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NEW BIOMEDICAL APPROACHES FOR STUDYING (PATHO)PHYSIOLOGICAL CONDITIONS OF HEALTHY AND INFLAMED SKIN *IN VITRO*

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by

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from Berlin

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I LEARNED THEN WHAT SCIENCE WAS ABOUT:
IT WAS PATIENCE

RICHARD P. FEYNMAN (1918–1988)

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LIST OF PUBLICATIONS AND CONTRIBUTIONS

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Khiao In, M.; Richardson, K.; **Löwa, A.**; Hedtrich, S; Kaessmeyer, S.; Plendl, J.: Histological and functional comparisons of four anatomical regions of porcine skin with human abdominal skin. *Anat Histol Embryol.* 48(3), 207-217 (2019).

Löwa, A.; Vogt, A.; Kaessmeyer, S.; Hedtrich, S.: Generation of full-thickness skin equivalents using hair follicle-derived human keratinocytes and fibroblasts. *J Tissue Eng Regen Med*. 12(4), 2134-2146 (2018).

Löwa, A.*; Jevtić, M.*; Gorreja, F.; Hedtrich, S.: Alternatives to animal testing in basic and preclinical research of atopic dermatitis. *Exp Dermatol*. 27(5), 476-483 (2018).

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CONFERENCES PROCEEDINGS

Jevtić, M.; **Löwa, A.**; Hedtrich, S.: *Intercellular crosstalk between fibroblasts and keratinocytes in skin models.* Poster presented at the 16th International Perspectives in Percutaneous Penetration Conference, La Grande Motte, France, April 3 – 6, 2018.

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Löwa, A.; Kováčik, A.; Vávrová, K.; Hedtrich, S.: *The impact of fibroblasts from atopic dermatitis patients on the epidermal differentiation of full-thickness skin equivalents.* Poster presented at the 18th Annual Congress of EUSAAT & 21th European Congress on Alternatives to Animal Testing, Linz, Austria, September 23 – 26, 2018.

Löwa, A.; Khiao In, M.; Kaessmeyer, S.; Hedtrich, S.: *Generation of in vitro skin models using primary hair follicle-derived and skin-derived keratinocytes and fibroblasts.* Poster presented at the JRC Summer School on Alternative Approaches for Risk Assessment, Ispra, Italy; May 16 – 19, 2017.

Löwa, A.: *With skin and hair – How you built skin with the help of hairs.* Presentation at Long Night of Sciences, Berlin, Germany; June 24, 2017.

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LIST OF ABBREVIATIONS

AD	atopic dermatitis
BSA	bovine serum albumin
CLDN1	claudin-1
CK	cytokeratin
CTS	connective tissue sheath
DC	dendritic cell
DS	dermal sheath
DED	de-epidermized dermis
DES	desmin
DMEM	Dulbecco's modified eagle's medium
DP	dermal papilla
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
Fb	fibroblast
FBS	fetal bovine serum
FFA	free fatty acid
FLG	filaggrin
FT	full-thickness
HF	hair follicle
HFDF	hair follicle-derived fibroblasts
HFDK	hair follicle-derived keratinocytes
HUVEC	human umbilical vein cord endothelial cells
IDEC	inflammatory dendritic epidermal cells
IFN-γ	interferon gamma
Ig	immunoglobulin
IL	interleukin
ILC2	group 2 innate lymphoid cell
iPSC	induced pluripotent stem cell
IRS	inner root sheath
IVL	involucrin
LEKTI	lymphoepithelial Kazal-type-related inhibitor
LIF	leukemia inhibitory factor
KLK	kallikrein
LOR	loricrin
MC	mast cell
NFκB	nuclear factor kappa B
NHDF	normal human dermal fibroblasts
NHEK	normal human epithelial keratinocytes
NMF	natural moisturizing factor
OCLN	occludin
OECD	Organization for Economic Co-operation and Development
ORS	outer root sheath
OX40L	OX40 ligand
PAR2	protease-activated receptor 2
PBS	phosphate-buffered saline
PCA	pyrrolidone carboxylic acid
RHE	reconstructed human epidermis
RNAi	RNA interference
SC	stratum corneum
SCF	stem cell factor
SPINK5	serine protease inhibitor Kazal-type 5
STAT	signal transducers and activators of transcription
TERT	telomerase reverse transcriptase
TEWL	transepidermal water loss
TGF	transforming growth factor
Th	T helper
TNF	tumor necrosis factor
TSLP	thymic stromal lymphopoietin
UCA	urocanic acid
VEGF	vascular endothelial growth factor
VIM	vimentin

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1 INTRODUCTION

Skin, the primary barrier of the human body, serves both as a sensor for environmental changes and as a physical barrier. The diversity of the cellular and molecular components within the skin ensures these functions, and so imbalances in their homeostasis can cause skin disorders. One of the most common inflammatory skin diseases is atopic dermatitis (AD). Over the past decades, great efforts have been made to better understand the pathogenesis of AD. At present, we know the pathogenesis of AD derives from a complex interplay between skin barrier defects and immune dysregulation. These insights have contributed to the development of effective therapeutic biologicals that specifically target dysregulated immune pathways, which in turn prevent skin barrier damage and minimize transcutaneous allergic sensitization. Nevertheless, AD is a highly heterogeneous disease displaying varied pathological features between patients. Indeed, findings from molecular research and therapeutics trials suggesting distinct AD endotypes (Paternoster et al., 2018; Bieber, 2012). There is an urgent need to find disease biomarkers that will support precision medicine principles on a global level. Therefore, studies investigating the etiology of AD in more depth are required. In the recent years, several *in vitro* skin equivalents mimicking features of AD were developed. These have proven to be useful as tools for understanding the pathogenesis of AD and as therapeutic screening platforms.

1.1 ATOPIC DERMATITIS

Atopic dermatitis (AD) is a chronic, heterogeneous, and often relapsing inflammatory skin disease. It is characterized by intense itch (pruritus), recurrent lesions, a fluctuating course (xerosis), and patches of cracked skin that leak exudate (eczematous lesions). At the clinical level, AD presentation is heterogeneous with a wide spectrum of possible features ranging from eczema limited to specific body regions or erythema affecting over 99% of the body surface (erythroderma). Typically, disease presentation is age-related (Fig. 1). In infants, lesions are mainly on the face, scalp and extensor surfaces of the limbs. In children, different types of skin lesions are seen that particularly affect the flexural folds. In adolescents and adults, lichenified and scraped plaques often affect visible parts of the body's flexural folds, wrists, ankles, eyelids, shoulders, and scalp (Weidinger & Novak, 2016; Lyons et al., 2015). These clinical features formed the basis for the development of diagnostic criteria for AD (Hanifin & Rajka, 1980). Today, enhanced and validated severity scales such as the SCORAD - Score in Atopic Dermatitis (Stalder et al., 2011) and or EASI - Eczema Area and Severity Index (Chopra et al., 2017) are available.

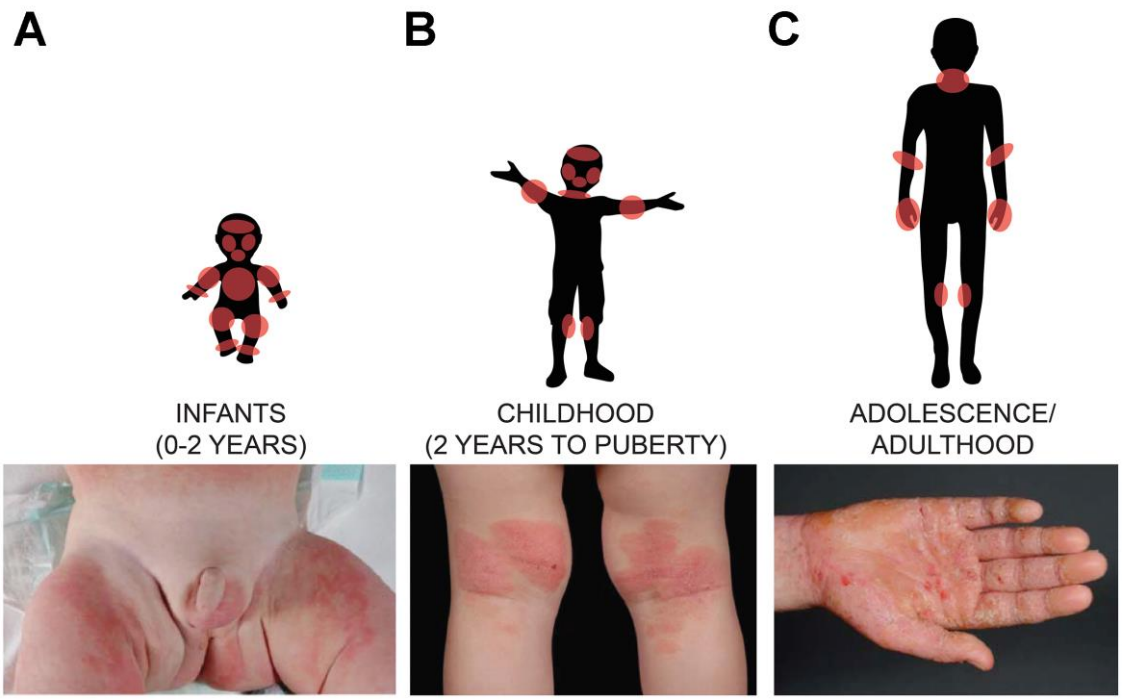

Figure 1. Age-related typical clinical appearance of atopic dermatitis. (**A**) In infantile phase, acute lesions mainly emerge on the face (cheeks), the scalp, and the extensor surfaces of the limbs. Although the trunk might be affected, the napkin area is typically spared. (**B**) During childhood, different types of skin lesions involve flexural folds, the nape and the face. (**C**) In adolescence, lichenified and excoriated plaques affect flexures, head and neck. Additionally, adults can have only chronic hand eczema. Reprinted by permission from Elsevier (Weidinger et al., 2016).

1.1.1 Epidemiology

Due to the already mentioned clinical heterogeneity of AD, e.g. in terms of lesion morphology and distribution, an accurate measurement of disease frequency is complicated. Nevertheless, there is evidence that AD is the most common chronic skin disease worldwide (Weidinger et al., 2018). Since AD was once regarded as a purely pediatric disease, several studies are dealing with AD in childhood (Deckers et al., 2012; Odhiambo et al., 2009). One of the largest studies is the International Study of Asthma and Allergies in Childhood (ISAAC). This study surveyed children aged 6-7 years and 13-14 years from different countries using validated questionnaires with questions on the symptoms of wheezing, rhinoconjunctivitis and eczema (Odhiambo et al., 2009). The ISAAC has already been performed three times over a period of five to 10 years (Phase One, Phase Two and Phase Three). Across Phase One and Three, the prevalence of AD symptoms in children aged 6-7 years increased over time in most geographical areas, particularly in developing countries such as Africa. For children aged 13-14 years, however, the prevalence remained stable at around 20%. Interestingly, for both age groups, significant differences in the prevalence of AD were observed in different regions of the

world (6-7-year-old children 0.9% in India to 22.5% in Ecuador; 13-14 year-old children 0.2% in China to 24.6% in Colombia) (Odhiambo et al., 2009), even between cities in the same country (Morocco: 13-14-year-old children 4.7% in Benslimane to 13.5% in Marrakesh) (Ait-Khaled et al., 2007). Overall, the prevalence of AD in children is estimated at 10% to up to 25% worldwide (Weidinger et al., 2018; Weidinger et al., 2016; Deckers et al., 2012; Williams, H. et al., 2008). Although, ISAAC and most other epidemiological studies have only included children, more-recent evidence points out the abundance of AD in adults. Studies including multiple countries show that 7-10% of adults are affected by AD (Abuabara et al., 2018; Silverberg & Hanifin, 2013; Deckers et al., 2012). In many cases, the onset of AD is during the first two years of life and patients experience relapsing-remitting episodes throughout childhood that improve towards adolescence (Garmhausen et al., 2013). Until recently, little was known about adult-onset AD or predictors of persistent disease (Weidinger et al., 2016; Williams, H. C., 2005). Interestingly, findings from a recent meta-analysis of seven birth cohort studies with follow-up times of up to 26 years, revealed no significant difference in AD prevalence before and after childhood (Abuabara et al., 2018). This supports the assumption that AD is a lifelong disease with variable phenotypic expression (Weidinger et al., 2016) and the proportion of patients suffering from the adult-onset disease with a persistent disease course or relapses from asymptomatic periods are much higher than previously thought (Margolis et al., 2014; Silverberg et al., 2013).

When looking at the epidemiological data, the strong differences in the prevalence of AD worldwide, even between highly genetically similar populations (Williams, H. et al., 2008) are particularly noticeable. This has led some to hypothesize that AD prevalence is associated with adverse environmental factors including industrialization and a western lifestyle (Williams, H. et al., 2008). Although, no key environmental risk factor is known, environmental and socioeconomic factors, as well as genetic predispositions seem to have an impact on the manifestation of AD. The strongest known risk factor is a family history of AD: disease risk increases 3- to 5-fold if one or both parents have AD (Apfelbacher et al., 2011; Wadonda-Kabondo et al., 2004). Environmental risk factors that are supported by strong epidemiological data include: urban environment, low ultraviolet light exposure, dry climate, western diet (i.e. high intake of sugar and polysaturated fatty acids), small family size, high level of family education, high socioeconomic status, and repeated treatment with broad-spectrum antibiotics before the age of five. Further factors like obesity, farm environment, household hair-bearing pets, hard water, some air pollutants, and active or passive exposure to tobacco smoke might play a role in AD. However, the data supporting the role of these in AD manifestation remain inconsistent (Bonamonte et al., 2019; Kantor & Silverberg, 2017; DaVeiga, 2012). AD patients, especially those with severe disease, are at high risk of immunoglobulin E (IgE)-mediated sensitization to common allergens, leading

to allergic comorbidities including asthma, food allergies, and allergic rhinoconjunctivitis (Manam et al., 2014).

1.1.2 Pathogenesis

The pathophysiology of AD involves genetic and environmental risk factors, which are further associated with dysregulation of the immune system and epidermal barrier. All these aspects contribute to the high complexity of this disease. The following sections will focus on the genetic, skin barrier, and immunological aspects.

GENETIC SUSCEPTIBILITY

Nowadays, the genetic aspect of AD is well-studied, and it is well-known that a positive family history of atopic diseases is the strongest AD risk factor. Early studies with twins helped make the genetic basis of AD apparent (Thomsen et al., 2007). Subsequently, genome-wide analyses and targeted high-throughput approaches were conducted to identify genetic disease associates (Paternoster et al., 2015; Schaarschmidt et al., 2015; Ellinghaus et al., 2013; Hirota et al., 2012; Paternoster et al., 2011; Sun et al., 2011; Esparza-Gordillo et al., 2009). More than 30 AD susceptibility loci were found that harbor genetic variants. These AD loci are enriched for genes that might affect the epidermal barrier, environmental sensing, immune regulation and tissue response (Weidinger et al., 2018; Paternoster et al., 2015).

The strongest genetic risk factor for AD is a loss-of-function mutation in the gene filaggrin (*FLG*) (Palmer et al., 2006). In Northern Europeans, the prevalence of loss-of-function mutations within *FLG* exon 3 is approximately 10% (Thyssen et al., 2013). These include the two most dominant genotypes, R501X and 2282del4, as well as the less common mutations S3247X and R2447X (Irvine et al., 2011; Smith et al., 2006). For now, more than 20 other rare loss-of-function mutations within *FLG* exon 3 have been discovered in European populations (Brown, S. J. & McLean, 2012). *FLG* is a polymorphic gene consisting of two small and a third large exon. The gene encodes the large (<400 kDa), insoluble polyprotein profilaggrin, the major component of keratohyalin granules found within the granular layer of the epidermis. Profilaggrin is composed of an N-terminal domain (S100-like calcium-binding and nuclear localization), 10-12 nearly identical filaggrin repeats (keratin-binding properties, third exon), and a C-terminal domain of unknown function (Sandilands et al., 2009; Presland et al., 1992; Gan et al., 1990). The polyprotein is dephosphorylated and proteolytically cleaved into 10-12 37 kDa filaggrin (FLG) monomers by serine proteases including matriptase, prostasin and kallikrein (Sakabe et al., 2013; Netzel-Arnett et al., 2006; List et al., 2003). This process is initiated by an increase intracellular Ca^{2+} levels (Markova et al., 1993). In healthy skin, FLG monomers bind to

keratin intermediate filaments such as cytokeratin 1 (CK1) and CK10, which are then cross-linked by transglutaminases within the keratinocyte cytoskeleton, creating tight bundles (Dale et al., 1997). This causes granular cells to collapse and become flattened anuclear corneocytes, within which a highly insoluble keratin matrix is assembled (Candi et al., 2005). This keratin matrix serves as a protein scaffold for the attachment of cornified-envelope lipids and proteins that, together, form the stratum corneum (SC). This ultimately forms the so-called 'skin barrier', which minimize entry of molecules such as bacterial antigens and transepidermal water loss (TEWL) (Sandilands et al., 2009). Upon loss-of-function mutations in *FLG*, cell flattening can still be observed, however disorganized keratin filaments, disturbed lamellar bilayer structures, and impaired lamellar body loading can also occur (Kawasaki et al., 2012; Gruber et al., 2011). Under healthy conditions, monomeric FLG is further degraded by cysteine peptidase, caspase-14, and other proteases to release its component amino acids. The mixture of hydroscopic amino acids including pyrrolidone carboxylic acid (PCA) and urocanic acid (UCA), the so-called natural moisturizing factor (NMF), contributes to epidermal hydration and barrier function (Rawlings & Harding, 2004). The other FLG breakdown products, together with organic acids may help to maintain human skin's surface pH within a range from 4.5 to 5.5 (Parra & Paye, 2003). The so-called 'acid mantle' of the SC has a well-known antimicrobial effect. Additionally, an acidic pH is required for the coordinated epidermal differentiation and functional activity of enzymes involved in ceramide metabolism for the cornified cell envelope formation (Kezic et al., 2011; Lee, S. H. et al., 2006). Hence, loss-of-function mutations within *FLG* exon 3 result not only in an almost complete absence of FLG monomers (Sandilands et al., 2007), but patients also show reduced NMF levels, skin dryness, and higher epidermal pH (Jungersted et al., 2010; Sergeant et al., 2009). Clinically, increased disease duration severity, and total IgE levels, as well as earlier disease onset are observed in patients with loss-of-function *FLG* mutations (Weidinger et al., 2007; Palmer et al., 2006; Sandilands et al., 2006; Smith et al., 2006). Interestingly, although individuals with loss-of-function *FLG* mutations are at an approximately 3-fold increased risk of developing AD (Irvine et al., 2011), only ~20% of patients with mild-to-moderate AD carry a *FLG* mutation (Weidinger et al., 2008; Baurecht, H. et al., 2007). Perhaps even more interesting is that over 50% of individuals carrying a *FLG* mutation do not develop AD (Weidinger et al., 2008).

Aside from *FLG*, mutations in other epidermal constituents and enzymes that maintain epidermal homeostasis have been linked to AD, such as serine protease inhibitor Kazal-type 5 (*SPINK5*) and tight junction protein claudin-1 (*CLDN1*) (De Benedetto et al., 2011; Barnes, 2010). Several genetic mutations related to adaptive and innate immunity have also been found in AD patients. These include gene polymorphisms in pathogen-associated molecular patterns such as toll-like receptors (TLR), antimicrobial peptides (AMPs), thymic

stromal lymphopoietin (TSLP), and the receptor for TSLP (Paternoster et al., 2015; Suarez-Farinas et al., 2015; Tamari & Hirota, 2014; Weidinger et al., 2013; Hirota et al., 2012; Paternoster et al., 2011). Another locus influencing AD predisposition is the type 2 T helper cell (Th2) cytokine cluster on chromosome 5q31.1 (Weidinger et al., 2013; Paternoster et al., 2011). This cluster includes genes encoding cytokines of type 2 immunity and the DNA repair protein *RAD50*, which is part of a locus control region. Through epigenetic changes, *RAD50* is involved in the regulation of the type 2 cytokine genes (Koh et al., 2010).

DISRUPTION OF SKIN BARRIER

Genetic mutations such as in the *FLG* gene belong to the primary intrinsic mechanisms that induce epidermal barrier dysfunction, allowing the entrance of pathogens and allergens that induce subsequent immune responses – the so-called outside-inside theory (Bieber, 2008; Cork et al., 2006). Epidermal barrier disruptions can also be mediated by secondary mechanisms such as the itch-scratch cycle, reduced lipid synthesis, or reduced expression of epidermal structural proteins as a result of type 2 immunity cytokines including interleukin (IL)-4, IL-13, and IL-33 – the so-called inside-outside theory (Seltmann et al., 2015; Cole et al., 2014). Certain impaired epidermal barrier functions become apparent in non-lesional (unaffected skin) as well lesional skin of AD patients. These include the manifestation of easily-irritated skin (Danby et al., 2018), increased skin-surface pH (Jungersted et al., 2010), increased TEWL (Flohr & Mann, 2014), and enhanced susceptibility to allergens that in turn promote allergenic sensitization and increased susceptibility to infections (Halling-Overgaard et al., 2017; Miajlovic et al., 2010; Gao, P. S. et al., 2009). These changes contribute to the initiation and exacerbation of AD inflammation (Fig. 2).

At the molecular level, reduced expression of epidermal structural proteins such as loricrin (LOR) and involucrin (IVL) (Jarzab et al., 2010; Guttman-Yassky et al., 2009; Kim, B. E. et al., 2008), and tight junction proteins such as CLDN1 (Yoshida et al., 2014; De Benedetto et al., 2011) in lesional and non-lesional AD skin can be observed compared to healthy skin. Moreover, the lipid composition and lamellar organization of epidermal lipids, which form the ‘mortar’ of the SC that enhances and maintains the hydration of epidermal barrier, is altered in atopic skin (Ishikawa et al., 2010). In AD skin, lipid levels, especially ceramide content, and the chain lengths of ceramides and free fatty acids (FFAs) are reduced, while the proportion of unsaturated FFAs is increased (van Smeden et al., 2014; Janssens et al., 2012; Park, Y. H. et al., 2012). Overall, the decreased lipid content leads to a less compact lipid organization and defective skin barrier function. Furthermore, the changes in epidermal lipid composition, especially the levels of long-chain unsaturated FFAs, has been shown to correlate strongly with the skin microbiome composition (Baurecht, Hansjörg et al., 2018), leading to a greater abundance of *Staphylococcus aureus* that frequently colonizes atopic

skin (Kong et al., 2012). Another molecular event that results in increased microbial colonization is the establishment of an imbalance between serine proteases and protease inhibitors (Igawa et al., 2017; Rawlings & Voegeli, 2013). Under normal conditions, the balance between proteases and protease inhibitors, as well as calcium and pH, regulates physiological desquamation (Rawlings et al., 2013; Ekholm et al., 2000). A lack of endogenous protease inhibitor activity causes, primarily, epidermal proteases hyperactivity and, secondary, SC detachment. For example, in a *Spink5* knockout mouse model, the activity of the secreted serine protease kallikrein 5 (KLK5) is enhanced. KLK5 then stimulates protease-activated receptor 2 (PAR2), which is highly expressed in granular layer as well as lower skin layers in lesional skin (Zhu et al., 2017), that in turn activates nuclear factor kappa B (NFκB)-mediated overexpression of TSLP, intercellular adhesion molecule 1 (ICAM1), tumor necrosis factor-alpha (TNF-α) as well as IL-8 overexpression and induced pruritus (Briot et al., 2009).

All listed events that contribute to epidermal barrier disruption promote inflammation. Here, immunomodulatory proteins and release of damage-associated molecular pattern molecules, including alarmins, are the main players. Alarmins are proteins released in response to tissue damage and induce inflammation (Yoon et al., 2016; Oyoshi et al., 2010). These include IL-1β, IL-25, IL-33 as well as the already mentioned TSLP. These mediators activate skin-resident group 2 innate lymphoid cells (ILC2s) and Th2 cell-mediated immune response (Salimi et al., 2013; Soumelis et al., 2002). TSLP itself induces the expression of OX40 ligand (OX40L) by dendritic cells (DCs), which binds to the OX40L receptor on naïve T cells. This event triggers Th2 cell-mediated response and stimulates the release of IL-4, IL-5 and IL-13. IL-4 and IL-13 promotes immunoglobulin switching in B cells, resulting in IgE production, which itself induces the expression of adhesion molecules and recruits various immune cells (Fig. 2) (Ito et al., 2005; Soumelis et al., 2002). Conversely, the inflammatory response reduces expression of epidermal barrier proteins, tight junction proteins as well as SC proteins, that in turn induce a disruption of the epidermal barrier (Omori-Miyake et al., 2014; Oyoshi et al., 2010).

IMMUNOPATHOGENESIS

A characteristic of AD is cutaneous inflammation that manifests as redness and/or swelling of the skin. Several cell types from the skin and immune system are involved in this, such as epidermal dendritic cells (Langerhans cells and inflammatory dendritic epidermal cells (IDEC)), T cells, B cells, eosinophils, mast cells, monocytes/macrophages, keratinocytes, and vascular endothelium (Werfel et al., 2016).

Although, the number of activated Langerhans cells is not increased in non-lesional AD skin, unlike lesional skin, such skin regions show immunohistological changes like

spongiosis and T cell infiltration, primarily $CD4^+$ Th2 cells that are defined by the production of IL-4, IL-5 and IL-13 (Gittler et al., 2012; Suarez-Farinas et al., 2011). In acute lesional sites, more Th2 cells and additional $CD4^+$ subsets, including Th22 (IL-22) and Th17 cells has been found in the skin (Gittler et al., 2012). In addition, the numbers of type 2 cytokine-producing $CD8^+$ T cells and ILC2s are also increased, though the precise role of these play is yet unknown (Hijnen et al., 2013; Salimi et al., 2013). Nevertheless, they take over the part of the recognition of tissue damage and initiation of inflammatory cascades (Tsakok et al., 2019). The initial Th2 inflammation results in the recruitment of additional immune cells such as eosinophils and mast cells. Subsequently, these contribute to pathological inflammation by liberating inflammatory mediators including histamine (Carrillo et al., 2017). Histamine, as well as IL-31, can stimulate directly or via release of pruritogens cutaneous somatosensory nerves (Sonkoly et al., 2006) and thereby contribute to the itch-scratch cycle (Mollanazar et al., 2016).

In chronic lesional skin, infiltration of Th1 and Th17 cells occurs, as defined by production of interferon-gamma (IFN-γ) (Th1) and/or IL-17 (Th17) (Suarez-Farinas et al., 2011). The role of Th1 and Th17 cell-mediated responses is still unclear, but it is postulated that the subset of activated Th1, Th2, and Th22 cells adopt a tissue-resident memory T cell phenotype in terms of tissue priming and limited capacity to recirculate out of the skin. In this fashion, the skin-resident lymphocyte may facilitate a rapid recall response to re-exposed antigens and contribute to the chronic AD state (Glatz et al., 2015). As mentioned before, the inflammatory response induces secondary mechanisms that contribute to the AD phenotype. For example, Th1, Th2 and Th22 cytokines downregulate genes of the epidermal differentiation complex (Gutowska-Owsiak et al., 2012; Gutowska-Owsiak et al., 2011; Kim, B. E. et al., 2008; Howell et al., 2007) and Th2 cytokines inhibit antimicrobial proteins (Kopfnagel et al., 2013).

AD is associated with systemic immune effects including IgE-mediated reactivity to food proteins, microbial antigens aeroallergens, and/or keratinocyte-derived auto-antigens (Sonesson et al., 2013; Tang et al., 2012; Virtanen et al., 1995). Interestingly, the severity of AD correlates with significant increases in serum levels of circulating IgE, both nonspecific and allergen-specific (Kumar et al., 2014; Wüthrich, 1978). IgE bind to skin-resident antigen-presenting cells, such as Langerhans cells, via their high affinity IgE receptor (FcεR1) and, in so doing, facilitate the uptake of allergens and initiate T cell-mediated type 4 (delayed type) hypersensitivity reactions (Novak, N., 2012). Furthermore, IgE can directly activate mast cells, which found in increased number in AD lesions (especially in chronic state). Although the role of mast cells is still not fully understood in AD (Irani et al., 1989; Soter, 1989), it is known that activated mast cells degranulate and cause acute inflammatory symptoms by releasing mediators such as histamine, prostaglandins,

serotonin, tryptase, heparin, and platelet-activating factor (Irani et al., 1989). Released histamine and tryptase elicit scratching behavior and secondary barrier disruptions. Additionally, histamine facilitates the migration of DCs to lymph nodes (Jawdat et al., 2004). Furthermore, mast cells directly regulate the recruitment and action of various inflammatory cells, T cells for example, by inducing expression of chemokines and adhesion molecules on endothelial cells. Mast cells themselves are a source of IL-4 and IL-13 (Obara et al., 2002; Horsmanheimo et al., 1994) and can be activated by overexpression of TSLP (Nagarkar et al., 2012).

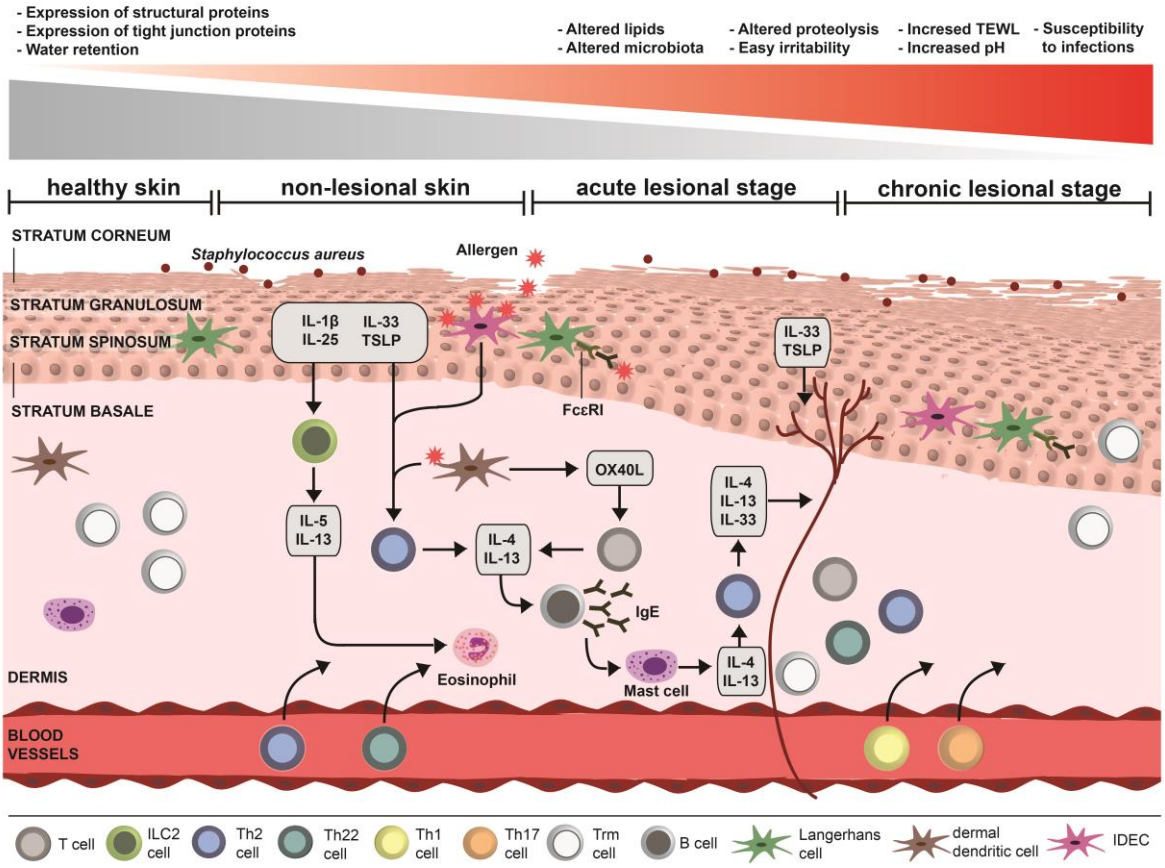

Figure 2. Pathogenesis of atopic dermatitis (AD). Following epidermal barrier disruption, keratinocytes express innate immune cytokines such as IL-1β, IL-25, IL-33 and thymic stromal lymphopoietin (TSLP). These alarmins activate skin-resident group 2 innate lymphoid cells (ILC2s) and type 2 T helper (Th2) cell-mediated immune responses. ILC2s release IL-5 and IL-13 leading to a further boost type 2 immunity and IgE production. The keratinocyte cytokine TSLP induces the OX40 ligand (OX40L) expression by dendritic cells. Subsequently, OX40L binds to OX40L receptor of naïve T cells and stimulates their release of IL-4, IL-5 and IL-13. Additionally, exogenous antigens and self-antigens, released by damaged cells, are taken up by dermal dendritic cells and dendritic epidermal cells (IDECs), which then promote type 2 immunity. Activated Th2 cells release IL-4 and IL-13, which promote a change in the immunoglobulin class produced by B cells to immunoglobulin E (IgE). Th2, Th22 cells, eosinophils, and mast cells are recruited to lesional skin. Antigen-primed T cells can remain as local pools of T resident memory (Trm) cells and are enable for a rapid recall

response. In contrast to healthy skin, AD skin has increased numbers of mast cells and dendritic cell subtypes, such as Langerhans cells, expressing high-affinity immunoglobulin-ε receptors (FcεR1s). IgE antibodies bound to mast cells by FcεR1 induces their degranulation. Furthermore, bound IgE to dendritic cells by FcεR1 can facilitate the uptake of allergens and initiate T cell-mediated type 4 (delayed type) hypersensitivity reactions. IL-33, TSLP and the downstream Th2 cytokines directly communicate with cutaneous sensory neurons to exacerbate pruritus. This mechanism highlights the dynamic interaction between barrier dysfunction, type 2 immunity and itch perception. While acute lesional skin are driven by Th2 and Th22 response, a mixed T cell population (Th2, Th22, Th1, Th17 cells) is present in chronic skin. Chronic lesions are characterized by a perpetuate skin inflammation, cutaneous remodeling and neuroinflammation.

1.2 3D *IN VITRO* SKIN EQUIVALENTS

The field of tissue engineering is closely connected to regenerative medicine and a main aspect in the field of alternatives to animal testing. Tissue engineering as alternative to animal testing is still in its early stages of development and mostly used in academic research or startup companies. Although, these startup companies are sprouting up and developing worldwide, large-scale industrial production has often not been achieved and regulatory acceptance is under heavy debate. Interestingly, however, the skin is unique in this regard, as described in more detail in the following chapter.

1.2.1 The History and Scope of Skin Equivalents

Traditionally, skin care products, cosmetics and other topical agents were tested using *in vivo* (animal) and *ex vivo* skin approaches (Holmes et al., 2017; Robinson et al., 2002). However, the 1986 EU Directive 86/609/EEC banned the use of animal experiments when scientifically validated alternatives exist (European-Commission, 1986). In the early 2000s, despite the fact, that there were no alternative to animal tests available, the EU's 7th amendment to the Cosmetics Directive was introduced and prohibited animal testing of finished products or cosmetic ingredients with the deadline of 2013 (Almeida et al., 2017; Holmes et al., 2017; Robinson et al., 2002). This amendment was a critical turning point and forced the cosmetic industry to find or develop equivalents to living skin that mimics the native physiological form and function of the human skin. Up to this point in time, *in vitro* studies were primarily conducted in two-dimensional cell culture systems in which cells were grown as a monolayer on flat surfaces like glass or polystyrene. But it was precisely due to this, that these models lacked important aspects, such as three-dimensional cell-cell-, cell-matrix- and cell-environmental-interactions. These are known to impact on cellular responses such as proliferation, differentiation, migration, biochemical signaling, gene transcription, and protein expression (Ali et al., 2015; Antoni et al., 2015; Bonnier et al.,

2015; Baker & Chen, 2012). Therefore, not all results from these two-dimensional are translatable to physiological *in vivo* systems (Langhans, 2018; Duval et al., 2017). This has given rise to the development of *in vitro* 3D skin equivalents. Although there were already patents existing for the generation of skin equivalents, the real impulse for their commercialization came from the cosmetic industry (Yu et al., 2019). There are now several commercially available reconstructed human epidermis (RHE) systems – including EpiDerm™ (Mattek Corp., USA), SkinEthic®, EPISKIN® (L`Oréal, France) – and several reconstructed full-thickness (FT) skin equivalents – such as EpiDerm-FT™ (Mattek Corp., USA) and Phenion® FT Skin Model (Henkel, Germany) – that have been the subject of several validation studies (Schäfer-Korting et al., 2008; Spielmann et al., 2007; Kandárová et al., 2006; Netzlaff et al., 2005). RHE models are now even partly integrated within certain OECD testing guidelines such as for skin irritation and corrosion testing (OECD, 2019a, 2019b). Additionally, FT skin equivalents have been used in the preclinical development and optimization of new drugs, and toxicological testing of drug formulations (Flaten et al., 2015). Beside their application in cosmetic and pharmaceutical industries, skin equivalents are increasingly used for preclinical and fundamental dermatological research dealing with skin absorption, skin physiology, and the study of skin diseases (Wallmeyer et al., 2017; Hönzke et al., 2016; van Smeden et al., 2014; Vávrová et al., 2014; Küchler et al., 2013; Küchler et al., 2011).

1.2.2 Generation of Skin Equivalents

There exists a wide spectrum of 3D *in vitro* skin equivalents and methods to generate these. They differ, primarily, with regard to their biological complexity. As above-mentioned, there are two main skin constructs available – RHE and FT skin equivalent. The RHE consists of the viable epidermis and the SC, but no dermal compartment. Therefore, keratinocytes are seeded on an inert polycarbonate scaffold/surface and cultivated submerged in growth media until the population matures. Afterwards, the keratinocytes are exposed to the air-liquid interface by removing the medium. Cultivation at the air-liquid interface and additives such as growth factors and calcium stimulate the keratinocytes to proliferate and differentiate into the epidermal layers (Bernstam et al., 1986). After a cultivation period of one to two weeks, a fully stratified epidermis is present. In close approximation to the *in vivo* situation, the engineered epidermis is composed of four epidermal strata: the stratum basale, stratum spinosum, stratum granulosum, and SC, running from deepest to most superficial layer (Frankart et al., 2012; Netzlaff et al., 2005; Boelsma et al., 2000; Boelsma et al., 1999; Rosdy & Clauss, 1990). The epidermal organization results from the maturation and differentiation of the mitotically active keratinocytes. CK5 and CK14 are typical makers for basal keratinocytes. When basal cells detach from the underlying basement membrane

they lose their mitotic potential, begin an outwards migration towards the skin surface, enter stratum spinosum, and switch from expressing CK5/14 to CK1/10 (Brohem et al., 2011; Poumay & Coquette, 2007). In the spinous layer, the cells strengthen their cytoskeletal and intercellular connections, and enter the granular layer. In the stratum granulosum, they produce the barrier precursor components consisting of glutamine- and lysine-rich cornified envelope precursor proteins including IVL, LOR, and profilaggrin, and lipid granules (Frankart et al., 2012). Following this, the cells enter the final phase of terminal differentiation and the SC is formed. Cells lose their nucleus, leaving completely differentiated 'dead' keratinocytes (corneocytes) interspersed with intercellular lipids, mainly ceramides and sphingolipids (Castiel-Higounenc et al., 2004). Overall, RHE exhibit a high reproducibility in terms of risk assessment and, as above-mentioned, are already validated for several routine tests such as skin irritation and corrosion testing (OECD, 2019a, 2019b; Kandárová et al., 2006).

FT skin equivalents are more complex. In addition to the epidermal part, these equivalents also contain a dermal compartment (Mathes et al., 2014). FT skin equivalents take cell-cell-interaction into account, better reproducing the morphological and physiological character of *in vivo* skin. There are different approaches to resemble the dermal compartment. One approach, which notably lacks cell-cell-interaction with fibroblasts, is the use of an acellular de-epidermized dermis (DED) (Tjabringa et al., 2008; Rezvani et al., 2007; Cario-André et al., 2002; Ponec, M. et al., 1988; Prunieras et al., 1983). To produce a DED, the epidermis is removed from skin pieces followed by a dermal sterilization using glycerol, ethylene oxide, alcohol or gamma irradiation. Afterwards, the keratinocytes are seeded on the epidermal side of the DED (Tjabringa et al., 2008; Rezvani et al., 2007; Cario-André et al., 2002; Ponec, M. et al., 1988; Prunieras et al., 1983). Cultivation at the air-liquid interface results in the formation of a stratified epidermis (Tjabringa et al., 2008; Rezvani et al., 2007; Cario-André et al., 2002; Ponec, M. et al., 1988; Prunieras et al., 1983). The interesting point about this method is that the complex basement membrane, which provides keratinocytes a good platform for growth, is retained. A major disadvantage, however, is the absence of fibroblasts and thus the cell-cell interaction. An advanced method compensating for this is the repopulation DED with fibroblasts (el-Ghalbzouri et al., 2002; Fleischmajer et al., 1993).

A more common method of dermal compartment formation is the embedding of fibroblasts into a scaffold formed of biocompatible materials. These materials include natural polymers such as collagen, fibronectin, glycosaminoglycans, and fibrin (Sahana & Rekha, 2018; Brougham et al., 2015), or synthetic polymers such as PEG (polyethylene glycol), PCL (polycaprolactone), and PLA (polylactic acid) (Chaudhari et al., 2016; Antoine et al., 2014). Because of their poor cell adhesive properties, synthetic polymers are regularly used in combination with natural polymers (Randall et al., 2018; Lotz et al., 2017). Yet, despite the

poor mechanical properties and high batch-to-batch variability, the natural polymer type I collagen is the most widely used biomaterial scaffold (Randall et al., 2018) and the one used for the generation of skin equivalents in this thesis (Fig. 3). One other noteworthy method to form a dermal compartment is through fibroblast-derived self-assembly. Here, fibroblasts are stimulated to synthesize their own extracellular matrix by cultivation with vitamin C (Hata & Senoo, 1989). Regardless of the chosen dermal compartment, the keratinocytes are seeded on the dermal equivalent and are cultivated at the air-liquid interface just like RHE models (Fig. 3). In comparison to the RHE, due to the presence of fibroblasts, FT skin equivalents show the following improvements: stimulation of keratinocytes proliferation, improved epidermal morphology, and enhanced formation of basal membrane proteins. All of these lead to a skin equivalent more closely mimicking the *in vivo* structure. (Wong, T. et al., 2007; el-Ghalbzouri et al., 2002; Ponec, Maria et al., 2002; Cooper et al., 1991).

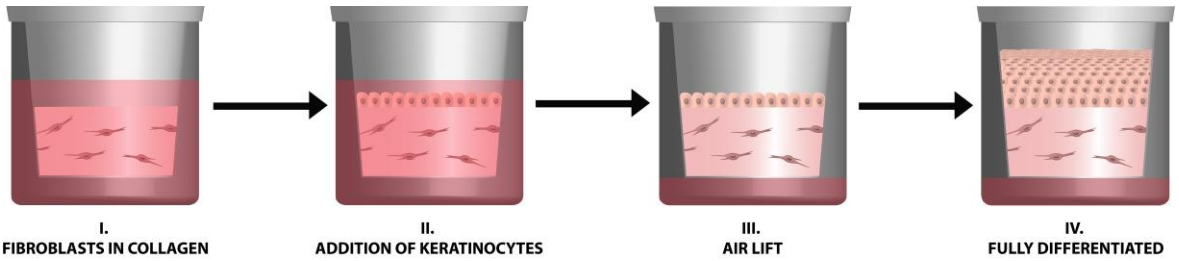

Figure 3. Generation of a full-thickness skin equivalent. Schematic illustration of the generation of a full-thickness skin equivalents used in this doctoral thesis, starting with the embedding of the fibroblasts in a collagen matrix. Subsequently, keratinocytes are seeded on top of the dermal equivalent. After 24 h, the skin equivalent is lifted to the air-liquid interface and medium is changed to a defined differentiation medium. After 14 days, a fully differentiated skin equivalent is generated and can be used for further investigations.

Over the last decade, huge advancements have been made in the field of engineered skin, not only in the generation of simple skin equivalents, but also in terms of the complexity. For instance, further cell types have been integrated, including immune cells such as T cells (Wallmeyer et al., 2017; van den Bogaard et al., 2014) and Langerhans cells (Kosten et al., 2015; Ouwehand et al., 2011). Other cell types include melanocytes (Choi et al., 2010; Gibbs et al., 2000) and endothelial cells (Khiao In et al., 2015; Shepherd, B. R. et al., 2006). Subcutaneous adipose tissue has also been integrated (Monfort et al., 2013; Bellas et al., 2012; Trottier et al., 2008). However, 3D models do not contain skin appendages such as hair follicles, and still show weaker barrier properties compared to *in vivo* skin (van den Broek et al., 2017). Moreover, these skin equivalents with higher complexity are mostly in-house developments used for purely fundamental research. Therefore, there is still an unmet need to develop more complex 3D skin equivalents for commercial applications.

1.2.3 Sources of Skin Cells

The basic unit for tissue engineering is the cell itself. For the generation of FT skin equivalents different possible cell sources exist. Some skin equivalents are based on the use of immortalized keratinocytes, for example the HaCaT cell line (Boelsma et al., 1999; Schoop et al., 1999), and Y-27632 (Rho kinase inhibitor)- (van den Bogaard et al., 2012), or telomerase reverse transcriptase (TERT)-immortalized keratinocytes (van Drongelen et al., 2014; van Drongelen et al., 2013; Vaughan et al., 2009; Dickson et al., 2000). In these cases, for the dermal counterpart dermal fibroblasts or immortalized mouse fibroblasts (3T3-J2) were used. Reijnders and colleagues developed the first FT skin equivalent using TERT-immortalized keratinocytes as well as TERT-immortalized fibroblasts (Reijnders et al., 2015). These equivalents are of great interest in industrial terms given the unrestricted proliferation and amplification capacity of these cells, the high reproducibility of the equivalents they generate, and the possibility to perform high-throughput screening of new drugs and drug formulations with them (van den Broek et al., 2017). However, most of these equivalents do not represent the native physiology of the human skin, since they show deficiencies in differentiation and stratification (Smits et al., 2017).

In recent years, evidence has emerged, that fibroblasts play an essential role in maintaining the structural integrity of several tissues by synthesizing structural proteins such as elastin and collagen and by integrating these in the extracellular matrix of connective tissue (Kalluri & Zeisberg, 2006; Parsonage et al., 2005; Tomasek et al., 2002). Since ‘typical’ fibroblasts markers, e.g. vimentin, are not expressed in all fibroblast types (Kalluri et al., 2006) and differences in gene expression (Rinn et al., 2008; Rinn et al., 2006), morphology and behavior of cultured non-dermal and dermal fibroblasts (Castor et al., 1962), and significant architectural differences in the dermides of different anatomical sites (Bayat et al., 2004) have now been reported, the most existing skin equivalents were generated using primary skin and fibroblasts and keratinocytes, which are mainly isolated from skin biopsies or juvenile foreskin after circumcision. Moreover, cutaneous fibroblasts display an important function in tissue homeostasis and support the proliferation of keratinocytes, as well as re-epithelization after wounding (Sorrell & Caplan, 2004). The importance of a complex crosstalk between fibroblasts and keratinocytes in terms of homeostasis, stratification, and deposition of the basement membrane and extracellular matrix constituents are also well documented (Wojtowicz et al., 2014; el-Ghalbzouri et al., 2002). These findings support the idea that fibroblasts are pivotal mediators of skin maintenance, either through secretion of soluble factors, or by matrix deposition. While full details of the mechanisms governing fibroblasts are not yet fully understood, it is already known that specific fibroblasts subtypes

play pathological roles in processes such as in cancer stroma, wound healing, and fibrosis (desJardins-Park et al., 2018; Lynch & Watt, 2018).

As mentioned before, skin equivalents are not only useful for cosmetic and drug testing, but also for studying skin diseases. Genetic disorders caused by a specific mutation can be easily studied in skin equivalents through simple approaches such as RNA interference (RNAi) that regulates the gene(s) of choice (Plank et al., 2019; Thomas et al., 2009). This approach is often commercially available and can be used in connection with normal human keratinocytes without the need to access clinical specimens. Another opportunity is the use of patient-derived cells, primarily obtained from patient skin biopsies. However, this invasive method can result in discomfort and may associate with scar formation, infections and impaired wound healing (Nischal et al., 2008). Hence, another method to obtain patient-derived cells would be an interesting solution to generate skin disease models with patient cells.

Interestingly, in terms of physicochemical traumas from the environment, the skin undergoes continual rejuvenation through homeostasis and wound repair in response to injury. For this, the skin relies on different stem cells residing not only in the interfollicular epidermis, but also in adult hair follicles (HFs) and sebaceous glands (Fuchs, 2008). The HF is easily obtainable without induction of major discomfort to the individual and can be sampled at any time. Before this aspect can be discussed in more detail, the anatomy as well the function of the HF must be understood, as described in the following chapter.

1.2.4 Hair Anatomy and Function

The skin consists of approximately five million HF with 100,000 of them on the scalp (Paus, R et al., 2008; Krause & Foitzik, 2006). Aside from representing an interesting stem cell niche, hairs exert various functional properties such as physical protection, thermoregulation, sensory activity, and social interaction (Schneider et al., 2009). In humans, three different types of hairs can be distinguished by their structure and localization: lanugo (fetal hairs), vellus (short body hair during childhood) and terminal hairs (e.g. scalp hair) (Duverger & Morasso, 2014).

The HF forms together with its associated structures: sebaceous, apocrine gland, and arrector pili muscle, the so-called pilosebaceous unit. Primarily, the HF serves as the production center of the hair shaft, a pigmented, multifunctional and extremely durable proteinaceous fibers (Schmidt-Ullrich & Paus, 2005). The HF can be divided into a permanent upper part that does not cycle visibly, and the lower part that is the actual ‘factory’ (Fig. 4A). The permanent part consists of the infundibulum, which is the opening of the hair canal to the skin surface, and the isthmus. The isthmus is the part from the insertion of the arrector pili muscle to the entrance of the sebaceous duct lower portion. Below the

isthmus is the so-called bulge region. In this site, a quiescent stem cell population of the follicle is located (Rompolas & Greco, 2014). Lineage tracing studies supported the idea of their potential to produce all cells required for hair regeneration and to repopulate the interfollicular epidermis following wounding (Cotsarelis, 2006; Ohyama et al., 2006). The bulge region is the end of the permanent, non-cycling area, separated from the lower part by a long stretch of suprabasal HF epithelium (Schneider et al., 2009). The lower part of the HF represents the anagen bulb, containing the hair matrix cells that give rise to the hair, the HF pigmentary unit (melanocytes) and the dermal papilla (DP) (Duverger et al., 2014). If cells from the bulge region become activated, they migrate downwards to the anagen bulb, colonize the matrix area, and start to proliferate rapidly. Their number as well as the volume and secretory activity of the DP determines hair bulb size, hair shaft diameter, and the duration of the growth phase of the HF (Krause et al., 2006; Paus, R. et al., 1999; Jahoda, C. A. & Reynolds, 1996). Additionally, the DP, the only mesoderm-derived part of the HF, is an essential source of growth factors (Buffoli et al., 2014; De Berker et al., 2004; Peus & Pittelkow, 1996). In the pre-cortical hair matrix, matrix cells stop proliferating and differentiate into the various cell lineages of the inner root sheath (IRS) and hair shaft to become, in the end, terminal differentiated trichocytes. Furthermore, these cells receive melanosomes from the melanocytes of the pigmentary unit. The other sheath of the HF, the so-called outer root sheath (ORS) is derived from separate progenitor cells (Cotsarelis, 2006; Legué & Nicolas, 2005).

A cross-section of a HF reveals a composition of at least eight concentric sheaths, each with specific functions as well as characteristics (Fig. 4B). Each layer is of a distinct lineage of epithelial differentiation, and display characteristic adhesion and matrix molecules, structural proteins such as hair keratins and trichohyalin, and enzyme activities (Langbein et al., 2001; Langbein et al., 1999; Powell & Rogers, 1997). This architecture ensures the stabilization of the growing hair and the guidance to the skin surface without penetrating the surrounding dermis. In the center is the outwards-moving hair shaft, which consists of three layers: cuticle (hair surface), cortex (hair bulk), and the medulla (Wolfram, 2003; Sperling, 1991). The hair cortex is the main structural component of the hair and is composed of densely packed, interdigitating, fully keratinized trichocytes. These cells stick together by proteinaceous material and contain pigment from the bulb melanocytes. The innermost medulla is characterized by the presence of cells that differentiate and are arranged into one or several columns, where follicular keratins are crosslinked with γ-glutamyl-ε-lysine peptide bonds (Lee, Y. J. et al., 2006). The surrounding layer of the hair shaft is the IRS that degenerates near the skin surface, thereby enabling the hair shaft to protrude on its own. The IRS consists of three compartments: the IRS cuticle, the Huxley's layer, and most peripherally the Henle's layer (Paus, R et al., 2008). In combination with the hair shaft, the

IRS moves outwards and uses the innermost layer of the following ORS layer, the so-called companion layer, an independent cell compartment as a slippage plane (Langbein et al., 2002; Stenn & Paus, 2001). The outermost non-keratinizing cell layer of the follicle is the ORS. This sheath is connected to the basal layer of the epidermis. The entire HF is surrounded by the connective tissue sheath (CTS), which consists of dermal sheath (DS) fibroblasts (Ohyama et al., 2006). The CTS maintains the dermal papilla at the base of the HF and is separated from the epithelial ORS by a specialized basement membrane, the so-called glassy membrane (Jahoda, C. A. B. & Reynolds, 2001).

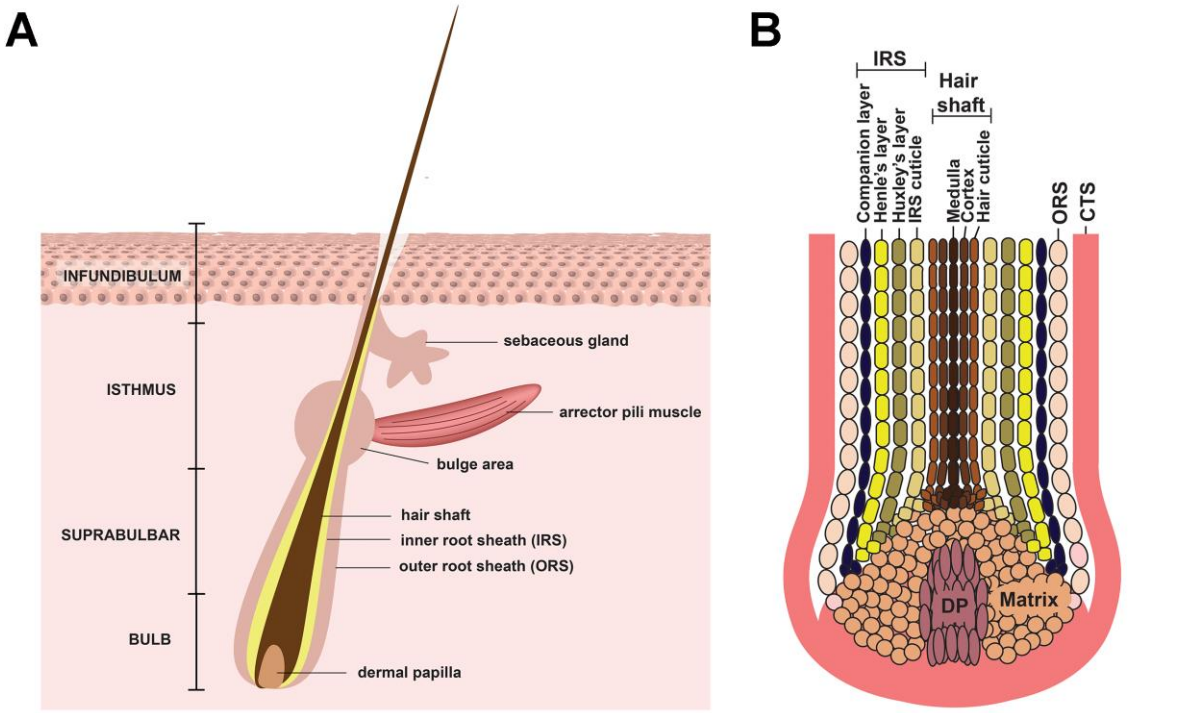

Figure 4. Microscopic anatomy of a hair follicle in the anagen phase. (**A**) Longitudinal section consisting of four segments: infundibulum, isthmus, suprabulbar, and bulb. (from top to bottom with respect to outer skin surface) with associated structures: sebaceous, apocrine gland, and arrector pili muscle. (**B**) Schematic drawing of a hair bulb cross-section illustrating concentric layers of the outer root sheath (ORS), inner root sheath (IRS) and hair shaft. The hair shaft consists of the medulla, cortex and hair cuticle, and is wrapped by a protective layer of overlapping scales. Further outwards, the hair shaft is surrounded and stabilized by the IRS, which is composed of four layers: the IRS cuticle, Huxley's layer, Henle's layer and the companion layer. The companion layer functions as a slippage plane between the stationary ORS and the upwards moving IRS, since the companion layer cells are tightly bound to Henle's layer. The entire hair follicle ORS is surrounded by a mesoderm-derived connective tissue sheath (CTS).

Overall, the HF is a fascinating mini-organ, and is the only organ to undergo continuous cycles of growth and retraction throughout life (Yi, 2017). This dynamic regeneration has three phases: anagen (growth), catagen (regression) and telogen (rest). Each phase is

regulated by distinct signals (Panteleyev et al., 2001; Stenn et al., 2001; Millar et al., 1999). In the anagen phase, stem cells from the bulge region differentiate in all cell lineages of the hair, which thereby induce hair elongation. The phase depends on the anatomic location of the hair – for example scalp hairs are 2-6 years in the growth phase, whereas the anagen phase for eyebrows last only three months (Cotsarelis, 2006; Krause et al., 2006). In the catagen phase, the lower compartment shortens and the distance between bulge and papilla becomes smaller due to apoptosis in the majority follicular cells (Lindner et al., 1997). The non-apoptotic cells in the upper ORS surround the reservoir and are responsible for the next anagen phase (Myung & Ito, 2012). At the end of this involution process, the HF enters the resting phase, the so-called telogen phase. Here, the proliferative and biochemical activity of the follicle reaches their lowest levels and the cells enter a period of quiescence waiting for the necessary signals to restart the anagen phase (Stenn et al., 2001; Paus, R. et al., 1990).

This life-long cyclic regression and regeneration activity is due to the presence of multipotent stem cells that have the capacity to develop multiple specialized cell types present in a specific tissue or organ and to self-renew by mitosis (Mistriotis & Andreadis, 2013). The HF constitutes an anatomically and molecularly well-defined stem cell niche (Rompolas et al., 2014). The broad differentiation capacity combined with the easy accessibility of the HF makes it an ideal stem cell source with high application potential for tissue engineering and regenerative medicine.

1.2.5 Plucked Hairs as Potential Cell Source

Hair follicles (HFs) present a perfect source for several cells including stem cells. To obtain an intact HF, an invasive method such as skin biopsy is necessary. Another, less invasive possibility to obtain cells from the HF is by plucking the corresponding hair. Interestingly, most of the epithelial structures from the HF, including the ORS, remain attached to plucked anagen hairs (Gho et al., 2004; Limat et al., 1991b). For the mesenchymal structures of an anagen hair bulb, different ‘break forms’ are described (Bassukas & Hornstein, 1989). These include the typical break conically surrounding the dermal papilla, rupture of the HF above the dermal papilla or around the upper third of the papilla, and total removal of the dermal papilla (Bassukas et al., 1989). Over the last decades, the possible usage of stem cells from plucked HF for the field of regenerative medicine has become more and more interesting. Various research groups have verified the attachment of the slow-cycling stem cells and demonstrated their presence in the central parts of the ORS close to and within the bulge region (Yamauchi & Kurosaka, 2010; Sasahara et al., 2009; Gho et al., 2004; Taylor et al., 2000; Lehrer et al., 1998; Michel et al., 1996; Moll, I., 1995; Cotsarelis et al., 1990). Another cell population with an enormous potential for regenerative medicine are

HF-derived keratinocytes, also known as bulge- or ORS-derived keratinocytes (Yoshikawa et al., 2013; Aasen & Izpisua Belmonte, 2010). These cells show a similar morphology and expression of keratin family genes before and after calcium-induced differentiation to those of interfollicular keratinocytes obtained from skin biopsies (Sasahara et al., 2009).
Furthermore, cultivated keratinocytes from plucked HF can be used for the generation of induced pluripotent stem cells (iPSCs) (Aasen et al., 2010; Aasen et al., 2008). Before, human iPSCs have been generated from various types of somatic cells, most commonly fibroblasts (Soldner et al., 2009; Huangfu et al., 2008; Lowry et al., 2008; Park, I. H. et al., 2008; Takahashi et al., 2007), isolated from tissue harvested via surgical intervention. This technique has facilitated the production of patient-specific cells for cell replacement therapy (Müller et al., 2009). The generation of iPSCs from plucked HF offers significant advantages over isolating skin fibroblasts through invasive surgical procedures. The reprogramming of keratinocytes-derived iPSCs from plucked HF was successfully achieved, generating cell types from inaccessible tissues such as functional cardiac myocytes (Novak, A. et al., 2010) and neural cells, including forebrain neurons (Petit et al., 2012). In addition to this potential, unmodified cells from plucked HFs have also become of great interest for regenerative medicine and basic research. For example, HF-derived keratinocytes can be used for the generation of fully differentiated epidermal equivalents (Tausche et al., 2003; Hoeller et al., 2001). Tausche and colleagues reported, in a randomized multicenter phase II study for Modex Therapeutics Ltd., that such autologous epidermal equivalents (called EpiDex^{TM}) were as effective as split thickness skin grafting, promoted healing and complete closure of recalcitrant vascular leg ulcers (Tausche et al., 2003). In another study, the potential of autologous *in vitro* reconstructed epidermal equivalents to heal one third of recurrent leg ulcers was shown (Limat & Hunziker, 2002). Furthermore, HF-derived keratinocytes have already been used for the generation of 3D FT skin equivalents (Guiraud et al., 2014; Hoeller et al., 2001). Because of their easily accessibility, the cells from plucked HF have already been used for study of skin diseases (Yoshikawa et al., 2013). For examples, Yoshikawa and colleagues performed microarray analysis and quantitative real-time PCR to generate gene expression signatures that can distinguish AD from healthy controls without skin biopsies (Yoshikawa et al., 2013).

1.3 *IN VITRO* DISEASE EQUIVALENTS OF ATOPIC DERMATITIS

Currently, the study of novel therapeutic approaches and pathophysiology for AD mostly rely on animal models, particularly mouse models (Martel et al., 2017). However, the high costs of drug development (Avci et al., 2013) and the ethical concerns of animal models (Martel et al., 2017; Törnqvist et al., 2014), as well as the fact that mice do not

spontaneously develop AD, have encouraged the use of *in vitro* models. Furthermore, murine and human skin differ in their anatomies and the genes associated with skin morphogenesis, growth and immunology (Gerber et al., 2014; Seok et al., 2013; Williams, F. M., 2006; Diembeck et al., 1999). Therefore, human-based 3D *in vitro* skin equivalents have gained increasing attention for the study etiological parameters of inflammatory cutaneous diseases such as AD. Human-based *in vitro* models constitute a perfect platform, since they can be easily adapted and mimic different characteristics of the AD etiology.

Today, a broad variety of human-based *in vitro* AD equivalents generated by use of varied protocols and cell sources are available. Detailed information to alternative methods to animal testing in basic and preclinical research of AD can be found in Löwa *et al.* 2018 as well as De Vuyst *et al.* 2017 (Löwa et al., 2018a; De Vuyst et al., 2017). This chapter will mainly focus on 3D *in vitro* skin equivalents of AD. Both RHE and FT skin equivalents, described in Chapter 1.2, are applied to create AD disease models. These *in vitro* equivalents can be altered to mimic epidermal AD conditions. This can be performed by silencing of genes involved in structure and development of the skin barrier, or by cultivation with interleukin cocktails overexpressed in AD. It can also be achieved through use of patient-derived cells in equivalent generation (Löwa et al., 2018a; De Vuyst et al., 2017).

Since FLG deficiency is often found in atopic skin (see Chapter 1.1.2), Mildner and colleagues developed the first FT *FLG* knockdown skin equivalent using siRNA (Mildner et al., 2010). They noted reduced barrier functions, an observation that was supported by the results from our group (Küchler et al., 2011). Moreover, our group observed inter- and intracellular spongiosis, and impaired epidermal differentiation and SC development (Wallmeyer et al., 2015; Küchler et al., 2011). Controversially, these last two observations were not observed by Mildner *et al.* possibly due to different cultivation periods of the skin equivalents. Additionally, our group also found alterations to skin acidification pathways, impaired lipid profiles. Unexpectedly, we also found a physiological skin surface pH in *FLG*-deficient equivalents revealing FLG does not play the crucial role in maintenance of skin surface pH (Vávrová et al., 2014).

As above-mentioned, another method to mimic AD *in vitro* is the cultivation of skin equivalents using interleukin cocktails. Kamsteeg and colleagues induced morphological features observed in lesional AD, such as spongiosis, by the cultivation of FT skin equivalents with the Th2 cytokines IL-4 and IL-13 (Kamsteeg et al., 2011). Another study from our group evaluated the combination of *FLG* knockdown and influence of the Th2 cytokines IL-4 and IL-13 in a FT skin equivalent (Hönzke et al., 2016). Histological changes such as epidermal thickness and significant increases in skin surface pH were observed. Additionally, a compensatory upregulation of the tight junction protein occludin (OCLN), and differentiation protein IVL occurred (Hönzke et al., 2016). Similar results were reported

following implementation of activated T cells in *FLG*-deficient equivalents. Interestingly, this study also demonstrated T cell migration in *FLG*-deficient equivalents but not normal equivalents, and identified the keratinocyte-derived cytokine TSLP as direct stimulator of T cell migration (Wallmeyer et al., 2017). Next to Th2 inflammatory processes, Gschwandtner and colleagues studied the influence of the mast cell-derived neurotransmitter histamine, which resulted in reduced expression of CK1, CK10, FLG, LOR, and tight junction proteins such as OCLN, CLDN1, and CLDN4, desmosomal junction proteins corneodesmosin and desmoglein-1. A loss of the granular layer was also seen, as well as thinning of epidermis and SC (Gschwandtner et al., 2013).

Although all the above approaches examined different variables that determine AD, only a few studies exist using patient-derived cells. One of these investigated how sensory nerve fibers and keratinocytes communicate with each other using FT skin equivalents grown from AD skin cells (Roggenkamp et al., 2013). For this, a collagen gel containing porcine dorsal root ganglia neurons was attached below the dermal equivalents. The authors observed epidermal thickening, increased neurite outgrowth into the dermal equivalent, and increased release of calcitonin gene-related peptide, which modulates cutaneous inflammation. These data indicate that the crosstalk of the cutaneous nerves and skin cells do indeed influence epidermal morphogenesis and extend the experimental options available to study the crosstalk between primary sensory neurons and skin cells *in vitro*.

Another study using patient-derived skin cells was conducted by Berroth and colleagues. This study investigated the role of fibroblasts derived from AD patients in the development of FT skin equivalents. Notably, impaired keratinocyte proliferation and terminal differentiation due to reduced expression of IVL, LOR and FLG were observed in skin equivalents grown from atopic fibroblasts and healthy keratinocytes. By contrast, skin equivalents using healthy fibroblasts and AD keratinocytes showed no epidermal alterations. Further analysis of AD fibroblasts showed a decreased expression of leukemia inhibitory factor (LIF). The effects of AD fibroblasts in the skin equivalent were improved by the addition of recombinant LIF. This was the first study indicating a potential role of the fibroblasts, and thereby LIF, in atopic skin (Berroth et al., 2013).

1.4 AIMS

Atopic dermatitis (AD) is the most common skin disease worldwide and its prevalence continues to increase in industrialized countries. However, the pathogenesis of AD is still not fully understood due to the complex interplay between barrier disruption and the cutaneous immune system. As already noted, many findings regarding the pathogenesis are derived from animal models, particularly mouse models. Even though murine and human skin exhibit numerous similarities, crucial differences exist, such as the number of epidermal layers and immune cell types present. Furthermore, mice do not spontaneously develop AD and the AD phenotype must, therefore, be induced experimentally. The translation of findings from experimental mice to humans remains questionable. As such, human-based *in vitro* skin equivalents seem to be a promising avenue for the study for central processes in the pathology of AD.

The overall goal of this thesis was to establish skin disease equivalents using AD patient-derived cells that would permit the study of underlying pathological mechanisms involved in AD. Typically, skin biopsies are taken to obtain patient-derived skin cells. However, this invasive method is associated with impaired wound healing, scar formation, and infections. Thus, the hair follicle as an alternative cell source and the use of hair follicle cells for the generation of skin equivalents was aimed. In the following, the aim was to investigate the impact of AD patient-derived fibroblasts on the skin equivalents and angiogenesis regarding clinical characteristics of atopic skin. Fibroblasts presumably contribute to pathogenic effects and a crosstalk between fibroblasts mast cells is well-known in inflammation, however the etiological role of this crosstalk in AD is still unknown. In order to support a possible continuation of the project, the isolation of skin mast cells, long-term cultivation and initial steps for the generation of a mast cell-competent skin equivalent were aimed.

2 MATERIALS & METHODS

This section lists those materials and methods that are not part of the publication (Löwa et al., 2018b) or submitted manuscripts.

Skin mast cell isolation via magnetic bead separation

Skin mast cells (MCs) were isolated from juvenile foreskins after circumcision surgeries (with ethical approval, EA1/081/13). Any adhering fat tissue from the underside of the skin was removed using scissors or scalpels, after which the skin was weighed under sterile conditions using a 50 ml tube (Sarstedt, Nürnbrecht, Germany). The skin pieces were then washed and cut in 3 mm^2 pieces using a scalpel. All pieces were placed in a tissue culture dish (Sarstedt, Nürnbrecht, Germany) and dispase type II solution (2.4 U/ml in phosphate buffered saline (PBS; Sigma-Aldrich, Munich, Germany); Roche Diagnostics, Mannheim) was added. The tissue culture dish was sealed with parafilm and incubated for 18-20 h overnight at 4 °C. The next day, the epidermis was carefully removed using forceps and could be used for keratinocyte isolation. The remaining pieces of dermis were washed twice in cold PBS (without magnesium and calcium; Sigma-Aldrich, Munich, Germany) and cut into small pieces. These were transferred into dispersing medium (10 ml per gram skin) consisting of PBS (with magnesium and calcium) supplemented with 1% penicillin/streptomycin (Sigma-Aldrich, Munich, Germany), 1% heat-inactivated fetal bovine serum (FBS Superior; Biochrom, Berlin, Germany), 5 mM $MgSO_4$ (Sigma-Aldrich, Munich, Germany), 10 µg/ml DNAse I (Roche, Prenzberg, Germany), 375 U/ml hyaluronidase (Sigma-Aldrich, Munich, Germany), 435 U/ml collagenase type II (Worthington, Lakewood, USA). The mix was placed in an incubator shaker (KS 4000 I control; IKA, Staufen, Germany) with a shaking frequency of 250-270 rpm at 37 °C. After 1-1.5 h, the solution was strained successively through a cell strainer with 100 µm pores and then another with 70 µm pores. The filtrate was centrifuged at 400 x g for 15 min at 4 °C. If the dermis was still not fully dispersed, the dispersing medium could be reapplied for a second cycle of the above protocol. The resulting cell pellet was resuspended with sterile, cold PBS (without magnesium and calcium) and centrifuged at 300 x g for 10 min at 4 °C (Centrifuge 5810R; Eppendorf, Wesseling-Berzdorf, Germany). Human CD117 antigen expressing cells, also known as MCs, were purified from the other dermal cells by positive selection using magnetic-activated cell sorting beads according to the manufacturer's instructions (MACS; Miltenyi Biotec, Bergisch-Gladbach, Germany). Briefly, 100 µl of FcR blocking reagent and 100 µl of CD117 MicroBeads were added. The mixture was carefully mixed and incubated in the dark at 4 °C for 15 min. Afterwards cells were washed by adding 2 ml MACS buffer (PBS supplemented with 0.5% bovine serum albumin (BSA; Carl Roth, Karlsruhe, Germany) and 2 mM ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich, Munich, Germany)). The cell suspension was centrifuged at 300 x g for 10 min at 4 °C. The buffer

was poured off, and the pellet loosened and resuspended in 500 µl MACS buffer. Meanwhile, MACS columns (large columns LS) were prepared. Here, a column was inserted in the appropriate magnet (MACS Separator; Miltenyi Biotec, Bergisch-Gladbach, Germany) and rinsed with 3 ml MACS buffer. Afterwards, the cell suspension (500 µl) was applied onto the column. The flow-through containing the unlabeled cells (negative selection) were collected. After three washing steps of column, each with 3 ml MACS buffer, the column was removed from the magnet, placed above a 15 ml tube (Sarstedt, Nürnbrecht, Germany), and 5 ml of MACS buffer was added. The magnetically labeled cells were then immediately flushed out by firmly pushing the plunger into the column. MCs were counted using a Neubauer counting chamber (Zeiss, Jena, Germany) and the cell suspension was centrifuged at 300 x g for 10 min at 4 °C. Buffer was poured off and the cells were resuspended at 1 x 10^6 cells/ml in MC medium consisting of Iscove's medium (Biochrom, Berlin, Germany) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Biochrom, Berlin, Germany), 50 U/ml penicillin, 50 µg/ml streptomycin (Sigma-Aldrich, Munich, Germany), 1x non-essential amino acid solution (Thermo Scientific, Waltham, USA) and 226.4 µM alpha-monothioglycerol (Sigma-Aldrich, Munich, Germany).

Long-term cultivation of skin mast cells

For long-term cultivation of MCs, double the volume of media additionally supplemented with 20 ng/ml human IL-4 and 100 ng/ml human stem cell factor (SCF) (both from PreproTech, Hamburg, Germany) were added to the culture media. After two days, cells were cultured at a cell density of 5 x 10^5 cells/ml with MC medium supplemented with SCF and IL-4. Medium changes were performed twice per week using medium 10 ng/ml human IL-4 and 50 ng/ml human SCF. Since the cell yield is small (approximately 1 x 10^5 cells per foreskin) and the proliferation of MCs begins around cultivation week two, the cells were used in week six.

Generation of mast cell-competent skin equivalent

Primary human keratinocytes and fibroblasts were isolated from juvenile foreskin after circumcision (with ethical approval, EA1/081/13) using standard procedure. Fibroblasts were cultivated in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich, Munich, Germany) supplemented with 10% FBS Superior (Biochrom, Berlin, Germany) and 2 mM L-glutamine (Sigma-Aldrich, Munich, Germany). Keratinocytes were cultivated in EpiLife with 60 µM calcium (Fischer Scientific, Schwerte, Germany). For *FLG* knockdown, keratinocytes were transfected with a mixture of HiPerFect® transfection agent and 50 nM target siRNA (Stealth RNAi™, siRNA ID: HSS177192, sequence: 5'-CAGCUCCAGACAAUCAGGCACUCAU-3'; Life Technologies, Darmstadt, Germany)

according to manufacturer's instruction 24 h before skin equivalent generation. Normal (*FLG*+) and filaggrin-deficient (*FLG*-) skin equivalents were generated according to previously published procedures (Wallmeyer et al., 2017; Hönzke et al., 2016; Wallmeyer et al., 2015). Briefly, primary human fibroblasts, heat-inactivated FBS Superior (Biochrom, Berlin, Germany) and bovine collagen I (PureCol, Advanced BioMatrix, San Diego, CA, USA) were brought to neutral pH. For MC-competent skin equivalents, MCs were added at 40 or 80 MC/mm^2 in addition to the fibroblasts. The mixture was poured into 12-well cell culture inserts (BD Biosciences, Heidelberg, Germany), incubated for 2 h at 37 °C followed by addition of EpiLife and another 2 h incubation at 37 °C and 5% CO_2 (CO_2 incubator HERAcell 240i; Heraeus, Hanau, Germany). Afterwards, normal or *FLG* knockdown keratinocytes, were seeded on the collagen matrix. After 24 h, the skin equivalents were lifted to the air-liquid interface and a differentiation medium was added. Media changes were performed every second day and skin equivalents were cultivated for 14 days in total.

Filaggrin (*FLG*) knockdown efficiency testing

A successful *FLG* knockdown was verified at day 4. Therefore, the epidermides of skin equivalents were gently peeled off the dermis and milled for 30 s at 25 Hz using a TissueLyzer (Qiagen, Hilden, Germany). Subsequently, RNA was isolated using an InnuPREP RNA Mini Kit (Analytik Jena, Jena, Germany) according to the manufacturer's instructions. For cDNA synthesis, the iScript cDNA Kit (Bio-Rad, Munich, Germany) was used following the manufacturer's instructions. Subsequently, qPCR was performed using the iTaq™ Universal SYBR® Green Supermix Kit (Bio-Rad, Munich, Germany), the sense and antisense primer for *FLG* (Table 2.1). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) served as the housekeeping gene (Table 2.1). All primers were acquired from the company TIB MolBiol (Berlin, Germany). The efficiency of *FLG* knockdown in skin equivalents was 89.42 ± 3.82% (n = 3).

Quantitative real-time polymerase chain reaction (qPCR)

Quantitative real-time PCR was performed as described in Chapter 3.1 and 3.2. The primer sequences used for the qPCR is listed in Table 2.1.

Table 2.1. Primer sequences for qPCR.

Gene	Sense primer (5' – 3')	Antisense primer (5' – 3')
AHR	CAAATCCTTCCAAgCggCATA	CgCTgAgCCTAAgAACTgAAAg
CLDN1	gCgCgATATTTCTTCTTgCAgg	TTCgTACCTggCATTgACTgg
FLG	AAggAACTTCTggAAAAggAATTTC	TTgTggTCTATATCCAAgTgATCCAT
GAPDH	CTCTCTgCTCCTCCTgTTCgAC	TgAgCgATgTggCTCggCT
GATA3	gAACCggCCCCTCATTAAg	ATTTTTCggTTTCTggTCTggAT

IL1B	TggAgCAACAAgTggTgT	TTgggATCTACACTCTCCAgC
IL6	CACAgACAgCCACTCACCTC	TTTTCTgCCAgTgCCTCTTT
IL8	CAAgAgCCAggAAgAAACCA	gTCCACTCTCAATCACTCTCAg
IVL	TCCTCCAgTCAATACCCATCAg	CAgCAgTCATgTgCTTTTCCT
KLK5	AgTCAgAAAggTgCgAggA	TAATCTCCCCAggACACgAg
LOR	TCATgATgCTACCCgAggTTTg	CAgAACTAgATgCAGCCggAgA
OCLN	TgCATgTTCgACCAATgC	AAgCCACTTCCTCCATAAgg
RORC	CAATggAAgTggTgCTggTTAg	gggAgTgggAgAAgTCAAAgAT
TBX21	TTgAggTgAACgACggAgAg	CCAAggAATTgACAgTTgggT
TNFA	CCCAgggACCTCTCTCTAATCA	gCTACAggCTTgTCACTCgg
TSLP	CCCAggCTATTCggAAACTCAg	CgCCACAATCCTTgTAATTgTg

Cryopreservation and preparation of cryosection of skin equivalents

For the histological evaluation of the skin equivalent, the insert membrane, including the skin construct was cut out of the insert using a scalpel. Together with the insert membrane the skin equivalent was transferred into disposable embedding molds (Sigma-Aldrich, Munich, Germany) filled with tissue freezing medium (Leica Biosystems, Nussloch, Germany) using forceps and then completely covered with tissue freezing medium. Air bubbles were avoided. The filled embedding mold was then held to the phase boundary of liquid nitrogen for shock freezing, resulting in a homogeneous cryobloc. The cryoblocs were stored at -80°C (HERAfreeze^{TM} HFU T Serie; Thermo Scientific, Schwerte, Germany). Prior to cryosection preparation, the respective cryoblocs were prewarmed to the cutting temperature of -18 °C to -21 °C in the cryotome (Leica CM 1510s; Leica Microsystems, Wetzlar, Germany). The cryosections (5 µm) were then prepared according to the manufacturer's specifications and were finally transferred to poly-L-lysine coated slides (Gerhard Menzel, Braunschweig, Germany). Subsequently, the sections were dried at room temperature for at least 30 min and further investigated as described below.

Histological analyses of skin equivalents

Hematoxylin and eosin (H&E) staining was used to examine the cellular and tissue structure of the skin sections. Before staining, skin sections fixed with 4% formaldehyde (Carl Roth, Karlsruhe, Germany). Subsequently, H&E staining (Carl Roth, Karlsruhe, Germany) was performed according to the technical procedure shown in Table 2.2.

Table 2.2. Procedure of H&E staining.

Solution	Application time [min]
Aqua bidest.	0.5
Hematoxylin solution (according Mayer)	5
Tap water	5
Eosin G solution	0.5

Ethanol 96% (v/v) I	2
Ethanol 96% (v/v) II	2
Ethanol 100% (v/v) I	2
Ethanol 100% (v/v) II	2
Roti®-Histol I	2
Roti®-Histol II	2

Room temperature dried cryosections were mounted with Roti®-Histokitt (Carl Roth, Karlsruhe, Germany) and covered with a thin cover glass. The slides with the skin sections were stored at 4 °C and analyzed by microscopy (BZ-8000; Keyence, Neu-Isenburg, Germany).

Immunofluorescence and western blot analyzes

Immunofluorescence staining and western blot analysis were performed as described in Chapter 3.1 and 3.2. Additional primary antibodies used for immunofluorescence staining and western blot analyses are presented in Table 2.3.

Table 2.3. Primary antibodies for immunofluorescence (IF) and/or western blot (WB).

Antibody	Isotype	Clone	IF	WB	Company
ß-actin	mouse IgG1	monoclonal (15G5A11/E2)	–	1:10,000	Sigma-Aldrich, Munich, Germany
ß-tubulin	rabbit IgG	monoclonal (9F3)	–	1:1,000	Cell Signaling Technology, Danvers, United States of America
CLDN1	mouse IgG2	monoclonal (1C5-D9)	1:300	1:500	Novus Biologicals Cambridge, United Kingdom
FLG	mouse IgG	polyclonal	1:1,000	1:1,000	BioLegend, San Diego, United States of America
GAPDH	rabbit IgG	monoclonal (14C10)	–	1:1,000	Cell Signaling Technology, Danvers, United States of America
IVL	rabbit IgG	polyclonal	1:1,000	1:1,000	Abcam, Cambridge, United Kingdom
Ki67	mouse IgG	monoclonal (Ki-67P)	1:200	–	Dianova, Hamburg, Germany
LOR	rabbit IgG	polyclonal	1:500	1:1,000	Life Technologies, Darmstadt, Germany
MC tryptase	mouse IgG1	monoclonal (AA1)	1:50	–	BioLegend, San Diego, United States of America
OCLN	mouse IgG1	monoclonal (OC-3F10)	1:300	1:500	ThermoFisher, Cambridge, United Kingdom

Skin absorption testing

Skin permeability studies were performed according to validated test procedures as described in Chapter 3.1.

For Lucifer yellow permeability assays, 4 µl Lucifer yellow (1 mM, Sigma-Aldrich, Munich, Germany) was added on top of the skin equivalents (day 14) and incubated at 37 °C for 2 hours. Afterwards, the skin equivalents were embedded in tissue freezing medium (Leica

Biosystems, Nussloch, Germany) and shock-frozen using liquid nitrogen. Sections (5 µm) were fixed with 4% formaldehyde, washed with PBS and embedded in 4',6-diamidin-2-phenylindol (DAPI) antifading mounting medium (Dianova, Hamburg, Germany). Skin sections were inspected using fluorescence microscopy (BZ-8000; Keyence, Neu-Isenburg, Germany).

Sample preparation for lipid analyses

The skin equivalents were placed on a filter paper (Schleicher&Schüll, Dassel, Germany) soaked with 0.25% trypsin in PBS (Sigma-Aldrich, Munich, Germany) and incubated for 4 h at 37 °C (CO_2 incubator HERAcell 240i; Heraeus, Hanau, Germany). After incubation, stratum corneum (SC) was carefully removed with forceps and placed in a 1.5 ml tube. The isolated SC sheets were washed with sterile water (Sigma-Aldrich, Munich, Germany) to remove any remaining keratinocytes. Subsequently, SC sheets were vacuum-dried using vacuum concentrator (Savant SpeedVac Plus; Thermo Scientific, Schwerte, Germany), subsequently perfused with nitrogen to avoid oxidative processes, and stored at 20 °C.

Lipid analyses

The lipid analyses including isolation of SC lipids, infrared spectroscopy and high-performance thin layer chromatography (HPTLC) were performed by our cooperation partner the lab of Prof. Dr. Kateřina Vávrová (Faculty of Pharmacy in Hradec Kralove, Charles University, Czech Republic). The organization of the skin lipids was determined by infrared spectroscopy. Skin lipid profiles were determined by high performance thin layer chromatography (Wallmeyer et al., 2015; Vávrová et al., 2014). Due to the completeness of this thesis, however, the methods will be described in the following.

Chemicals

Ceramides (Cer) NS, AS, NP, AP, glucosylceramide, and sphingomyelin were purchased from Avanti Polar Lipids (Alabaster, AL, USA); Cer EOS, Cer EOP and Cer NH were prepared according to published procedures (Kováčik et al., 2016; Opálka et al., 2015). Cholesterol, lignoceric acid, L-α-phosphatidylcholine, and solvents were from Merck (Darmstadt, Germany). Silica gel 60 HPTLC plates (20 × 10 cm) were obtained from Merck (Darmstadt, Germany).

Isolation of SC lipids

For the extraction of the SC intercellular lipids, a modified method was used (Bligh & Dyer, 1959). SC samples were extracted with 1 ml $CHCl_3$/MeOH 2:1 (v/v) per mg of SC for 2 h, and then with additional 0.5 ml $CHCl_3$/MeOH 2:1 (v/v) per mg of SC for 1 h. Filtered organic solutions were combined and concentrated under a stream of nitrogen. The lipids were dried under reduced pressure and stored at -20 °C under argon.

Infrared spectroscopy

IR spectra of the samples were collected on a Nicolet 6700 FTIR spectrometer (Thermo Scientific) equipped with a single-reflection MIRacle attenuated total reflectance (ATR) germanium crystal at 23 °C. The spectra were generated by coaddition of 256 scans collected at 2 cm^{-1} resolution and analyzed with the Bruker OPUS software (Bruker Corp, Billerica, MA). The exact peak positions were determined from second derivative spectra.

High-performance thin layer chromatography (HPTLC) lipid analysis

The lipid analysis was performed on silica gel 60 HPTLC plates (20 × 10 cm^2, Merck, Darmstadt, Germany) as previously described (Wallmeyer et al., 2015; Vávrová et al., 2014). To generate calibration curves, the lipids were mixed in ratios that approximately correspond to the composition of human SC (Pullmannová et al., 2014). Standard lipid mixtures (at least eight calibration points for each lipid, for concentrations, see Table 2.4) were analyzed along with the samples on the same HPTLC plate. Lipids were applied on a HPTLC plate under stream of nitrogen using Linomat IV (Camag, Muttenz, Switzerland).

Table 2.4. Calibration curve range of lipid standards used for HPTLC analysis.

Lipid standard	Calibration curve range [µg]
Cholesterol	0.5 – 10
Lignoceric acid	0.4 – 8
Ceramide EOS	0.03 – 0.6
Ceramide NS	0.2 – 4
Ceramide EOP	0.04 – 0.8
Ceramide NP	0.1 – 2
Ceramide AS	0.08 – 1.6
Ceramide AP	0.08 – 1.6
Cholesterol sulfate	0.1 – 2.5
Glucosylceramide	0.2 – 5
Sphingomyelin	0.2 – 5
Phospholipid	0.5 – 12.5

The plates were developed in an automatic developing chamber ADC 2 (Camag, Muttenz, Switzerland) with controlled humidity (33 – 36% RH) and temperature (25 – 27 °C). To separate the main barrier lipids (Chol, FFA and Cer), the plate was developed first to 85 mm and then to 60 mm with a $CHCl_3$/MeOH/acetic acid (190:9:1.5 v/v/v) mobile phase. To separate more polar lipids (GlcCer, CholS, PL, SM), the plate was developed once to the 85 mm using a $CHCl_3$/MeOH/acetic acid/H_2O (66:25:6:3 v/v/v/v) mobile phase. The lipids were visualized by dipping in a derivatization reagent (7.5% $CuSO_4$, 8% H_3PO_4, and 10% MeOH in water) for 10 s, and heating at 160 °C for 20 min. The lipids were then quantified by densitometry using TLC scanner 3 and VisionCats software (Camag, Muttenz, Switzerland).

Cell isolation from the dermal compartment of skin equivalents

To examine the number of MCs that still expressed MC markers, mast cells as well as fibroblasts from the dermal equivalent were gently isolated. The epidermis was removed, and the dermis was washed twice in cold PBS (without magnesium and calcium). Subsequently, the dermis was cut into small pieces. These were transferred in Iscove's medium (Biochrom, Berlin, Germany) supplemented with 1 mg/ml collagenase type I (Worthington, Lakewood, USA) and incubated in an incubator shaker with a shaking frequency of 160 rpm at 37 °C. After 20-30 min, the solution was strained through a 70 µm cell strainer. The filtrate was centrifuged at 300 x g for 10 min at 4 °C. The cell pellet was resuspended in 20 ml sterile, cold PBS (without magnesium and calcium) and centrifuged at 4 °C for 10 min at 300 x g. Afterwards, the cell pellet was resuspended in cold FACS buffer (PBS without magnesium and calcium supplemented with 0.5% BSA and 2 mM EDTA) and used for flow cytometric analysis.

Flow cytometry analyses

Purity and activation status of the isolated MCs and the expression level of the two surface markers FcεRI and CD117 (c-Kit) on MCs was assessed by flow cytometry. At least 100,000 cells were resuspended in 100 µl FACS buffer. 10 µl of the monoclonal antibodies PE anti-human CD117 (clone: 104D2) or PE anti-human FcεRI (clone: AER-37(CRA-1)) or corresponding isotype control (all from BioLegend, London, United Kingdom) were added and incubated for 10 min in the refrigerator at 4 °C. Afterwards, 2 ml of PBS was added, and the cell suspension centrifuged at 300 x g for 10 min at 4 °C. The cell pellet was resuspended in 100 µl FACS buffer and directly used for flow cytometric analysis. A total of 20 x 10^3 events were counted and examined using CytoFLEX research flow cytometer (Beckman Coulter, Indianapolis, USA). Debris and dead cells were excluded by population gating within forward scatter by side scatter plots.

Enzyme-linked immunosorbent assay (ELISA)

The culture medium from the skin equivalents was collected and the release of IL-4, IL-6 and IL-8 in the culture medium were quantified using ELISA Ready-Set-Go kits (eBioscience, Hatfield, United Kingdom). Furthermore, histamine was measured in the culture medium using Histamine ELISA Kit (Enzo Life Science, Lausen, Germany).

Statistical analyses

The unpaired student's t-test was used for direct comparisons of two independent groups. For comparisons of more than two independent groups, one-way analysis of variance followed by Bonferroni's correction for multiple comparisons was performed using GraphPad Prism 8.0 (GraphPad Software Inc., La Jolla, CA). Asterisks (*) indicate statistical

significance over skin equivalents using normal fibroblasts (Part 2) or normal (*FLG*+) skin equivalents without MCs (Part 3), plus (+) signs indicate statistical significance over skin equivalents using AD fibroblasts (Part 2) or *FLG* knockdown (*FLG*-) skin equivalents without MCs (Part 3). $p \leq 0.05$ was considered statistically significant. Data from at least three independent experiments are presented as means ± standard error of the mean (SEM).

3 RESULTS

3.1 PART 1

—

Generation of full-thickness skin equivalents using hair follicle-derived human keratinocytes and fibroblasts

Human skin equivalents are increasingly used as an alternative to animal experiments. Currently, most of the established *in vitro* skin equivalents are grown from primary keratinocytes and fibroblasts that were either isolated from excised human skin or from juvenile foreskin following circumcision. In recent years, the human hair follicle has received increasing attention in the field of regenerative medicine, due to its stem cell reservoir (Rompolas et al., 2014; Mistriotis et al., 2013; Zhang et al., 2013). Current diagnostic and clinical applications of cells derived from plucked hair follicles include generation of three-dimensional (Nakano et al., 2016; Guiraud et al., 2014; Hoeller et al., 2001) and/or autologous epidermal equivalents (Limat et al., 2002). Notably, the plucking hairs is relatively painless and compared to invasive methods such as skin biopsies, which makes this method interesting for the isolation of patient cells. Hence, the following publication presents a novel method to isolate and cultivate keratinocytes and fibroblasts from plucked hair follicles that are then successfully used in the generation of skin equivalents.

3.1.1 Publication

This is the peer reviewed version of the following article:

Anna Löwa, Annika Vogt, Sabine Kaessmeyer, Sarah Hedtrich**.** Generation of full-thickness skin equivalents using hair follicle-derived human keratinocytes and fibroblasts. *J Tissue Eng Regen Med*. 12 (4), 2134-2146 (2018).

, which has been published in final form at https://doi.org/10.1002/term.2646. This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Use of Self-Archived Versions.

Personal contribution:

Planning, design, and conducting of isolation and cultivation of hair follicle-derived cells, generation of skin equivalents, histological investigations including immunofluorescence staining, protein quantification by western blot, gene analysis using quantitative real-time PCR, and skin absorption testing with subsequent data evaluation. Manuscript design and preparation under 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. Sabine Kaessmeyer performed ultrastructural analyses.

3.1.2 Supplementary Data

Occurrence of hair bulb 'break forms' following the plucking of hair follicles

Before outgrowth of hair follicle-derived cells, the plucked hair follicles were examined for their growth phase using a phase contrast microscope. Hair follicles with a clearly visible ORS were used for outgrowth (Fig. 1.1a). Furthermore, different plucking effects were visible on the bulbs. Typically, a break at the mid-level of the dermal papilla was observed (Fig. 1.1a). Breaks at the level of the glassy membrane and subsequently intact dermal papilla were rarely been observed (Fig. 1.1b).

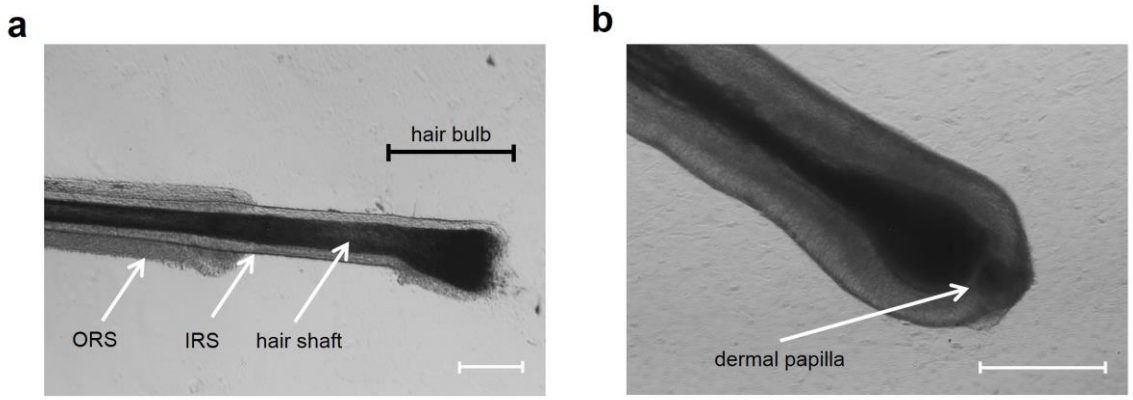

Figure 1.1. Microscopically visible structures of plucked hair follicles in the anagen growth phase. (**a**) Representative light microscopic image of a plucked hair follicle showing clearly distinguishable structures of outer root sheath (ORS), inner root sheath (IRS), hair shaft, and hair bulb. (**b**) In some cases, the dermal papilla was still intact after plucking procedure. Scale bar = 200 µm. Modified from (Löwa, 2015b).

Hair follicle-derived keratinocytes (HFDK) from AD patients already show differentiation characteristics in passage 2

For healthy donors, ~0.9 to 2.5 x 10^6 cells in passage 0 can be obtained following outgrowth plucked hair follicles (n = 10). During outgrowth cultivation, follicle-derived cells from healthy donors grew into a compactly arranged monolayer of small-sized cells (Fig. 1.1aI). In passage 1, HFDK are less compactly arranged with numerous cells of larger size (Fig. 1.1aII). This effect becomes even more apparent when HFDK are cultured in keratinocytes-specific medium in the absence of feeder cells (Fig. 1.1aIII). Although similar cell numbers (~0.6 to 2.2 x 10^6 cells) and cell appearances were observed during outgrowth of AD patient-derived cells (Fig. 1.1bI), AD HFDK were less compactly arranged and formed a variety of larger cells at passage 1 on feeder cells (Fig. 1.1bII) compared to healthy HFDK (Fig. 1.1aII). Furthermore, HFDK from AD patients also lose their proliferative capacity *in vitro* after passage 0 regardless of cultivation with or without feeder cells, as indicated by

the large flatten differentiated cells and low cell yield (Fig. 1.1bII, III). This effect was even more apparent if the AD HFDK were cultivated in keratinocyte-specific medium (Fig. 1.1bIII).

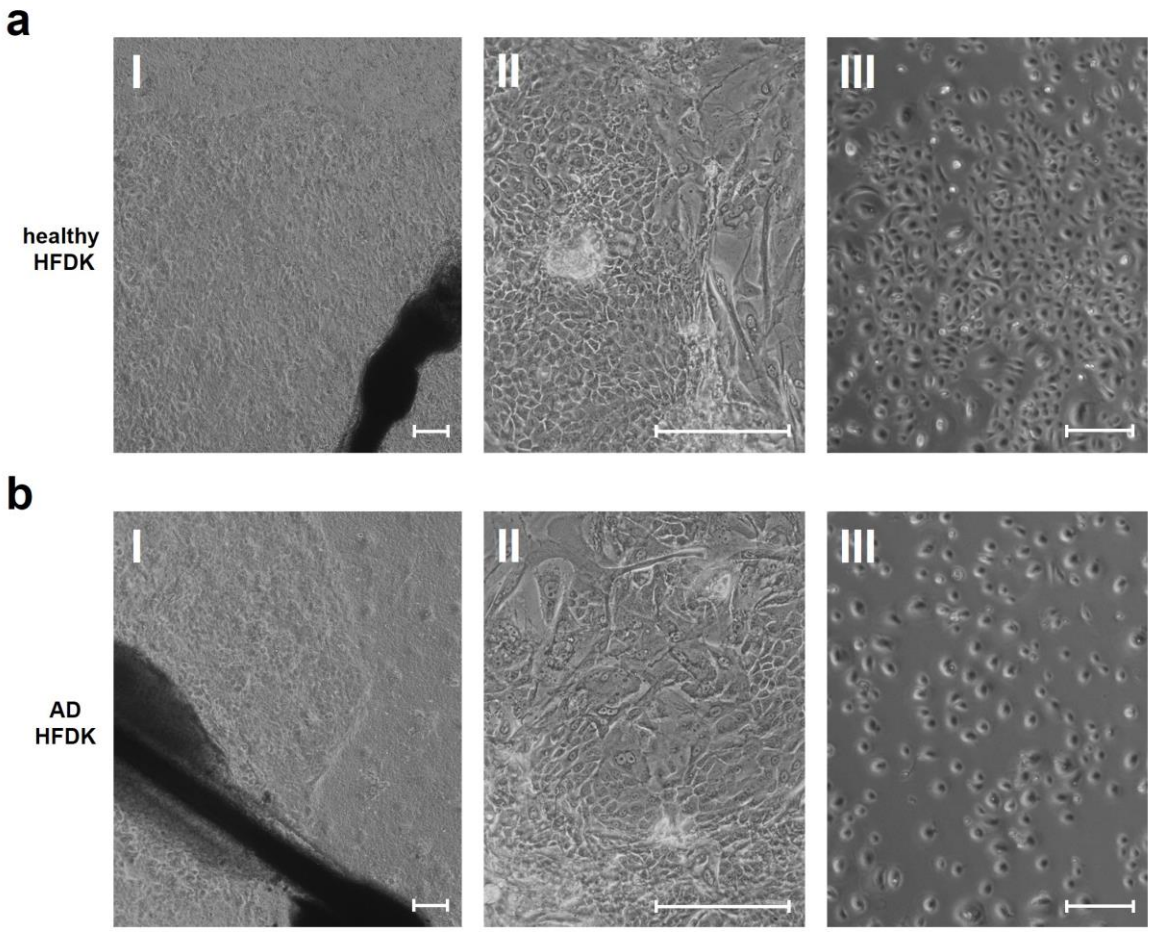

Figure 1.2. Microscopic appearance of hair follicle-derived keratinocytes (HFDK) from healthy donors and atopic dermatitis (AD) patients. (**a**, **b**) Representative light microscopic images of HFDK from (**a**) healthy donors and (**b**) AD patients during (**I**) outgrowth (passage 0), (**II**) co-cultivation with feeder cells (passage 1, day 5), and (**III**) cultivation in keratinocyte-specific medium (passage 1, day 5). Scale bar = 200 µm. n = 4.

3.2 PART 2

—

Fibroblasts from atopic dermatitis patients impair the epidermal homeostasis of skin equivalents

Atopic dermatitis (AD) is the most common inflammatory skin disease in industrialized countries (Weidinger et al., 2018). However, its underlying pathomechanism is still not fully understood. AD is a highly heterogenic disease characterized by misdirected immune reactions and impaired skin barrier function, the complexity of which has hampered our understanding of the disease (Tsakok et al., 2019). Over the past years, the impact of fibroblasts for disease induction and/or maintenance has been increasingly recognized (Gardiner et al., 2018; Kühbacher et al., 2017; Teves et al., 2017). However, little is known about their role in the etiology of AD. The use of human-based *in vitro* skin equivalents grown from patient-derived fibroblasts, which can be isolated from plucked hair follicles by the established less invasive method from Chapter 3.1, is a promising alternative approach to investigate pathogenic parameters in AD.

Hence, we investigated the impact of AD patient-derived fibroblasts isolated from plucked hair follicles on the tissue homeostasis of human-based skin equivalents and their angiogenic effect.

3.2.1 Manuscript

The following manuscript has been submitted to the *Journal of Pathology*:

Anna Löwa, Patrick Graff, Anna Nováčková, Andrej Kováčik, Kateřina Vávrová, Sabine Kaessmeyer, Sarah Hedtrich. Fibroblasts from atopic dermatitis patients trigger hyperproliferation and inflammation in skin equivalents.

Personal contribution:

Planning, design, and conducting of isolation and cultivation of hair follicle-derived cells and T cells, generation of skin equivalents, histological investigations including immunofluorescence staining, protein quantification by western blot, gene analysis using quantitative real-time PCR, *FLG* mutation analysis using Taqman allelic discrimination assays and restriction fragment length polymorphism (RFLP) analysis, cytokine measurement by enzyme-linked immunosorbent assay, and skin absorption testing's with subsequent data evaluation. Sample preparation for lipid analysis (stratum corneum isolation). Manuscript design and preparation under 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. Patrick Graff performed analyses of leukemia inhibitory factor by performing cytokine measurement by enzyme-linked immunosorbent assay and gene analysis using quantitative real-time PCR. Sabine Kaessmeyer conducted angiogenesis assay with subsequent data evaluation. Anna Nováčková, Andrej Kováčik, and Kateřina Vávrová performed stratum corneum lipid analyses with subsequent data evaluation.

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Artikel type : Brief definitve report

Fibroblasts from atopic dermatitis patients trigger hyperproliferation and inflammation in skin equivalents

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ABSTRACT

Atopic dermatitis (AD) is a highly complex and heterogenic skin disease which hampers the understanding of its underlying pathomechanism. Interestingly, the impact of fibroblasts on the induction and maintenance of various diseases has been increasingly recognized just recently; however, very little is known about their actual impact on AD. Hence, in the present study we investigated how AD patient-derived fibroblasts influence the tissue homeostasis of human-based skin equivalents. A subset of AD patient-derived fibroblasts induced characteristic features of atopic skin such as hyperproliferation, increased angiogenesis and upregulated levels of TSLP and PAR2 in the skin equivalents. These effects seem to be linked to a reduced secretion of the differentiation-associated cytokine leukemia inhibitor factor (LIF). Subsequent exposure of the hyperproliferative skin equivalents to $CD4^+$ T cells resulted in T cell infiltration and attenuated PAR2 expression; the latter likely being a result of increased LIF signaling due to the T cells. Overall, this study reports new findings on the effect fibroblasts may have on the induction and maintenance of an atopic phenotype.

Keywords: skin equivalents, atopic dermatitis, patient-derived fibroblasts, inflammation, LIF

INTRODUCTION

Atopic dermatitis (AD) is the most common inflammatory skin disease in industrialized countries and is characterized by recurrent eczematous lesions, intense itch and a fluctuating course of disease [1,2]. The underlying pathomechanism of AD is still not fully understood due to the complex interplay between skin barrier defects, environmental factors and immune dysregulations [3,4]. The strongest known genetic risk factor for AD are loss-of-function mutations in the filaggrin gene (*FLG*), which encodes the epidermal barrier protein filaggrin (FLG) [5]. However, decreased expression of barrier proteins such as FLG and T helper type 2 (Th2)-driven inflammation are commonly observed in AD patients, irrespective of any mutation, due to down-stream effects of Th2 cytokines such as interleukin (IL-) 4 and IL-13 [6-8]. Furthermore, key inflammation markers such as protease-activated receptor 2 (PAR2) and thymic stromal lymphopoietin (TSLP) are signifcantly upregulated in atopic skin [9,10] and angiogenesis is triggered by keratinocyte and mast cell-derived cytokines such as vascular endothelial growth factor (VEGF) and tumor necrosis factor alpha (TNFα) [11,12].

Interestingly, still very little is known about the actual impact of fibroblasts on AD, although their role in disease induction and/or maintenance of many other diseases has been increasingly recognized [13-16]. A landmark paper was published by Berroth et al. who demonstrated that AD fibroblasts induce epidermal hyperproliferation and reduce the expression of skin barrier proteins such as FLG due to reduced levels of leukemia inhibitory factor (LIF) [17]. Despite these findings, it is still unclear which other effects AD fibroblasts may trigger. Hence, to obtain a more detailed understanding about the role of diseased fibroblasts in tissue homeostasis, we investigated the impact of AD patient-derived fibroblasts on the epidermal differentiation, maturation and inflammation of full-thickness skin equivalents in the presence and absence of activated $CD4^+$ T cells.

METHODS

Fibroblasts from 6 healthy volunteers and 6 atopic dermatitis patients (age between 20-30; with ethical approval, EA1/345/14) were isolated from plucked hair follicles [18] and were screened for the most common *FLG* mutations R501X, 2282del4, R2447X and S3247X using Taqman allelic discrimination assays (Light SNiP Assays, TIB MOLBIOL, Berlin, Germany). Subsequently, skin equivalents were generated using either normal keratinocytes (KC) and fibroblasts (Fb) (isolated from juvenile foreskin; with ethical approval, EA1/081/13) or normal KC and AD patient-derived Fb as described previously [8].

For the generation of T cell-competent skin equivalents, 0.35×10^6 activated $CD4^+$ T cells/cm^2 were applied directly underneath the skin equivalents onto the culture insert membrane at day 12 of tissue cultivation [19]. Skin equivalents treated with medium only served as control. At day 14, the skin equivalents were embedded in tissue freezing medium (Leica Biosystems, Nussloch, Germany) and shock-frozen using liquid nitrogen. Subsequently, 5 µm skin sections were cut and stained for histological analysis (H&E; Carl Roth, Karlsruhe, Germany) according to standard protocols. Immunofluorescence staining, western blot analysis and quantitative PCR were performed according to standard procedures (Supplemental Materials, Table S1). Protein expression was semi-quantified by densitometry and normalised to ß-actin and ß-tubulin levels using ImageJ version 1.46r (NIH, Bethesda, USA). The release of cytokines IL-6, IL-13, TNFα and LIF were determined in the cell culture media using ELISA (Life Technologies, Darmstadt, Germany) according manufacture's instructions.

Furthermore, normal or AD fibroblasts were co-cultivated with primary human dermal microvascular endothelial cells (Lonza, Walkersville, USA). After 10 days, the co-cultures were fixed (methanol/acetone, 1:1) and immunolabelled with anti-CD31, endothelial tube were counted and VEGF levels in the culture medium of the skin equivalents was quantified

using ELISA kits (Life Technologies, Darmstadt, Germany) according manufacture's instructions.

RESULTS & DISCUSSION

Atopic dermatitis (AD) is a highly complex and heterogenous disease. The underlying pathological mechanism of the epidermal barrier abnormalities and immunological hyperreactivity are still not fully understood. While many studies explored the impact of diseased epithelial cells on the skin homeostasis, little is known about the role of fibroblasts, although a growing body of evidence demonstrated their central role in skin homeostasis and epidermal regeneration [20-22]. Hence, in the present study, we investigated the impact of AD patient-derived fibroblasts on the tissue homeostasis and differentiation using human-based skin equivalents. Most interestingly, fibroblasts isolated from three out of six AD patients (**group 1 AD fibroblasts**) triggered significant epidermal thickening, parakeratosis and hyperproliferation in the skin equivalents (Fig. 1aII). One of these AD patients carried a heterozygot 2282del4 *FLG* mutation (Fig. S1a). Furthermore, these hyperproliferative skin equivalents showed a significant downregulation of filaggrin (FLG) expression and a clear trend towards increased involucrin (IVL, $p = 0.0577$) (Fig. 1b, c), which is in line with previous work [17] and data derived from *FLG* knockdown skin equivalents [8,19,23]. The inflammatory master regulators TSLP and PAR2 were also significantly upregulated in the hyperproliferative skin equivalents by 40% for TSLP and 50% for PAR2 (Fig. 1b, c). Here, PAR2 was detected throughout the entire epidermis whereas in the control equivalents, PAR2 was exclusively expressed in upper epidermal layers (Fig. 1c). Notably, skin equivalents generated from three different AD patients (**group 2 AD fibroblasts**) did not show any histological differences compared to normal skin equivalents (Fig. 1aI, III; b, c). A potential explanation for these findings may be the significantly reduced expression of the differentiation-associated cytokine LIF in the hyperproliferative skin equivalents (**group 1**)

(Fig. 1d, e) which is in line with Berroth et al. and *in vivo* data [17]. LIF signaling is important for adequate skin proliferation and differentiation and, thus if altered, potentially contributing to the aberrant dermal-epidermal communication in AD patients. If these findings in skin equivalents correlate with the disease activity of the cell donors *in vivo* remains unclear since the ethical vote did not allow to obtain any information about the type and disease severity of the patients.

In addition, our data also indicate a potential involvement of fibroblasts in angiogenesis in atopic skin since we found increased levels of VEGF in the culture medium of the hyperproliferative skin equivalents (**group 1 AD fibroblasts**). Again, no changes in VEGF secretion were seen for the skin equivalents generated from **group 2** AD fibroblasts. In addition, co-cultivation of AD-patient derived fibroblasts and dermal microvascular endothelial cells facilitated the formation of a significantly enhanced number of endothelial tubes (Fig. 2, S2). This finding is interesting since AD is characterized by excessive angiogenesis and high serum concentrations of VEGF, which ultimately facilitates immune cell infiltration. So far, keratinocytes and especially mast cells are considered the main drivers of angiogenesis [24].

Aiming to investigate the potential impact of diseased fibroblasts onto immune cell infiltration, we exposed the skin equivalents to activated $CD4^+$ T cells by adding these onto the membrane of the culture insert. Interestingly, the T cells exclusively migrated into the dermis equivalent of the hyperproliferative skin equivalents (generated from **group 1 AD fibroblasts**) (Fig. 3a). Most likely, T cell migration was triggered by the high TSLP levels in the hyperproliferative skin equivalents; a down-stream effect which has been previously reported by our group [19]. Further analysis suggests a Th2 polarization of the T cells after exposure to the disease equivalents as indicated by upregulated *GATA3* and charactertic Th2 cytokine IL-13 (Fig. 3b, S2).

Most interestingly, the PAR2 expression was significantly diminshed (Fig. 3c; $p = 0.0098$) and TSLP levels slightly attenuated (Fig. 3d; $p = 0.621$) after exposure to the T cells in the hyperproliferative skin equivalents (**group 1 AD fibroblasts**). This may be surprising, however, Strid and colleagues previously discussed potential homeostatic effects of Th2 cytokines like IL-13 in the human skin. IL-13 activates two complex receptor systems consiting of IL-4Rα and IL-13Rα1 or IL-13Rα2, all of which are expressed by keratinocytes. Their expression pattern may change due to, for example, aberrant or low filaggrin expression [25]. In addition, IL-13Rα2 is highly upregulated in keratinocytes from human AD lesions [26] and its expression can be induced by IL-13 [27]. Nevertheless, the role of IL-13Rα2 whether it functions as a "decoy" receptor or has actually signals is still unknown [28]. Interestingly, the deletion of IL-13Rα2 in mice resulted in cutaneous inflammation and increased IgE levels [27]. Overall, these data indicate that IL-13Rα2 may exert protective functions at increased IL-13 levels, which may explain the attenuation of inflammatory markers such as PAR2 in the hyperproliferative skin equivalents after T cell exposure. Alternatively, the anti-inflammatory effects may be also connected to LIF since the exposure of the skin equivalents to the T cells significantly increased the gene encoding for LIF in the dermal compartment as well as LIF secretion into the culture medium of the hyperproliferative skin equivalents (Fig. S4a, b). It is known that activated $CD4^+$ T cells secrete relevant levels of LIF [29]. However, the underlying reasons for the attenuation of the PAR2 are speculative at the moment and require further evaluations. In addition, future studies need to focus on a more detailed characterization of AD patient-derived Fb including the analysis of secretory profiles to better understand the effects reported here.

Nevertheless, this study clearly highlights the impact fibroblasts may have on the epidermal homeostasis in normal and diseased states and further underlines the heterogeneity and multifactoriality of AD phenotypes.

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AUTHOR CONTRIBUTIONS

A.L., P.G., A.N., and A.K. performed the experiments. S.H. supervised the work. A.L., P.G. A.N., A.K., K.V., S.K. and S.H. designed the experiments, analyzed data and wrote the manuscript. All authors provided critical review of the manuscript.

ADDITIONAL INFORMATION

Supplementary information accompanies this paper at

Competing financial interests: The authors declare no competing financial interests.

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FIGURES

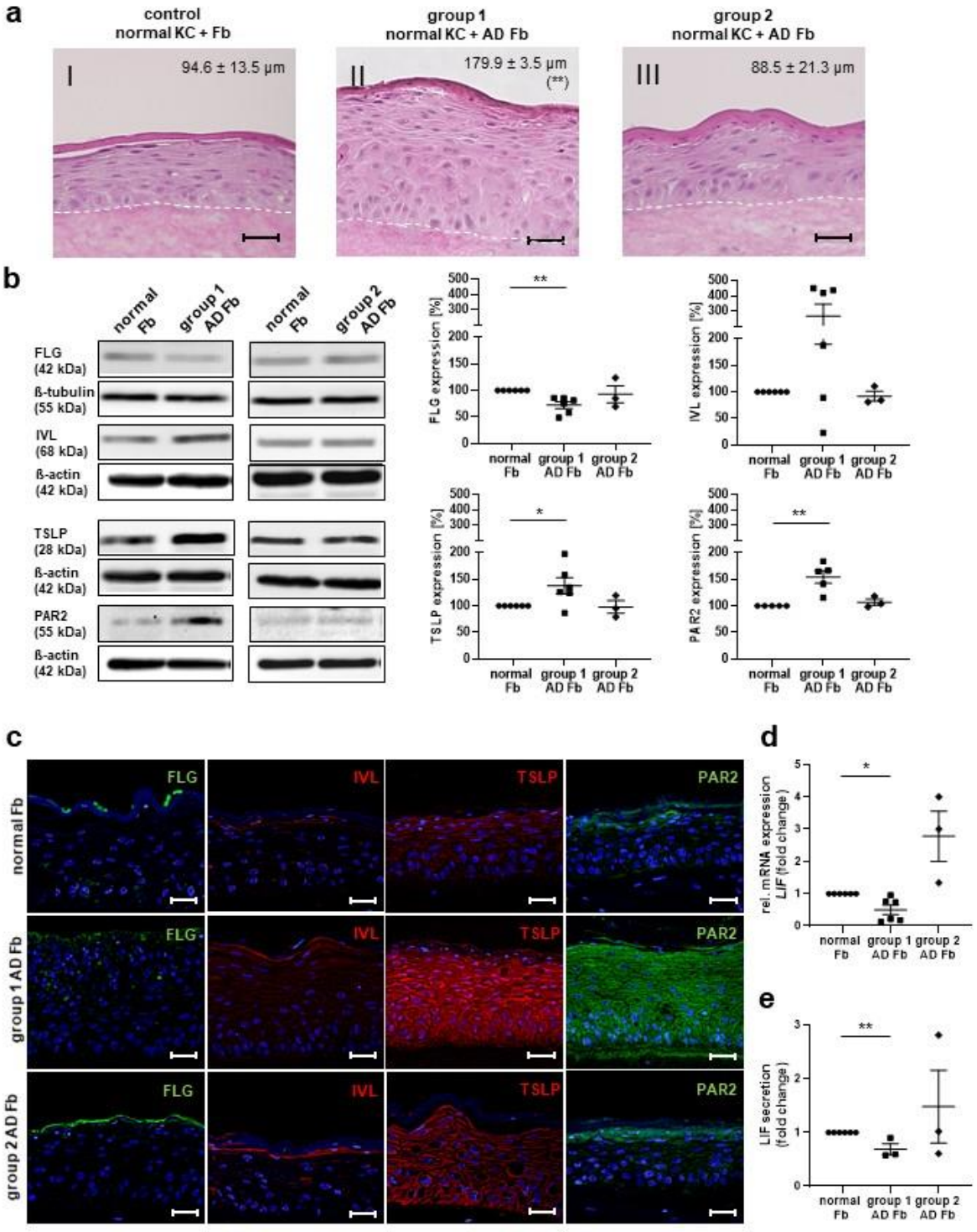

Figure 1. Donor-dependent impact of atopic dermatitis (AD) patient-derived fibroblasts on the epidermal differentiation of skin equivalents. (**a**) Representative histological staining of skin equivalents generated from normal keratinocytes (KC) and

normal fibroblasts (Fb) or AD patient-derived Fb. Three out of six skin equivalents cultivated from AD patient-derived Fb showed significant epidermal thickening and hyperproliferation (**referred to as group 1**). White dotted line = epidermal-dermal junction. (**b**) Western blots and relative protein expression of filaggrin (FLG), involucrin (IVL), thymic stromal lymphopoietin (TSLP) and protease-actived receptor 2 (PAR2) semi-quantified by densitometry and (**c**) corresponding representative immunostaining against FLG (green), IVL (red), TSLP (red) and PAR2 (green) of normal and hyperproliferative (**group 1 AD Fb**) as well as non-hyperproliferative skin equivalents **(group 2 AD Fb**). Cell nuclei were counterstained with 4',6'-diamin-2-phenylindol (DAPI, blue). Scale bar = 50 µm. (**d**) Expression of leukemia inhibitory factor (LIF) on gene and (**e**) protein level in normal, hyperproliferative (**group 1 AD Fb**) and non-hyperproliferative skin equivalents (**group 2 AD Fb**). Values are given as mean ± SEM. n = 3-6. Statistical analysis was performed by unpaired student's *t*-test for direct comparisons of two independent groups. * indicates statistically significant differences between skin equivalents generated from normal and AD patient-derived fibroblasts (*$p \leq 0.05$; **$p \leq 0.01$).

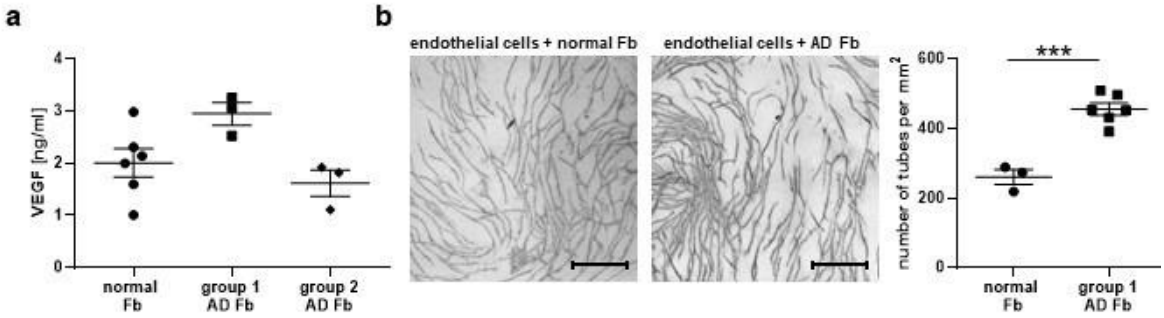

Figure 2. Endothelial tube formation of primary human dermal microvascular endothelial cells is significantly stimulated by AD fibroblasts. (**a**) Levels of vascular endothelial growth factor (VEGF) in the culture medium of normal, hyperproliferative skin equivalents (**group 1**) and non-hyperproliferative skin equivalents (**group 2**). (**b**) Representative images and numbers of endothelial tube formation of endothelial cells after

co-cultivation with normal and group 1 AD fibroblasts. Scale bar = 1,000 nm. Values are given as mean ± SEM. n = 2-6. Statistical analysis was performed by unpaired student's *t*-test. * indicates statistically significant differences between skin equivalents generated using normal or AD patient-derived fibroblasts (***$p \leq 0.001$).

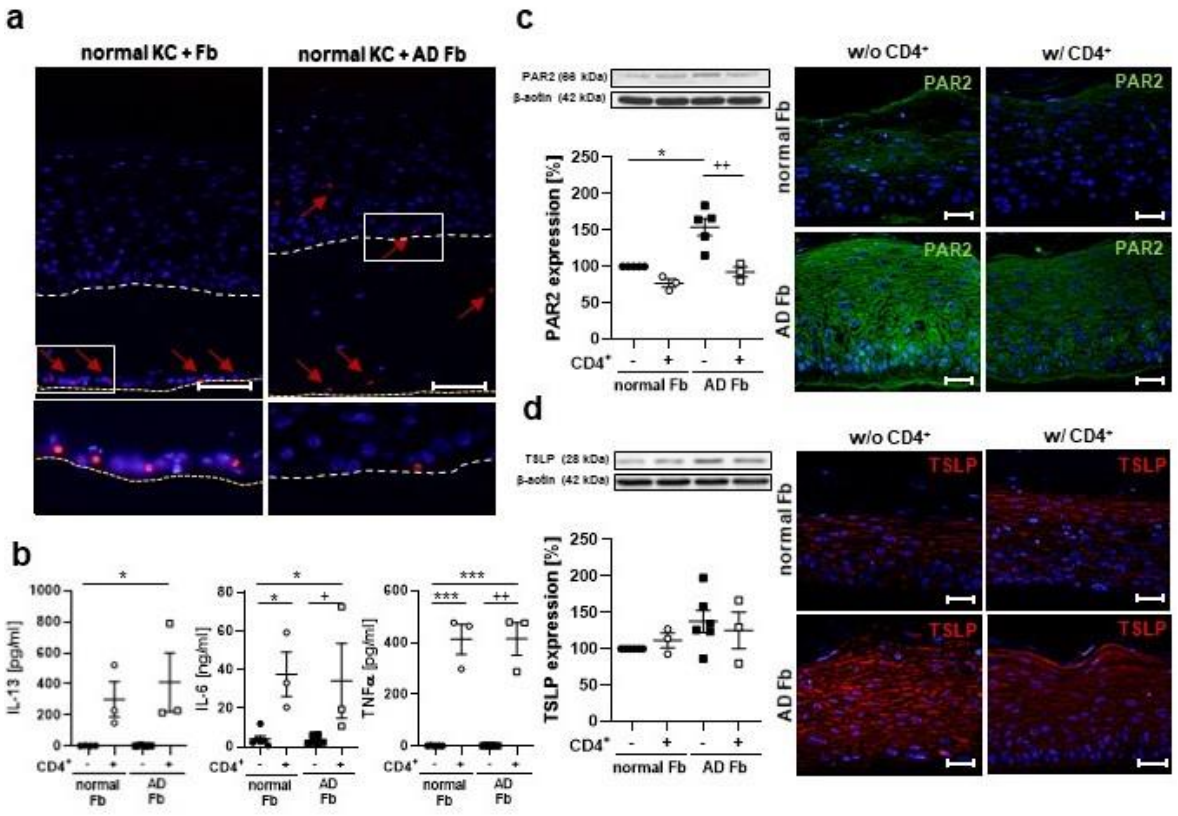

Figure 3. T cells migrate into the hyperproliferative skin equivalents and attenuate PAR2 and TSLP expression. (**a**) Representative immunostaining against $CD4^+$ T cells in skin equivalents generated using normal or AD fibroblasts (**group 1 AD Fb**) after exposure to T cells. (**b**) Protein levels of the Th2 cytokine IL-13 and the pro-inflammatory cytokines IL-6 and tumor necrosis factor alpha (TNFα) in skin equivalents cultivated from normal and AD patient-derived fibroblasts (**group 1 AD Fb**) before and after exposure to activated $CD4^+$ T cells. (**c**, **d**) Representative western blots and relative protein expression semi-quantified by densitometry and corresponding representative immunostaining against (**c**) protease-actived receptor 2 (PAR2) PAR2 and (**d**) thymic stromal lymphopoietin (TSLP) in skin equivalents before and after exposure to T cells. Cell nuclei were counterstained with DAPI

(blue). White dotted line = epidermal-dermal junction, green dotted line = insert membrane. Scale bar = 50 μm. Statistical analysis was performed by one-way analysis of variance followed by Bonferroni's correction. * indicates statistically significant differences between skin equivalents generated from normal and AD patient-derived fibroblasts (*$p \leq 0.05$; **$p \leq 0.01$). + indicates statistically significant differences between skin equivalents generated from AD patient-derived fibroblasts before and after exposure to acitivated $CD4^+$ T cells (+$p \leq 0.05$; ++$p \leq 0.01$).

SUPPLEMENT OF THE MANUSCRIPT

MATERIALS AND METHODS

Immunofluorescence staining

Skin sections were fixed with 4% formaldehyde, washed with PBS containing 0.0025% BSA and 0.025% Tween 20 (Carl Roth, Karlsruhe, Germany) and blocked with normal goat serum (1:20 in PBS) followed by incubation with primary antibodies overnight at 4 °C (in PBS, 0.0025% BSA, 0.025% Tween 20; Table S1). After washing, fixed sections were incubated for 1 h at room temperature with secondary antibodies IgG Alexa Fluor®488 and IgG Alexa Fluor®594 (Abcam, Cambridge, UK) (1:400 in PBS, 0.0025% BSA, 0.025% Tween 20), embedded in 4',6-diamidin-2-phenylindol (DAPI) antifading mounting medium (Dianova, Hamburg, Germany) and analyzed by fluorescence microscopy (BZ-8000; Keyence, Neu-Isenburg, Germany).

Western blot analysis

For western blot analysis, the epidermides of skin equivalents were gently peeled off, lysed in radioimmunoprecipitation assay buffer supplemented with protease/phosphatase inhibitor cocktail (Cell Signaling, Frankfurt/Main, Germany), and the total protein concentrations determined using the Pierce® BCA Protein Assay Kit (Thermo Scientific, Waltham, USA). Subsequently, the samples (~ 30 µg protein) were boiled in standard SDS-PAGE sample buffer supplemented with 100 mM DTT, boiled at 95 °C and separated by 10% tris-glycine protein gels for SDS polyacrylamide gel electrophoresis (Bio-Rad, Munich, Germany). Samples were then blotted onto nitrocellulose membranes (Bio-Rad, Munich, Germany) and blocked in Tris-buffered saline containing 0.1% Tween-20 (TBST; Sigma-Aldrich, Munich, Germany) supplemented with 5% skimmed milk for 1 h at room temperature. Subsequently, the membranes were incubated with the primary antibodies (Table S1) diluted in TBST with

5% skimmed milk at 4 °C overnight and after washing in TBST, incubated with anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibody (Cell Signaling, Frankfurt/Main, Germany) for 1 h at room temperature. Blots were then developed with SignalFire™ ECL reagent or for filaggrin detection with SignalFire™ elite ECL reagent (Cell Signaling, Frankfurt/Main, Germany) and visualised by PXi/PXi Touch gel imaging system (Syngene, Cambridge, UK).

Quantitative real-time polymerase chain reaction (qPCR)

The epidermides of skin equivalents were gently peeled off the dermis and milled for 30 s at 25 Hz using a TissueLyzer (Qiagen, Hilden, Germany). Subsequently, RNA was isolated using an InnuPREP RNA Mini Kit (Analytik Jena, Jena, Germany) according to the manufacturer's instructions. For cDNA synthesis, the iScript cDNA Kit (Bio-Rad, Munich, Germany) was used. Subsequently, qPCR was performed using the iTaq™ Universal SYBR® Green Supermix Kit (Bio-Rad, Munich, Germany) and forward primer LIF F (5'-CAAGAATCAACTGGCACAGC-3') and reverse primer LIF R (5'-AGTGGGGTTCAGGACCTTCT-3'). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward primer: 5'-CTCTCTgCTCCTCCTgTTCgAC-3'; reverse primer: 5'-TgAgCgATgTggCTCggCT-3') served as the housekeeping gene.

TABLES

Table S1. Primary antibodies for immunofluorescence (IF) and western blot (WB).

Antibody	Isotype	Clone	IF	WB	Company
ß-actin	mouse IgG1	monoclonal (15G5A11/E2)	–	1:10,000	Sigma-Aldrich, Munich, Germany
ß-tubulin	rabbit IgG	monoclonal (9F3)	–	1:1,000	Cell Signaling Technology, Danvers, United States of America
CD31	mouse IgG1	monoclonal (JC/70A)	1:50	–	DAKO, Hamburg, Germany
CD4	mouse IgG1	monoclonal (4B12)	1:50	–	DAKO, Hamburg, Germany
FLG	mouse IgG	polyclonal	1:1,000	1:1,000	BioLegend, San Diego, United States of America
IVL	rabbit IgG	polyclonal	1:1,000	1:1,000	Abcam, Cambridge, United Kingdom
PAR2	mouse IgG2a	monoclonal (SAM11)	1:200	1:50	Santa Cruz Biotechnology, Dallas, United States of America
TSLP	rabbit IgG	polyclonal	1:1,000	1:1,000	Abcam, Cambridge, United Kingdom

FIGURES

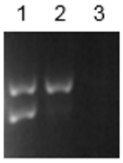

Figure S1. Analysis of the 2282del4 mutation by PCR following restriction fragment length polymorphism (RFLP) with the *DraIII* enzyme; lane 1: AD donor carries a heterozygous 2282del4 mutation; lane 2: healthy homozygous donor; lane 3: no template control.

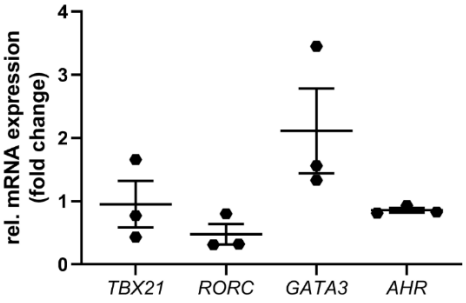

Figure S2. Relative mRNA expression of T cell master regulators *TBX21* (Th1), *RORC* (Th17), *GATA3* (Th2) and *AHR* (Th22) normalized to their expression in control equivalents after T cell exposure. Values are given as mean ± SEM. n = 3.

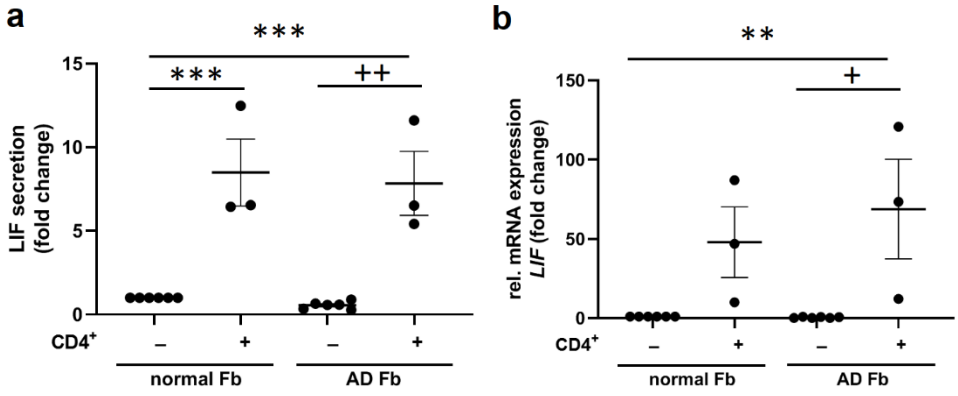

Figure S3. Expression of leukemia inhibitory factor (LIF) in the dermal compartment of skin equivalents cultivated from normal and AD patient-derived fibroblasts (group 1) before and after exposure to activated $CD4^+$ T cells on (**a**) protein and (**b**) gene level. Values are given as mean ± SEM. n = 3-6. Statistical analysis was performed using the unpaired student's *t*-test for direct comparisons of two independent groups and by one-way analysis

of variance followed by Bonferroni's correction for multiple comparisons. * indicates statistically significant differences between skin equivalents generated from normal and AD patient-derived fibroblasts (**$p \leq 0.01$; ***$p \leq 0.001$). + indicate statistically significant differences between skin equivalents generated from AD patient-derived fibroblasts before and after exposure to activated $CD4^+$ T cells (+$p \leq 0.05$; ++$p \leq 0.01$).

3.2.2 Supplementary Data

AD patient-derived fibroblasts induce hyperproliferation and alter the expression of skin barrier and tight junction proteins

Skin equivalents grown from group 1 AD Fb, inducing hyperproliferative status in skin equivalents, showed clear hyperproliferation indicated by an increased Ki67 expression in the basal layer of the epidermis compared to skin equivalents grown from group 2 AD Fb and normal Fb (Fig. 2.1).

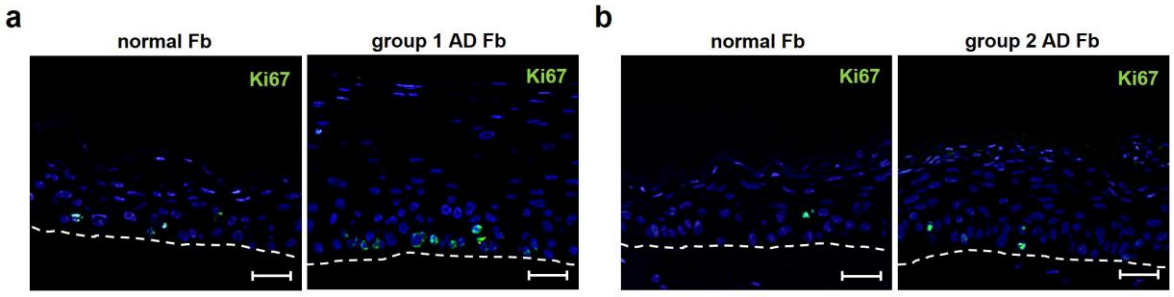

Figure 2.1. Representative immunostaining against the proliferation marker Ki67 (green) in (**a**) normal and hyperproliferative (group 1 AD Fb) skin equivalents and (**b**) normal and non-hyperproliferative (group 2 AD Fb) skin equivalents. Counterstaining of cell nuclei was performed with 4',6'-diamin-2-phenylindol (DAPI, blue). Scale bar = 100 µm. n = 3-6.

Skin equivalents characterized by hyperproliferation (group 1 AD Fb) showed a trend towards increased loricrin expression (LOR, 2.2-fold, $p = 0.1174$) (Fig. 2.2) as well as an increased expression of the tight junction proteins occludin (OCLN, 1.4-fold, $p = 0.2746$) and claudin-1 (CLDN1, 1.2-fold, $p = 0.3336$) (Fig. 2.3). Interestingly, no major differences in LOR, OCLN and CLDN1 expression were detected in the skin equivalents generated from group 1 AD Fb with no apparent hyperproliferative state relative to the skin equivalents grown from normal Fb (Fig. 2.2, 2.3). Corresponding regulations at the mRNA level are presented in Fig. 2.4. Notably, no significant differences in the skin barrier function of normal and diseased skin equivalents grown either from group 1 or group 2 AD Fb were detected (Fig. 2.5). Lipid analyses revealed no changes in the stratum corneum (SC) lipid composition between normal and non-hyperproliferative skin equivalents (group 2 AD Fb), whereas infrared spectroscopy indicated a slightly disturbed lipid chain order in the hyperproliferative skin equivalents (group 1 AD Fb) (Fig. 2.6).

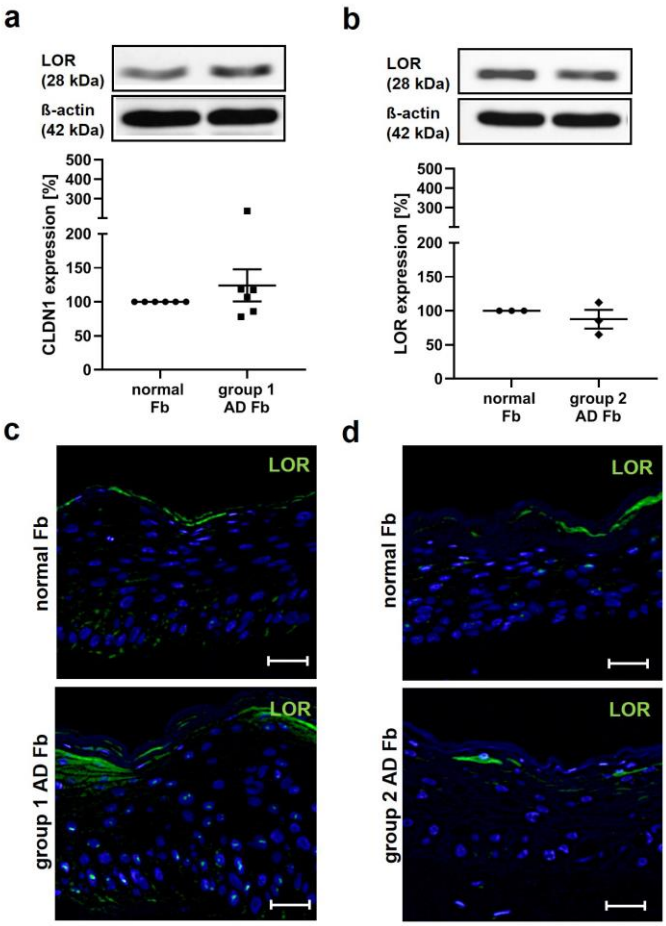

Figure 2.2. (**a**, **b**) Western blots and relative protein expression of loricrin (LOR) semi-quantified by densitometry of (**a**) normal and hyperproliferative (group 1 AD Fb) skin equivalents and (**b**) normal and non-hyperproliferative (group 2 AD Fb) skin equivalents. (**c**, **d**) Representative immunostaining against LOR (green) in (**c**) normal and hyperproliferative (group 1 AD Fb) skin equivalents and (**d**) normal and non-hyperproliferative (group 2 AD Fb) skin equivalents. Counterstaining of cell nuclei was performed with 4',6'-diamin-2-phenylindol (DAPI, blue). Scale bar = 50 µm. Values are given as mean ± SEM. n = 3-6.

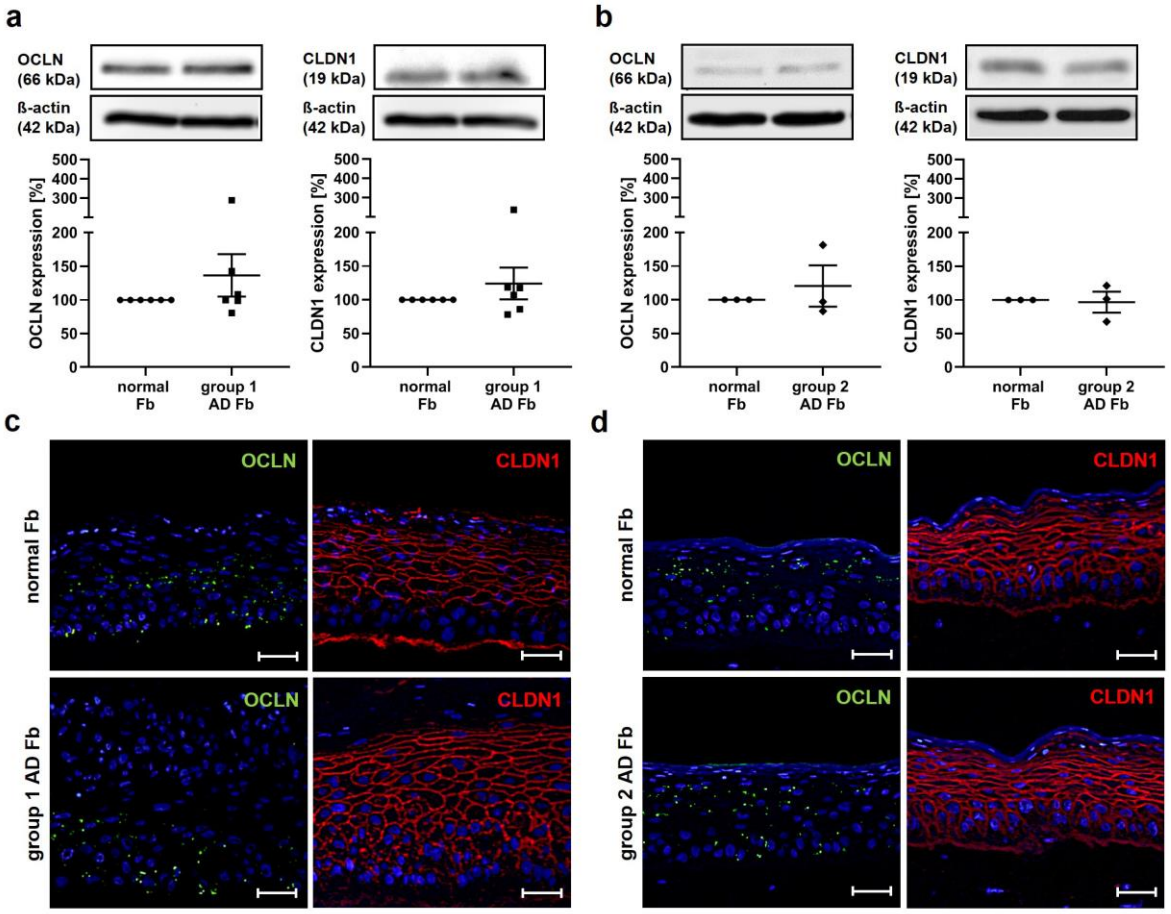

Figure 2.3. (**a**, **b**) Western blots and relative protein expression of (**a**, **b**) occludin (OCLN) and claudin-1 (CLDN1) semi-quantified by densitometry and (**c**, **d**) representative immunostaining against OCLN (green) and CLDN1 (red) in (**a**, **c**) normal and hyperproliferative (group 1 AD Fb) skin equivalents and (**b**, **d**) normal and non-hyperproliferative (group 2 AD Fb) skin equivalents. Counterstaining of cell nuclei was performed with 4',6'-diamin-2-phenylindol (DAPI, blue). Scale bar = 50 µm. Values are given as mean ± SEM. n = 3-6.

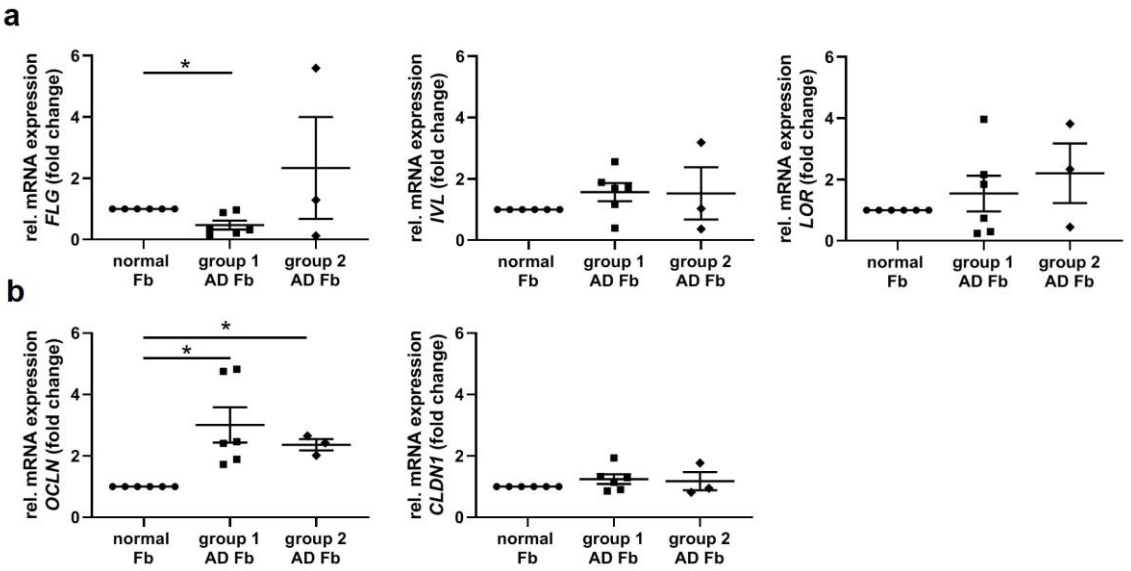

Figure 2.4. Relative mRNA expression of (**a**) the differentiation markers filaggrin (*FLG*), involucrin (*IVL*), loricrin (*LOR*) and (**b**) the tight junction proteins occludin (*OCLN*) and claudin-1 (*CLDN1*) in normal (●), hyperproliferative (group 1 AD Fb; ■), and non-hyperproliferative (group 2 AD Fb; ♦) skin equivalents. Values are given as mean ± SEM. n = 3-6. * indicates statistical significance over skin equivalents grown from normal fibroblasts (*$p \leq 0.05$; **$p \leq 0.01$).

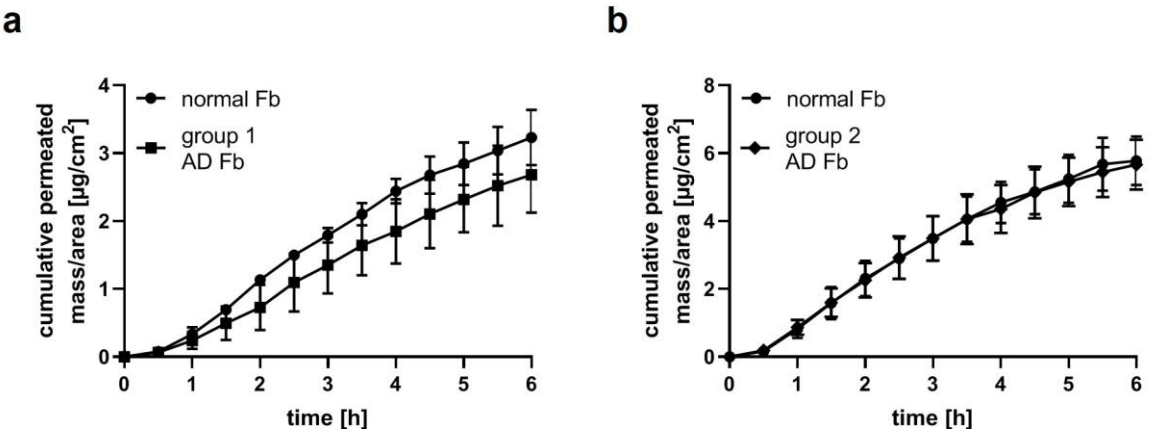

Figure 2.5. Skin permeability of (**a**) normal (●) and hyperproliferative (group 1 AD Fb; ■) skin equivalents as well as (**b**) normal (●) and non-hyperproliferative (group 2 AD Fb; ♦) skin equivalents. Values are given as mean ± SEM. n = 3.

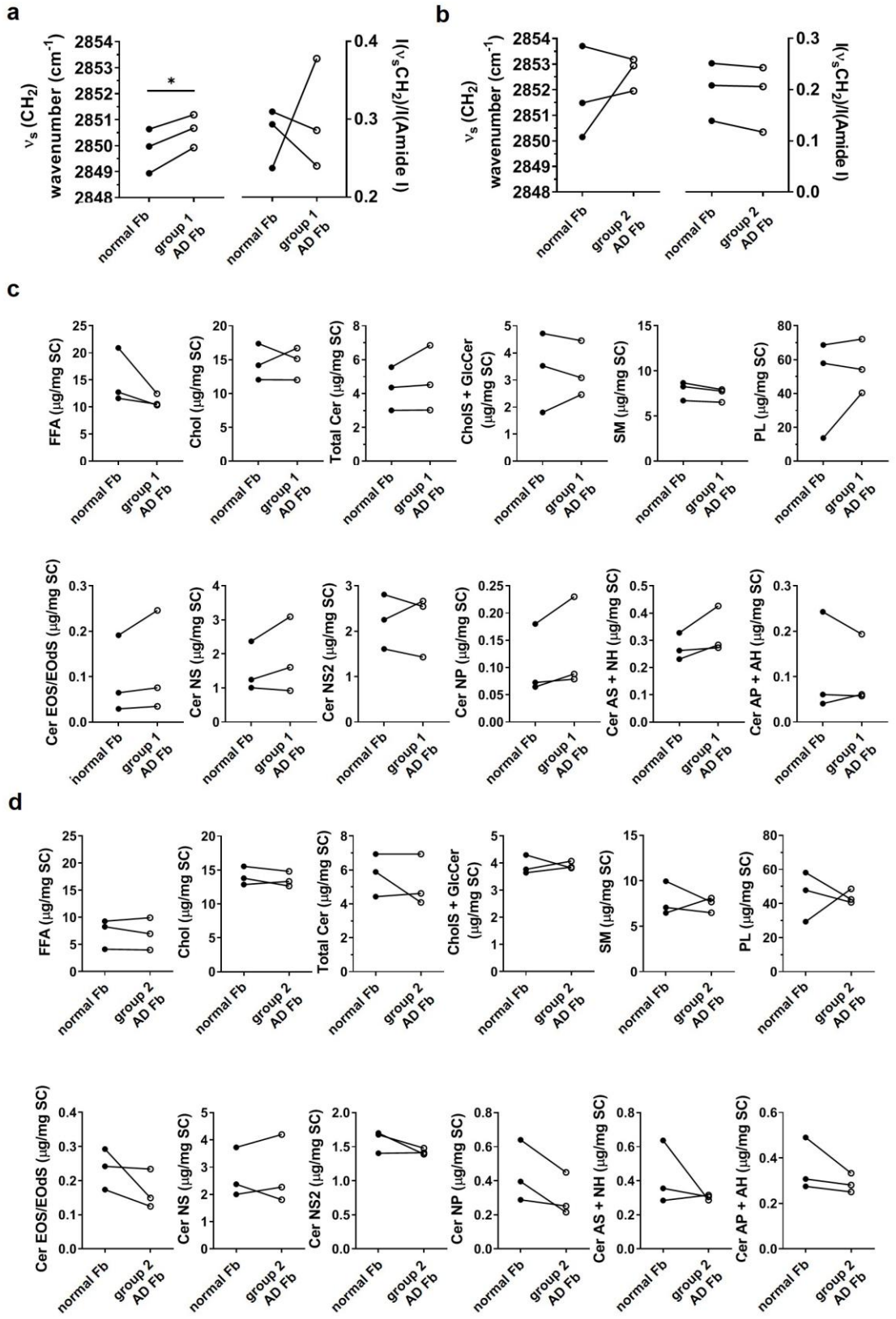

Figure 2.6. Stratum corneum (SC) lipid chain order (panel left) and apparent lipid/protein ratios (panel right) of (**a**) normal and hyperproliferative (group 1 AD Fb) skin equivalents and (**b**) normal and non-hyperproliferative (group 2 AD Fb) skin equivalents. The hydrated

SC samples isolated from the skin equivalents were examined by attenuated total reflectance-Fourier transform infrared spectroscopy by coaddition of 256 scans at 2 cm^{-1} resolution at 23 °C, each point represents the mean of at least two values recorded at different areas of the sample. The lipid chain order (left) was probed by methylene symmetric stretching vibrations – the higher wavenumber indicated less ordered lipids. The ratios of the intensities of methylene symmetric stretching and amide I vibrations were used as a simple lipid/protein characteristics (right). (**c**, **d**) SC lipids of (**c**) normal and hyperproliferative (group 1 AD Fb) skin equivalents, and (**d**) normal and non-hyperproliferative (group 2 AD Fb) skin equivalents analyzed by high-performance thin layer chromatography analysis: free fatty acids (FFA), cholesterol (Chol), ceramides (Cer), cholesterol sulfate (CholS), glucosylceramides (GlcCer), sphingomyelins (SM) and phospholipids (PL). Values are given as mean ± SEM. n = 3. * indicates statistically significant differences over skin equivalents grown from normal fibroblasts (*$p \leq 0.05$).

TSLP and PAR2 are significantly upregulated in skin equivalents generated from AD patient-derived fibroblasts

In addition to the upregulation of the inflammatory master regulators thymic stromal lymphopoietin (TSLP) and protease-activated receptor 2 (PAR2) (manuscript, Fig. 1b, c), mRNA expression of *TSLP*, interleukin-6 (*IL6*), -8 (*IL8*) and tumor necrosis factor-alpha (*TNFA*) were significantly increased and a clear trend towards an increased expression of kallikrein 5 (*KLK5*; 1.5-fold, $p = 0.0811$) was observed in hyperproliferative (group 1 AD Fb) skin equivalents compared to the normal skin equivalents (Fig. 2.7a, b). No significant differences, however, were observed for the expression of interleukin-1 beta (*IL1B*) as well as for the cytokine levels of IL-6, IL-8, and TNFα in the culture medium (Fig. 2.7c). In non-hyperproliferative disease equivalents (group 2 AD Fb), no significant differences in the expression of *TSLP* or *KLK5* were detected on the gene level, nor were they for the pro-inflammatory cytokines on the gene and protein levels other than IL-6 (Fig. 2.7). A significant downregulation of IL-6 gene expression was seen in non-hyperproliferative skin equivalents (group 2 AD Fb) as compared to normal skin equivalents (Fig. 2.7b).

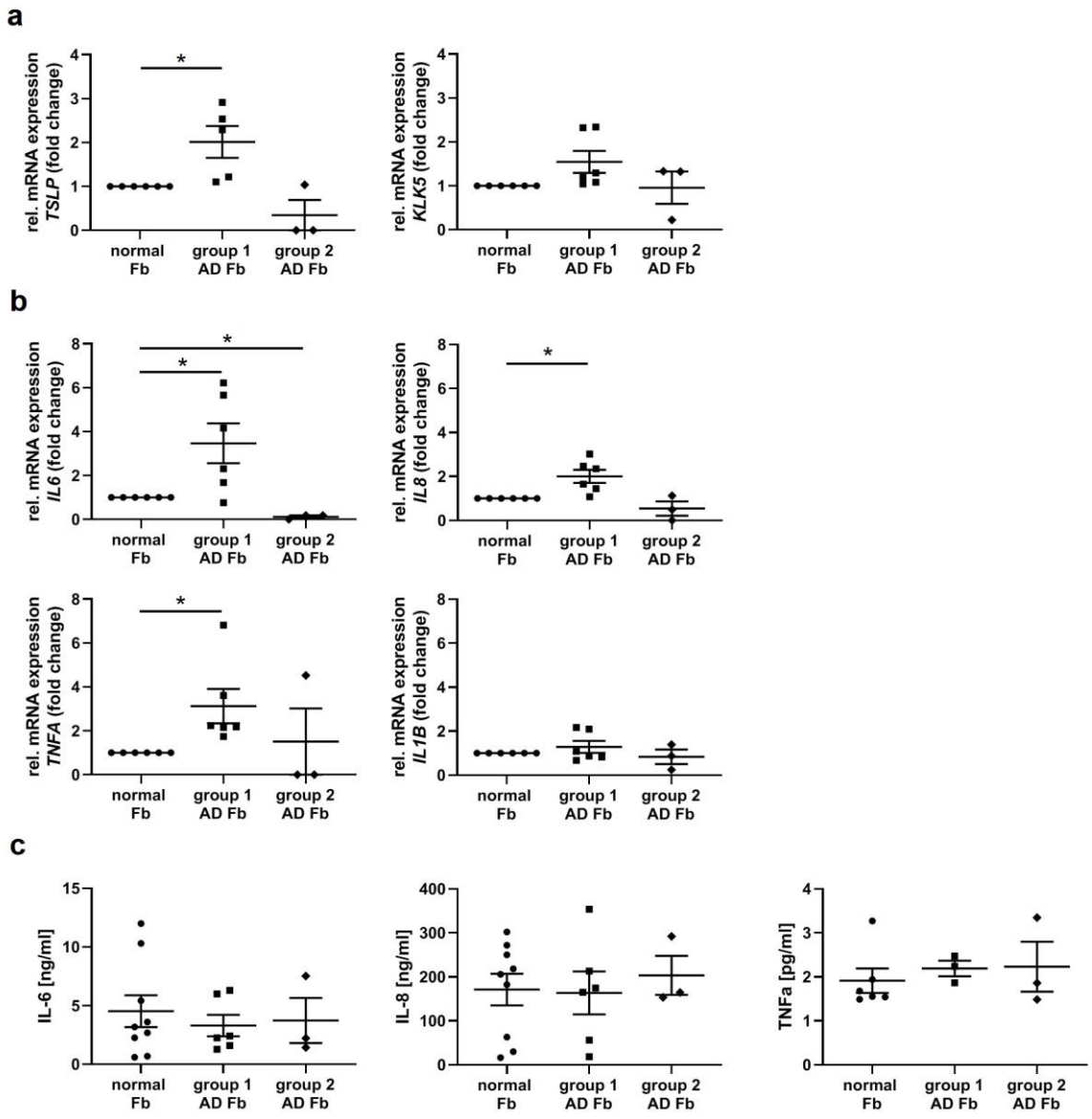

Figure 2.7. (**a**, **b**) Relative mRNA expression of (**a**) AD-specific markers thymic stromal lymphopoietin (*TSLP*) and kallikrein-related peptidase 5 (*KLK5*) and (**b**) interleukin-6 (*IL6*), interleukin-8 (*IL8*), tumor necrosis factor alpha (*TNFA*) and interleukin-1beta (*IL1B*) in normal (●), hyperproliferative (group 1 AD Fb; ■), and non-hyperproliferative (group 2 AD Fb; ♦) skin equivalents. (**c**) Levels of the pro-inflammatory cytokines IL-6, IL-8 and tumor necrosis factor-alpha (TNF-α) in culture media of normal (●), hyperproliferative (group 1 AD Fb; ■), and non-hyperproliferative (group 2 AD Fb; ♦) skin equivalents. Values are given as mean ± SEM. n = 3-6. * indicates statistical significance over skin equivalents grown from normal fibroblasts (*$p \leq 0.05$).

T cell migration occurs exclusively in skin equivalents generated from AD fibroblasts

Since previous studies from our group have shown that TSLP may directly activate T cell migration in inflammatory skin models (Wallmeyer et al., 2017), activated $CD4^+$ T cells were added to the hyperproliferative disease equivalents (group 1 AD Fb) and their controls (normal Fb). In accordance with our previous work, the $CD4^+$ T cells actively migrated into the dermis equivalent of the disease equivalents. No T cell migration was observed in the controls (manuscript, Fig. 3a). As expected, levels of the Th2 cytokine IL-13 as well as the pro-inflammatory cytokines IL 6, IL-8, TNFα (manuscript, Fig. 3b; Fig. 2.8a) were increased in the culture media of all skin equivalents after T cell exposure, being in line with their regulation on the gene level (Fig. 2.8b).

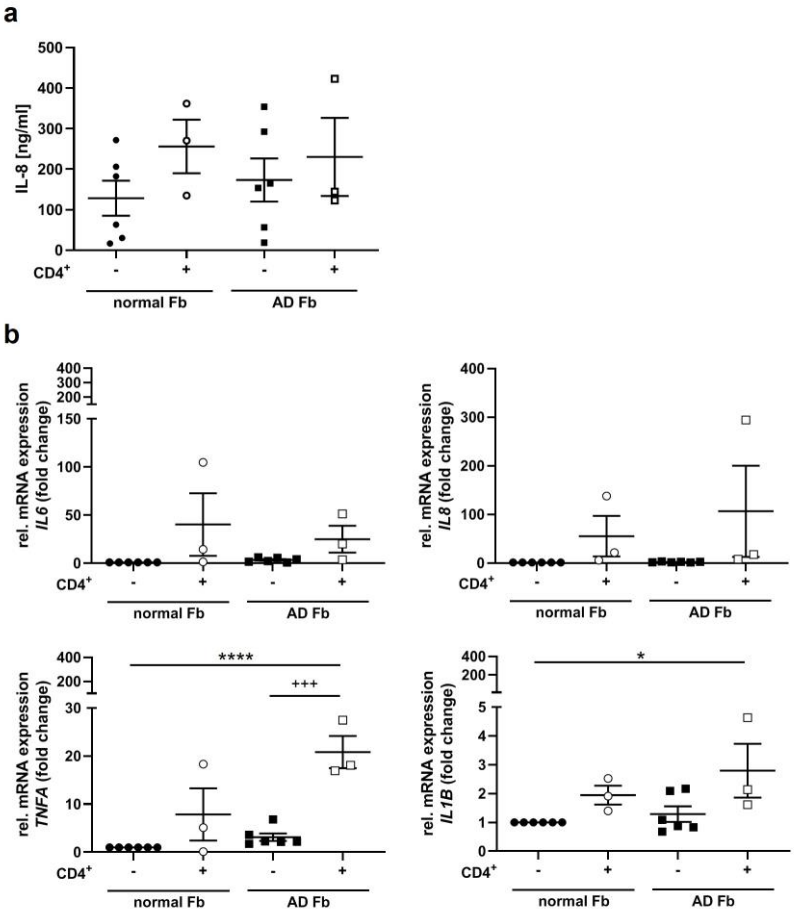

Figure 2.8. (**a**) Protein levels of the pro-inflammatory cytokines IL-8 in the culture media of skin equivalents cultivated from normal and AD patient-derived fibroblasts (group 1 AD Fb) before and after exposure to activated $CD4^+$ T cells. (**b**) Relative mRNA expression of interleukin-6 (*IL6*), interleukin-8 (*IL8*), tumor necrosis factor alpha (*TNFA*) and interleukin-1 beta (*IL1B*) in skin equivalents cultivated from normal and AD patient-derived fibroblasts (group 1 AD Fb) before and after exposure to activated $CD4^+$ T cells. Values are given as

mean ± SEM. n = 3-6. * indicates statistical significance over skin equivalents grown from normal fibroblasts alone (*$p \leq 0.05$; ****$p \leq 0.0001$). + indicates statistical significance over skin equivalents grown from AD fibroblasts alone (+++$p \leq 0.001$).

T cell exposure did not affect the protein expression of FLG, IVL, LOR, OCLN or CLDN1 in the equivalents grown from normal keratinocytes and Fb (Fig. 2.9). Skin equivalents grown from AD Fb (group 1) showed similar FLG expression after T cell exposure compared to skin equivalents alone (Fig. 2.9a, b). However, a significant downregulation of *FLG* was observed in the control and the disease equivalents after T cell exposure on the gene level (Fig. 2.10a), clearly indicating effects on skin barrier proteins. However, these have not yet been detected on the protein level. Interestingly, in all skin equivalents grown from AD Fb, a trend towards diminished expression of LOR, OCLN and CLDN1 after T cell exposure was observed (Fig. 2.9a, b). Most interestingly, T cell exposure significantly diminished PAR2 protein expression (0.6-fold, $p = 0.0098$) and slightly attenuated TSLP expression (0.9-fold, $p = 0.621$) in the hyperproliferative skin equivalents (manuscript, Fig. 3c, d). Corresponding regulations at the mRNA level are presented in Fig. 2.10.

With regard to the SC lipids, T cell exposure increased the levels of ceramide EOS, NS, NP and AS+AH, and decreased sphingomyelin levels in skin equivalents grown from AD Fb (group 1) (Fig. 2.11a). The relative proportion of the shorter-chain ceramide NS2 to its very long chained counterpart ceramide NS decreased with T cell exposure. Trends towards diminished free fatty acid and phospholipid levels were also observed in T cell-exposed skin equivalents. Cholesterol levels, however, remained unaffected. Despite these changes in the lipid composition after T cell exposure, the lipids of the SC did not organize properly as indicated by approximately 1.5 cm^{-1} shifts of the methylene symmetric stretching bands to higher wavenumbers (Fig. 2.11b). The skin barrier function was not significantly altered after T cell exposure as assessed by permeation assays using radioactively labelled testosterone (Fig. 2.11c) and Lucifer yellow (Fig. 2.11d).

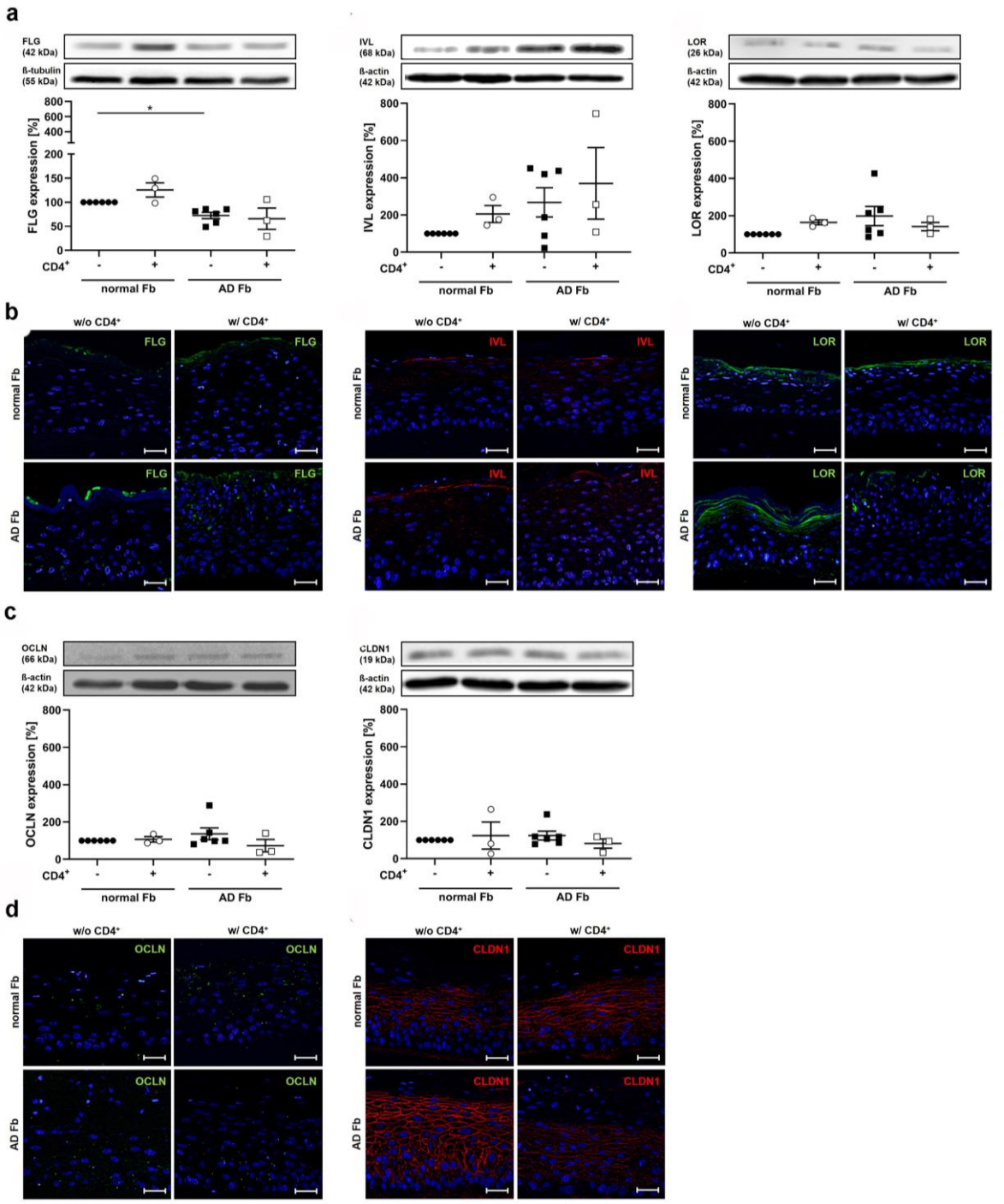

Figure 2.9. (**a**) Western blots and relative protein expression of differentiation markers filaggrin (FLG), involucrin (IVL) and loricrin (LOR) semi-quantified by densitometry of skin equivalents grown from normal and AD patient-derived fibroblasts (group 1 AD Fb) before and after exposure to activated $CD4^+$ T cells. (**b**) Representative immunostaining against FLG (green), IVL (red) and LOR (green) in normal and hyperproliferative skin equivalents before and after exposure to activated $CD4^+$ T cells. (**c**) Western blots and relative protein expression of tight junction proteins occludin (OCLN) and claudin-1 (CLDN1) semi-quantified by densitometry of skin equivalents grown from normal and AD patient-derived

fibroblasts (group 1 AD Fb) before and after exposure to activated CD4$^+$ T cells. (**d**) Representative immunostaining against OCLN (green) and CLDN1 (red) in normal and AD patient-derived fibroblasts (group 1 AD Fb) before and after exposure to activated CD4$^+$ T cells. Cell nuclei were counterstained with 4',6'-diamin-2-phenylindol (DAPI, blue). Scale bar = 50 µm. Values are given as mean ± SEM. n = 3-6. * indicates statistical significance over skin equivalents generated from normal fibroblasts alone (*$p \leq 0.05$).

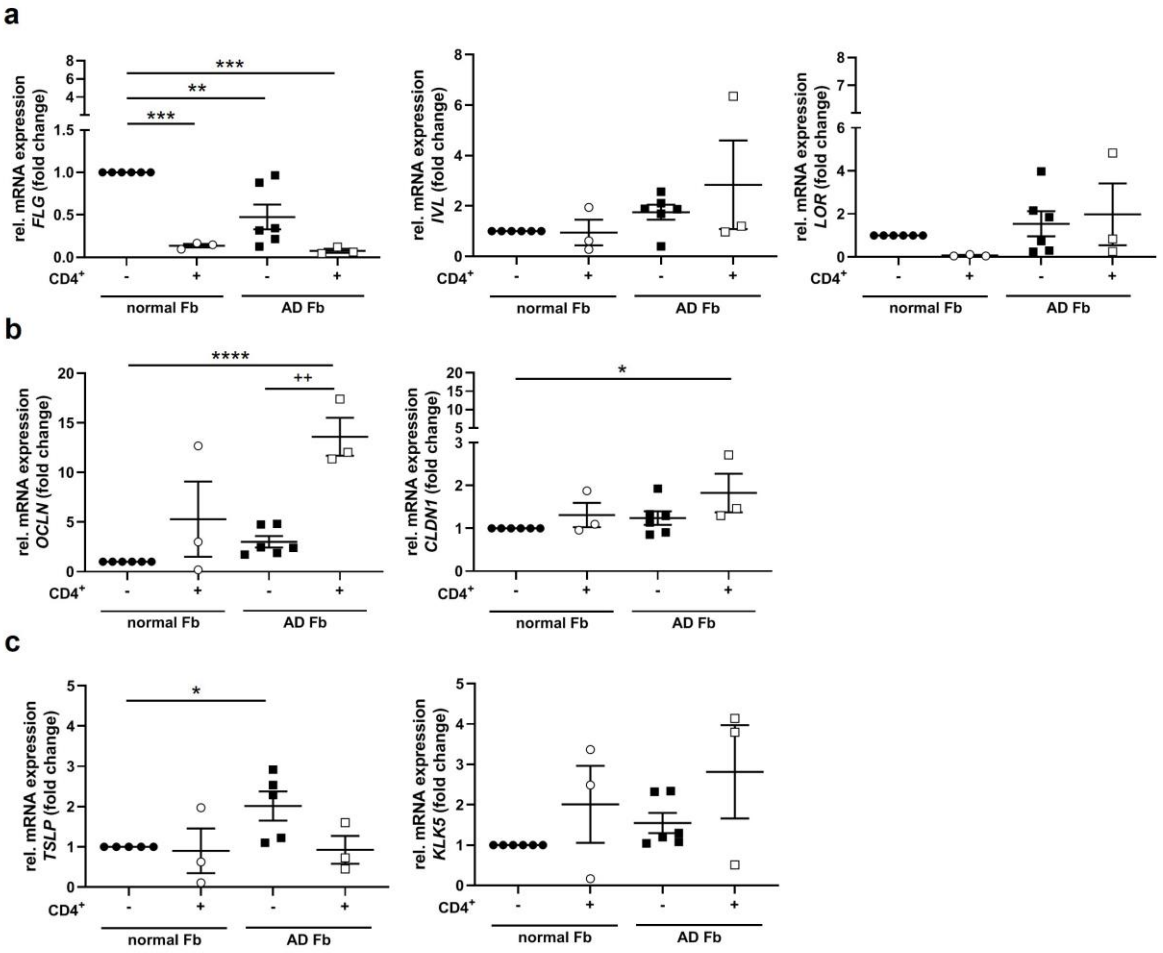

Figure 2.10. Relative mRNA expression of (**a**) differentiation markers filaggrin (*FLG*), involucrin (*IVL*), loricrin (*LOR*), (**b**) tight junction proteins occludin (*OCLN*) and claudin-1 (*CLDN1*) and (**c**) AD-specific markers thymic stromal lymphopoietin (*TSLP*) and kallikrein-related peptidase 5 (*KLK5*) in skin equivalents grown from normal and AD patient-derived fibroblasts (group 1 AD Fb) before and after exposure to activated CD4$^+$ T cells. Values are given as mean ± SEM. n = 3-6. * indicates statistical significance over skin equivalents grown from normal fibroblasts alone (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$). + indicates statistical significance over skin equivalents grown from AD fibroblasts alone (++$p \leq 0.01$).

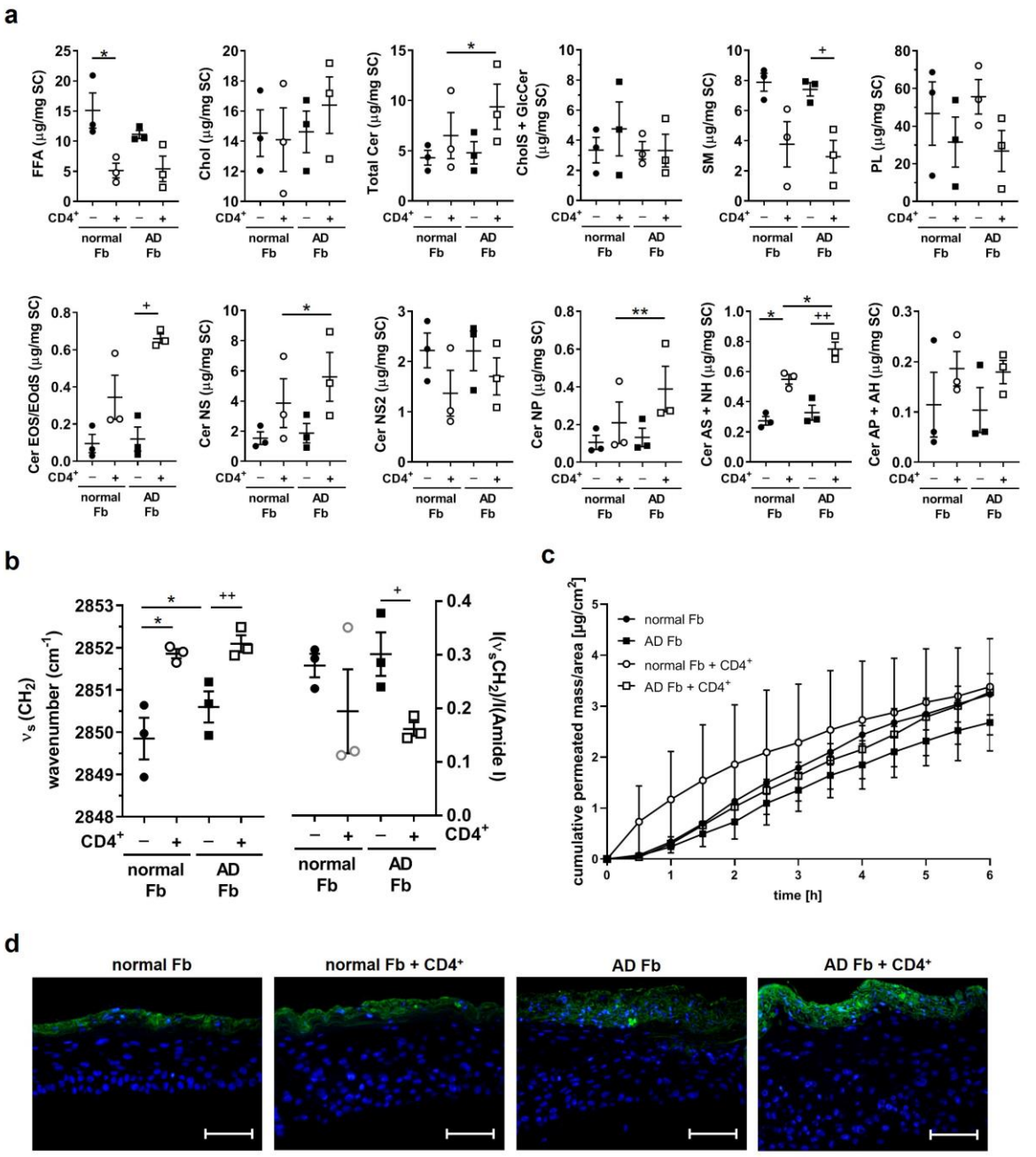

Figure 2.11. (**a**) Stratum corneum (SC) lipid composition of skin equivalents grown from normal and AD patient-derived fibroblasts (group 1 AD Fb) before and after exposure to activated $CD4^+$ T cells analyzed by high-performance thin layer chromatography analysis: free fatty acids (FFA), cholesterol (Chol), ceramides (Cer), cholesterol sulfate (CholS), glucosylceramides (GlcCer), sphingomyelins (SM) and phospholipids (PL). (**b**) SC lipid chain order (panel left) and apparent lipid/protein ratios (panel right) of skin equivalents grown from normal and AD patient-derived fibroblasts (group 1 AD Fb) before and after exposure to activated $CD4^+$ T cells. The hydrated stratum corneum samples isolated from the skin equivalents were examined by attenuated total reflectance-Fourier transform infrared spectroscopy by coaddition of 256 scans at 2 cm^{-1} resolution at 23 °C. Each point

represents mean of at least two values recorded at different areas of the sample. The lipid chain order was probed by methylene symmetric stretching vibrations – the higher wavenumber indicated less ordered lipids. The ratios of the intensities of methylene symmetric stretching and amide I vibrations were used as a simple lipid/protein characteristic. (**c**, **d**) Permeability of skin equivalents grown from normal and AD patient-derived fibroblasts (group 1 AD Fb) before and after exposure to activated $CD4^+$ T cells assessed by permeation assays using (**c**) radioactively labelled testosterone and (**d**) Lucifer yellow. Values are given as mean ± SEM. Scale bar = 100 nm. n = 3. * indicates statistical significance (*$p \leq 0.05$; **$p \leq 0.01$). + indicates statistical significance over skin equivalents grown from AD fibroblasts alone (+$p \leq 0.05$; ++$p \leq 0.01$).

3.3 PART 3

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Preliminary development of a mast cell-competent skin equivalent

Mast cells (MCs) contribute to homeostasis in the immune system and play an important role in innate and adaptive immunity. MCs can be sensitized by IgE-dependent mechanism when an antigen crosslinks IgE molecules that are bound to the MC high-affinity IgE receptor (FcεRI) (allergic reaction). In addition, MCs can also be activated independently of IgE by complement cascade products and various cytokines (Krystel-Whittemore et al., 2015). Following activation, MCs release granular mediators (e.g. histamine, tryptase) and produce a large variety of cytokines, chemokines and growth factors, whereby they regulate recruitment, trafficking and functions of other immune cells and affect the function of keratinocytes (Kanda & Watanabe, 2007; Nakae et al., 2006; Kohda et al., 2002; Walsh et al., 1991).Due to the increased MC numbers in lesional skin, especially in the chronic state, IgE elevation, broad array of pro-inflammatory mediators secreted from mast cells as well as sensitization to allergens, MCs seems to play a pivotal role in the pathogenesis of AD (Weidinger et al., 2018; Kawakami et al., 2009). However, their role in AD pathogenesis is not completely understood (Sehra et al., 2016).

Hence, this chapter focuses on the establishment of mature skin MC isolation, their long-term cultivation and subsequent integration into our in-house skin equivalent using MC numbers comparable to healthy and atopic skin. In addition to normal skin equivalents, MCs were also integrated into our filaggrin-deficient skin equivalent to study the possible impact of MCs on diseased equivalents.

Mast cell (MC) cultivation in presence of IL-4 and stem cell factor (SCF) ensures stable expression of the mature MC markers FcεRI and c-Kit

Skin MCs were isolated according to previously published procedures (Guhl et al., 2011). Low cell numbers of skin MCs were isolated from foreskin (4 x 10^5 cells per mg). Due to their *in vitro* cultivation in presence of IL-4 and SCF, the MCs began to proliferate at about week 2. Hereafter, they doubled in cell number with each week of cultivation. In the first passages, several adherent mesenchymal cells were present (Fig. 3.1a), however the MC purity increased with every passage since the MCs are not adherent and can be easily passaged without using trypsin (Fig. 3.1b). After a cultivation period of six weeks, MC purity exceeded 70%, as assessed by expression of c-Kit (CD117) and FcεRI (Fig. 3.1c).

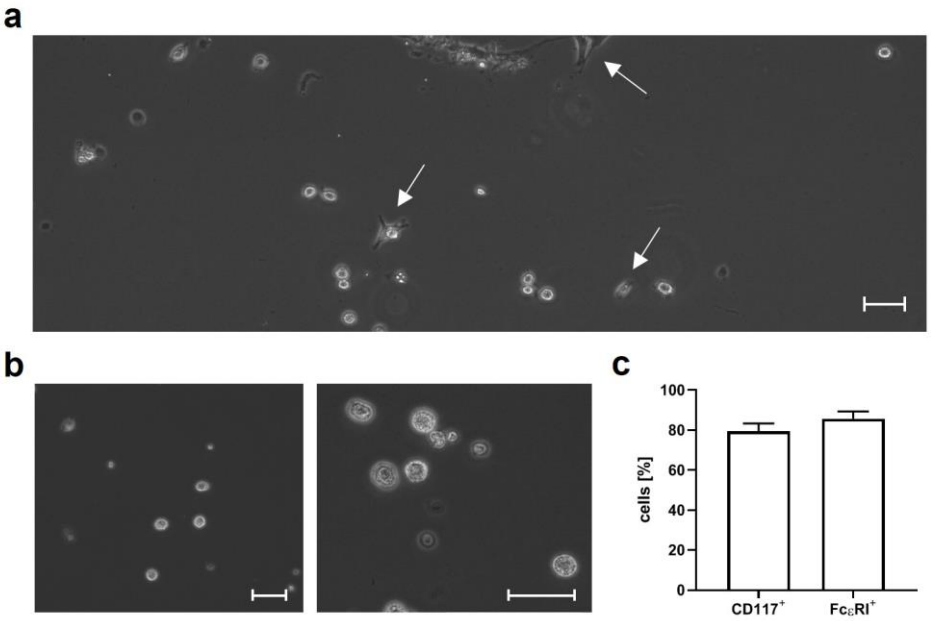

Figure 3.1. Mast cell (MC) appearance and their expression of c-Kit (CD117) and FcεRI is stable over 4 weeks *in vitro* cultivation. (**a**, **b**) Phase contrast microscopy images from MCs (**a**) in passage 1 (24 h after isolation) with visible mesenchymal cells (white arrows) and (**b**) in passage 4. (**c**) Expression of the MC surface marker c-Kit (CD117) and FcεRI of MCs after a cultivation period of six weeks assessed by flow cytometry. Scale bar = 50 nm. Values are given as mean + SEM. n = 4.

Integration of mast cells (MCs) increased thickness of stratum corneum only in filaggrin-deficient skin equivalents

After cultivation of six weeks, MCs were integrated in the dermal compartment of the skin equivalent at a density of either 40 MCs/mm^2 or 80 MCs/mm^2. In either case, the skin equivalents revealed no distinct histological differences in the epidermal differentiation of normal skin equivalents with integrated MCs compared to normal skin equivalents alone (Fig. 3.2a). Histological analysis demonstrated a well differentiated epidermis with all

epidermal layers expressed in native human skin, starting with the erected cells of the stratum basale, differentiating through to stratum spinosum, granulosum, and finally the stratum corneum (Fig. 3.2a, upper row). Filaggrin-deficient skin equivalents were characterized by epidermal thickening, spongiosis and parakeratosis (Fig. 3.2a, lower row). Interestingly, a thickened stratum corneum (hyperkeratosis) was observed in filaggrin-deficient skin equivalents in a manner apparently proportional to MC number (Fig. 3.2a, lower row).

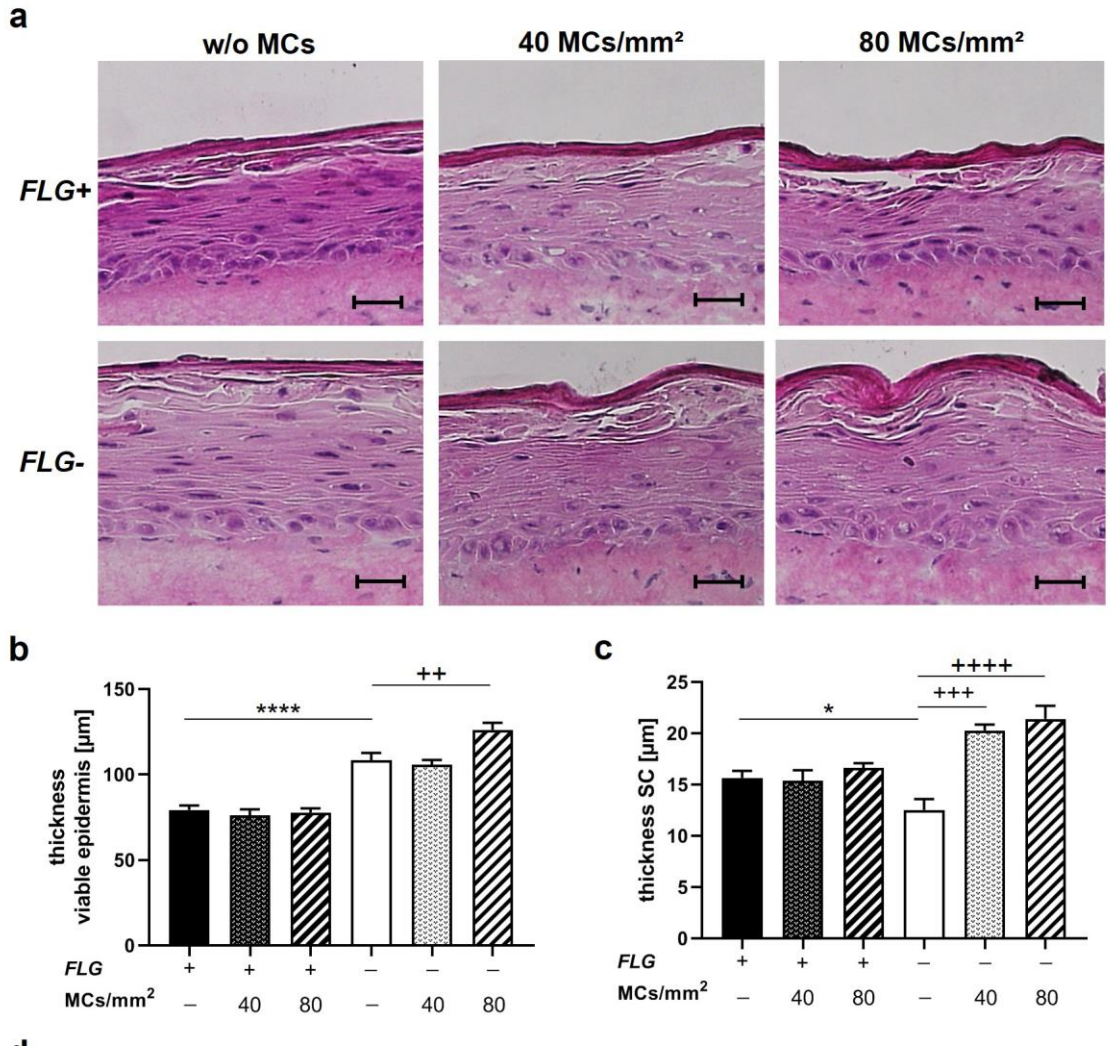

Figure 3.2. Histological analysis of normal and filaggrin knockdown skin equivalents alone and with integrated mast cells (MCs). (**a**) Representative hematoxylin & eosin (H&E) staining of normal (*FLG*+) and filaggrin-deficient (*FLG*-) skin equivalents grown alone or with integrated 40 MCs/mm^2 or 80 MCs/mm^2. (**b**, **c**) Thickness of (**b**) the viable epidermis and (**c**) the stratum corneum (SC) of normal (*FLG*+) and filaggrin-deficient (*FLG*-) skin equivalents grown alone or with integrated 40 MCs/mm^2 or 80 MCs/mm^2. * indicates statistical significance over normal (*FLG*+) skin equivalents without MCs (*$p \leq 0.01$; ****$p < 0.0001$). + indicates statistical significance over FLG knockdown (*FLG*-) skin

equivalents alone. (++$p \leq 0.01$; +++$p \leq 0.0001$; ++++$p < 0.0001$). Scale bar = 50 µm. Values are given as mean + SEM. n = 3.

H&E staining cannot distinguish MCs in the skin equivalents. Therefore, immunofluorescence staining of tryptase, an enzyme stored in MC granules, was conducted. In normal skin equivalents (*FLG*+) with 80 MCs/mm^2, clear staining of tryptase-positive cells was observed (Fig. 3.3, left). Notably, in filaggrin-deficient equivalents (*FLG*-), cells with only small amounts of tryptase were detected (Fig. 3.3, right).

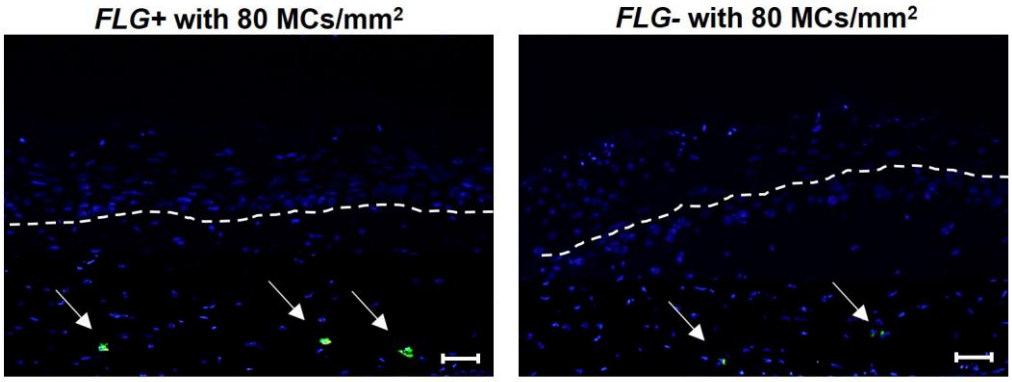

Figure 3.3. Immunolocalization of tryptase to detect mast cells (MCs) in normal and filaggrin (*FLG*) knockdown skin equivalents. Representative immunostaining against MC content tryptase (green) of normal (*FLG*+) and filaggrin-deficient (*FLG*-) skin equivalents grown with 80 MCs/mm^2. Cell nuclei were counterstained with 4',6'-diamin-2-phenylindol (DAPI, blue). White dotted line = epidermal-dermal junction. Scale bar = 50 µm. n = 3.

Trend towards an inflammatory status was observed as a result of mast cell integration

A trend towards an increased level of the pro-inflammatory cytokine IL-6 in the culture medium was observed in MC-competent skin equivalents, whereby higher MC numbers enhanced the IL-6 level (Fig. 3.4a). This effect occurred in addition to the impact of filaggrin-deficiency upon IL-6 release. Although MCs are one major source of IL-4, skin equivalents without MCs secreted higher levels of IL-4 compared to skin equivalents with integrated MCs (Fig. 3.4b). Histamine could not be detected in culture medium of skin equivalents with or without MCs.

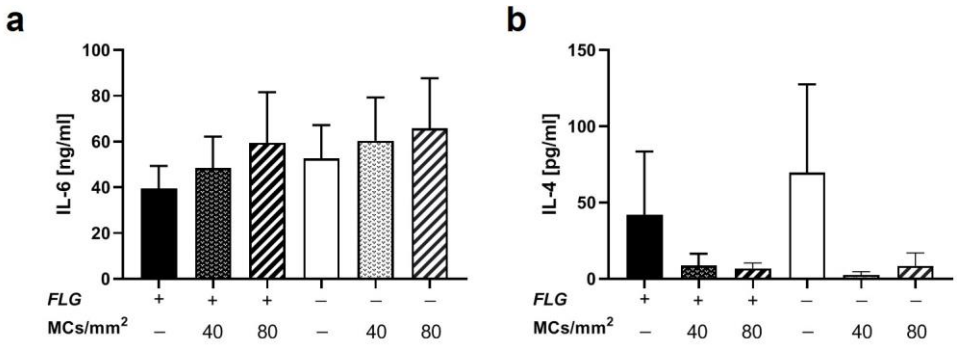

Figure 3.4. Integration of mast cells (MCs) induces a trend towards an inflammatory status in normal (*FLG*+) and filaggrin-deficient (*FLG*-) skin equivalents. Levels of (**a**) the pro-inflammatory cytokines IL-6 and (**b**) the anti-inflammatory cytokine IL-4 in *FLG*+ and *FLG*- skin equivalents alone and with integrated 40 MCs/mm^2 or 80 MCs/mm^2. Values are given as mean + SEM. n = 3-4.

Decreased expression of mature MC surface markers either after cultivation in skin equivalents or in monoculture in absence of SCF and IL-4

To assess the cell number of mature MCs after two weeks of cultivation in skin equivalents, the dermal cells (fibroblasts and MCs) were isolated from the dermal equivalent and the expression of FcεRI and CD117 (c-Kit) was assessed by flow cytometry. Theoretically calculated, if integrated MCs are still mature in skin equivalents, 40 MCs/mm^2 should mean 5.3% of the cells of the dermal equivalent express both surface markers, and 80 MCs/mm^2 10% of the cells. In normal (*FLG*+) skin equivalents grown with 40 MCs/mm^2, only 3.7% of the cells from the dermal compartment still expressed the surface marker CD117 (Fig. 3.5a). For skin equivalents grown with 80 MCs/mm^2, even fewer cells expressed CD117 (3.5%). By contrast, filaggrin-deficient skin equivalents (*FLG*-) gave a higher number CD117 positive cells in skin equivalents grown with 80 MCs/mm^2 compared to skin equivalents grown with 40 MCs/mm^2 (3,7% in skin equivalents grown with 80 MCs/mm^2; 2.3% in skin equivalents grown with 80 MCs/mm^2). Interestingly, no FcεRI positive cells were detected in skin equivalents with integrated MCs. Due to the small cell numbers expressing the surface marker CD117 and the lack of FcεRI positive cells, the effect of IL-4 and SCF absence on MC surface markers in monocultured MCs was examined. After a cultivation period of six weeks, mature MCs were cultivated in the absence of SCF and IL-4 for two weeks. Subsequently, the expression of FcεRI and CD117 (c-Kit) was assessed by flow cytometry. Here, significantly decreased mast cell numbers expressing CD117 or FcεRI c-Kit) were detected (Fig. 3.5b).

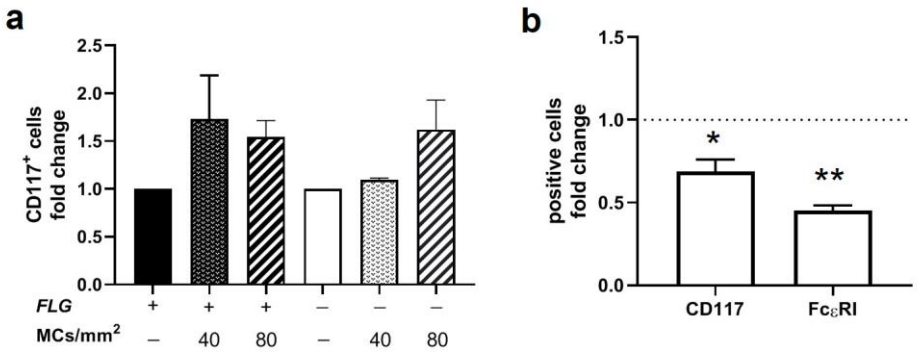

Figure 3.5. Number of FcεRI and CD117 (c-Kit) positive cells decrease following cultivation in skin equivalents and in monoculture in absence of stem cell factor (SCF) and IL-4. (**a**) Ratio of cells in dermal equivalent expressing MC surface marker c-Kit (CD117) and FcεRI after cultivation in normal (*FLG*+) and filaggrin-deficient (*FLG*-) skin equivalents. (**b**) Ratio of MCs expressing the surface marker c-Kit (CD117) and FcεRI cultured in monoculture in absence of SCF and IL-4 against those of MCs cultured in the presence of SCF and IL-4 after a cultivation period of two weeks. Values are given as mean + SEM. n = 3-4. * indicates statistical significance over MCs cultivated in presence of SCF and IL-4 (*$p \leq 0.01$; **$p \leq 0.01$).

4 FINAL DISCUSSION AND OUTLOOK

4.1 FINAL DISCUSSION

Both the EU Directive 2010/63/EU and the EU Cosmetic Directive 76/768/EEC encourage the replacement, reduction, and refinement of animal testing, whereby the EU Cosmetic Directive 76/768/EEC has banned the testing of finished cosmetic products and cosmetic ingredients on animals from March 11, 2013. As such, there is an ever more urgent need for physiologically relevant *in vitro* skin equivalents. Today, organotypic skin equivalents are integrated in OECD guidelines such as for the assessment of skin corrosion (OECD, 2019a) and irritation (OECD, 2019b). In addition, skin equivalents have gained increasing attention in fundamental research for the study of normal and abnormal skin biology (Popov et al., 2014; van den Broek et al., 2012; Xie et al., 2010; Shepherd, J. et al., 2009; Coolen et al., 2008; Breetveld et al., 2006). Thus, it is not surprising, that skin equivalents have been used to model various skin diseases – such as psoriasis, ichthyosis vulgaris and AD – for preclinical and fundamental research (Eckl et al., 2011; Küchler et al., 2011; Jean et al., 2009).

In the field of AD research, there are several established *in vitro* skin equivalents. Overviews of these can be found in the review from de Vuyst and colleagues (De Vuyst et al., 2017) and from my own group (Löwa et al., 2018a). To generalize, disease models are currently generated by modulation of disease-associated genes (Wallmeyer et al., 2015; Vávrová et al., 2014; Eckl et al., 2011; Küchler et al., 2011), addition of disease-associated stimuli such as cytokines (Hönzke et al., 2016; Gschwandtner et al., 2013; Kamsteeg et al., 2011), and/or co-cultivation with immune cells (Wallmeyer et al., 2017; Engelhart et al., 2005). Generation of skin disease equivalents using patient-derived cells is another possibility (Berroth et al., 2013; Roggenkamp et al., 2013), an approach not often used in AD models. Aquisition of patient-derived skin cells mostly relies on skin biopsies, an invasive method associated with complications in healing process, scar formation as well as infections (Nischal et al., 2008), all of which are less than desirable to AD patients. Thus, the intention behind the first part of this thesis was to extend the non-invasive isolation method of the hair follicle-derived cells, the basis of which was established in my diploma thesis, to a comprehensive characterization of the isolated cells and to use these for the generation of full-thickness skin equivalents.

Plucked hair follicle as potential cell source for 3D full-thickness skin equivalents

In the last decade, plucked hair follicles have gained increasing attention in the field of regenerative medicine (Schembri et al., 2013). The hair follicle not only represents an interesting stem cell source but also offers a simple and accessible route to obtain keratinocytes from the outer root sheath (ORS) (Aasen et al., 2010). Autologous

organotypic cultures of ORS cells from plucked hair follicles have been already used to cover recalcitrant chronic leg ulcers, demonstrating for the first time the innovative application of hair follicle-derived cells (Limat et al., 2002; Limat et al., 1996b). Various methods exist for the isolation of ORS keratinocytes from plucked hair follicles (Aasen et al., 2010; Detmar et al., 1993), either through direct outgrowth on tissue culture plastic (Limat et al., 2002; Moll, I., 1996) or by enzymatic digestion (Limat & Noser, 1986). The method with the highest cell yield should be used for the generation of skin equivalents. According to the results of my diploma thesis, direct outgrowth approach (Löwa, 2015a) following the protocol from Limat and colleagues showed the best results (Guiraud et al., 2014; Limat et al., 2002). After the primary cultivation, the cells were frozen, and then subcultured in keratinocyte growth medium, as described by Limat *et al.* (Limat & Hunziker, 1996a; Limat et al., 1996b). In passage 2, the cultured hair follicle-derived keratinocytes (HFDK), as well as normal human keratinocytes (NHK) expressed cytokeratin (CK)5 and CK14, molecular markers of the basal epidermal layer (Chapter 3.1, (Löwa et al., 2018b), Fig. 1b) in line with a previous study (Alam et al., 2011). Both cell types were negative for CK10, a marker of the suprabasal layer of the epidermis, indicating an active mitotic status for both cell types (Moll, R. et al., 2008). In addition, CK17 was expressed by both cell types, which shows that HFDK originate from the ORS (Troyanovsky et al., 1989) and NHK are activated (Proby et al., 1993) (Chapter 3.1, (Löwa et al., 2018b), Fig. 1b).

Interestingly, secondary cultivation of hair follicle-derived cells without feeder cells in serum-enriched cell culture medium led to a spindle-shaped cell type that look microscopically like fibroblasts (Löwa, 2015a); sometimes these were already visible during outgrowth (Chapter 3.1, (Löwa et al., 2018b), Fig. S1b). This observation was unexpected, since the isolation of mesenchymal cells such as dermal papilla or dermal sheath cells has only been described using micro-dissected hairs and rather than plucked hairs (Higgins et al., 2017; Wu et al., 2010; Limat et al., 1993; Messenger, 1984). The opportunity to obtain keratinocytes and fibroblasts from one donor without use of an invasive method would be a perfect starting point to study skin diseases. However, the use of the here isolated cells should be considered with caution, since the precise subtype of the isolated ‘fibroblasts’ was not clear. Several publications have described the fibroblast heterogeneity, in terms of gene expression profile, according to anatomical origin (Lynch et al., 2018). This heterogeneity reflects a combination of intrinsic differences and the role of environmental factors such as differences in mechanical stresses between body regions (Wong, V. W. et al., 2011). Different characteristics have been described of skin fibroblasts derived from papillary and reticular skin layers and fibroblast subpopulations associated with distinct hair follicle regions such as hair follicle dermal papilla and dermal sheath fibroblasts (Sorrell et al., 2004; Jahoda, C. A. et al., 1996; Sorrell et al., 1996; Jahoda, C. A. et al., 1991; Schafer

et al., 1985; Azzarone & Macieira-Coelho, 1982; Harper & Grove, 1979). Thus, characterization of our hair follicle-derived fibroblast was crucial, especially regarding their intended use in the generation of skin equivalents. However, no specific skin fibroblast marker has yet been described (Kalluri et al., 2006). Hence, 'fibroblasts' from the hair follicle, termed hair follicle-derived fibroblasts (HFDF), have been evaluated with regard to cell-specific markers and gene expression profiles as compared to normal human dermal fibroblasts (NHDF). This has already been discussed in the publication, but I would like to take up this again in more detail as the thesis is mainly based on the AD fibroblasts. As expected, no differences in the expression between interfollicular fibroblasts (NHDF) and HFDF were observed for vimentin (VIM) and desmin (DES) expression (Löwa, 2015a), which are expressed in all fibroblast types (Jahoda, C. A. et al., 1991; Mork et al., 1990; Schmid et al., 1982). The weak-positive expression of versican (VCAN) as well as alpha smooth muscle actin (alpha SMA) in *in vitro* cultured NHDF (Chapter 3.1, (Löwa et al., 2018b), Fig. 1a) suggests a mixed population of papillary and reticular fibroblasts, since VCAN and in some cases alpha SMA are only expressed by reticular fibroblasts (Sriram et al., 2015). The number of HFDF expressing alpha SMA and VCAN were slightly higher compared to NHDF. Notably, alpha SMA is a characteristic marker for the expression of dermal sheath fibroblasts *in vivo*. Dermal papilla fibroblasts also become alpha SMA positive upon *in vitro* cultivation (Jahoda, C. A. et al., 1991). Hence alpha SMA cannot be used to distinguish between these cells. The same applies to versican. Though fresh isolated dermal papilla cells show high VCAN expression while dermal sheath fibroblasts exhibit only weak VCAN immunoreactivity (Sriram et al., 2015; Ohyama et al., 2012), Ohyama and colleagues showed a downregulation of VCAN expression following longer *in vitro* cultivation period (Ohyama et al., 2012). Thus, due to the lack of a stable marker for *in vitro* culture, the origin of these mesenchymal cells from the plucked hair follicle could not be identified. Nevertheless, I was able to refute the assumption of my diploma thesis that these cells could be derived from stem cells, since a differentiation of hair follicle-derived cells into adipocytes or chondrocytes was not successful. Instead, there are two points in favor of their being dermal sheath cells. Firstly, plucking of hair follicles with dermal papilla was observed only in a few cases, similar to results from Bassukas and colleagues (Chapter 3.1, Fig. 1.1) (Bassukas et al., 1989). Secondly, during the outgrowth of hair follicle-derived cells for certain donors, spindle-shaped cells were already visible at the edge of the ORS (Chapter 3.1, (Löwa et al., 2018b), Fig. S1b). These could indicate the here mentioned HFDF originate from the dermal sheath. The difficulties to determine the cell type HFDFs indicates, that the simple definition of cellular identity represented by the expression of a distinct set of macromolecules at a specific time, omits the possibility, that cells with identical gene expression patterns at a particular time may react differently to an external

stimulus or differentiate spontaneously due to epigenetic or other factors (Lynch et al., 2018). This is an important point for *in vitro* cell cultivation, that has received more attention in recent years. However, this thesis is not intended to shed any further light on this issue.
It focused instead on the use of both hair follicle-derived cell types for the generation of full-thickness skin equivalents (Chapter 3.1). Therefore, the following cell combinations were used: NHDF and NHEK, NHDF and HFDK, HFDF and NHEK, as well as HFDF and HFDK. Histological analysis of all generated skin equivalents showed normal epidermal phenotypes, comparable to *in vivo* stratification, as characterized by correct immunolocalization of epidermal markers: the basal keratinocyte marker CK14, the tight junction proteins claudin-1 (CLDN1) and occludin (OCLN), and the differentiation proteins CK10, filaggrin (FLG), involucrin (IVL) and loricrin (LOR) (Chapter 3.1, (Löwa et al., 2018b), Fig. 2, 4, 5, S5) (Wang et al., 2016; Kirschner & Brandner, 2012). These findings were also previously described for skin equivalents grown from HFDK (Guiraud et al., 2014; Limat et al., 2002; Wiszniewski et al., 2000; Limat et al., 1996b; Limat et al., 1991a). As already discussed in the publication, minor differences were observed in terms of epidermal organization, expression levels of tight junction proteins and differentiation proteins, as well as basal lamina formation. However, a clear epidermal stratification and correct immunolocalization of the markers in all generated skin equivalents elucidate the possibility to use all combinations with patient-derived cells with the requirement the control equivalent is grown using cells from the same cell source.
In summary, a new non-invasive procedure for obtaining keratinocytes and fibroblasts from plucked hair follicles was developed. Since, plucked hair follicles are samples that can be easily taken and cause little to no discomfort for patients, this study laid the basis for the generation of skin equivalents using patient-derived cells.

Cultivation of AD patient cells from plucked hair follicle

Approximately, 0.9 to 2.5 million cells in passage 0 (p0) can be obtained from 10 plucked anagen hair follicles of healthy donors. In line with Limat *et al.*, isolated HFDK appeared smaller and compactly arranged during outgrowth (p0) than HFDK after a trypsinization step (in p1) irrespective of cultivation on feeder cells and/or alone. In p1, HFDK are less compactly arranged with numerous cells of larger size (Limat et al., 1996b; Limat et al., 1986) (Chapter 3.1, Fig. 1.2). This indicates that cells should already be used in lower passages, but this is also associated with a lower cell yield. Therefore, the established protocol for skin equivalents in a 6-well format was adjusted to a 12-well format to accommodate the low cell yield from the hair follicles, requiring 0.9 million keratinocytes instead of 4.2 million. Nevertheless, it must be noted that for each analysis, e.g. histology, or protein expression, one equivalent is needed. This means that a detailed analysis

including histology, barrier function, gene and protein expression requires about 4 million cells in total. However, even fewer keratinocytes (~0.6 to 2.0 million cells in p0) can be obtained from 10 plucked hair follicles of AD patients compared to healthy donors. Additionally, hair follicle-derived cells of AD patients showed a much lower proliferative capacity and increased cell size as early as p1 (Chapter 3.1, Fig. 1.2). Although, the high sensitivity of HFDK to calcium was taken into account when choosing the keratinocyte growth medium, testing of different keratinocyte media did not lead to better results. Interestingly, fibroblasts grew well irrespective whether derived from either healthy or AD donors.

For the generation of skin equivalents using hair follicle-derived cells from AD patients, one possibility would be to have an ethical approval with permission to pluck more hairs from one donor allowing to use the cells directly after outgrowth for the skin equivalent generation. However, this was not the case in our study. Owing to this and the ever-growing attention fibroblasts have received in the literature surrounding diseases and the underlying mechanisms cellular components communicate in homeostatic and inflammatory conditions is key to understanding the skin's biology and immunity, this thesis focused on the impact of AD fibroblasts in skin equivalents.

Fibroblasts have a major impact on the epidermal homeostasis

AD is the most common skin disease. Although its incidence is increasing worldwide, there is still no satisfying therapy. This is mainly due to the lack of the understanding surrounding underlying pathogenic mechanism. Its complex etiology involves abnormal immunological pathways, and skin barrier disruption, as well as environmental and neurophysiological factors (Tsakok et al., 2019; Baurecht, Hansjörg et al., 2018; Weidinger et al., 2018; Kim, J. E. et al., 2016) complicating efforts to unravel the pathological mechanisms of AD. Because animal models allow in-depth investigation of pathogenesis and provide valuable tools for pharmaceutical purposes, many studies are conducted on animals such as mice. Although we owe several findings to mice models, the relevance of these studies is increasingly questioned. Not only growing political and social pressure, but also an increasing awareness of the often low predictivity animal models hold for the human situation (Leist & Hartung, 2013; Seok et al., 2013) has created an ever increasing interest in alternative methods to animal experiments. Especially, for the AD research, mouse models show many deficiencies. For example, normal mice do not spontaneously develop AD, and the anatomy of rodent skin differs significantly from humans in terms of the number of epidermal cell layers, hair follicles and presence of an additional muscle layer in mice (Löwa et al., 2018a; Williams, F. M., 2006). Furthermore, only ~30% of skin-associated genes overlap between mice and humans (Gerber et al., 2014), and major differences in genes associated with skin

morphogenesis, growth and immunology were found (Williams, F. M., 2006; Diembeck et al., 1999). Therefore, human-based skin equivalents offer a good platform to unravel the underlying pathological mechanism of epidermal barrier abnormalities and T cell-driven skin inflammation. Since *FLG* gene mutations have been identified to increase the odds of developing AD by more than 3-fold (Brown, S. J. et al., 2012), several *in vitro* studies explored the impact of *FLG* knockdown in epithelial cells (Wallmeyer et al., 2017; Hönzke et al., 2016; Vávrová et al., 2014; van Drongelen et al., 2013; Küchler et al., 2011; Mildner et al., 2010). Interestingly, less is known about the role of fibroblasts, although it is widely accepted that fibroblasts exert significant effects on the epidermal regeneration in normal (el-Ghalbzouri et al., 2002) and diseased states (Lynch et al., 2018; Kühbacher et al., 2017; Jean et al., 2009). Furthermore, already commercially available 3D skin diseased models of psoriasis based only on psoriatic fibroblasts and healthy keratinocytes and exhibit a notable psoriatic phenotype (Mattek Corp., USA). Hence, the impact of AD fibroblasts on the epidermal maturation of skin equivalents was investigated in this thesis (Chapter 3.2). AD donors (in total 20 patients) were selected based on a medical diagnosis. Following successful plucking of hair follicles and isolation of HFDF – which was only possible for 10 donors – the *FLG* mutation status of each donor was analyzed. Interestingly, only one donor of the AD patients (10%) carried the most common loss-of-function mutation (2282del4). This value corresponds to values from the epidemiological study of Decker and colleagues (10-50%) (Deckers et al., 2012). As already discussed in the manuscript (Chapter 3.2), independent of the *FLG* mutations, three out of six skin equivalents generated from AD patient-derived fibroblasts showed significant histological changes such as epidermal thickening, parakeratosis, and hyperproliferation (referred to as group 1 AD Fb) (Chapter 3.2, Fig. 1a and Fig. 2.1). Furthermore, a significant downregulation of FLG expression and a trend for a compensatory upregulation of epidermal barrier proteins (IVL, LOR) and tight junction proteins (OCLN, CLDN1) was observed, in line with previous work (Berroth et al., 2013) and data derived from *FLG* knockdown skin equivalents (Wallmeyer et al., 2017; Hönzke et al., 2016; Pendaries et al., 2015; Dang et al., 2014) (Chapter 3.2, Fig. 1b, c and Fig. 2.2, 2.3, 2.4). Interestingly, skin equivalents using fibroblasts from three other AD patients (referred to as group 2 AD Fb) did not differ phenotypically from normal equivalents (normal keratinocytes and fibroblasts). One major fibroblast-specific factor for which differential regulation between atopic and normal conditions has recently been described is leukemia inhibitory factor (LIF) (Berroth et al., 2013). In line with *in vivo* data, a reduced expression of LIF on gene and protein level was observed only in hyperproliferative skin equivalents grown from AD Fb (group 1) (Chapter 3.2, Fig. 1d, e), (Berroth et al., 2013). This result suggests LIF as a key among the epidermal factors triggering the atopic phenotype. The different phenotypes (group 1 vs. group 2 AD Fb)

highlight the heterogeneity and multifactorial nature of AD. Potential explanations as to why equivalents with normal LIF expression and normal epidermal histology came from patients manifesting AD would, at this point, be speculative. One could be a high impact of the recently discussed multiple environmental factors and the mode of action by which they induce the AD-typic type 2 immune deviation in these patients (Matthias et al., 2019; Kantor et al., 2017) or, as already mentioned in the manuscript (Chapter 3.2), a possible correlation of our findings with the disease activity *in vivo*.

In addition to the study of Berroth and colleagues, the expression of key markers for epithelial inflammation thymic stromal lymphopoietin (TSLP) and protease-activated receptor 2 (PAR2) were analyzed. Both markers are highly expressed by keratinocytes in lesional skin of AD patients (Zhu et al., 2017; Sakai et al., 2016; Stefansson et al., 2008; Soumelis et al., 2002) and are significantly upregulated in hyperproliferative skin equivalents (Chapter 3.2, Fig. 1b, c). In line with AD patient data, PAR2 expression was also apparent in lower epidermal layers of hyperproliferative skin equivalents (Frateschi et al., 2011; Caubet et al., 2004; Steinhoff, Martin et al., 2003; Steinhoff, M. et al., 1999). A high expression of PAR2 in keratinocytes and cutaneous immune cells, induces nuclear factor kappa B (NFκB)-mediated overexpression of TSLP, intercellular adhesion molecule 1, TNF-α, and IL-8, all of which amplify leukocyte infiltration and activation (Barr et al., 2019; Briot et al., 2009; Seeliger et al., 2003; Steinhoff, Martin et al., 2003). However, it should be noted above relies on PAR2 activation rather than upregulation alone (Steinhoff, Martin et al., 2003). The activity of PAR2 can be assessed by intracellular calcium mobilization in living cells (Zhu et al., 2017). Since we analyzed snap frozen tissue, which is not suitable for use of a calcium mobilization assay, the activity status of PAR2 could not be determined. Therefore, an indirect proof was chosen. PAR2 is activated by specific serine and cysteine proteases such as kallikreins (KLKs), tryptase, cathepsin, dust mice allergen proteases (Lee et al., 2010). Kallikreins, especially kallikrein 5 (KLK5), have frequently been implicated in AD (Moniaga et al., 2013; Frateschi et al., 2011; Briot et al., 2009). Gene expression analysis showed increased expression of *KLK5* ($p = 0.0811$) in hyperproliferative skin equivalents (group 1 AD Fb), as well as significant increases in *TSLP*, *KLK5*, *IL6*, *IL8,* and *TNFA* gene expression as well as TSLP on protein expression (Chapter 3.2, Fig. 2.7a, b), all of which indicates the presence of active PAR2 (Frateschi et al., 2011; Briot et al., 2009; Hou et al., 1998). Nevertheless, there exist a controversial study, that showed that PAR2 has a higher response to transient KLK5 stimulation, but a weaker response to persistent KLK5 stimulation. Interestingly, despite the low activity of PAR2 in cells overexpressing KLK5, these cells upregulated and secreted pro-inflammatory and Th2-polarizing cytokines, including IL-8, IL-10, and TSLP, which indicate that persistent KLK5 induce IL-8, IL-10, and

TSLP (Zhu et al., 2017). However, a mathematic model for KLK5 activation system showed PAR2 is cleaved by active KLK5 to be activated and internalized (Tanaka et al. 2011).
The reason for the increased expression of *KLK5*, PAR2 and finally TSLP can only be speculated. In *in vivo* skin, KLKs are tightly regulated by pH, with peak activity between pH 7 and 8, and their inhibitors including the canonical Kazal-type inhibitors from the SPINK/LEKTI family (Serine Proteinase Inhibitor Kazal-type/Lympho-Epithelial Kazal-Type Inhibitor) (Jones et al., 2015). Interestingly, *SPINK5* gene dysfunction correlates with the development of AD and Netherton syndrome, a severe genetic skin disease (Walley et al., 2001). Briot and colleagues showed that the lack of endogenous protease inhibitor LEKTI activity in a *Spink5* knockout mouse model resulted in increased KLK5 stimulated PAR2, and PAR2 subsequently activates NFκB-induced overexpression of TSLP inducing pruritus (Briot et al., 2009). Therefore, a downregulation of LEKTI directly or an alkaline pH (Eberlein-König et al., 2000) could be one explanation. Altered LIF activity is another potential candidate, given it seems to play an important role in AD and has been shown to induce pro-inflammatory effects and altered protease expression (TIMP, caspase) (Fitzgerald et al., 2005) that could influence epidermal pH. Usually, increases in pH and in serine protease activity result in increased microbial colonization and accelerated degradation of the ceramide synthesis enzymes (Hachem et al., 2006). Interestingly, although we observed increased skin permeability and lipid disordering in skin equivalents grown from AD patients, consistent with clinical findings in AD patients (Janssens et al., 2012), we could not confirm a decrease of Cer levels in the SC (Chapter 3.2, Fig. 2.5, 2.6). Since we could only observe histological differences in equivalents derived from group 1 of AD Fb, we ultimately focused on this group when introducing activated $CD4^+$ T cells to explore immunological component in AD skin equivalents. Exposure of the skin equivalents to $CD4^+$ T cells resulted in active migration of the T cells into the dermis equivalent (Chapter 3.2, Fig. 3), well in line with previous reports from our group (Wallmeyer et al., 2017). Here, T cell migration was directly triggered by TSLP, which is also significantly enhanced in the AD skin equivalents with histological changes. Our data clearly demonstrate that AD diseased fibroblasts may trigger and/or maintain inflammatory conditions. In line with T cell competent *FLG* knockdown disease equivalents of AD, the upregulation of the Th2 cytokine IL-13 and Th2 master regulator *GATA3* in skin equivalents grown from AD fibroblasts (Chapter 3.2, Fig. 3b, S2) indicates a Th2-profile of the T cells (Wallmeyer et al., 2017). This observation supports the assumption that the release of alarmins from the epithelial barrier initiate a Th2 cell-mediated response (Weidinger et al., 2018). In line with previous findings, the initial compensatory upregulation of the epidermal barrier protein LOR and the tight junction proteins OCLN and CLDN1 in AD fibroblasts skin equivalents were abolished after exposure to T cells or their cytokines (Chapter 3.2,

Fig. 2.9) (Wallmeyer et al., 2017; Hönzke et al., 2016). Previously, the disturbed compensatory upregulation was interpreted T cell-derived cytokines inhibiting the expression of barrier proteins through a STAT-6 dependent mechanism (Kim, B. E. et al., 2008) or the S100 calcium-binding protein A11 (Howell et al., 2008). Most interestingly, we observed a significant attenuation of PAR2 expression and slightly diminished TSLP expression in skin equivalents grown from AD fibroblasts after T cell exposure. This effect was already described for TSLP expression in FLG-deficient skin equivalents supplemented with Th2 cytokines (Hönzke et al., 2016) and seemed to be unexpected at the time. However, as already discussed in the manuscript (Chapter 3.2), Strid and colleagues' postulate Th2 cytokines like IL-13 in the skin may induce physiological homeostatic activities in skin. IL-13 activates two complex receptor systems consisting of IL-4Rα and IL-13Rα1, or IL-13Rα2, all of which are expressed by keratinocytes. Their expression pattern may change as a result of stress resulting in e.g. downregulation upon disturbed FLG expression (Strid et al., 2016). In addition, IL-13Rα2 is highly upregulated in keratinocytes from AD lesions (Lü et al., 2009) and its expression in keratinocytes can be induced *in vitro* by IL-13 treatment. Interestingly, the deletion of IL-13Rα2 in mice resulted in cutaneous inflammation and increased transepidermal water loss as well as increased STAT6 signaling in keratinocytes (Sivaprasad et al., 2010). Hence, IL-13Rα2 seems to exert protective functions at increased IL-13 levels. This hypothesis may explain the attenuation of, in particular, PAR2 expression in the diseased skin equivalents. Nevertheless, consistent with the suggested homeostatic role of IL-13 skin barrier function (Strid et al., 2016), several ceramides subclasses (including ω-O-acylceramide EOS) increase upon T cell exposure (Chapter 3.2, Fig. 2.11), indicating enhanced ceramide synthesis and/or their release from precursors. In addition, the increased ratio of the very long chain ceramide NS to shorter ceramide NS2 suggested enhanced elongation of fatty acids. These changes in SC lipid profiles are in strong contrast with the diminished and shortened ceramides found in AD skin (van Smeden & Bouwstra, 2016) where the sustained expression of high IL-13/IL-4 levels damage the skin barrier. Furthermore, these effects may also be connected to the anti-inflammatory effects of LIF (Berroth et al., 2013). In line with previous studies, increased expression of LIF was observed following activation of T cells (Gao, W. et al., 2009). Increased LIF expression in the T cells and fibroblasts containing dermal compartment of skin equivalents, and the secretion of LIF in culture medium of skin equivalents after T cell exposure (Chapter 3.2, Fig. S3) could explain the diminished expression of PAR2. However, this hypothesis requires further evaluation.

Overall, our results show AD fibroblasts have an impact on epidermal differentiation including compensatory upregulation of skin barrier proteins in full-thickness skin equivalents. We observed similar events compared to AD lesional skin, indicating

fibroblasts are a key factor in the development of AD, independent of *FLG* mutation status. Furthermore, the AD-like features in the skin equivalents grown from AD fibroblasts were sufficient for T cell stimulation. In the following section, the influence of AD fibroblasts on angiogenesis and extracellular matrix (ECM) will be discussed.

Angiogenic effects of AD fibroblasts

Angiogenesis describes the formation of new capillaries from pre-existing blood vessels that contributes to organ growth during embryogenesis. In steady state adult tissue, angiogenesis is mostly absent, occurring only in the cycling ovary and placenta (Carmeliet & Jain, 2011; Norrmén et al., 2011). During wound healing and repair, however, angiogenesis is reactivated (Staton et al., 2010; Nguyen et al., 2009). In wounded skin, angiogenic factors including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), angiogenin, IL-8 and IL-17 are released (Genovese et al., 2012; Sismanopoulos et al., 2012; Detoraki et al., 2009; Groneberg et al., 2005). These are also released by inflammatory cells such as mast cells, though to a lesser degree. In malignancies and inflammatory skin disorders, these factors are released excessively, contributing to the 'angiogenic switch' in skin (Nguyen et al., 2009), wherein 'sprouting angiogenesis' forms new capillaries from pre-existing blood vessels (Genovese et al., 2012). One example for an extensively studied skin diseases in terms of angiogenesis is psoriasis. The role of angiogenesis in AD has, by contrast, been largely neglected (Guttman-Yassky et al., 2011a, 2011b). Today, we know excessive angiogenesis and increased VEGF plasma levels occur in AD patients as compared to healthy controls (Brockow et al., 2002). In recent years, the role of fibroblasts in physiological and pathological angiogenesis became well recognized. Although several studies have shown the possible secretion of soluble angiogenic growth factors e.g. VEGF (Kellouche et al., 2007; Fukumura et al., 1998), transforming growth factor-beta (TGF-β; (Paunescu et al., 2011)), and platelet-derived growth factor (PDGF; (Antoniades et al., 1991)) – the precise mechanisms underlying their action and, to our best knowledge, the connection between angiogenic factors and AD fibroblasts have yet to be determined. To study this aspect *in vitro*, established vascularized skin equivalents grown from normal keratinocytes and the already studied AD fibroblasts would be ideal. However, vascularization in tissue engineering remains a long-term goal rather than a reproducible reality. As of the time of writing, the only existing vascularized skin model uses a perfused vascular network combining a biological vascularized scaffold (BioVaSc) formed a decellularized segment of a porcine jejunum and a tailored bioreactor system (Groeber et al., 2016) and rather than a self-assembled extracellular matrix.

To study the effect of atopic fibroblasts on the organization of endothelial cells, an *in vitro* angiogenesis assay was established using AD fibroblasts and dermal microvascular endothelial cells. The fibroblasts were first stimulated to produce their own extracellular matrix, subsequent to which dermal microvascular endothelial cells were added. Consistent with clinical findings from AD patients, we observed an increased number of endothelial tubes (Varricchi et al., 2015) indicative of pro-angiogenic effects derived from the AD fibroblasts themselves (Chapter 3.2, Fig. 2). Additionally, increased VEGF levels were found in the culture medium of hyperproliferative skin equivalents grown from group 1 AD Fb, suggesting that the fibroblasts trigger and/or release VEGF.

Since the fibroblasts were stimulated to produce their own ECM in the angiogenesis assay and effects on the endothelial cells were observed, the idea arose to take a closer look at the ECM of the fibroblasts. Therefore, fibroblasts were cultured in presence of ascorbic acid in a monoculture system, since ascorbic acid has been shown to be effective in cell proliferation and collagen expression (Chojkier et al., 1989; Murad et al., 1981). Although, a production of ECM could be observed by H&E staining, strong donor differences were observed regarding the thickness of produced ECM layer. This may be related to different proliferation rates of the fibroblasts as well as the formation of the ECM. ECM is a complex mix of fibrillar proteins and polysaccharides synthesized and secreted by the cells into the extracellular space. Quantification of these ECM components is still very technically challenging due to the complexity of the structure. There are very few labs capable of such an analysis. However, since ECM directs cellular growth, metabolism and differentiation as well as adhesion and migration (Valiente-Alandi et al., 2016), all of which are highly relevant to disease state, this would be an interesting point to study.

Mast cell-competent skin equivalent

Fibroblasts from atopic dermatitis (AD) patients showed alterations in epidermal differentiation, maturation and inflammation state of full-thickness skin equivalents (Chapter 3.2). With the help of T cells, an immunological component could also be examined. However, regarding immunological aspects, the pathogenesis of AD is far more complex, since also other skin-resident cells such as mast cells (MCs) are involved (Tay et al., 2014). Following their activation, mast cells degranulate and release of a diverse spectrum of biologically active mediators, including tryptase and histamine, into the extracellular environment. The activation is induced by either IgE-dependent or IgE-independent mechanisms (Bulfone-Paus et al., 2017). IgE-independent mechanisms include activation by the c-Kit ligand stem cell factor (SCF), immune complexes of IgG, various complement peptides, cytokines and chemokines (Galli et al., 2008; Galli et al., 2005). Interestingly, overexpression of TSLP in AD lesions can also activate mast cells to

generate Th2 cytokines (Nagarkar et al., 2012). However, the most common physiological pathway for mast cells activation is via their expressed high-affinity IgE receptor (FcεR1) following crosslinking of surface-bound IgE by allergens (Sibilano et al., 2014). Overall, the released mediators increase the permeability of capillaries, evoke scratching behavior and recruit leukocytes to the site of allergic insult (Kim, J. E. et al., 2016; Bischoff, 2007).

Hence, it is assumed that MCs contribute to skin inflammation and have been already implicated in a wide variety of skin diseases including AD. To further increase the complexity of our in-house skin equivalent, the preliminary development of a MC-competent skin equivalent was performed. Although MCs are of hematopoietic origin, their complete maturation occurs in the tissue from which they need to be extracted for the specific research purpose. Therefore, skin MCs were isolated from juvenile foreskin for this project. One of the main disadvantages of this method is the small mean number of cells that can be obtained – 38.8 $\pm$ 3.5 MCs/mm^2 in normal skin (Rabenhorst et al., 2012). The expansion and long-term cultivation mature skin MCs expressing c-Kit (CD117) and high-affinity IgE receptor (FcεRI) were achieved according to a protocol of Guhl *et al.* by cultivation in the presence of stem cell factor (SCF) and IL-4 (Chapter 3.3, Fig. 3.1) (Guhl et al., 2011). In addition to the integration of mast cells into normal skin equivalents, this project aimed to investigate histological effects of mast cells in AD models, that have never been done before. Due to the donor differences of primary AD fibroblasts, it was decided not to use them for the first establishment steps. Instead, filaggrin-deficient in-house equivalent was used. Furthermore, since the MC number (40 MCs/mm^2 for normal skin and 80 MCs/mm^2 for chronic skin lesions (Rabenhorst et al., 2012)) is increased in atopic skin, both features were combined in the skin equivalent. MCs seemed to have neither an ameliorating nor exacerbating effect on the epidermal differentiation in normal (*FLG*+) skin equivalents, however, further evaluation dealing with assessment of differentiation markers such as CK10, FLG, IVL and LOR, tight junction proteins, and the skin barrier function are still required. In line with previous studies, filaggrin-deficient skin equivalents exhibit histological features such as epidermal thickening and parakeratosis comparable to *in vivo* AD skin (Chapter 3.3, Fig. 3.2a) (Hönzke et al., 2016; Küchler et al., 2011). Interestingly, the hyperkeratosis, a characteristic of chronic lichenified lesions of AD (Weidinger et al., 2018), observed only in filaggrin-deficient skin equivalents, may be an indicator of MC activation. As result of an activation, MCs release pro-inflammatory cytokines, such as TNF-α, which are known to alter the stratum corneum properties in skin equivalents (Danso et al., 2014). Furthermore, a possible degranulation, indicated by a diffuse tryptase staining in filaggrin-deficient skin equivalents, implies a degranulation of MC (Chapter 3.3, Fig. 3.2b). However, this is speculative. The level of tryptase in culture medium and ultrastructural analysis of the degranulation should be addressed in the next study to clarify the activation status of the

MCs. Notably, MCs can be activated by overexpression of TSLP in AD lesions (Nagarkar et al., 2012), that is also upregulated in filaggrin-deficient skin equivalents (Wallmeyer et al., 2017). A connection between this fact and the activation of mast cells should be examined.

Alongside tryptase expression, viability of MCs was indirectly assessed by quantifying IL-6 and IL-4 levels in culture medium (Chapter 3.3, Fig. 3.3). As expected, integration of MCs enhanced the levels of the pro-inflammatory cytokine IL-6 in normal and filaggrin-deficient skin equivalents, the latter already demonstrates increased IL-6 release over controls (Babina et al., 2004). Since IL-4 is produced by MCs (Okayama et al., 1995; Brown, M. A. et al., 1987), the decreased level in skin equivalents with integrated MCs was unexpected. However, previous studies have shown that IL-4 prolongs the survival of skin-derived MCs (Babina et al., 2004; Thienemann et al., 2004), which could be an indicator for the assumption of IL-4 remains sequestered within the skin equivalents. Furthermore, the survival of MCs is critically dependent on signaling via the SCF receptor c-Kit (CD117), a member of the receptor tyrosine kinase family (Okayama & Kawakami, 2006). The detected CD117 positive cells in the dermal compartment of skin equivalents after two weeks cultivation are an indicator for the presence of viable MCs (Chapter 3.3, Fig. 3.4a). However, fewer cells were found expressing the mast cell marker than were originally integrated into the equivalent. Fibroblasts are known to produce SCF (Shen et al., 2017), which stimulates c-Kit and maintains the viability of MCs. If SCF release was insufficient owing to low fibroblast numbers and/or their viability, mast cell viability would be expected to suffer. In contrast to Artuc and colleagues who showed positive immunohistological staining of FcεRI, no FcεRI positive cells were detected after isolation from the dermal compartment of the skin equivalent (Chapter 3.3, Fig. 3.4a) (Artuc et al., 2002). However, the expression level of FcεRI in skin MCs is highly variable (Guhl et al., 2010) and can also change after isolation through collagenase treatment, among other methods (Ravindran et al., 2018). Furthermore, the presence of SCF and IL-4 promote not only MC proliferation, but also robust CD117 and FcεRI cell surface expression (Guhl et al., 2011), that are significantly decreased following monoculture of MCs in absence of SCF and IL-4 (Chapter 3.3, Fig. 3.4b). The lack of FcεRI positive cells could explain the undetectable histamine level seen in the equivalent culture medium. Additionally, a possible crosslinking of FcεRI by anti-IgE treatment should be addressed in future studies to examine the functionality of MCs.

The presented procedure for isolating skin MCs, their *in vitro* expansion, and subsequent integration into normal or *FLG* knockdown skin equivalents could serve as a perfect platform to study the impact of MCs and the mastocytosis observed in AD. Nevertheless, future

studies clearly need to focus on the functionality of the MCs in the dermal compartment and the epidermal differentiation of skin equivalents.

In summary, the results described in this thesis demonstrate the possibility to study AD in skin equivalents using patient-derived cells from plucked hair follicle. Even though, skin equivalents using patient cells appear relevant to the investigation of new treatment options in AD, this study clearly substantiates that AD is a highly complex disease and many of the building blocks remain unclear. An increase the numbers of patients and the use of advanced analytical methods are necessary. Furthermore, in order to map this multifactorial disease, a mast cell competent skin equivalent of AD patient cells is a promising biomedical approach for the investigation of complex cell-cell interactions in AD lesions, which is currently still poorly understood.

4.2 OUTLOOK

In vitro skin equivalents are promising tools for the modeling of several physiological and pathological skin conditions. Over the past decades, many different protocols have been developed for the generation of full-thickness skin equivalents. The presented possible use of hair follicle-derived cells from one donor for the generation of skin equivalents not only enables the possibility to generate disease equivalents, but also provides an ideal foundation for immunocompetent models. In particular, functional skin equivalents with Langerhans cells (LCs) remain a challenge, as most LCs are no longer found in the equivalents after a cultivation period of two weeks, possibly due to rejection reaction (Ouwehand et al., 2011; Bechetoille et al., 2007). Therefore, the possibility of taking blood samples from the respective hair donors and finally combining hair follicle cells and immune cells (blood) in one skin equivalent could help to avoid this effect.

Since the cultivation of diseased hair follicle-derived keratinocytes from patients is still challenging, for future studies the initial number of plucked hairs should be enhanced. Firstly, to increase the cell yield after the initial outgrowth phase. Secondly, in order to reduce the number of passages and thus, the differentiation state of the cells. Another approach that could enhance personalized high-throughput screening is the use of small-scale spheroid skin organoids. Although, to my best knowledge, skin organoids have not been described yet, they could have great potential for the prediction of patient-specific response to therapies, as already shown for cancer-derived organoids (Diaby et al., 2015; DeMatteo et al., 2013). Organoids are mostly generated from pluripotent embryonic stem (ES) cells and induced pluripotent stem cells (iPSCs) as well as organ-restricted adult stem cells (aSCs) (Clevers, 2016). Therefore, plucked hairs are particularly interesting, as they are a source of iPSCs (Aasen et al., 2010), that have the unique ability to differentiate into various skin and skin-related cells such as keratinocytes (Kogut et al., 2014), fibroblasts (Hewitt et al., 2011), melanocytes (Ohta et al., 2011), and endothelial cells (Abaci et al., 2016). The use of iPSCs is also interesting for the generation of several organs and their implementation in multi-organ-chips, as these cells can not only differentiate in skin-related cells, but also in various cell types of the body including ectodermal (neuron (Denham & Dottori, 2011), epidermal keratinocytes (Kogut et al., 2014)), endodermal (lung alveolar cells (Ghaedi et al., 2013)) and mesodermal (adipocytes (Mohsen-Kanson et al., 2014)) lineage. Especially, a multi-organ-chip including skin and lung model would be a promising approach to study the atopic march. Furthermore, multi-organ-chip and the prevailing dynamic cultivation could improve one of the greatest limitations of skin equivalents, namely the weak skin barrier compared to *in vivo* skin.

Throughout my studies, major differences were observed between *in vitro* skin disease equivalents grown from different patients. Secretory profile of fibroblasts is still missing and should be included in further studies. Moreover, the donor differences not only make clear that AD is a multifactorial disease, but also underline the importance of placing patients at the center of the drug and treatment development process. The use of single-cell transcriptomics, Science's 2018 "Breakthrough of the Year", in combination with patient-specific organoids is a perfect method to examine the reconstructed tissue at the cellular level and could dramatically improve our understanding of disease from patient to patient. This would be a major step towards personalized medicine (Berg, 2018). Another promising method is the intravital photon microscopy that already enabled visualization of cell-cell-communication during inflammation in living mice (Gaudenzio et al., 2018; Dudeck et al., 2017). To transfer this method to human *in vivo* skin but also to *in vitro* skin equivalents in order to visualize the crosstalk of keratinocytes/fibroblasts and immune cells such as T cells would be helpful. Speaking of the immunological part of AD: An important requirement of skin disease equivalents is their need to reflect the complexity of AD pathogenesis including not only the epidermal barrier dysfunction, altered penetration of chemicals and type 2 immunity, but also the complex interplay with other immune cells. Therefore, the development of MC-competent skin equivalents presented in this thesis should be continued and improved. The combination of T cells and MCs should be aimed at the long term to further increase the complexity of skin equivalents. In addition, fibroblasts contribute to the survival and development of MCs (Piliponsky et al., 2003). The influence of diseased fibroblasts on MC could therefore be of interest for future studies. Furthermore, it is well known that MC-fibroblast interactions induce remodeling of extracellular matrix (ECM) (Abel & Vliagoftis, 2008). Regarding diseased fibroblasts, ECM is an interesting subject to study, since fibroblasts are able to modify their formation of ECM components. In recent years, ECM has gained more attention as a physiologically active component of living tissue, and is responsible for cell–cell communication, cell adhesion and proliferation (Frantz et al., 2010). Particularly, an enormous impact is awarded to ECM of diseased tissue. Therefore, several projects dealing with disease research not only focus on the correction on gene level using CRISPR/Cas, but also on soft tissue correction. One example is the project RegMedXB Cardiac Moonshot dealing with heart failure. In the event, that anomalies can be found in ECM that could potentially affect immunological cells in AD, transferring of the focus from RegMedXB to AD would be a novel and interesting approach.

Overall, the development of more advanced human equivalents of AD using patient-derived cells offers the potential for a deeper understanding of molecular mechanisms of the underlying pathogenic events of AD and for predicting therapeutic outcomes at the individual patient level.

5 SUMMARY

5.1 SUMMARY

Atopic dermatitis (AD) is the most common skin disease worldwide and the prevalence continues to increase in industrialized countries. However, due to the complex interplay between barrier disruption and the skin immune system, the pathogenesis of AD is still not fully understood. The use of human-based *in vitro* skin equivalents grown from patient-derived cells is a promising alternative approach to investigate pathogenic parameters in AD. However, skin biopsies, that are necessary for the collection of patient cells, are often associated with wound healing complications and scar formations. In this thesis, it was shown for the first time that both keratinocytes and fibroblasts can also be isolated from plucked scalp hair follicles and successfully used for generation of skin equivalents. Moreover, the established method laid the foundation for the generation of skin disease models with the help of patient cells. Over the past years, the impact of fibroblasts for disease induction and/or maintenance has been increasingly recognized. However, little is known about their actual contribution in AD. Hence, the impact of AD patient-derived fibroblasts, isolated from plucked hair follicles, on the tissue homeostasis of human-based skin equivalents was investigated. Interestingly, a subset of AD patient-derived fibroblasts induced characteristic features of AD in the skin equivalents such as hyperproliferation, altered expression of tight junction and skin barrier proteins. Notably, the expression of AD-related proteins such as thymic stromal lymphopoietin (TSLP) and protease-activated receptor 2 (PAR2) were significantly increased. A reduced expression of differentiation-associated cytokine leukemia inhibitor factor (LIF) seems to be linked to these effects. Exposure of hyperproliferative skin equivalents to $CD4^+$ T cells resulted in T cell migration into the dermal equivalent, which was not observed in the equivalents grown from normal fibroblasts. Surprisingly, the addition of T cells to the disease equivalents improved the stratum corneum lipid profiles and distinctly attenuated PAR2 expression, probably as a result of increased LIF signaling due to the T cells. In addition to their effects in the skin equivalents, their angiogenic impact was investigated by co-cultivation with dermal microvascular endothelial cells. Interestingly, AD fibroblasts facilitated to the formation of an increased number of endothelial tubes and thus appear to be involved in angiogenesis. Overall, the results of this doctoral thesis demonstrate the pathogenic effects of AD fibroblasts. The major donor differences among the patients support the opinion that AD is a highly heterogeneous disease, which increases the difficulty of unraveling the pathomechanism of AD. In order to a better understanding of this multifactorial disease, the development of a mast cell-competent skin equivalent was introduced. This model might be a promising biomedical approach for the investigation of complex cell-cell interactions in AD lesions, which is currently poorly understood.

5.2 ZUSAMMENFASSUNG

Die atopische Dermatitis (AD) ist die weltweit häufigste Hautkrankheit, und die Prävalenz in den Industrieländern steigt weiter. Aufgrund des komplexen Zusammenspiels zwischen Barrierestörung und dem Immunsystem der Haut ist die Pathogenese der AD jedoch immer noch nicht vollständig verstanden. Die Verwendung von humanbasierten *in vitro* Hautäquivalenten, die aus Patienten-abgeleiteten Zellen generiert werden, ist ein vielversprechender alternativer Ansatz zur Untersuchung pathogener Parameter in der AD. Für die Gewinnung von Patientenzellen werden jedoch Hautbiopsien benötigt, welche oft mit Wundheilungsstörungen und Narbenbildungen verbunden sind. In dieser Arbeit wurde erstmals gezeigt, dass sowohl Keratinozyten als auch Fibroblasten von gezupften Kopfhauthaaren isoliert und erfolgreich für die Generierung von Hautäquivalenten eingesetzt werden können. Die entwickelte Methode legte den Grundstein für die Generierung von Hautkrankheitsmodellen mit Hilfe von Patientenzellen. In den letzten Jahren wurde der Einfluss von Fibroblasten auf die Krankheitsinduktion und/oder -erhaltung zunehmend ersichtlich. Jedoch ist über ihren tatsächlichen Einfluss bei AD erst wenig bekannt. Daher wurde der Einfluss von patienteneigenen Fibroblasten, die vom gezupften Haarfollikel isoliert werden, auf die Gewebehomöostase von humanbasierten Hautäquivalenten untersucht. Interessanterweise induzierte eine Gruppe von Patienten-abgeleiteten Fibroblasten charakteristische Merkmale der AD in den Hautäquivalenten, wie unter anderem Hyperproliferation, veränderte Expression von Tight Junction-Proteinen und Hautbarriereproteinen. Insbesondere die Expression von AD spezifischen Proteinen wie das thymische stromale Lymphopoietin (TSLP) und der Protease-aktivierte Rezeptor 2 (PAR2) waren signifikant erhöht. Eine verminderte Expression des differenzierungs-assoziierten Zytokins leukämiehemmender Faktor (LIF) scheint mit diesen Effekten verbunden zu sein. Die Exposition hyperproliferativer Hautäquivalente gegenüber $CD4^+$ T-Zellen führte zu einer Migration der T-Zellen in das Dermisäquivalent. Ein Effekt der bei gesunden Hautäquivalenten nicht beobachtet wurde. Überraschenderweise normalisierte die Zugabe von T-Zellen zu den Krankheitsäquivalenten die Lipidprofile des Stratum corneums und reduzierte die PAR2-Expression deutlich, vermutlich als Folge einer erhöhten LIF-Signalisierung durch die T-Zellen. Zusätzlich zu ihrer Wirkung in den Hautäquivalenten wurde die angiogene Wirkung der Fibroblasten mittels einer Co-Kultivierung mit dermalen mikrovaskulären Endothelzellen untersucht. Interessanterweise haben AD-Fibroblasten die Bildung einer erhöhten Anzahl von Endothelröhrchen induziert und scheinen daher an der Angiogenese beteiligt zu sein.

Zusammenfassend zeigen die Ergebnisse dieser Doktorarbeit die pathogenen Effekte von AD-Fibroblasten. Die großen Spenderunterschiede zwischen den Patienten unterstützen

die Aussage, dass AD eine sehr heterogene Krankheit ist, was die Schwierigkeit erhöht, die Pathomechanismen der AD zu entschlüsseln. Um diese multifaktorielle Erkrankung zu untersuchen, wurde die Entwicklung eines Mastzell-kompetenten Hautäquivalents initiiert. Dieses Modell ist ein vielversprechender biomedizinischer Ansatz zur Untersuchung komplexer Zell-Zell-Interaktionen bei AD-Läsionen, die derzeit noch wenig verstanden werden.

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7 STATEMENT OF AUTHORSHIP

(SELBSTSTÄNDIGKEITSERKLÄRUNG)

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Berlin, den 30.10.2019

……

Anna Löwa

8 CURRICULUM VITAE

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