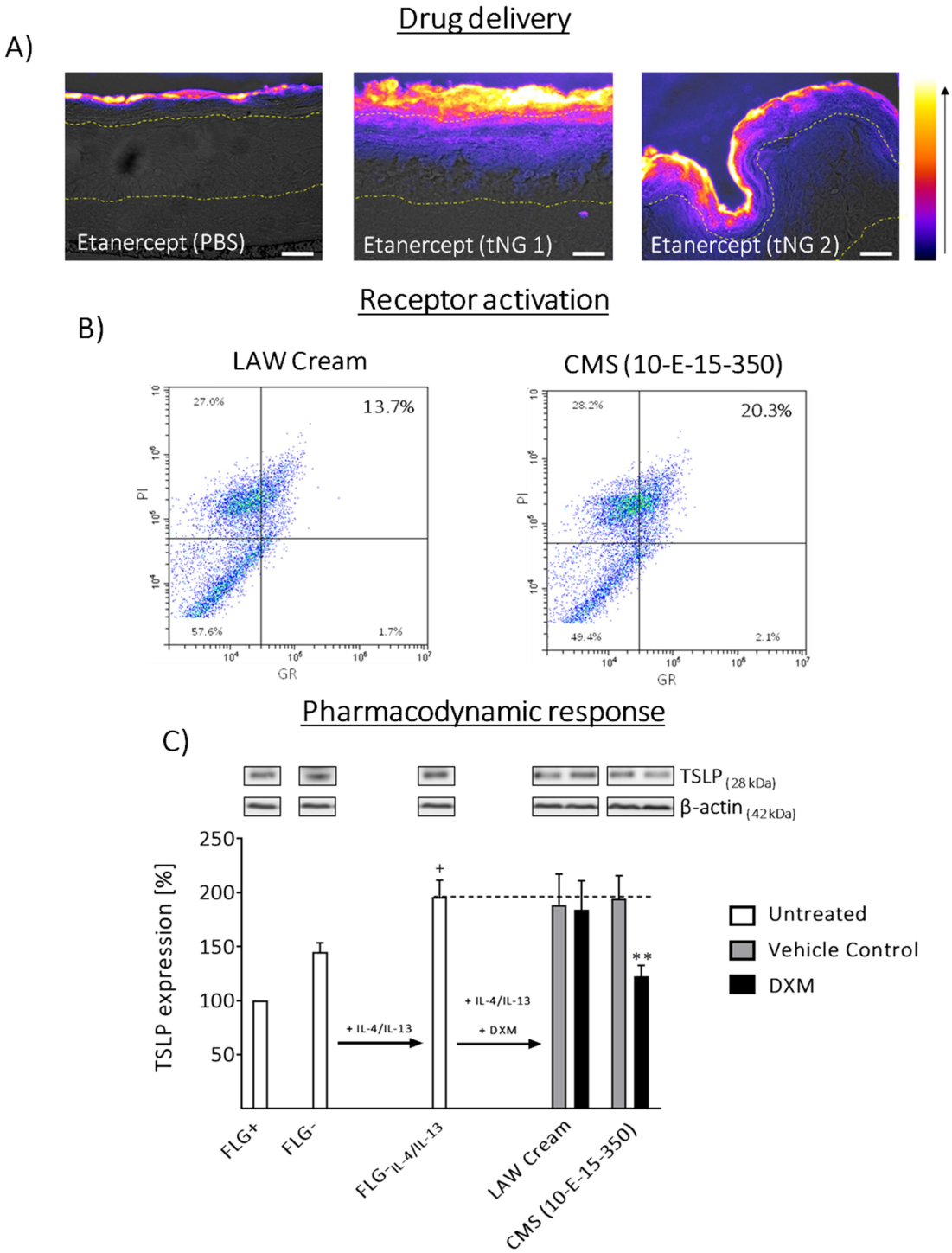

Figure 11. Evaluation opportunities for topical delivery on skin disease equivalents. *In vitro* skin disease equivalents can be used to monitor requirements for successful treatment. Stronger drug delivery (A), receptor activation (B) and anti-inflammatory effects (C) compared to conventional approaches are necessary for a more effective treatment. (Skin sections with immunostained Etanercept (A) were reprinted with permission from Theranostics using the terms of the Creative Commons Attribution License CC BY-NC 4.0, copyright 2018, (Giulbudagian et al., 2018a). GR activation using flow cytometry (B) and TSLP expression using Western blot (C) in treated skin equivalents were shown from Chapter 5.3). (CMS = core multi-shell nanocarrier, GR = glucocorticoid receptor, PBS = phosphate buffered saline, PI = propidiumiodid, tNG = thermoresponsive nanogel, TSLP = thymic stromal lymphopoetin).

Similar challenges are present for the *in vitro* evaluation of topically applied GCs. Again, the correlation of penetration rates with biologic effects in *ex vivo* skin is a challenge. In addition to the already mentioned limited culture period of the excised skin, typical short-term clinical read-outs for GC bioavailability, such as blanching as a result of vasoconstriction (Wiedersberg et al., 2009), or inhibition of UV-induced erythema (Reuter et al., 2008) are not applicable in the absence of active blood flow. Hence, the establishment of appropriate parameters in skin disease equivalents was necessary. First, the altered pharmacokinetics of DXM absorption after topical nanocarrier application were retraced with an innovative approach combining keratinocyte isolation from the equivalent (Bock et al., 2018) with subsequent nuclear isolation and immunohistochemical staining of, in this case, the glucocorticoid receptor (GR), which was then quantified by flow cytometry (Rosner et al., 2013). Stronger GR nuclei translocation after DXM application loaded onto CMS nanocarrier clearly proved the enhanced bioavailability of the delivered DXM (Figure 11B). The translocation of the GR into the nuclei of keratinocytes presents the fundamental parameter for successful anti-inflammatory treatment (Ratman et al., 2013). Investigation and visualization of this relevant effect in the treated skin disease equivalents using imaging and protein quantification methods (Chapter 5.3, Figure 3 and 4) supported the hypothesis.

Quantification of TSLP – a key cytokine to AD – presented a successful way to demonstrate the superiority of CMS nanocarriers (Figure 11C) and βCD decorated tNGs (Chapter 4, (Giulbudagian et al., 2018b), Figure 7) even after longer treatment. TSLP is highly up-regulated in the skin disease equivalents (Chapter 2, (Hönzke et al., 2016a), Figure 4), is known to be regulated via GR (Surjit et al., 2011), and has already been used in animal studies as a reliable readout parameter for GC efficacy (Mizuno et al., 2015). A significant decrease of TSLP expression was achieved after application of DXM every second day, which presents a relevant improvement in dose reduction. In the treatment of acute flares, daily application of DXM is recommended to clear the eczema within 1-2 weeks, notable when the drug's side effects are considered (Werfel et al., 2016). Additionally, the same results were found for the expression of pro-inflammatory cytokines IL-6/IL-8, an additional anti-inflammatory parameter, after DXM treatment using CMS nanocarriers (Chapter 5, (Hönzke et al., 2016b), Figure 8). Due to the carriers molecular weight of 70,000 g/mol, concerns may rise about systemic accumulation (renal cut-off: 50,000 g/mol), but the carrier themselves did not overcome the stratum corneum, which was consistent to parallel investigations (Pischon et al., 2016; Yamamoto et al., 2016).

It should be noted that also potential limitations of the skin equivalents were found for drug efficacy evaluation. Skin atrophy as side effect could not be investigated properly due to distinct effects of the drug free cream (Chapter 5.3, Figure S3), which served in all

experiments as a vehicle control. The results confirmed studies of DXM-treated skin equivalents from Weindl and colleagues (Weindl et al., 2011), where as well vehicle controls had a great impact on read-out parameters. This queried in summary study results on the atrophic effects of different GC loaded creams in reconstructed skin where no appropriate vehicle controls were included (Zöller et al., 2008). It has to be kept in mind that the use of the collagen matrix does not resemble the network-like fiber structure of the native human extracellular matrix, which forms the basic framework for the dermis. This also includes aspects of bio-mechanical properties of the matrix, which might exert major impact on cell response (Planz et al., 2016). The application of the cream onto the models implied further limitations. First, the equal distribution of cream on the model was a challenge and informative studies e.g. permeation assay with radio labeled testosterone to assess improvements in barrier function were not applicable due to the lipophilic character of the cream. Additionally, the drug-free cream increased expression of barrier protein IVL (Chapter 5.3 , Figure 1) explainable due to high amounts of glycerol and the penetration enhancer propylene glycol, both known to influence epidermal proliferation and differentiation (Short et al., 2007).

In conclusion, the results of the thesis encourage the implementation and use of *in vitro* reconstructed human skin in preclinical AD research. The developed skin disease equivalents emulating characteristics of atopic skin helped to identify pathological pathways that otherwise would have remained inaccessible in complex *in vivo* systems, and were useful to examine promising topical delivery systems based on the corresponding pharmacodynamic effects of the delivered agent. Despite the described limitations, the here presented equivalents showed similarities with the situation in AD lesions and the detected read-out parameters might be relevant for pharmaceutical development in terms of local drug efficacy and safety. This might hold true for other drugs used for other indications, too.

6.2 PROSPECTS

With regard to the complexity of the skin and to the diversity of factors involved in the pathogenesis of AD, the development of biomimetic *in vitro* human skin equivalents with more physiological functions would be useful for preclinical research, in particular drug discovery, disease modeling and basic research of skin biology. Promising cell sources for reconstructed skin, especially when aiming for personalized medicine, are induced pluripotent stem cells (iPSCs) (Itoh et al., 2013). The use of iPSC technology can circumvent the limitation of cell diversity/availability and so the introduction of vascular structures, pigmentation, skin appendages or nerves will increase the possibility of the *in vitro* approaches for pharmaceutical and biologic research (Abaci et al., 2017). For example, inclusion of hypodermis – one of the most understudied components of the skin – will raise more functional equivalents for wound therapy (Huber et al., 2016) and may also be useful to elucidate the role of adipose tissue in maintenance of epidermal and dermal cell homeostasis or regeneration. Another promising cell source and approach for personalized skin equivalents constitutes the use of hair follicle-derived cells (Löwa et al., 2018). Although juvenile normal human keratinocytes and fibroblasts are the current state-of the-art in tissue engineering, such age-matched and patient-derived cells would depict a more accurate reference than cells from foreskin. As mentioned in Chapter 1, most of the skin toxicity and immunological studies are carried out using animal models, although it is clear that these animal models often poorly represent the human immune system. Such concerns have led to the development of *in vitro* skin equivalents containing various immune cells, such as T cells (Wallmeyer et al., 2017) or LC´s (Kosten et al., 2016). These approaches would also increase the possibilities of efficacy evaluation, especially for agents that modulate the immunological response and that are used for inflammatory skin disease treatment. Thus, oral drug candidates such as Tofacitinib (Janus kinase inhibitor) or biologics such as Fezakinumab (IL-22 antibody), which are currently in clinical trials for AD treatment (Paller et al., 2017), could be evaluated as possible topical treatments with all the corresponding advantages relative to systemic administrations.

To further increase the read-out possibilities, skin disease equivalents need to be combined with other test systems in the future. The development of organ-on-a-chip platforms that connects the skin with other tissues/organs of interest would be highly beneficial in terms of recapitulating interactions of skin with other organs, detecting unknown side-effects of drugs, evaluating the effects of drugs metabolized by other organs (e.g. liver) and estimating systemic pharmacodynamics/pharmacokinetics of topically administered drugs (Maschmeyer et al., 2015). Probably the most faithful systems to study either pathological effects or drug

related effects on the skin and/or systemic environment are based on xenotransplantation. Therefore, either human skin biopsies or *in vitro* skin equivalents were grafted onto immunodeficient mice followed with injection of the disease related T cell populations (Carretero et al., 2016). Those xenografts in part overcome the limitations of the individual approaches and will enter more the preclinical research to study the role of immune cells and/or to predict the efficacy of therapeutic compounds.

The production of the equivalents is done by hand, which is costly and time-consuming. Miniaturization (scaling) may indeed be required for drug development, because it will allow to reduce the costs, perform high-throughput screening and minimize the variability between different skin equivalents (Abaci et al., 2017).

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7

SUMMARY/ZUSAMMENFASSUNG

7.1 SUMMARY

Atopic dermatitis, also referred as atopic eczema, is one of the most common chronic inflammatory skin diseases and the phenotype adversely affects the quality of life of patients significantly. Despite the high predictive power of reconstructed human skin, *in vitro* approaches for AD have not been widely introduced into preclinical drug research. Our lacks of understanding of the disease pathogenesis as well as the unmet clinical need for new therapeutic options in the field of AD ask for the development of predictive skin disease equivalents that accurately reflect the disease. In order to create reliable skin equivalents that contribute to the understanding and treatment of skin diseases, appropriate tools and the current knowledge of the pathogenesis are necessary to transfer the condition of the disease into the *in vitro* approach.

In the first step, cytokine exposure in skin equivalents was combined with a genetically derived *FLG* deficiency for the first time, permitting the influences and interdependencies of these two key AD hallmarks to be investigated systematically. Histology, surface pH and disease related protein expression (FLG ↓, TSLP↑) in the exposed skin equivalents closely resemble acute lesions in AD patients. Distinct up-regulation of different skin barrier and tight junction proteins initially compensate for the lack of functional FLG, resulting in a similar phenotype (e.g. surface pH and skin barrier function) as in healthy models. For the first time, a direct link between FLG deficiencies and the innate immune response were demonstrated by the upregulation of AMP expression. Dramatic downstreaming effects after cytokine exposure found for all compensatory mechanism (except TSLP) strengthened the hypothesis that Th2 inflammation might constitute the main trigger for the disease within the body.

To identify the optimal topical drug delivery system for evaluation in the skin disease equivalents, promising nanocarrier candidates were characterized in terms of their physicochemical properties, biocompatibility and, in particular, drug deliver efficacy into human skin. Thus, essential differences between the various carrier systems were revealed, primarily dependent on their chemical composition. Unlike other carriers that exhibited drawbacks in one or two of the necessary requirements for efficient drug delivery systems, the CMS nanocarriers and tNG turned were biocompatible, stable, and effectively delivered small molecules into excised human skin. They clearly outperformed the conventional and widely used cream formulation in terms of drug delivery and demonstrated excellent biocompatibility towards skin derived cells and immune cells.

Finally, the developed skin disease equivalents were able to prove the promising results of both carrier systems from the *ex vivo* evaluation by establishment of various read-out parameters. The successful delivery of therapeutic proteins through the skin barrier by tNG

was shown along with the first demonstration of their anti-inflammatory pharmacological activity in the skin disease equivalents. After chemical modification with cyclodextrin, the tNGs were even able to deliver DXM in an efficient manner. The altered pharmacokinetics of DXM absorption after topical application of loaded CMS nanocarriers was verified with faster glucocorticoid receptor activation in the viable epidermis. Consequently, as seen in several different studies, stronger anti-inflammatory effects were seen compared to the standard treatment as demonstrated by significant reduction of key pathological AD proteins like TSLP and pro-inflammatory cytokine such as IL-6/IL-8.

In summary, the results, described in the thesis show that the developed AD skin disease equivalents present certain characteristics of lesional AD. They also demonstrate reconstructed diseased skin is valuable for basic biological research as well greatly useful for pharmacodynamic evaluation. In terms of *in vitro* AD research, relevant pathways, read-out parameters and corresponding techniques were demonstrated for efficacy assessment of topical pharmaceutical approaches/drugs.

7.2 ZUSAMMENFASSUNG

Atopische Dermatitis, auch bekannt als atopisches Ekzem, ist eine chronisch-entzündliche Hauterkrankung mit hoher Prävalenz, deren Erscheinungsbild die Lebensqualität der betroffenen Patienten massiv beeinträchtigt. Der klinische Bedarf an wirksamen Arzneimitteln für Patienten sowie die Notwendigkeit eines besseren Verständnisses der zugrundeliegenden Pathogenese erfordert die Entwicklung prädiktiver Hautmodelle mit entsprechenden Eigenschaften. Trotz der hohen Aussagekraft von rekonstruierten humanen Hautmodellen, werden solchen *in vitro* Methoden zur Erforschung der Erkrankung bisher nur begrenzt eingesetzt, meist aufgrund von Mangel an geeigneten Krankheitsmodellen. Um prädiktive und verlässliche Modelle der atopischen Dermatitis generieren zu können, welche in der Lage sind einen Beitrag für erfolgreiche präklinische Forschung zu leisten, ist es notwendig den aktuellen Wissenstand der Forschung zu kennen und diesen in ein solches *in vitro* Konzept zu überführen.

Im ersten Schritt wurden die pathologischen Parameter der FLG Defizienz und der Zytokinexposition zum ersten Mal gleichzeitig in einem Hautmodell integriert, vor allem um deren Wechselbeziehung systematisch zu untersuchen. Kompensatorische Hochregulierungen von Hautbarriere- und Tight Junction Proteinen, bedingt durch das Fehlen von FLG, führen dazu, dass zunächst kaum eine Veränderung des Phenotypes im Vergleich zu gesunden Hautmodellen festzustellen war. Durch die gezeigte Hochregulation von antimikrobiellen Peptiden, wurde erstmals eine direkte Verbindung zwischen der angeborenen Immunantwort und der FLG Defizienz gezeigt. Die charakterisierten Hochregulationen in den Hautmodellen wurden durch die Zytokinexposition gravierend gestört, was die Hypothese bekräftigt, dass die Entzündung innerhalb des Körpers als ein Hauptstimulus für das Fortschreiten der Erkrankung zu sehen ist. In den krankheitsspezifischen Merkmalen Histologie, Hautoberflächen pH und Proteinexpression (FLG ↓, TSLP ↑) ist vor allem in den zytokinbehandelten Hautmodellen eine deutliche Ähnlichkeit zu akuten Läsionen in Patienten zu erkennen.

Um das bestmögliche topische Transportsystem für die pharmakologische Evaluierung auf dem Krankheitsmodell zu identifizieren, wurden verschiedenste Nanotransportersysteme in Bezug auf deren Eigenschaften (Physikochemie, Toxizität, Transporteffizienz in Humanhaut) charakterisiert. Dabei wurden essentielle Unterschiede der verschiedenen Systeme identifiziert, vor allem in Abhängigkeit der chemischen Zusammensetzung. Einige Nanotransporter zeigten dabei deutliche Nachteile in wichtigen Voraussetzungen für den Einsatz als Transportsystem, aber thermoresponsive Nanogele (tNG) und CMS Nanotransporter stellten sich als die vielversprechendsten Konzepte heraus. Sie übertrafen

deutlich die konventionelle Creme Formulierung in Sachen Transporteffizienz und zeigten eine herausragende Komptabilität gegenüber epidermalen Zellen und Immunzellen.
Die positiven Ergebnisse der Evaluierung beider topischer Transportsysteme konnten abschließend auf den entwickelten Krankheitsmodellen mithilfe verschiedener Parameter bestätigt werden. Durch messbare anti-entzündliche Effekte konnte zum ersten Mal der durch die Nanogele ermöglichte Transport eines wirksamen Proteinarzneistoffs in seiner aktiven Form verifiziert werden. Die veränderte Pharmakokinetik des topischen Dexamethasontransportes, bedingt durch die Applikation mittels CMS Nanotransporter konnte durch eine schnellere Aktivierung des Glucocorticoidrezeptors auch pharmakologisch überprüft werden. Mithilfe der Reduktion von TSLP, einem Schlüsselprotein in der AD Pathogenese, sowie der Reduktion der pro-inflammatorischen Zytokine IL-6 und IL-8, wurden stärkere anti-entzündliche Effekte im Vergleich zur entsprechenden Standardtherapie mit Dexamethason Creme beobachtet.
Die Ergebnisse der Dissertation veranschaulichen die Entwicklung von Krankheitsmodellen der Haut, welche bestimmte Charakteristika von Läsionen der atopischen Dermatitis aufweisen. Es wird deutlich, dass die rekonstruierten Hautmodelle einen wertvollen Beitrag zur biologischen Forschung als auch zur pharmakologischen Evaluierung leisten können. Zum ersten Mal konnte die Effektivität neuer topischer Therapieansätze mittels relevanter Parameter und entsprechenden Techniken in einem humanbasierten *in vitro* System krankhafter Haut gezeigt werden.

8

STATEMENT OF AUTHORSHIP

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Hiermit versichere ich, Stefan Hönzke, 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, den 15.02.2018

Stefan Hönzke

9

CURRICULUM VITAE

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.