Potential drivers of the atopic march – unraveling the skin-lung crosstalk

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Patrick Graff

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1st Reviewer	Prof. Dr. Sarah Hedtrich BIH-Standort Berlin Buch, Charité Universitätsmedizin Käthe-Beutler-Haus Lindenberger Weg 80 13125 Berlin
2nd Reviewer	Prof. Dr. Charlotte Kloft Freie Universität Berlin Institut für Pharmazie Kelchstraße 31 12169 Berlin
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STATEMENT OF AUTHORSHIP

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Patrick Graff

LIST OF ABBREVIATIONS

AA	allergic asthma
AD	atopic dermatitis
AHR	airway hyperresponsiveness
ALI	air-liquid interface
APC	antigen-presenting cell
AR	allergic rhinitis
BALF	bronchoalveolar lavage fluid
BSA	bovine serum albumin
C3	complement factor C3
CD44	cluster of differentiation 44
COL	collagen
DC	dendritic cell
DE	differentially expressed
DMEM	Dulbecco's modified eagle's medium
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EMT	epithelial-to-mesenchymal transition
EoE	eosinophilic esophagitis
FA	food allergy
Fb	fibroblast
FBS	fetal bovine serum
FLG	filaggrin
GCH	goblet cell hyperplasia
GCM	goblet cell metaplasia
GWAS	genome-wide association study
IFN	interferon
Ig	immunoglobulin
IL	interleukin
ILC2	group 2 innate lymphoid cell
KC	keratinocyte
KRT	cytokeratin
LDH	lactate dehydrogenase
IVL	involucrin
LC	langerhans cell
LOR	loricrin
MHC	major histocompatibility complex
MMP	matrix metalloproteinase
NFκB	nuclear factor kappa B
NHBE	normal bronchial epithelial cells
NHDF	normal human dermal fibroblasts
NHEK	normal human epithelial keratinocytes
NHLFb	normal human lung fibroblasts
NMF	natural moisturizing factor
OVA	ovalbumin
PAR-2	protease-activated receptor 2
PBS	phosphate-buffered saline
qRT-PCR	quantitative real-time polymerase chain reaction
SD-4	syndecan-4
siRNA	small interfering RNA
TEWL	transepidermal water loss
TGF	transforming growth factor
TIMP	tissue inhibitor of metalloproteinase
Tc	cytotoxic T cell
Th	T helper cell
TNF	tumor necrosis factor
Treg	regulatory T cell
TSLP	thymic stromal lymphopoietin
TSP-1	thrombospondin-1
VEGF	vascular endothelial growth factor
WT	wild type

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SUMMARY

Atopic diseases such as atopic dermatitis (AD), food allergy, allergic rhinitis, and allergic asthma are not only an economical burden to the healthcare system but also a highly physiological and psychological burden for patients. Similar pathophysiological patterns like increased IgE plasma concentration as well as Th2-dominant inflammation characterize these diseases. By now, it is well-accepted that AD is the “entry point“ for a successive development of further atopic diseases during the first 10 years of life - a process known as the atopic march. However, the underlying mechanisms are still not fully understood and by far not all responsible factors have been identified; a drawback resulting in poor options for prevention.

In this thesis, a human-based two-organ co-culture of skin disease equivalents, mimicking hallmarks of AD, and healthy bronchial epithelial equivalents was established for studying the pathophysiological crosstalk between skin and bronchi. Already a short co-cultivation period of six days induced a clear hyperproliferative phenotype with elevated mucus-secretion and an increase of inflammatory markers as determined on gene and protein level. Consequently, this led to the suggestion that either epithelial or dermal factors might play a role due to missing immune cells in this co-culture. Secretome of skin disease equivalents as well as proteome and transcriptome analysis patient-derived fibroblasts revealed significant changes of extracellular matrix (ECM)-related genes and proteins. In an exemplary study, the effect of five ECM-related compounds (complement factor C3, fibronectin, syndecan-4 (SD-4), cluster of differentiation 44 (CD44), and thrombospondin-1 (TSP-1)) was tested on bronchial epithelial equivalents and naïve activated CD4+ T cells. For all compounds, an asthma-like inflammation was induced in bronchial epithelial equivalents. Furthermore, a compound-specific polarization of naïve activated CD4+ T cells into different Th subsets was observed.

In addition to the human-based *in vitro* co-culture model, three out of five ECM-related compounds (SD-4, CD44, and TSP-1) were tested *in vivo* in healthy BALB/c mice in the context of a translational study. In line with the *in vitro* experiments, a polarization of murine CD4+ T cells towards Th1, Th17, and Th22 subtypes was observed in a first low-dose approach. Interestingly, no histological changes in skin and lung were observed. However, treated mice had an increased size and weight of spleen.

Taken together, the established human-based two-organ co-culture system not only enables investigation of the crosstalk between healthy skin and bronchus, but also of the pathophysiological communication in the context of the atopic march by using diseased skin equivalents. The results of this thesis demonstrate the importance of ECM in the complex pathophysiology of the atopic march - a hitherto underestimated feature. In this regard, further research is needed to finally assess new targets for the prevention of the atopic march.

ZUSAMMENFASSUNG

Atopische Erkrankungen wie atopische Dermatitis (AD), Nahrungsmittelallergie, allergische Rhinitis und allergisches Asthma stellen nicht nur eine wirtschaftliche Belastung für das Gesundheitssystem dar, sondern sind auch eine große physiologische und psychologische Belastung für die Patient*innen. Ähnliche pathophysiologische Muster wie eine erhöhte IgE-Plasmakonzentration und eine Th2-dominante Entzündung kennzeichnen dieses Krankheitsbild. Es ist inzwischen allgemein anerkannt, dass AD der Ausgangspunkt für die sukzessive Entwicklung weiterer atopischer Erkrankungen in den ersten 10 Lebensjahren ist - ein Prozess, der als atopischer Marsch bekannt ist. Die zugrundeliegenden Mechanismen sind jedoch noch immer nicht vollständig geklärt und es sind bei weitem nicht alle relevanten Faktoren identifiziert, welches in mangelnden Präventionsmöglichkeiten resultiert.

In dieser Arbeit wurde eine humane Zwei-Organ-Kokultur aus Haut-Modellen mit AD-Merkmalen und gesunden Bronchialepithel-Modellen etabliert, um die pathophysiologische Wechselwirkung zwischen Haut und Bronchien zu untersuchen. Bereits eine kurze Kokultivierungszeit von sechs Tagen führte zu einem hyperproliferativen Phänotyp mit erhöhter Schleimsekretion und einem Anstieg von Entzündungsmarkern auf Gen- und Proteinebene. Daraus lässt sich schließen, dass entweder epitheliale oder dermale Faktoren eine Rolle spielen könnten, da Immunzellen in dieser Kokultur fehlten. Das Sekretom von AD Haut-Modellen sowie die Proteom- und Transkriptomanalysen von Patientenfibroblasten ergaben signifikante Veränderungen der Expression von Genen und Proteinen, die mit der extrazellulären Matrix (ECM) in Zusammenhang stehen. In einer beispielhaften Studie wurde die Wirkung von fünf ECM-verwandten Mediatoren (Komplementfaktor C3, Fibronektin, Syndecan-4 (SD-4), Cluster of differentiation 44 (CD44) und Thrombospondin-1 (TSP-1)) auf Bronchialepithel-Modellen und naiven CD4+ T-Zellen getestet. Alle Mediatoren induzierten eine Asthma-ähnliche Entzündung in den Bronchialepithel-Modellen und eine Mediator-spezifische Polarisierung von naiven CD4+ T-Zellen in verschiedene Th Subtypen.

Darüber hinaus wurden drei der fünf ECM-verwandten Mediatoren (SD-4, CD44 und TSP-1) im Rahmen einer translationalen Studie *in vivo* an gesunden BALB/c-Mäusen getestet. In Übereinstimmung mit den *in vitro* Experimenten wurde in einem ersten Niedrigdosis-Ansatz eine Polarisierung muriner CD4+ T-Zellen in Richtung der Subtypen Th1, Th17 und Th22 beobachtet. Interessanterweise wurden keine histologischen Veränderungen in der Haut und Lunge beobachtet, allerdings waren Größe und Gewicht der Milz erhöht.

Insgesamt ermöglicht das etablierte humane Zwei-Organ-Kokultursystem nicht nur die Untersuchung der Interaktion zwischen gesunder Haut und Bronchien, sondern auch der pathophysiologischen Kommunikation im Rahmen des atopischen Marsches durch die

Verwendung von AD Haut-Modellen. Die Ergebnisse dieser Arbeit zeigen die Bedeutung der bisher unterschätzen ECM in der komplexen Pathophysiologie des atopischen Marsches. In dieser Hinsicht sind weitere Forschungen erforderlich, um neue Targets für die Prävention des atopischen Marsches zu ermitteln.

1. INTRODUCTION

1.1 Atopy

Atopy (from Greek “strangeness”) describes the genetic tendency to develop allergic disorders such as atopic dermatitis (AD), food allergy (FA), allergic rhinitis (AR), and allergic asthma (AA) and was first introduced in 1923 by Coca and Cooke (Coca and Cooke 1923). Whereas AD as life-long and non-lethal condition has the highest disease burden of all skin diseases (Laughter et al. 2020; WHO Global estimates 2020) and often leads to psychological stress (e.g., sleep disorder, depression) and economic consequences (Drucker et al. 2017), asthma ranks 3rd among global causes of death with around 400,000 people dying from asthma each year (Vos et al. 2017; WHO Global estimates 2020). Patients suffering from atopic diseases are more likely to react to natural or synthetic environmental factors (e.g., indoor and outdoor allergens, pollutants) with an immediate hypersensitivity (Type 1 allergy). This type 1 allergy is characterized by increased immunoglobulin E (IgE) secretion and a Th2-dominant immune response. The responsible environmental factors may cause symptoms through cutaneous, aerogenic, or intestinal contact. Most prominent allergens are dust mite, trees, pollens, and dander. After a symptom-free first contact, a second contact might lead to characteristic type 1 allergy symptoms such as histamine-induced redness, swelling, and itching. Other symptoms, depending on the affected tissue, can also be (mucus-)hyper-secretion and hyper-responsiveness.

These diseases do not inevitably occur together or at the same time. Often, a sequential progression is observed starting with the “entry point“ AD and continuing with AR and AA. This process is called the atopic march.

Although several animal models of the atopic march exist, the need for human-based alternatives is high. As shown with human-based *in vitro* skin (disease) equivalents, especially mimicking features of AD, it is possible to generate highly valuable data concerning biomarkers, pathophysiological mechanisms, and improve and expand treatment options. The development of further reconstructed organs and the gradual expansion from a single- to a multi-organ co-culture might be a highly useful tool for understanding inter-tissue communication on a fundamental level, hence identifying key mediators and developing options for prevention as well as the treatment of the atopic march.

1.1.1 From early to late stage of the atopic march

The atopic march classically begins with AD, followed by IgE-mediated FA, AR, and AA (Hill et al. 2016). Other common diseases in the spectrum of atopy are considered to be eosinophilic esophagitis (EoE) and allergic conjunctivitis (Sicherer and Leung 2012; Burks et al. 2012; Abonia and Rothenberg 2012; Rancé 2008). Although the term “atopy“ is highly associated with IgE, this pathophysiologic mediator plays a role

in just some atopic diseases, but not all of them. All atopic diseases have a highly complex pathophysiology and emerge from genetic, environmental as well as immunological sources. This multifactorial group of diseases is connected on several different levels. Even though no clinical symptoms can be observed immediately after birth, a genetic predisposition for the development of allergies might already be present (Wahn 2000). The most frequent diseases evolving during childhood are AD, AR, and AA, also known as the “allergic triad“ (Rhodes et al. 2001, 2002). The natural history of atopic diseases classically starts with AD within the first 6-24 months of life (Eller et al. 2010; Kulig et al. 1999). Depending on the allergen, FA follows at varying age. Whereas allergies against cow´s milk and hen´s egg often occur within the first year of life, soy-, wheat-, and peanut butter-allergy develop in later childhood (Kulig et al. 1999). With rising prevalence from early childhood to adolescence, the development of AR follows (Keil et al. 2010) (**Fig. 1**). Ultimately, AA completes the picture of atopic diseases in manifesting before the age of 5 (Yunginger et al. 1992; Phelan, Robertson, and Olinsky 2002; Morgan et al. 2005). This classic progression of atopic diseases for sure cannot be generalized. The atopic march (or allergic march) depends on many factors. It is clear that children suffering from AD more likely develop FA, AR, and AA than children not suffering from AD (Lack et al. 2003; Tsakok et al. 2016; Brough et al. 2015, 2014). The risk for FA increases by 6-fold for AD patients compared to healthy children (Tsakok et al. 2016). Nevertheless, an additional distinction has to be made concerning the age of onset, severity, and chronicity of AD. Children with mild AD for example have a 20% risk for developing asthma, whereas it is a 60% risk for children with severe AD (Gustafsson, Sjöberg, and Foucard 2000; Lewis 1998; Beasly 1998). The proof for a temporal relationship between atopic diseases and the concept of the atopic march was clearly provided by several epidemiological studies. Also, some factors are already known to play a role in the atopic march. Nevertheless, the underlying molecular mechanisms are poorly understood and partly not known at all. Therefore, we have a high need for more detailed research and for the development of reliable human-based methods and models.

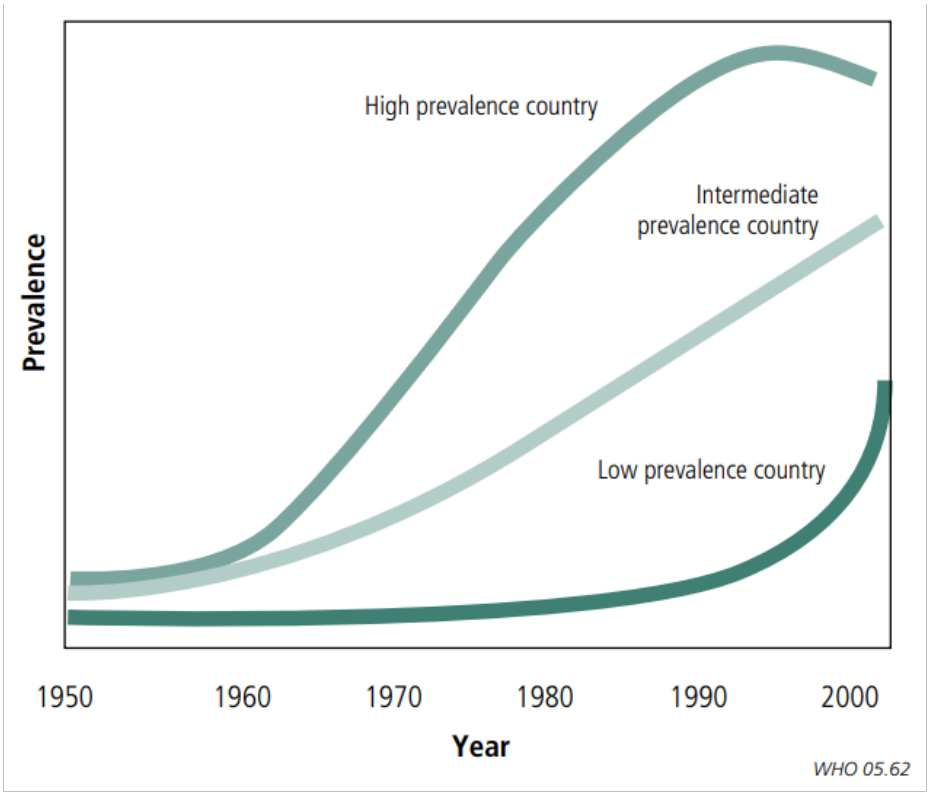


Figure 1. Development of atopic diseases
Successive development of atopic diseases such as AD, FA, AA, and AR depending on age according to (Hill and Spergel 2018). Reprinted with permission from John Wiley and Sons.

1.1.2 Epidemiology

Atopic Dermatitis

Despite great clinical heterogeneity, AD is one of the biggest health problems and the most common inflammatory skin disease in the western world with a prevalence of up to 30% affected children and 7-10% adults and an increasing incidence over the past few decades all over the world (Deckers et al. 2012; Dalgard et al. 2015; Schmitt et al. 2015; Abuabara et al. 2018; Langan, Irvine, and Weidinger 2020). For a long time, AD has been the disease of children (up to 7 years of age) in developed countries with 45% affected children before 6 months of age, approximately 60% before reaching 1 year of age, and almost 85% before 5 years of age (Spergel 2005; Gawkrodger, Mortimer, and Jaron 1994), whereas nowadays more than 230 million people worldwide across all ages and ethnicities suffer from AD according to data from the WHO Global Burden of Diseases initiative. An incidence peak in infancy shows an early onset of AD before 6 years of age with decreasing prevalence in early adulthood and another slight increase in older generations (>75 years of age) (Laughter et al. 2020). The International Study of Asthma and Allergy in Childhood (ISAAC) shows a highly variable AD prevalence across the world. It highlights the importance of environmental factors by showing differences even in

genetically similar populations. Moreover, it shows an increasing prevalence in 6- to 7-year-old children all over the world, especially in low-income countries such as Africa and southeast Asia, while the prevalence of >12-year-old children remained stable. These datasets were collected by measuring AD symptoms of 6- to 7- as well as 13- to 14-year-old AD patients using uniform questionnaires at two different timepoints 5-10 years apart (M. Asher et al. 2006). An annual prevalence of approximately 15% in adults living in developed countries meanwhile shows that AD may persist, relapse or onset in adulthood; thus, may be considered as a lifelong disease (Abuabara et al. 2018; Margolis et al. 2014). Worldwide there is a prevalence of 15-20% among children, varying between countries, regions, and age. For children of 6-7 years of age, prevalence lies between 0.9% (India) and 22.5% (Ecuador). In several Asian, African, and Latin-American countries prevalence is also high ranging between 7-14%. For 13- to 14-year-old children, numbers between 0.2% (China) and 24.6% (Columbia) were determined with a likewise high prevalence in in Africa, Latin-America, and Europe (>15%).

Asthma

Worldwide >300 million children and adults are affected by asthma, making asthma one of the most common major non-communicable chronic diseases (WHO Global surveillance 2008) characterized by airway inflammation and airway remodeling. The number of individuals suffering from asthma is likely to increase to 400 million by 2025 (Global Asthma Network 2018). The worldwide increase of asthma prevalence started around 1970 with still unclear cause and a continues trend towards 1995-2000 (Bousquet et al. 2005). In 2000, after a peak in high-income countries, first studies observed declining prevalence, whereas prevalence in middle- and low-income countries is still rising (M. I. Asher et al. 2006). In the late 70s, not only asthma but also the prevalence of other immune-mediated diseases such as type 1 diabetes mellitus and multiple sclerosis started increasing. A possible reason epidemiological researchers started to focus on is the change of maternal diet during pregnancy which is possibly leading to altered airway and gut microbiota (Jackson et al. 2014). As seen with other atopic diseases, not only a large geographical variation in prevalence but also huge variation in severity of asthma can be observed. In low-income countries and rural areas, the prevalence is <1%, whereas a mean prevalence of 10% (1-18%) is seen in developed countries and urban areas (D. Wang et al. 2013; Bousquet et al. 2005). In contrast to the non-fatal diseases AD and FA, asthma is additionally characterized by relatively high mortality, paradoxically being predominant in low-income countries (To et al. 2012). Considerable and potentially avoidable reasons for this high morbidity and mortality in low- as well as middle-income countries might be under- and misdiagnosis as well as insufficient treatment (**Fig. 2**).

Not only is there a difference in prevalence between urban-rural/developed-developing countries but also in age and sex. Although asthma predominantly begins in early

childhood, spontaneous remission or a late first-onset in adulthood is not uncommon (Butland and Strachan 2007). Asthma is also known for its sex-reversal. Whereas incidence, prevalence, and hospitalization rate are higher for pre-pubertal boys than for girls, there is asthma remission for boys/men in post-pubertal childhood and early adulthood with a rise of incidence and prevalence for women (Arshad et al. 2014; Szefler 2015; Yunginger et al. 1992; Fuhlbrigge, Jackson, and Wright 2002). A population-based survey found an asthma-onset for boys to be at the median age of 3 years, whereas for girls it was at 8 years (Yunginger et al. 1992).

Nevertheless, there is a huge problem in consistency due to multiple various definitions. Across 122 published articles, around 60 definitions of “childhood“ asthma have been used which leads to a huge heterogeneity of asthma as well as a challenge for epidemiological research (Van Wonderen et al. 2010).

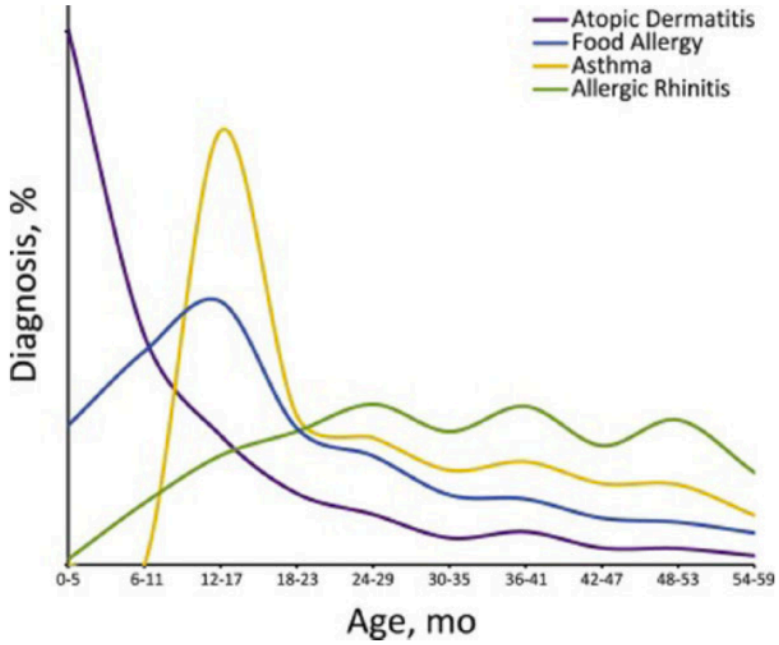


Figure 2. Asthma prevalence
Prevalence of asthma depicted in low-, intermediate- and high-income countries from 1950 to 2000 according to (Bousquet et al. 2005).

1.1.3 Late stage: allergic asthma

Asthma, as the most common inflammatory airway disease and “endpoint” of the atopic march, involves both, the large- and small-conducting airways, and is increasing in prevalence worldwide depicting a substantial global health and economic burden. This disease is highly heterogenous, strongly linked with allergic sensitization, and characterized by chronic (type 2-dominant) inflammation and remodeling of airways. Nevertheless, several sub-phenotypes of asthma need to be considered. The early-onset (childhood-onset) asthma is a T2-type asthma including AA, eosinophilic asthma, Aspirin-

exacerbated respiratory disease, and exercise-induced asthma. The late-onset (adulthood-onset) asthma on the other hand is a non-T2-type asthma and includes obesity-associated asthma, smoking-related neutrophilic asthma, and smooth muscle-mediated paucigranulocytic asthma (S. E. Wenzel 2012). Asthma has a variety of respiratory symptoms like wheeze, cough, breath shortness, and hyperresponsiveness leading to airflow obstruction and decline in lung function over time. A possible trigger for childhood-onset asthma beside susceptibility for atopic diseases are viral infections (RSV or rhinovirus) with already underlaying deficits in lung function and immune system.

Airway inflammation and remodeling

The most prominent features of asthma pathophysiology are airway inflammation and airway remodeling. Analogous to other atopic diseases such as AD and FA, in 50-80% of children and adults suffering from asthma a type 2 inflammation occurs (Fahy 2015), which is associated with sensitization to pollens, pets, dust mites, and fungi (Sporik et al. 1990). In addition to an infiltration with Th2 cells, eosinophils, basophils, and neutrophils are involved in mucosal inflammation (Djukanovic et al. 1990). An important distinction has to be made here concerning T cell profile in asthmatic airways. In classic AA, a dominant role of Th2 CD4+ T cells is known, whereas other T cell subtypes play a role in other asthmatic sub-phenotypes. With neutrophilic asthma, Th1 und Th17 cells are associated (Truyen et al. 2006; Chesné et al. 2014). For non-allergic and eosinophilic asthma, it is known that TSLP, interleukin (IL-)25, and -33, the currently known key mediators in the atopic march, trigger type 2 inflammation by inducing group 2 innate lymphoid cells (nuocytes, ILC2s) with subsequent IL-5, -9, and -13 secretion (Neill et al. 2010; H. Y. Kim, Dekruyff, and Umetsu 2010; Martinez-Gonzalez, Steer, and Takei 2015).
The reasons for an activation of the airway immune system are diverse. Beside the already mentioned systemically acting T cells from allergen-induced skin sensitization, a local activation is possible. Therefore, either bacterial and viral infections or pollutants disturb the mucosal epithelium of the airways inducing the secretion of chemokines (e.g., TSLP, IL-25, IL-33, GM-CSF, IL-1β, and TNF) subsequently attracting immature dendritic cells (DCs) to the epithelium. Some of the typically known asthmatic allergens such as fungi, dust mite, and cockroach are able to directly penetrate epithelial barrier through their enzymatic properties (Jacquet 2011). Analogous to allergen-induced inflammation in the skin, immature DCs maturate into competent antigen presenting cells (APCs) through activation of pattern recognition receptors (PRRs). These competent antigen-presenting myeloid-type DCs phagocytose, process, and present allergen peptides via MHC class II to naïve T cells after migrating to local lymph nodes. Subsequent to the interaction of MHC II with TCR and several co-stimulatory molecules, differentiated and mature Th2 cells migrate back to the airway epithelium with additional influence of several cyto- and

chemokines such as TSLP, IL-25, IL-33, CCL-17, CCL-22, GM-CSF, and TNF (Holgate 2012).

These activated Th2 cells trigger further immune responses (e.g., IgE, eosinophils, mast cells) by secretion of pro-inflammatory cytokines such as IL-3, -4, -9, and -13 (Lambrecht and Hammad 2015; Kay 2006), whereas IL-4, -9, and -13 increase results in injury and epithelial stress followed by metaplasia of mucus-secreting epithelial goblet cells hand in hand with excessive mucus production (Boucherat et al. 2013). This process may result in infections, intraluminal accumulation of mucus, and obstruction of the airways (Rose and Voynow 2006). As soon as an individual is sensitized, further allergen exposure to the airways results in two types of responses. The early and late response. The first, the early-type broncho-constrictor response (EAR), is mast cell-driven, lasts for up to 90 minutes, and is characterized by high levels of IgE-dependent histamine release, prostaglandin D2, and leukotriene C4 (LTC4) (Bradding, Walls, and Holgate 2006). The subsequent late-phase response (LAR) is characterized by infiltration of eosinophils and other leukocytes, high levels of LTC4, cytokine release from T cells and mast cells with a duration of 3-12 hours, and an increase of airway hyperresponsiveness (AHR) (Hargreave et al. 1986). In addition to these well-known pathophysiological processes in the asthmatic airway inflammation, further potential inflammatory mediators such as in asthmatic airways reduced lipoxins (especially lipoxin A4) — normally inducing eosinophil apoptosis and decreasing ILC2- and NK-activity — or resolvins were identified (Barnig et al. 2013).

The next important feature of the asthmatic airways is the process of remodeling. It is known that disease severity and duration correlate with the thickness of the airway wall (Barbers et al. 2012; Al-Muhsen, Johnson, and Hamid 2011). This process is characterized by several changes in the lung tissue such as metaplasia of epithelial mucus, neuronal proliferation, angiogenesis, sub-epithelial reticular lamina thickening, smooth muscle increase, and matrix deposition throughout the airway wall (**Fig. 3**) (Wadsworth, Sin, and Dorscheid 2011; Bjermer 2014). A metaplasia or hyperplasia of goblet cells for example results from injury, epithelial stress, and is dependent on several factors (IL-4, -9, -13, -17, ROS, EGFR) (Shimbara et al. 2000; Humbert et al. 1997; Al-Ramli et al. 2009; Di Stefano et al. 2002; Janssen-Heininger et al. 2009; Louahed et al. 2000; Wills-Karp et al. 1998; Takeyama, Fahy, and Nadel 2001; Takeyama et al. 1999). The two mechanisms behind that are either selective cellular proliferation (goblet cell hyperplasia – GCH) or cell trans-differentiation (goblet cell metaplasia - GCM). Especially club cells were found to transdifferentiate into goblet cell-like mucus producing cells as observed in secretory granules expressing club cell marker CC10 by confocal imaging and *in vivo* lineage tracing in antigen-challenged mice (Hayashi et al. 2004; Reader et al. 2003; Boucherat, Chakir, and Jeannotte 2012). Another cell type found to transdifferentiate into mucus-producing cell are ciliated cells. Here, a co-expression of acetylated β-tubulin, a marker for ciliated cells, and the goblet cell mucin MUC5AC was

observed in mice after inducing metaplasia with Sendai virus (Tyner et al. 2006). Another study supports this data. Here, ovalbumin (OVA-)treated mice had a 75% decrease in club cells and a 25% decrease in ciliated cells (Reader et al. 2003). Nevertheless, GCH remains the main mechanism (T. Shimizu et al. 1996). In addition to an increase in the number of goblet cells, myofibroblasts produce several collagens of the so-called “repair type“ (I, III, V, and VI), periostin, tenascin, and fibronectin leading to thickening of the subepithelial basement membrane (Roche et al. 1989; Kanemitsu et al. 2014; Liesker et al. 2009). Epithelial cell injury moreover leads to the establishment of an epithelial-mesenchymal trophic unit located between smooth muscle and epithelial layers (Holgate et al. 2004), in which the epithelium secrets growth factors like TGFβ, PDGF, FGF, and VEGF resulting in smooth muscle proliferation, fibrosis, angiogenesis (Chanez et al. 1995; Puddicombe 2000; Olgart Höglund et al. 2002; Lopez-Guisa et al. 2012), and migration of subepithelial microvascular pericytes (Johnson et al. 2015). All these processes lead to a chronic wound-like scenario (Puddicombe et al. 2003; Alcala et al. 2014) promoting mucosal fibrosis, neuronal and microvascular proliferation, and smooth muscle hyperplasia; thus, resulting in airway remodeling (Harkness, Ashton, and Burgess 2015; Desideria et al. 2008; Roche et al. 1989).

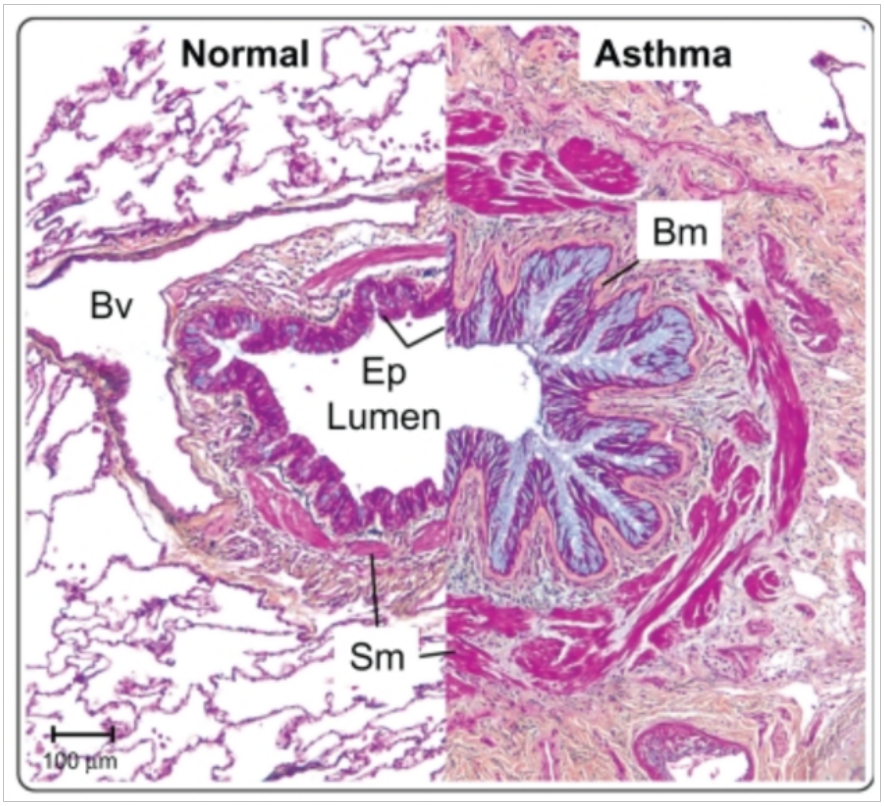


Figure 3. Structural remodeling of asthmatic airways
Sections from healthy and severe asthmatic airways stained with Movat´s pentachrome stain show increased volume of smooth muscle (Sm), hyperplasia of mucus, thickened basement membrane (Bm), and reduced lumen (Wadsworth, Sin, and Dorscheid 2011).

Another important feature of asthma is the immediate hypersensitivity mediated by the IgE–FcεRI (high-affinity Fc receptor for IgE) complex on mast cells not only playing a role in the lung, but also in the skin (AD), in the gut (FA), and in the nose (AR) (Gould et al. 2003; Kraft and Kinet 2007). The FcεRI is expressed on mast cells, basophils, and human (not mouse) APCs, eosinophils, smooth-muscle cells, and monocytes as αγ2 trimer (Kinet 1999; Kraft and Kinet 2007). IgE itself is synthesized by B cells. The process of allergen-induced T cell activation towards Th2 response mentioned above is followed by the secretion of IL-4 and -13 as well as the expression CD40 ligand (CD40L) on T cell surface, resulting in initiation of B cell-to-plasma cell differentiation, a heavy-chain class switch from IgM to IgE, and the synthesis of allergen-specific IgE antibodies (Takhar et al. 2005; Gould et al. 2006; Takhar et al. 2007). Although the main mechanism of mast cell degranulation occurs through binding and crosslinking of several allergen-IgE complexes on mast cell surface inducing the early phase of allergic reaction, studies found that also specific monomeric IgE molecules are able to activate mast cells and either lead to a full degranulation (highly cytokinergic mast cells) or to enhanced mast cell survival (poorly cytokinergic mast cells) (Kraft and Kinet 2007; Kawakami and Galli 2002). Here, beside a histamine release that leads to bronchospasm, edema, and mucous secretion in the lower airways (Stone, Prussin, and Metcalfe 2010), mast cells also secrete and express type 2 cytokines such as IL-4, -13, and CD40L; thus, amplifying IgE-production in B cells (MacGlashan 2005). Around 6-24 h after the early phase of allergic reaction, the late phase peaks leading to local recruitment and activation of further inflammatory cells. Eosinophils, recruited by IL-5, are the most important subtype of leukocytes involved in the late phase leading to edema, tissue damage, and maintaining chronic inflammation (Stone, Prussin, and Metcalfe 2010).

Interestingly, with 4% IgE-expressing B cells and 12-19% IgE-expressing plasma cells in respiratory mucosa of asthmatic and allergic individuals, these populations are increased compared to healthy individuals with only 1% of IgE-expressing B and plasma cells (Kleinjan et al. 2000).

In asthma diagnosis and monitoring, several different biomarkers are available. Currently applied methods include non-invasive pulmonary function tests (PEV, AHR) via spirometry, exhaled nitirc oxide as well as invasive methods such as sputum analysis (inflammatory cell count) and tissue biopsies. Alternative options include further non-invasive techniques such as nuclear magnetic resonance spectroscopy (NMR) for >70 urinary metabolites (Saude et al. 2011), the analysis of exhaled breath condensate for pH and proteins (IL-6, -8, TNF, H_2O_2, and leukotriens, actin, cytokeratins, albumin) as well as invasive analysis of serum proteins (chemokines, chitinases, leptin, adiponectin) (Wadsworth, Sin, and Dorscheid 2011).

1.2 Underlying mechanisms of the atopic march

For infants suffering from severe, persistent, and extrinsic AD within their first 2 years of age, the incidence for developing AA is significantly increased between age 6-7 as shown by several longitudinal studies (Dharmage et al. 2014). From a total of 50 children suffering from AD (diagnosed at median age of 1.2 years), 34% were diagnosed with AA at a median age of 3 years as described in a report from Thailand (Somanunt et al. 2017). An early onset as well as an atopic family history even increased the risk for AA development with common sensitizing allergens *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*. Additionally, an early onset (<2 years of age) increased the risk for FA towards egg white (58%) and cow´s milk (91%). A Canadian birth cohort study with 2,311 children suffering from AD at the age of 1 supports that report with observing a 7-fold increased AA prevalence 2 years later (Tran et al. 2018). Interestingly, an association with an increased AA risk was only found in infants additionally sensitized to allergens, not for AD without sensitization. The Dampness in Building and Health (DBH) study, a prospective cohort study, including 3,124 children aged 1-2 years and a follow-up parental questionnaire based on an International Study of Asthma and Allergies in Childhood protocol, 5 years later found an AD prevalence of 17.6% and a 3-fold increase for developing AA in the AD group (von Kobyletzki et al. 2012). Additionally, different subgroups were formed based on severity, age of onset, and persistence. A severe, early-onset, and persistent AD with a parental history of atopy was found to even increase the odds for developing AA (and AR). Thus, the atopic march presents itself as a multi-factorial und multi-morbid disease (**Fig. 4**).

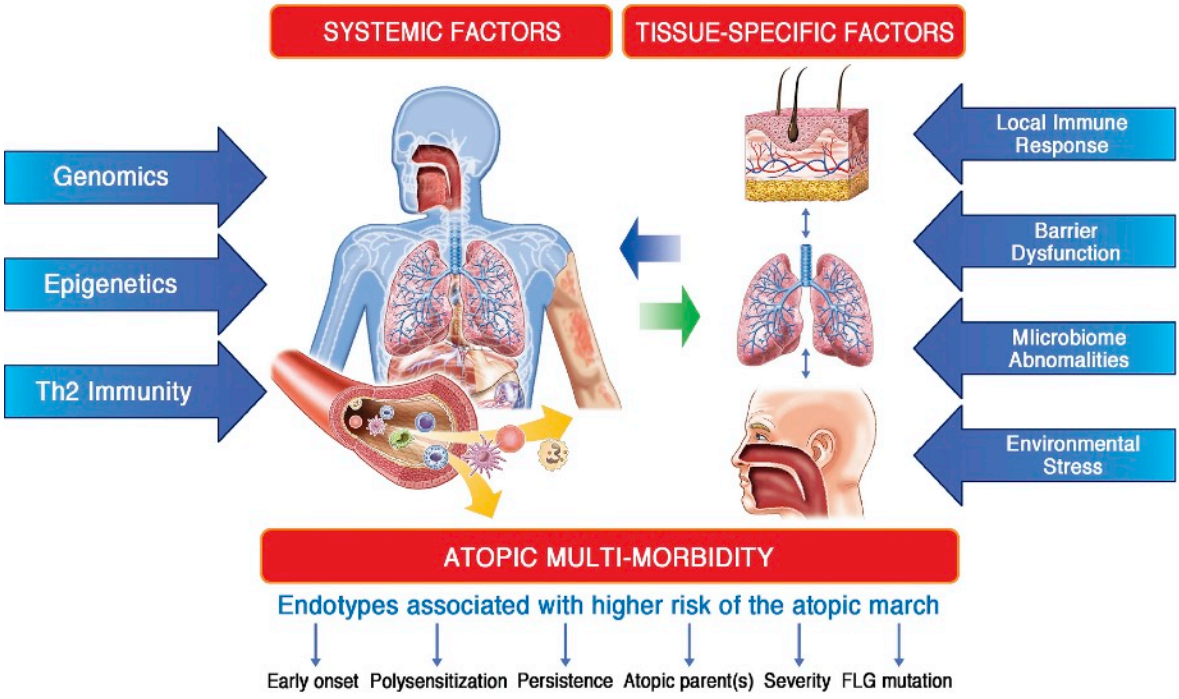


Figure 4. Atopic multimorbidity
Systemic and tissue-specific factors triggering the atopic march are shown according to (Paller et al. 2019). Reprinted with permission from John Wiley and Sons.

1.2.1 Environmental factors

Especially in urban areas of developed countries several environmental and individual factors come together that are increasing the risk for AD. The former includes a sterile environment (hygiene-hypothesis), the western lifestyle (nutrition, obesity), air pollution, urban vs. rural setting, the family size, and a high socio-economic status as increasing risk factors, while the latter mostly includes factors linked to microbial exposure (C. Flohr, Pascoe, and Williams 2005; Carsten Flohr and Yeo 2011). Family size and rate of atopic diseases behave contrary to one another. A fewer number of (older) siblings and a higher standard of personal cleanliness often come together with reduced microbial exposure; thus, reduced infections (Strachan 1989). Also, in urban and wealthier areas - even in less-industrialized countries - the incidence for atopic diseases is higher than in rural and poorer areas (Addo-Yobo et al. 2007; Endara et al. 2015; Addo Yobo et al. 1997; Stevens et al. 2011; Addo-Yobo et al. 2001). Concluding, it can be stated that decreased microbial exposure in early childhood is linked to an increased risk for AD. Thus, decreasing the risk can be achieved by early attendance of day care (<2 years old), dog exposure (Langan, Flohr, and Williams 2007), a diverse gut microbiome, and a farm environment (Bengt Björkstén et al. 2001; B. Björkstén et al. 1999). For a long time, studies linked the increased exposure of children to microorganisms on farms to a reduced risk for wheeze and asthma (Ege et al. 2011; Stein et al. 2016; Schuijs et al. 2015). Nowadays, it is clear

that “farm environment“ in specific means, on the one hand, the direct contact of pregnant mothers to farm animals and, on the other hand, the consumption of unpasteurized farm milk during the first two years of life (Bråbäck, Hjern, and Rasmussen 2004).
The exact underlying mechanisms for positive effects on the development of atopic diseases after microbial exposure are still not fully understood. However, the basic idea is the assumption that exposure to microorganisms trains the innate and adaptive immune system. A study analyzed the prevalence of asthma and allergic sensitization in two communities with genetically similar ancestry. On the one hand, the Amish with traditional farming practice, on the other hand, the Hutterites with industrialized faming practice. Interestingly, prevalence for asthma and allergic sensitization was lower in the Amish community. This leads to the assumption that modulation of innate and adaptive immune system after microbial exposure protects against atopic diseases as proven by analysis of gene expression and immune profiling (Stein et al. 2016). A positive effect can already occur during pregnancy, since the above-mentioned maternal farm exposure seems to be protective. The underlying mechanism is supposed to be either mediated by Th1 or regulatory T cells (Tregs) (Lluis et al. 2014).

1.2.2 Genetics

A family history of atopic diseases is the strongest risk factor known for developing AD. Depending on the number of atopic diseases from one parent, a possible risk of a child for developing AD increases by 1.5-fold. If both parents on the other hand suffer from atopic diseases, the risk for AD even increases by 3- to 5-fold (Apfelbacher, Diepgen, and Schmitt 2011; Wadonda-Kabondo et al. 2004). In several genome wide association studies (GWAS) with high throughput approaches, more than 30 genes have been identified to be associated with AD and atopic diseases in general (Paternoster et al. 2015; Ellinghaus et al. 2013; Paternoster et al. 2012; Sun et al. 2011; Esparza-Gordillo et al. 2009). These include susceptibility loci with functions in e.g., cytokine-mediated signaling, antigen processing and presentation, regulation of cytokine production, leukocyte differentiation, structural molecule activity, extracellular structure organization, autophagy, and vitamin D receptor signaling.
Although genes involved in the above-mentioned processes are linked to AD, the strongest known genetic risk factor is a semi-dominant null mutation in the filaggrin gene (*FLG*) (Irvine, McLean, and Leung 2011; Baurecht et al. 2007). FLG is expressed in the stratum corneum, the outermost layer of the skin and is important for the epidermal barrier, hence protects against environmental factors (e.g., pollutants, bacterial antigens, allergens) prevents transepidermal water-loss (TEWL) (Sandilands et al. 2007, 2009), forming the so-called “natural moisturizing factor“ (NMF) and, maintains pH of the skin surface (Rawlings and Harding 2004).

The most common *FLG* mutations are known to be R501X and 2282del4 with several other *FLG* mutations (e.g., S1695X, Q1701X, Q1745X) existing depending on the population. Whereas more than 50% of people carrying a *FLG* mutation do not develop AD, just 20% of mild-moderate AD patients carry a *FLG* mutation (Baurecht et al. 2007; Weidinger et al. 2008). This leads to the conclusion, that *FLG* mutations are neither necessary nor sufficient for developing AD. Additional interactions between gene-environment and/or gene-barrier function are considered to contribute to the pathogenesis of AD.

It is also known that *FLG* null mutations are a risk for each step of the atopic march. Several studies have shown a correlation of *FLG* null mutation with AD (Palmer et al. 2006), AR (Schuttelaar et al. 2009; Weidinger et al. 2008), peanut butter allergy (Brown et al. 2011), allergic sensitization, EoE, and AA (Van Den Oord and Sheikh 2009; Marenholz et al. 2015; Henderson et al. 2008; Palmer et al. 2007; Basu et al. 2008). Finally, the *FLG* null mutation results in complete absence of FLG monomers which leads to reduced NMF levels and, consequently, to dry skin with higher epidermal pH and barrier dysfunction (J. M. Jungersted et al. 2010; Jakob Mutanu Jungersted et al. 2010). These results support the idea of the diseased skin being the entry point and the driver of atopic diseases, since *FLG* mutation seem to be associated with higher risk for FA or EoE (Brown et al. 2011; Venkataraman et al. 2014) and AA (Basu et al. 2008; Palmer et al. 2007). Several GWAS found association between numerous other genes and atopic diseases. Whereas some genetic susceptibility loci are associated to just one specific disease, other loci share similar pathophysiological pathways. Thus, not only mutation affecting barrier function itself play a role in driving the atopic march but also single nucleotide polymorphisms (SNP) at loci of epithelial-derived cytokines and their receptors for example.

The most prominent epithelial cytokines, together with their receptors, known to play an essential role in the atopic march are keratinocyte (KC-)derived thymic stromal lymphopoietin (TSLP), IL-25, and IL-33. The former two being associated with AD, AA, and EoE (Sherrill et al. 2010; Miyake et al. 2015; Harada et al. 2011), the latter with AD and AA (M. Shimizu et al. 2005; Savenije et al. 2014; Moffatt et al. 2010). Beside *FLG*, *TSLP*, *IL-25*, and *IL-33*, AD-associated genes involved in Th2 inflammation and its pathways were identified (Leung 2016). Further GWAS and meta-analysis identified 99 loci being shared by AD, AA, and AR, hence supporting the theory of a shared genetic foundation of the atopic diseases (Marenholz et al. 2015; Weidinger et al. 2013; Ferreira et al. 2017). Most prominent in that case were *FLG* (skin barrier), *GARP* (T cell regulator gene), and *GATA3* (Th2 lineage) (Manz et al. 2016; Barton et al. 2017).

Taken together, numerous loci were identified to be associated with specific atopic diseases only, others are associated with more than just one atopic disease by sharing underlying pathophysiological mechanisms. However, knowing the genetics and pathophysiology of AD, particularly defects of the skin barrier in connection with a Th2-

dominant immune reaction seem to be pointing towards a strong link between disrupted skin barrier and increased risk for the development of further atopic diseases.

1.2.3 Immunological mechanisms

The initiating step of the atopic march is often suspected in allergen exposure through inflamed skin. A disrupted skin barrier as it is known in AD, enables simplified entrance of allergens through the skin presumably with subsequent innate and adaptive immune response. Animal studies support this theory by showing that transcutaneous exposure to allergens stimulates certain T and B cell responses resulting in AA- and FA-phenotypes in lung and gut. (Tordesillas et al. 2014). This also connects with the knowledge of the skin accommodating a highly heterogenous and highly dynamic population of immune cells, hence being a highly immunoactive organ. Whereas langerhans and CD8+ T cells are present in the epidermis, the dermis contains DCs, mast cells, macrophages, and several lymphocyte subtypes (Kashem, Haniffa, and Kaplan 2017). Consequently, there is a complex interplay between transcutaneous entrance of allergens, the immune system, and epithelial-derived cytokines in barrier-deficient skin. The latter, namely TSLP, IL-25, and IL-33 are potent inducers of type 2 immune response at barrier site with subsequent increase of Th2-derived cytokines such as IL-4, -5, and -13 (Tait Wojno and Artis 2016). Although TSLP, IL-25, and IL-33 have distinct functions and are regulated by numerous factors, the interplay between these three cytokines is highly complex with partly overlapping pathways (**Fig. 5**). Differences can be observed in expression kinetics with *IL-33* mRNA being upregulated at early and late time points, *TSLP* mRNA upregulated at earlier time points, and *IL-25* mRNA upregulated at late time points in a model of chronic house dust mite (HDM) exposure (Vannella et al. 2016). Beside differences in kinetics, requirements for TSLP, IL-25, and IL-33 differ depending on the allergen and its dose, the duration of exposure (Vannella et al. 2016; Chu et al. 2013; Dewas et al. 2015), the investigated mouse strain (Salimi et al. 2013; B. E. Kim et al. 2013), and model of inflammation (Chu et al. 2013; Hongwei Han et al. 2014; Ziegler and Artis 2010; Barlow et al. 2013).

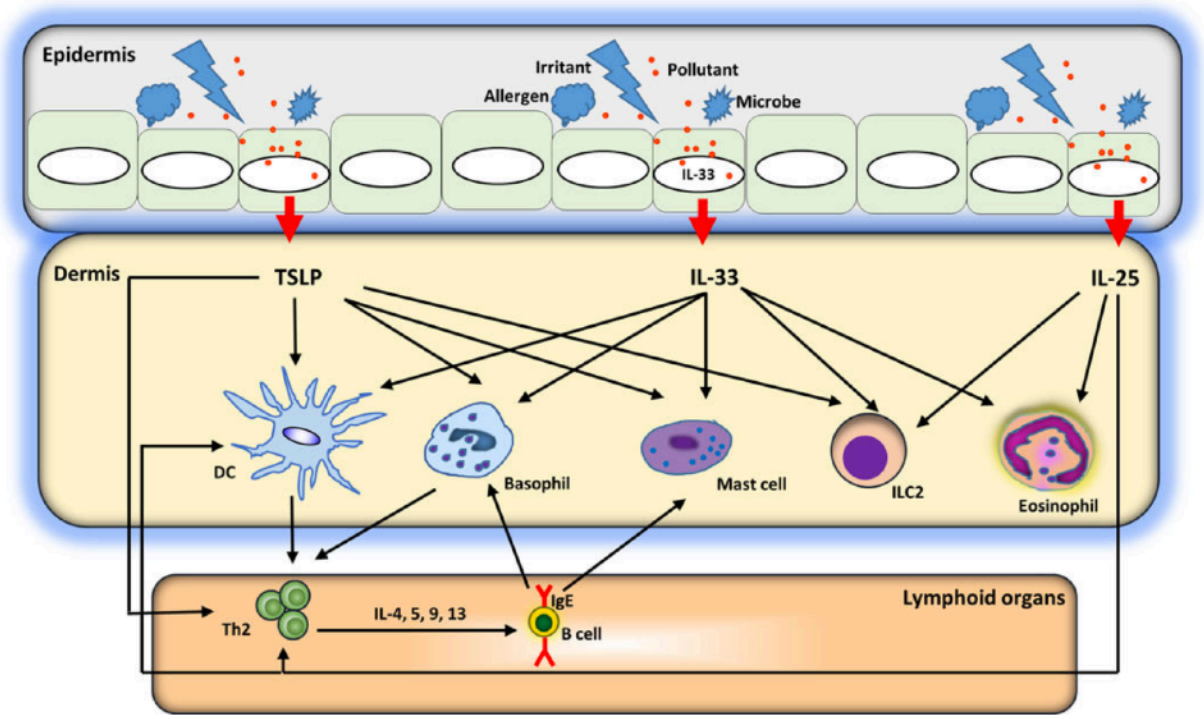


Figure 5. Cell-cytokine crosstalk
Depiction of the crosstalk between epithelial cells as well as skin resident and circulating immune cells with focus on epithelial cytokines TSLP, IL-33, and IL-25 according to (H. Han, Roan, and Ziegler 2017). Reprinted with permission from John Wiley and Sons.

TSLP

TSLP, a member of the IL-2 cytokine family, is primarily produced by epithelial cells at barrier surfaces (skin, airways, gastrointestinal tract), stromal cells, and to lesser amounts by DCs, mast cells, fibroblasts (Fbs), basophils, and smooth muscle cells (Soumelis, Reche, Kanzler, Yuan, Edward, Homey, Gilliet, Ho, Antonenko, Lauerma, Smith, Gorman, Zurawski, Abrams, Menon, McClanahan, Waal-Malefyt, et al. 2002; Watanabe et al. 2004; Ying et al. 2005; Moon, Choi, and Kim 2011; Kashyap et al. 2011). It is associated with AD (Yoo et al. 2005; Gao et al. 2010; Briot et al. 2009), asthma and COPD (Ying et al. 2005, 2008; K. Zhang et al. 2007; Shikotra et al. 2012), and EoE (Rothenberg et al. 2010; Sherrill et al. 2010). Several hematopoietic cell populations including DCs, CD4+ and CD8+ T cells, B cells, mast cells, basophils, eosinophils, and natural killer cells (NKT) as well as non-hematopoietic populations like epithelial cells respond to TSLP (Ziegler et al. 2013; Reche et al. 2001; Zhou et al. 2005; Rochman et al. 2007). In healthy individuals, TSLP maintains Th2 homeostasis at barrier surfaces (Ziegler and Artis 2010), whereas overexpression of TSLP leads to indirect (via MHC class II expression on CD11c+ DCs) priming and differentiation of naïve CD4+ T cells (Soumelis, Reche, Kanzler, Yuan, Edward, Homey, Gilliet, Ho, Antonenko, Lauerma, Smith, Gorman, Zurawski, Abrams, Menon, McClanahan, Waal-Malefyt, et al. 2002; Ito et al. 2005; Liu et al. 2007) as well as

direct interaction with CD4+ and CD8+ T cells as well as Tregs (Omori and Ziegler 2007; Kitajima et al. 2011; Di Piazza et al. 2012; Demehri et al. 2012; Kashiwagi et al. 2017).
TSLP is highly overexpressed in lesional areas of AD skin (Yoo et al. 2005; Demehri et al. 2008) and correlates with disease severity in asthma as shown by *in situ* hybridization and immunohistochemistry from 20 asthmatics and 15 healthy controls (Ying et al. 2005). Interestingly, a mouse model of skin sensitization with artificially induced TSLP overexpression specifically in the skin has shown airway inflammation after antigen challenge in the lung (Z. Zhang et al. 2009; Leyva-Castillo et al. 2013) as well as esophageal inflammation after oral antigen challenge (Noti et al. 2014).

IL-33

Interleukin 33 (IL-33), a member of the IL-1 family, was identified as ligand of the suppression of tumorigenecity 2 (ST2) receptor. ST2R can be found on numerous cell types like Th2 cells, Tregs, DCs, macrophages, basophils, mast cells, and type 2 innate lymphoid cells (ILC2) (D. Xu et al. 1998; Allakhverdi et al. 2007; Kondo et al. 2008; Salimi et al. 2013). On the one hand, IL-33 promotes inflammation by activating eosinophils, mast cells, and basophils (Stolarski et al. 2010; C. Y. Chen et al. 2015; Komai-Koma et al. 2012; Pecaric-Petkovic et al. 2009; Suzukawa et al. 2008), whereas on the other hand, it also might act as regulator of systemic inflammation through its action on Tregs; thus, having protective function (Schiering et al. 2014; Alves-Filho et al. 2010). In human and mice, IL-33 is overexpressed in AD-skin as well as inflamed airway epithelium, hence the main source for IL-33 appear to be non-hematopoietic cells (Hardman, Panova, and Mckenzie 2013; Molofsky, Savage, and Locksley 2015; Savinko et al. 2013, 2012). Overexpression of IL-33 in the skin of mice, just like with TSLP, induces spontaneous AD with a strong association of ILC2 (Imai et al. 2013). Beside driving a Th2-dominant inflammation it also affects FLG expression in KCs leading to a disrupted skin barrier (Seltmann et al. 2015). Moreover, a mice study highlighted its TSLP-independent role in the atopic march. The intradermal application of IL-33+OVA resulted in local skin inflammation followed by inflammation of airways upon subsequent intranasal allergen challenge as well as allergic diarrhea upon oral antigen challenge.

IL-25

Interleukin 25 (IL-25/IL-17E), member of the IL-17 cytokine family, is secreted by epithelial cells, endothelial cells, and Th2-polarized CD4+ T cells (Fort et al. 2001; Angkasekwinai et al. 2007; Corrigan et al. 2011). Its receptor, a heteromeric complex of IL-17RA and IL-17RB (J. Lee et al. 2001; Rickel et al. 2008), is expressed on several cells such as DCs, eosinophils, macrophages, T cells, type-2 myeloid cells, ILC2s, and epithelial cells (Gratchev et al. 2004; Cheung et al. 2006; Tworek et al. 2016; Saenz et al. 2010; Neill et al. 2010; Angkasekwinai et al. 2007). The increased IL-25 expression together with increased levels of Th2 cytokines in AD skin reduces expression *FLG* mRNA

and protein; thus, just like IL-33, leading to enhanced barrier disruption (Y. H. Wang et al. 2007; B. E. Kim et al. 2013; Hvid et al. 2011). Also, tuft cells in the gut (Von Moltke et al. 2016; Howitt et al. 2016; Gerbe et al. 2016) as well as the lung (Hurst et al. 2002; Kang et al. 2005; Tamachi et al. 2006) secret IL-25 upon exposure to allergens or pollutants.
OVA-sensitized mice have shown up-regulation of *Il-25* mRNA in models of allergic airway disease subsequent to intranasal allergen challenge (J. B. Lee et al. 2016; Kang et al. 2005). An IL-25 overexpression in the lung epithelium resulted in increased mucus production and infiltration of eosinophils and macrophages (Angkasekwinai et al. 2007); reversing these effects when inhibiting IL-25 function (Angkasekwinai et al. 2007).
Nevertheless, the role of IL-25 in the atopic march differs from TSLP and IL-33, since, in contrast to TSLP/IL-33, IL-25 failed to promote an asthmatic or FA-phenotype upon intradermal application followed by intranasal and oral antigen challenge (Hongwei Han et al. 2014).

Role of immune cells in the atopic march

The transcutaneous allergen exposure is one of the main amplifiers of the atopic march. As mentioned above, barrier disruption simplifies entrance of allergens and enables interaction with the immune system. In addition to an already existing inflammation through the epithelial-derived key cytokines TSLP, IL-25, and IL-33 and their activation and recruitment of several immune cells, allergens trigger the next step in this process. Entering allergens get recognized and phagocytosed by immune cells of the innate immune system, represented by the APCs, such as DCs (Kashem, Haniffa, and Kaplan 2017; Tait Wojno and Artis 2016) expressing the TSLP receptor (TSLPR) as well as the high affinity IgE receptor (FCRI); thus, promoting inflammation. Upon receptor binding, pro-inflammatory cytokines as well as cytokines favoring Th2 (e.g., IL-1, -8, -16, CCL2) as well as Th1 environment (IL-18, IFNγ, IL-12p70) are produced (Soumelis, Reche, Kanzler, Yuan, Edward, Homey, Gilliet, Ho, Antonenko, Lauerma, Smith, Gorman, Zurawski, Abrams, Menon, McClanahan, Waal-Malefyt, et al. 2002; Novak et al. 2004). APCs, subsequently to allergen contact, become activated, migrate from skin to draining lymph nodes, present phagocytosed antigens (AG) via major histocompatibility complex class II (MHC II) on DC surface, and increase secretion of co-stimulatory cytokines (den Haan, Arens, and van Zelm 2014; Brzostek, Gascoigne, and Rybakin 2016). A matching MHC II/AG-T cell receptor complex together with further co-stimulatory signals (ligation of CD28, CD27, OX40, IL-12 and IFNα/β) (Croft 2003; Curtsinger and Mescher 2010; Agarwal et al. 2009) stimulates naïve T cells to differentiate into allergen-specific Th2 cells (Tait Wojno and Artis 2016; Kambayashi and Laufer 2014) resulting in systemic induction of allergen-specific IgE and inflammation in esophagus, gut, and lung (Galand et al. 2016; Spergel et al. 1998; Noti et al. 2013).

1.3 Preclinical models of atopic diseases

1.3.1 The gold standard: animal models

Animal models are a fundamental tool in life sciences and still the gold standard in preclinical research. In the past, these models made a great contribution to the current knowledge and understanding of pathophysiological processes and the assessment of new drug candidates.

A well characterized and validated animal model for AD and AA research is the mouse, precisely the BALB/c mouse. In contrast to AD, also other animal species are available for asthma research like fruit fly (*Drosophila melanogaster*), rats, guinea pigs, sheep, cats, dogs, horse, pigs, primates, and equines with several (dis-)advantages (Blume and Davies 2013; Kirschvink and Reinhold 2008; Zosky and Sly 2007). However, mice are a comparably cheap animal, easy to bread, to hold, and to handle with different strains being available, a huge knowledge about their genetics, and they are easy to manipulate with transgenic technology (Shapiro 2008; Nials and Uddin 2008; Kumar and Foster 2002). Numerous different methods of artificially inducing AD in mice are established nowadays. One possible way used in most of the models is repeated tape stripping; thus, altering the outermost layer of the skin, the protective stratum corneum. Additionally, to enhance the penetration of allergens and sensitizers, occlusive patches are used causing damage to stratum corneum by preventing water loss (Martel et al. 2017). Further, highly valuable options are genetically engineered (transgenic or knockout) mice.

Currently emerging canine models of AD are getting relevant because of spontaneous development of AD in dogs (Bizikova, Pucheu-Haston, et al. 2015). Moreover, dogs often share same environments as humans e.g., living indoors, being exposed to similar environmental factors and allergens, comparable AD features as observed in human, and a similar response to treatment (Bizikova, Santoro, et al. 2015; Olivry et al. 2015; Favrot et al. 2010). Also, mouse strains with spontaneous, natural AD development under certain conditions like the NC/Nga or flaky tail mice are available (Sasaki et al. 2013; Kohara et al. 2001).

Atopic march models

Just a few groups established models for the atopic march by using the skin as sensitization site and airways as challenge site. As for AD and AA models, BALB/c mice were also used as models for investigating mechanisms of the atopic march.

Han et al. treated wild type (WT) BALB/c mice with OVA+TSLP intradermally for two weeks and subsequently challenged with OVA intranasally. An increase in OVA-specific IgE in serum, goblet cell metaplasia with mucus hypersecretion, and eosinophil infiltration into bronchoalveolar lavage fluid (BALF) were observed (H. Han et al. 2012).

Leyva-Castillo et al. on the other hand started with epicutaneous treatment of WT BALB/c mice with calcipotriol (MC903)+OVA on shaved, tape-stripped dorsal skin in two phases; sensitizing mice every other day from D0-D10 and from D26-D32. Here, also an increase of OVA-specific IgE was observed, as well as and increase of type 2 and type 17 cytokines. The subsequent intranasal challenge from D50-D53 with OVA led to the exhibition of AA symptoms with an increase of Th2 cytokines, mucus hypersecretion, and infiltration of eosinophils (Leyva-Castillo et al. 2013).

In another study from Akei et al. BALB/c mice were sensitized via epicutaneous route with either 3 repeating cycles of OVA or 2 repeating cycles of *Aspergillus fumigatus*, whereas one cycle consisted of 1 week of sensitization via patch and a resting time of two weeks (no patch), inducing an AD-like phenotype. Two days after the end of epicutaneous sensitization, mice were challenged intranasally once with the respective allergen. An increase in total and allergen-specific IgE, type 2 cytokines, mucosal infiltration of eosinophils and neutrophils, and AHR to methacholine was observed. This study additionally shows that *A. fumigatus* led to a stronger type 2 response compared with OVA and that eosinophilia is regulated by STAT6 (Akei et al. 2006).

The main requirement for preclinical validation of targets and drug testing are reproducibility and predictability. Therefore, artificially inducible models are preferred over spontaneous ones since the former are more controllable and predictable concerning disease onset. Moreover, models representing just the specific target of interest are often considered sufficient in most cases leaving out many other characteristics of the human disease. For the latter, the models should basically mimic the characteristics of the human disease as close as possible, whereby an extensive understanding of human AD and AA pathophysiology is essential. Additional drawbacks in the use of animal models are variations in skin architecture and immunology (Haley 2003; Pasparakis, Haase, and Nestle 2014) which are moreover sex-dependent (Dao and Kazin 2007; Klein and Flanagan 2016) and often not being considered in mice studies. More precisely, due to the absence of protective hair coat compared to mice and dogs, the human epidermis is thicker (Jung and Maibach 2015). Additionally, a careful selection of the specific mouse model has to be considered, since e.g., C57BL/6 mice develop a Th1-dominant immune response, whereas the AD-characteristic Th2 response is known in BALB/c mice (Sellers et al. 2012).

Taken together, the low predictability and the artificial induction of AD-like symptoms with numerous different protocols highlights the need for development and establishment of reproducible human-based models for the investigation of the atopic march.

1.3.2 Human-based alternatives: 3D reconstructed tissues

The low predictive power of animal models that results in high failure of drug candidates in clinical trials of >90% highlights the necessity for improved preclinical models that are in line with the 3R principle (Perrin 2014; Aske and Waugh 2017). From a total of 179 drug approvals in Europe, the European Medicines Agency (EMA) recommended approval of 38 new active substances in 2020. The time needed for drug development between 2008-2018 ranged from 5.8 to 15.2 years with costs of $0.314-$2.8 billion, and solely one out of ten candidates entering the first phase of clinical trials being approved in the end (Wouters, McKee, and Luyten 2020; Prasad and Mailankody 2017).

Reconstructed human AD models

In addition to the wide variety of existing protocols for skin equivalent generation, there are numerous established methods for generating skin equivalents with characteristics of AD as well. Mainly, a barrier deficiency and/or inflammation are induced. In most cases, AD-characteristic Th2 cytokines are supplemented such as IL-4, -13, -25, and -31 to induce TSLP inflammation in KCs since immune cells are non-existent (Danso et al. 2014; do Nascimento Pedrosa et al. 2017). Since expected TSLP secretion was not fully achieved throughout all these stimuli, TNF as well as synthetic inducers of TLR-3 (polyinosinic-polycyti-dylic acid) or TLR-4 (Pam3CKS4) were added to achieve a more intense immune response (Danso et al. 2014; Castex-Rizzi et al. 2014; Rouaud-Tinguely et al. 2015). These approaches induced inflammation through increased TSLP as well as IL-8 secretion and decreased barrier function by altering the expression of epidermal proteins FLG, claudin (CLDN-)1, loricrin (LOR), involucrin (IVL), and cytokeratin (KRT) 10 with induction of AD typical spongiosis and a disorganized lipid matrix. A further approach, the use of *Staphylococcus aureus* (*S. aureus*) extracts and IL-6 and -8 resulted in reduced KRT1 and KRT10 (Son et al. 2014).

The second approach, the induction of barrier deficiency, was induced with small hairpin RNA (shRNA). Here, no inflammation was studied but only protein expression. Interestingly, altered expression of FLG, hornerin, KRT10, LOR, CLDN-1, and corneodesmosine were determined (Valérie Pendaries et al. 2014; V Pendaries et al. 2015). It must be mentioned that these findings are based on reconstructed human epidermis; thus, all the above-mentioned models consisting of KCs.

The skin equivalents used in this thesis are based on long experience and expertise in this field and are far more complex. These equivalents consist of primary normal human dermal fibroblasts (NHDF) embedded in bovine collagen type I with primary normal human epidermal keratinocytes (NHEK) seeded on top, lifted to the air-liquid interface, and cultivated for up to 14 days (**Fig. 6**). In order to generate a diseased, AD-like phenotype, a knockdown in the *FLG* gene in NHEK was induced (Küchler et al. 2011; Eckl et al. 2011) in a first step resulting in a compensatory up-regulation of skin barrier proteins

such as LOR and IVL; thus, contradictory improvement of the skin barrier and decreased permeability. The supplementation of the Th2-derived cytokines IL-4 and -13 (=inflammatory model), hence mimicking an AD-typical immune environment, diminished these compensatory effects and led to an increased barrier disruption and permeability towards hydrophilic and lipophilic substances (Hönzke, Wallmeyer, et al. 2016). In addition, this inflammatory model is characterized by increased pH of skin surface, reduced expression of barrier and tight junction proteins IVL, LOR, FLG, occludin, and CLDN-23 as well as increased expression of pro-inflammatory KC-derived cytokine thymic stromal lymphopoietin (TSLP). In a next step, *FLG*- equivalents were exposed to activated CD4+ T cells (=immunocompetent model) resulting in increased skin pH, reduced expression of FLG, IVL, claudin-1, and a disturbed skin lipid organization (Wallmeyer et al. 2017). Additionally, it was proven that KC-derived TSLP, which is not only increased in the already mentioned established AD-equivalents but also in lesional skin *in vivo* (Ziegler 2010), directly triggers T cell migration without DCs as intermediary being necessary.
Accordingly, bronchial epithelial equivalents were generated with normal human bronchial epithelial cells (NHBE) and normal human lung fibroblasts (NHLFb), and cultivated for up to 21 days (**Fig. 6**)

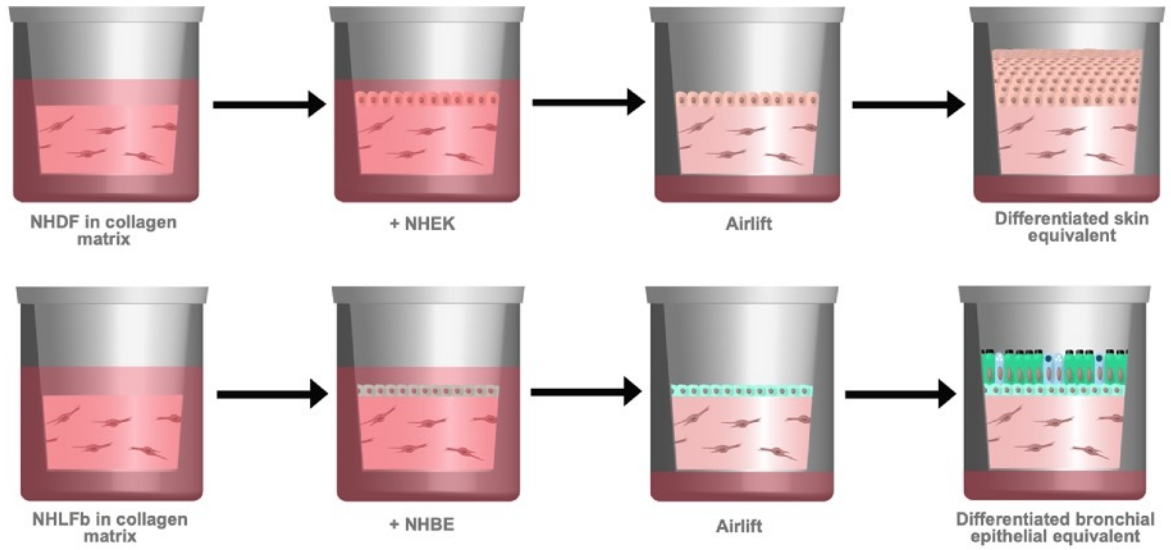


Figure 6. Skin and bronchial epithelial equivalent scheme
Scheme for generation of skin (disease) and bronchial epithelial equivalents. Briefly, primary skin (NHDF) or lung fibroblasts (NHLFb) are mixed with bovine collagen type I, keratinocytes (NHEK) or normal human bronchial epithelial cells (NHBE) are seeded on top, and are air-lifted 24 h later. Graphic designed and kindly provided by Dr. Anna Löwa.

1.4 Aims

The crucial role of atopic skin in the highly complex pathophysiological disorder, the atopic march, is well-known. Several diseases such as atopic dermatitis (AD), allergic asthma (AA), and food allergy (FA) are linked through similar pathophysiological mechanisms making the atopic march a global health burden with increasing prevalence in industrialized and developing countries. Furthermore, underlying pathophysiological mechanisms are still not fully understood and little is known concerning the direct interplay between diseased skin and airways, although several animal models of AD and AA exist. However, a reliable and translatable model of the atopic march does not exist. Moreover, animal models exhibit crucial differences compared to human, such as skin thickness, present immune cell types, and the fact that AD needs to be artificially induced in most animals.

This bottleneck highlights the lack of predictability and translation of findings from animal to human. A complex interaction between genetics, the immune system, and the environment are stated so far, not being able to decipher the connection due to missing research systems. Therefore, the development of human-based alternatives is the ultimate goal to understand pathophysiological mechanisms and key players of the atopic march.

The main aim of this thesis was to unravel the role of AD skin in the atopic march and prove that a direct skin-lung crosstalk exists. A first aim on this way was to establish a human-based two-organ co-culture between AD skin and bronchial epithelial equivalents. With the help of this system, it was aimed to identify skin-derived factors and, furthermore, test their impact on bronchial epithelial equivalents as well atopy-relevant Th cells. In order to prove the suitability of this human-based system, it was aimed to perform a translational study in BALB/c mice.

Overall, this thesis aimed to identify possible skin-derived key mediators within the framework of the atopic march in order to understand a direct skin-lung crosstalk and identify possible targets as prevention options.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Cells and human material

Primary human fibroblasts and keratinocytes were isolated from juvenile (<10 years old) human foreskin according to standard procedures (with permission and informed consent; EA1/081/13).

Primary bronchial epithelial cells and normal human bronchial fibroblasts were purchased from STEMCELL Technologies (Vancouver, Canada). CD4+ T cells were isolated from buffy coat preparations from whole human blood (2.2.1.2). Buffy coats were kindly provided by Dr. Lucie Loyal and Prof. Andreas Thiel from BCRT Berlin who purchased female and male buffy coats from DRK Blutspendedienst (DRK-Blutspendedienst Ost, Berlin, Germany; with informed consent and ethical approval EA2/067/15).

Blood plasma samples from atopic patients and non-atopic controls were obtained through the BC Children's Hospital biobank (CREB approval #H19-00446).

Excised human skin from healthy patients was obtained from plastic surgery with informed consent and ethical approval (#CREB H19-03096).

2.1.2 Equipment

Analytical balance XS 205 dual range	Mettler Toledo, Gießen, GER
Autoclave V95	Systec, Wettenberg, GER
Centrifuge, 5072R Eppendorf,	Eppendorf AG, Hamburg, GER
Centrifuge, Heraeus Megafuge 1.0R	Thermo Scientific, Waltham, USA
Centrifuge, Heraeus Pico 17	Thermo Scientific, Waltham, USA
CO_2 Incubator	Heraeus, Hanau, GER
CO_2-free incubator	Heraeus, Hanau, GER
Cryotome Leica CM1510 S	Leica Biosystems, Wetzlar, GER
CytoFlex, flow cytometer	Beckmann Coulter, Krefeld, GER
Fluorescence microscope Biozero	Keyence, Neu-Isenburg, GER
FLUOstar Optima	BMG Labtech, Ortenberg, GER
Fridge 4°C	Quelle, Fürth, GER
Freezer -80°C, HERAfreeze	Heraeus, Hanau, GER
Freezer -80°C	Thermo Scientific, Waltham, USA
Freezer -20°C	Siemens, München, GER
Gel documentation system, PXi/PXi touch	Syngene, Cambridge, UK
Haemocytometer, Neubauer improved	Carl Zeiss, Jena, GER
Incubator, HERAcell 240i	Thermo Scientific, Waltham, USA
Laboratory scale AK 160	Mettler Toledo, Gießen, GER
Liquid nitrogen tank, Arpege 70	Air Liquid, Paris, F
Light microscope	Axiovert 135 Carl Zeiss, Jena, GER
Microtome Hyrax M40	Carl Zeiss, Jena, GER
Mini-PROTEAN Tetra System	Bio-Rad, Munich, GER
Mr. Frosty	Thermo Scientific, Waltham, US
Paraffinization station (Microm EC 350)	Carl Zeiss, Jena, GER

PCR thermocycler Tgradient	Biometra, Jena, GER
pH meter 766	Knick, Berlin, GER
Pipettes (10, 20, 100, 300, 1000 µl)	Eppendorf, Hamburg, GER
Paraffin stretching bath	Thermo Scientific, Schwerte, GER
Rocking tumbler, TH 10	Edmund Bühler, Hechingen, GER
RT PCR-System, LightCycler 480	Roche, Mannheim, GER
Sterile workbench, LaminAir HB2472	Heraeus, Hanau, GER
Vacuum concentrator SCA 210	Thermo Scientific, Waltham, USA
Vortex Genie 2TM	Bender & Hobein, Zürich, SWI
Waterbath	GFL, Burgwedel, GER
TissueLyzer	Qiagen, Hilden, GER
Trans-Blot Turbo Blotting System	Bio-Rad, Munich, GER

2.1.3 Consumables

Alvetex well insert holder and deep petri dish	REPROCELL Europe Ltd., Glasgow, UK
ALZET® Osmotic Pumps 1007D	Charles River, Sulzfeld, GER
Blotting pad	VWR, Pennsylvania, USA
Cell culture flask (25, 75, 150 cm^2)	TPP, Melbourn, UK
Cell culture insert 6-well 3.0 µm pore size	Corning, Amsterdam, NL
Cell culture insert 12-well 3.0 µm pore size	Corning, Amsterdam, NL
Cell strainer (70, 100 µM)	VWR, Pennsylvania, USA
Centrifuge tubes (15, 50 ml)	Sarstedt, Nümbrecht, GER
Coverslip (18x18 mm)	Menzel, Braunschweig, GER
Cryotubes, NUNC	Sarstedt, Nümbrecht, GER
Deep 6-well plate	Corning, Amsterdam, NL
Deep 12-well plate	Corning, Amsterdam, NL
Disposable microtome blade, SB Type 819	Leica Microsystems, Wetzlar, GER
Disposable syringe (1, 5, 10, 20 ml)	Braun, Kronberg, GER
Forceps	Carl Roth, Karlsruhe, GER
LS columns	Miltenyi Biotec, Bergisch-Gladbach, GER
MACS Separator + MIDI MACS	Miltenyi Biotec, Bergisch-Gladbach, GER
Multi-well plates (6-, 12-, 24-, 48-well)	VWR, Pennsylvania, USA
Nitril gloves	VWR, Pennsylvania, USA
Optical seal for PCR plates, Microseal ’C’ Film	Bio-Rad, Munich, GER
Parafilm	Carl Roth, Karlsruhe, GER
Hard-shell 96-well 480 PCR plates	Bio-Rad, Munich, GER
PCR reaction tubes	Sarstedt, Nümbrecht, GER
PCR strips	Carl Roth, Karlsruhe, GER
Peel-A-Way embedding molds	Sigma Aldrich, Munich, GER
Petri dishes	TPP, Melbourn, UK
Pipettes (5, 10, 25 ml)	Sarstedt, Nümbrecht, GER
Pipette tips (filtered) (10, 20, 100, 1000 µl)	Sarstedt, Nümbrecht, GER
Pipette tips (not filtered) (10, 20, 100, 1000 µl)	Sarstedt, Nümbrecht, GER
Poly-lysine slides	Menzel, Braunschweig, GER

PVDF membrane	Bio-Rad, Munich, GER
Sterile scalpel	Aesculap-Werke, Tuttlingen, GER
Syringe filter 0.2 µm	Sarstedt, Nümbrecht, GER

2.1.4 Reagents

1,4-Dithiothreitol (DTT)	Carl Roth, Karlsruhe, GER
α-Nuclear red	Sigma Aldrich, Munich, GER
Adenine	Sigma Aldrich, Munich, GER
Alcian blue	Sigma Aldrich, Munich, GER
Ammonium persulfate	Carl Roth, Karlsruhe, GER
β-Mercaptoethanol	Sigma Aldrich, Munich, GER
Bovine collagen type 1	Cellsystems, Troisdorf, Germany
Bovine serum albumin	Carl Roth, Karlsruhe, GER
Choleratoxin	Sigma Aldrich, Munich, GER
Collagenase type I	Worthington, New Jersey, USA
DAPI mounting medium	Dianova, Hamburg, GER
Dexamethasone	Sigma Aldrich, Munich, GER
Dimethyl sulfoxide	Carl Roth, Karlsruhe, GER
DMEM high glucose, no glutamine	Sigma Aldrich, Munich, GER
DMEM low glocose, Glutamax	Sigma Aldrich, Munich, GER
Nutrient Mixture F-12 Ham	Sigma Aldrich, Munich, GER
EDTA	Sigma Aldrich, Munich, GER
Eosin G	Carl Roth, Karlsruhe, GER
EGF	Sigma Aldrich, Munich, GER
EpiLife	Thermo Scientific, Schwerte, GER
Ethanol, absolute	Merck, Darmstadt, GER
Fetal bovine serum superior	Biochrom, Berlin, GER
Glycine	Sigma Aldrich, München, GER
Goat serum	Dianova, Hamburg, GER
Ham's F-12 Nutrient Mix, GlutaMAX™ Sup	Thermo Scientific, Schwerte, GER
Hank’s Buffered Salt Solution (HBSS)	Life Technologies, Darmstadt, GER
Hematoxilin	Carl Roth, Karlsruhe, GER
HiPerFect transfection reagent	Qiagen, Hilden, GER
HKGS	Thermo Scientific, Schwerte, GER
Insulin	Roche, Mannheim, GER
Isopropanol	VWR, Darmstadt, GER
L-glutamine	Sigma Aldrich, Munich, GER
Methanol	VWR, Darmstadt, GER
Nycoprep	Axis-Shield, Oslo, NOR
Paraformaldehyde 4%	Carl Roth, Karlsruhe, GER
Penicillin/streptomycin	Sigma Aldrich, Munich, GER
Phosphate Buffered Saline (PBS)	Sigma Aldrich, Munich, GER
Protease/Phosphatase Inhibitor Cocktail (x100)	CST, Massachusetts, USA
Protein marker VI (10-245 kDa), pre-stained	AppliChem GmbH, Darmstadt, GER
rhCD44	Bio-Techne GmbH, Wiesbaden-Nordenstadt, GER

rhComplement factor C3	Bio-Techne GmbH, Wiesbaden-Nordenstadt, GER
rhCD44	Bio-Techne GmbH, Wiesbaden-Nordenstadt, GER
rhFibronectin	Bio-Techne GmbH, Wiesbaden-Nordenstadt, GER
rhInterleukin-4, -13	Miltenyi Biotec, Bergisch-Gladbach, GER
rhSyndecan-4	Bio-Techne GmbH, Wiesbaden-Nordenstadt, GER
rhTGFβ	Miltenyi Biotec, Bergisch-Gladbach, GER
rhThrombospondin-1	Bio-Techne GmbH, Wiesbaden-Nordenstadt, GER
rmCD44	Cederlane, Burlington, CA
rmSyndecan-4	Cederlane, Burlington, CA
rmThrombospondin-1	Cederlane, Burlington, CA
Rotiphorese gel 40 (37,5:1)	Carl Roth, Karlsruhe, GER
Roti® Histofix	Carl Roth, Karlsruhe, GER
Roti® Histokit	Carl Roth, Karlsruhe, GER
Roti® Histol	Carl Roth, Karlsruhe, GER
RPMI 1640	Sigma Aldrich, Munich, GER
Sodium chloride (NaCl)	Carl Roth, Karlsruhe, GER
Sodium dodecyl sulfate (SDS)	Sigma Aldrich, Munich, GER
Sodium hydroxide (NaOH)	Carl Roth, Karlsruhe, GER
TEMED	Carl Roth, Karlsruhe, GER
Tissue freezing medium	Leica Biosystems, Nussloch, GER
TRIS	Carl Roth, Karlsruhe, GER
Trypsin/EDTA	Biochrom, Berlin, GER
Tween-20	Carl Roth, Karlsruhe, GER

2.1.5 Antibodies

Anti rabbit IgG, HRP conjugated	CST, Massachusetts, USA
Anti mouse IgG, HRP conjugated	CST, Massachusetts, USA
IgG Alexa Fluor® 488	Abcam, Cambridge, UK
IgG Alexa Fluor® 594	Abcam, Cambridge, UK
Mouse anti α-acetylated Tubulin	Sigma Aldrich, München, GER
Mouse anti α-SMA	Novus Biol, Minneapolis, USA
Mouse anti ß-actin	Abcam, Cambridge, UK
Mouse anti Cytokeratin 10	Dianova, Hamburg, GER
Mouse anti Cytokeratin 14	Dianova, Hamburg, GER
Mouse anti E-cadherin	Dianova, Hamburg, GER
Mouse anti Ki-67	Dianova, Hamburg, GER
Mouse anti MUC5AC	Merck, Darmstadt, GER

Mouse anti SCCA-1	Bio-Techne GmbH, Wiesbaden-Nordenstadt, GER
Mouse anti Tenascin C	Abcam, Cambridge, UK
Mouse anti Vimentin	Dianova, Hamburg, GER
Rabbit anti β-tubulin	CST, Massachusetts, USA
Rabbit anti Filaggrin	Biolegend, California, USA
Rabbit anti GAPDH	CST, Massachusetts, USA
Rabbit anti Involucrin	Abcam, Cambridge, UK
Rabbit anti Loricrin,	Life Technologies, Darmstadt, GER
Rabbit anti PAR-2	Santa Cruz Biotechnology, Heidelberg, GER
Rabbit anti sPLA2	Abcam, Cambridge, UK
Rabbit anti TSLP	Abcam, Cambridge, UK
Rabbit anti Uteroglobin	Abcam, Cambridge, UK
Rabbit anti ZO-1	Thermo Scientific, Schwerte, GER

2.1.6 Primers

Gene	Sequence (5’ — 3’)
ADAM33	ff 5’ - TCTTTCggATggAgCAgCTg rv 5’ - gACgCTgTTTggTgTggTTC
ASMA	ff 5’ - TgggCTCTgTAAggCCggCT rv 5’ -CACCCCCTgATgTCTgggACg
GAPDH	ff 5’ - CTCTCTgCTCCTCCTgTTCgAC rv 5’ - TgAGCgATgTggCTCggCT
FLG	ff 5’ - AAggAACTTCTggAAAAggAATTTC rv 5’ - TTgTggTCTATATCCAAgTgATCCAT
FN	ff 5’ - ggTgACACTTATgAgCgTCCTAAAA rv 5’ - AACATgTAACCACCAgTCTCATgTg
LIF	ff 5’ - ACAgAgCCTTTgCgTgAAAC rv 5’ - TggTCCACACCAgCAgATAA
MUC5AC	ff 5’ - CCTTCgACggACAgAgCTAC rv 5’ - TCTTgATggCCTTggAgCAg
PAR2	ff 5’ - TCATTgTCACTgTCCTggCC rv 5’ - AAgggTAgAgAggCAgAggg
SSCA1	ff 5’ - ggAgCCAAAgACAACACTgC rv 5’ - gCTTgTTggCgATCTTCAgC
tenascin C	ff 5’ - TCAAAgACgTgCCAggAgAC rv 5’ - CTgTCTgggAAACACgTCgA
TSLP	ff 5’ - CCCAggCTATTCggAAACTCAg rv 5’ - CgCCACAATCCTTgTAATTgTg
UG	ff 5’ - CCCCTCCTCCACCATgAAAC rv 5’ - AAAgTTCCATggCAgCCTCA
ZO1	ff 5’ - TCCTgCTTgACCTCCCTAAA rv 5’ - ACAACACggAACACCTCTCC

TBX21	ff 5’ - TTgAggTgAACgACggAgAg rv 5’ - CCAAggAATTgACAgTTgggT
GATA3	ff 5’ - gAACCggCCCCTCATTAAg rv 5’ - ATTTTTCggTTTCTggTCTggAT
RORC	ff 5’ - CAATggAAgTggTgCTggTTAg rv 5’ - gggAgTgggAgAAgTCAAAgAT
AHR	ff 5’ - CAAATCCTTCCAAgCggCATA rv 5’ - CAAATCCTTCCAAgCggCATA
YHWAZ	ff 5’ - AgACggAAggTgCTgAgAAA rv 5’ - gAAgCATTggggATCAAgAA
Tbx21	ff 5’ - TCAACCAgCACCAgACAgAgATg rv 5’ - CACCAAgACCACATCCACAAACA
Gata3	ff 5’ - AgAACCggCCCCTTATgAA rv 5’ - AgTTCgCgCAggATgTCC
Rorc	ff 5’ - CCgCTgAgAgggCTTCAC rv 5’ - TgCAggAgTAggCCACATTACA
Il-22	ff 5’ - ggTgACgACCAgAACATCCAgA rv 5’ - AgAgACATAAACAgCAggTCCAgT
Hprt	ff 5’ - AggCCAgACTTTgTTggATTTgAA rv 5’ - CAACTTgCgCTCATCTTAggCTTT
Yhwaz	ff 5’ - AAgACAgCACgCTAATAATgC rv 5’ - TTggAAggCCggTTAATTTTC

2.1.7 siRNA

Filaggrin, Sequence	Invitrogen, Darmstadt, Germany (NM_002016) CAGCUCCAGACAAUCAGGCACUCAU For T150: 2.379 ml EpiLife 156 µl HiPerFect 65 µl siRNA ad 26 ml EpiLife

2.1.8 Kits

Allprep mini DNA/RNA/Protein Isolation Kit	Qiagen GmbH, Hilden, GER
$CD4^+$ T Cell Isolation Kit, mouse	Miltenyi Biotec, Bergisch-Gladbach, GER
CytoTox-ONE™ Homogeneous Membrane Integrity Assay	Promega, Madison, USA
Dynabeads Human T-Act CD3/CD28	Thermo Scientific, Schwerte, GER
ELISA IL-1β, -6, -8, -10, -33, TSLP, TNFα	Thermo Fisher, Schwerte, GER
ELISA IL-25, C3, SD-4, TSP-1, CD44, FN	Bio-Techne GmbH, Wiesbaden-Nordenstadt, GER
innuPREP RNA mini Kit 2.0	Analytik Jena, Jena, GER
iScript™ cDNA Synthesis Kit	Bio-Rad, Munich, GER
iTaq™ Universal SYBR® Green Supermix Kit	Bio-Rad Munich, GER

Naive $CD4^+$T Cell Isolation Kit II, human	Miltenyi Biotec, Bergisch-Gladbach, GER
Naive $CD8^+$T Cell Isolation Kit II, human	Miltenyi Biotec, Bergisch-Gladbach, GER
Pierce® BCA Assay Kit	Thermo Fisher, Schwerte, GER
Signal Fire™ ECL reagent	Cell Signaling, Frankfurt/Main, GER
Signal Fire™ ECL elite reagent	Cell Signaling, Frankfurt/Main, GER

2.1.9 Buffers

Blotting buffer (10x)	1000 ml dH_2O supplemented with: 144 g glycin, 10 g tris base
Buffer for blood preparation	D-PBS supplemented with: 2 mMol EDTA
Freezing medium	Basal medium supplemented with: 10% (v/v) FBS, 10% (v/v) DMSO
Loading buffer	3x Blue Loading Buffer Pack 187.5 mM tris HCl, 6% (w/v) SDS, 30% glycerol, 0.03% (w/v) bromphenolblue, 1.25 M dithiothreit
Running buffer (10x)	1000 ml dH_2O supplemented with: 30.2 g tris base, 144 g glycin, 10 g SDS
Running gel buffer	250 ml dH_2O supplemented with: 56.2 g tris base
PBS	dH_2O supplemented with: 0.2 g/l KCl, 8.0 g/l NaCl, 0.2 g/l KH_2PO_4, 1.44 g/l Na_2HPO_4
Stacking gel buffer	250 ml dH_2O supplemented with: 15.0 g tris HCl
TBS (10x)	1000 ml dH_2O supplemented with: 12.144 g tris HCl, 87.66 g NaCl
TBST	900 ml dH_2O supplemented with: 100 ml TBS (10x), 1 ml tween-20
Washing buffer (ELISA)	PBS supplemented with: 0.025% tween-20
Washing buffer (immunofluorescence)	PBS supplemented with: 0.0025% BSA, 0.025% tween-20

2.1.10 Cell culture media

Crossover medium	DMEM high glucose supplemented with: 10% (v/v) FBS, 5 µg/ml insulin, 10 ng/ml epidermal growth factor, 0.4 µg/ml hydrocortisone, 0.18 mM adenine, 4 mM L-glutamine
Endothelial cell growth medium (EGM Bulletkit)	EBM 500 ml supplemented with: 10 ml FBS, 0.2 ml hydrocortisone, 2.0 ml hFGF-B 1, 0.5 ml VEGF, 0.5 ml R3-IGF-1, 0.5 ml ascorbic acid, 0.5 ml hEGF, 0.5 ml GA-1000, 0.5 ml heparin
Fibroblast growth medium (FGM)	DMEM high glucose supplemented with: 7.5% (v/v) FBS, 1% (v/v) penicillin/streptomycin, 1 % (v/v) L-glutamine
Fibroblast medium (ECM models) (DMEM+)	DMEM, low glucouse, Glutamax supplemented with: 7.5% (v/v) FBS, 1% (v/v) penicillin/streptomycin
Keratinocyte differentiation medium (KDM)	DMEM supplemented with: 21.5% (v/v) sodium pyruvate/Ham's F12, 10% (v/v) FBS, 5 µg/ml insulin, 10 ng/ml epidermal growth factor, 0.4 µg/ml hydrocortisone, 0.18 mM adenine, 0.1 nM cholera toxin, 4 mM L-glutamine
Keratinocyte growth medium	500 ml EpiLife with (60 µM Ca^{2+}) supplemented with: 1% (v/v) HKGS
Minimal medium	DMEM high glucose supplemented with: 10% (v/v) FBS, 1% (v/v) penicillin/streptomycin
PneumaCult™-ALI medium (P-ALI)	PneumaCult™-ALI Basal Medium supplemented with: 10% PneumaCult™-ALI Medium 10x, 1% PneumaCult™-ALI Maintenance 100x, 0.2% hydrocortisone, 0.5% heparin
PneumaCult™ Ex Plus medium	PneumaCult Ex Plus Basal Medium supplemented with: 2% PneumaCult™ 50 x sup

RPMI++	RPMI supplemented with: 10% (v/v) AB-Serum, 1% (v/v) penicillin/streptomycin

2.1.11 Software

GraphPad Prism 6	GraphPad Software, La Jolla, CA, USA
ImageJ 1.46r	National Institute of Health, MD USA

2.1.12 Mice

BALB/c mice (female and male) were purchased from Charles River (Sulzfeld, Germany), kept and treated at the Institute of Veterinary Pharmacology, FU Berlin in the lab of Prof. Dr. Wolfgang Bäumer. The animal experiment was approved by the LaGeSo (Berlin, Germany, Reference no. G 0204/20).

2.2 Methods

2.2.1 Cell culture

2.2.1.1 ISOLATION OF NHEK AND NHDF FROM JUVENILE FORESKIN[1]

Normal human epidermal keratinocytes (NHEK) and fibroblasts (NHDF) were isolated from juvenile foreskin following circumcision (approved by the ethics committee of the Charité–Universitätsmedizin Berlin, Germany, EA1/081/13; with informed consent); patient age did not exceed 10 years. Therefore, juvenile foreskin was washed in PBS 3-5 times, cleared from fatty tissue, cut into 3-4 cm thick pieces, and incubated in a dispase solution (1.2 U/ml dispase in PBS) overnight at 4°C. After 18-20 h the epidermis was peeled off the dermis by using forceps. Subsequently, the dermis pieces were placed in a 6-well cell culture plate for 30 min in order to attach before adding 1.5 ml FGM. Epidermis meanwhile was collected in trypsin-EDTA and incubated at 37°C for 20 min with periodical shaking. Subsequently, the reaction with trypsin was stopped by adding 10 ml of FGM, pipetting cell suspension up and down several times, and filtrating through a cell strainer (70 µm). After a centrifugation step (130 g, 5 min, 25°C) cells were counted and seeded into 6-well plates or T75 cell culture flaks depending on cell count (P0). Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. After one week in culture, the dermis pieces were removed and the outgrown NHDF (P0) sub-cultivated at a confluency of about 70-80%. NHEK meanwhile were kept in culture for 1-2 weeks before sub-cultivating once. NHEK and NHDF in passage 1 were trypsinized, resuspended in DMEM supplemented with 10% DMSO, and frozen until needed for further experiments. For generation of skin equivalents, cells of max. P2 were used.

2.2.1.2 GENERATION OF IN VITRO 3D SKIN (DISEASE) EQUIVALENTS

For generation of skin equivalents primary human epidermal keratinocytes (NHEK) and primary human dermal fibroblast (NHDF) isolated from juvenile foreskin were used (2.2.1.1). In a first step, a mix of bovine collagen and 10x HBSS was brought to neutral pH. Subsequently, a total of $0.3x10^6$ NHDF per equivalent were added and poured into 6-well cell culture inserts with a growth are of 4.2 cm^2. After 2 h of incubation (37°C, CO_2-free), medium was added into the well and on top of the collagen for another incubation step (2 h, 37°C, 95% humidity, 5% CO_2). Subsequently, $4.2x10^6$ NHEK were added on top of the collagen matrix. For generation of diseased skin equivalents, NHEK were treated with *FLG* siRNA (2.1.7) 24 h prior to generation of equivalents. 24 h after adding NHEK onto collagen matrix, medium was changed to a differentiation medium (KDM) and skin equivalents were

[1] The isolation was mainly performed by Carola Kapfer and Uta Hirt

lifted to the air-liquid interface for a cultivation period of 14 days with medium change every second day. For skin disease equivalents, IL-4 and -13 (15 ng/ml each) were added at day 10 and 12. Eventually, the equivalents were analyzed for viability, histology, and gene and protein expression.

2.2.1.3 ISOLATION OF HUMAN NAÏVE CD4+/8+ T CELLS FOR PRIMING EXPERIMENTS

Human naïve T cells were isolated and treated with complement factor C3 (C3), fibronectin (FN), syndecan-4 (SD-4), CD44, and thrombospondin-1 (TSP-1). Therefore, CD4+ and CD8+ T cells were isolated from peripheral blood mononuclear cells (PBMCs) from buffy coats by density gradient centrifugation. Buffy coat preparations from female and male donors were purchased from the German Red Cross (DRK-Blutspendedienst Ost, Berlin, Germany; with informed consent and ethical approval EA2/067/15). For that, blood was diluted with buffer (~2.5x the volume of blood) and 35 ml of that dilution were carefully layered over 15 ml NycoPrepTM 1.077 (Axis-Shield plc, Oslo, Norway) in a 50 ml tube. After a density gradient centrifugation (45 min, 1,400 rpm, RT, without brake), PBMC layer was carefully aspirated, transferred to a new 50 ml tube, and mixed with buffer. Subsequently to centrifugation (10 min, 1,200 rpm, RT), PBMC pellet was resuspended in pre-cooled buffer and centrifuged again (15 min, 1,000 rpm, 4°C). That step was repeated for up to 3 times until supernatant appeared clear. Naïve human $CD4^+$ and $CD8^+$ T cells were then purified from PBMCs by negative selection using magnetic-activated cell sorting beads according to the manufacturer's instructions (MACS; Miltenyi-Biotec, Bergisch-Gladbach, Germany). T cells were cultivated with RPMI containing 10% human AB-serum and 1% pen/strep (RPMI++). Subsequently, the T cells were activated using CD3/CD28 antibody (BioLegend, San Diego, USA). Therefore, 96-well round bottom plates were pre-coated with CD3/CD28 antibody for 2 h at 37°C and naïve T cells were added along with 50 ng/ml C3, 1,000 ng/ml FN, 5 ng/ml SD-4, 50 ng/ml CD44, or 5 ng/ml TSP-1, respectively.
After 48 h, the T cells were transferred to CD3/CD28-free wells and stimulated with each compound for 8 more days with medium change every 2-3 days. At day 10, compounds were removed and T cells were cultivated in RPMI++ for 2 more days. T cells were lysed in lysis solution for further mRNA isolation using the innuPREP RNA Mini Kit 2.0.

2.2.1.4 ISOLATION OF PATIENT-DERIVED FIBROBLASTS[2]

For generation of (atopic) ECM models (2.2.1.5), a total of 10-15 hair follicles were plucked from the scalp of healthy volunteers (20-30 years of age, with informed consent

[2] The isolation was performed by Dr. Anna Löwa

and ethical approval EA1/345/14). After identifying follicles in the anagen growth phase based on the presence of an intact epithelial ORS via inverted microscopy, the distal keratinized hair shaft was cut off and immersed 4 times in DMEM (Sigma-Aldrich, Germany) buffered with 25 nM HEPES (Life Technologies, Darmstadt, Germany) and supplemented with 400 U/ml penicillin, 400 µg/ml streptomycin, and 250 ng/ml amphotericin B (Sigma- Aldrich, Munich, Germany). After coating cell culture inserts (Corning, Costar 3450, USA) with post-mitotic 3T3-J2 fibroblasts ($3x10^4$ cells/cm^2) on their basal side, the hair follicles were then placed on the apical side of these inserts and cultivated submerged for 2-3 weeks in a defined outgrowth medium consisting of 10% FBS (Biochrom, Berlin, Germany), 5 µg/ml insulin (Roche, Prenzberg, Germany), 10 ng/ml EGF, 0.4 µg/ml hydrocortisone, 0.135 mM adenine, 2 nM triiodothyronine, 0.1 nM choleratoxin, 2 mM L-glutamine (all from Sigma-Aldrich, Munich, Germany), 50 U/ml penicillin, and 50 µg/ml streptomycin in DMEM with sodium pyruvate/Ham's F12 (3:1; all from Life Technologies, Darmstadt, Germany). Subsequently, cells were trypsinized and cultivated in the outgrowth medium without postmitotic 3T3-J2 fibroblasts to obtain hair follicle-derived fibroblasts (HFDF). After 4 days, HFDF were further cultivated in FGM. The cells were used at passage 3–4.

2.2.1.5 GENERATION OF SELF-ASSEMBLED ECM MODELS[3]

For generation of self-assembled ECM, donor-derived fibroblasts (healthy or atopic; 2.2.1.4) were cultivated in a mixed media of 50% DMEM+ and 50% endothelial cell (EC) (EGM-2 MV BulletKit, Lonza) medium before seeding 2,800 Fbs into 0.4 µm 12-well transwell inserts (1.12cm^2) (Corning 3460), and cultured submerged for 26 days with medium change every third day. Subsequently, instead of seeding NHEK on top according to the regular protocol for self-assembled skin equivalents, an air-lift was performed without NHEK and 50% DMEM+/EC medium was mixed with 50% KDM. Fbs were cultivated for 14 more days in order to generate ECM self-producing dermal equivalents.
Excised human skin from healthy patients served as control for an *in vitro-ex vivo* comparison of ECM.

2.2.1.6 GENERATION OF IN VITRO 3D BRONCHIAL EPITHELIAL EQUIVALENTS

Bronchial epithelial equivalents were generated using primary normal human bronchial epithelial cells (NHBE) and primary normal human lung fibroblasts (NHLFb) purchased from STEMCELL Technologies (Vancouver, Canada). In a first step, a mix of bovine collagen and 10x HBSS was brought to neutral pH. Subsequently, a total of 64,300 NHLFb per equivalent were added and poured into 12-well cell culture inserts. After 4 h of incubation (2 h CO_2-free; 2 h 5% CO_2 with medium in the well and on top

[3] These models were generated by Zheng Tan (Hedtrich Lab, UBC, Vancouver, Canada)

of the matrix), 0.9x10^6 NHBE were added on top of the collagen matrix. 24 h after adding NHBE, medium was changed to differentiation medium (PneumaCult™-ALI medium = P-ALI) and bronchial epithelial equivalents were lifted to the air-liquid interface for a cultivation period of 21 days with medium change every second day. Eventually, the models were analyzed for viability, histology, mucus secretion, and gene and protein expression.

2.2.1.7 MEDIA ADAPTATION

Media adaptation for skin equivalents started at day 5 and day 7. Skin equivalents were either directly mixed with 50% KDM and 50% P-ALI (S5 - 50%; S7 - 50%) or they were slowly adapted to P-ALI. For that, at day 5 or day 7 10% P-ALI was added and proportion was either raised by 10% (S5 - 10%; S7 - 10%) or 20% (S5 - 20%; S7 - 20%) daily until a mix of 50% KDM and 50% of P-ALI were reached. Another approach was to mix 50% KDM with either 50% “minimal“ (S7 Mini) or 50% “crossover“ (S7 Crossover) at day 7 and increase its proportion to 100% after 24 h. After media adaptation, media change was performed every second day.

Media adaptation for bronchial epithelial equivalents started at day 14. For “minimal“ medium, P-ALI was replaced for 50% P-ALI and 50% „minimal“ at day 14. After 24 h, that medium was replaced by 100% “minimal“ and medium change was performed every second day until day 21.

For “crossover“ medium, the same procedure was performed. In the case of “S14 - 10%“, P-ALI was replaced by 90% P-ALI and 10% KDM on day 14. Every 24 h proportion of KDM was increased by 10% until 50% were reached.

2.2.1.8 CO-CULTURE OF SKIN AND BRONCHIAL EPITHELIAL EQUIVALENTS

For “direct“ co-culture, skin equivalents at day 10 and bronchial epithelial equivalents at day 14 were used. Diseased skin equivalents were generated by adding 15 ng/ml of IL-4 and IL-13 at day 7 and day 9 to *FLG*- equivalents. At day 10 of skin equivalents and day 14 of bronchial epithelial equivalents, medium was replaced to 50% P-ALI and 50% “minimal“ in bronchial equivalents. The next day (day 11 skin, day 15 bronchial epithelial) both were placed into Alvetex deep petri dishes (REPROCELL Europe Ltd, Glasgow) with 33 ml of “minimal“ medium for 6 days (**Fig.7**).

For “indirect” co-culture, skin disease equivalents were prepared as already mentioned for direct co-cultivation with the difference, that after 4 days of IL-4/-13 stimulation, medium was replaced with IL-4/-13-free “minimal” medium for 24 h in order to obtain conditioned medium. Conditioned medium from healthy control skin equivalents was obtained the same way. Bronchial equivalents were supplemented

with this conditioned medium mixed 1:1 with fresh “minimal” medium and cultivated from day 15-21 with medium change every second day (**Fig.7**).

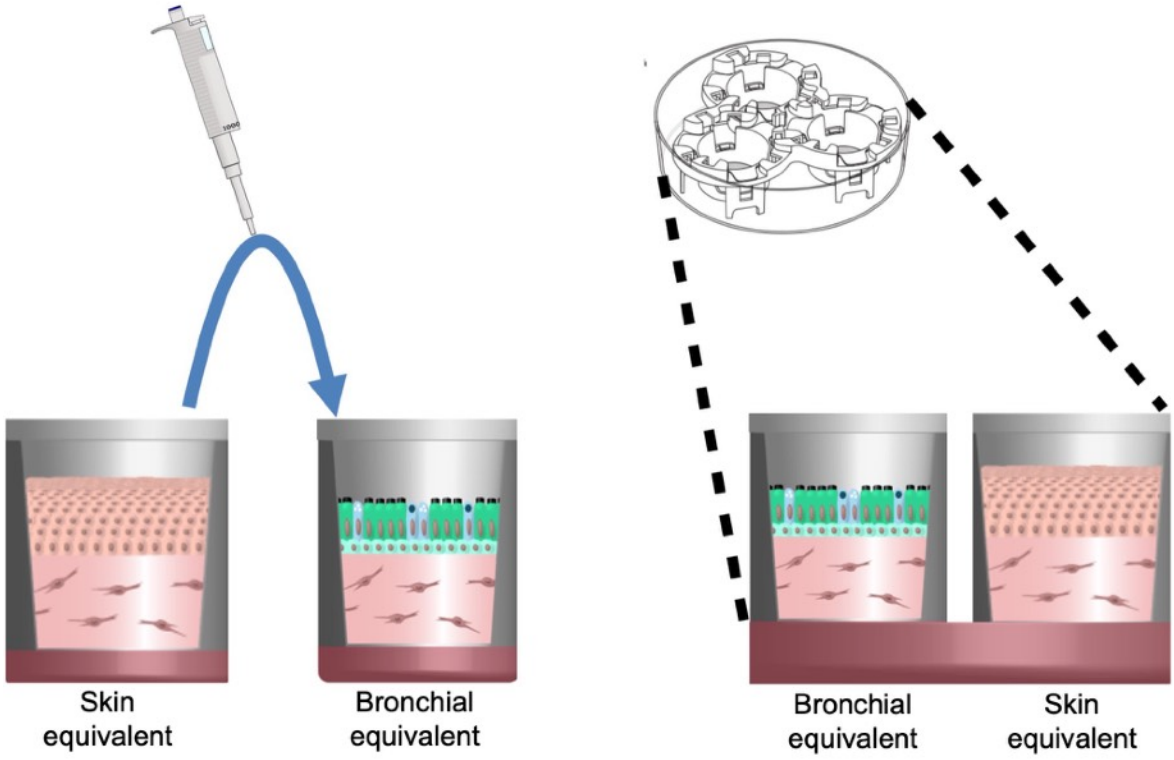


Figure 7. Co-culture scheme
In vitro co-culture of skin and bronchial epithelial equivalents was performed indirectly via conditioned medium (**left**). Conditioned medium from healthy and diseased (FLG-$_{IL-4/-13}$) skin equivalents was collected, mixed with 50% fresh “minimal” medium and added to bronchial epithelial equivalents every other day for 6 days. Direct co-culture (**right**) was performed in Alvetex deep petri dishes in “minimal” medium for 6 days (skin and bronchial equivalent schemes kindly provided by Dr. Anna Löwa).

2.2.2 Cryo- and micro-sectioning

Cryo-sectioning of skin equivalents

Skin equivalents were cultivated for 7-14 days, cut out the insert with a scalpel, placed into embedding molds pre-filled with tissue freezing medium, and covered with additional tissue freezing medium. These were shock frozen in liquid nitrogen and stored at -80°C. Vertical tissue sections of 7 µm thickness were prepared using a

cryotome (Leica Microsystems, Wetzlar, DE) and processed according to further protocols (2.2.3, 2.2.4, 2.2.6).

Micro-sectioning of bronchial epithelial equivalents[4]

After 21 days of tissue culture, bronchial epithelial equivalents were apically treated with 100 µl of 4% PFA for 1-2 min, membrane and equivalent were cut out of the insert, and placed into 2 ml of 4% PFA. After 24 h in 4% PFA, equivalents were embedded into paraffin automatically with a Tissue-Tek VIP embedding device (Skura, Umkirch, GER) according to the following protocol (**Tab. 1**):

Table 1. Paraffin-embedding protocol:

Step		Time [min]	Step		Time [min]
1	Formalin	60	8	Ethanol 100%	0.5
2	H_2O	0.5	9	Xylol	0.5
3	Ethanol 70%	0.5	10	Xylol	0.5
4	Ethanol 80%	0.5	11	Paraffin	0.5
5	Ethanol 96%	0.5	12	Paraffin	0.5
6	Ethanol 96%	0.5	13	Paraffin	60
7	Ethanol 100%	0.5	14	Paraffin	60

Vertical tissue sections with a thickness of 1 µm were prepared using a manual rotary microtome (Zeiss, Oberkochen, Germany). Directly after obtaining the section, it was placed into tap water (RT) for a few seconds, transferred into a paraffin stretching bath (40-50°C), and attached to an objective slide afterwards. Subsequently, those slides were dried (37°C, 24 h). The next day, standard protocols for staining were performed (2.2.3-2.2.6).

2.2.3 De-waxing of paraffin sections

After sections were dried overnight, hydration steps were performed immediately before staining. For that, slides were first placed into Roti®-Histol (2x5 min), ran through a dilution series of ethanol (100% 5 min, 95% 5 min, 90% 5min, 70% 5 min, 50% 5 min) afterwards, and finally placed into double-distilled (dd) H_2O for 1 min before moving on with hematoxilin and eosin (H&E-) or immunofluorescence staining. For the latter, the immunofluorescence staining, sections were incubated in citrate buffer (10 mM, pH 6) at 95°C for 20 min for antigen retrieval after de-waxing (**Tab. 2**) before following the protocol (2.2.4-2.2.6).

[4] The embedding was performed by cooperation partners from the Institute of Veterinary Pathology at FU Berlin (Prof. Joachim Gruber)

Table 2. De-waxing protocol:

Solution	Time [min]
Roti®-Histol	2x5
Ethanol 100%	5
Ethanol 95%	5
Ethanol 90%	5
Ethanol 70%	5
Ethanol 50%	5
ddH_2O	1

2.2.4 Hematoxilin and eosin staining

After fixing cryo-sections (4% PFA, 5 min) or de-waxing micro-sections (2.2.2), slides were placed into ddH_2O for 30 s before dipping those into Mayer's hematoxylin (5 min, agitation). Slides were then rinsed in tap water (5 min, agitation) and stained with 1% eosin G solution for 30 s with agitation. Subsequently, dehydration steps were performed (2x2 min 96% ethanol, 2x2 min 100% ethanol, 2x2 min Roti®-Histol) before air-drying, mounting with 1 drop of Roti®-Histokitt, and covering with cover slips. Whereas hematoxylin stains nuclei, eosin stains eosinophilic structures as e.g., the cytoplasm (**Tab. 3**).

Table 3. H&E-staining protocol:

Solution	Time [min]
ddH_2O	0.5
Meyer's hematoxylin	5
Tap water	5
Eosin G solution 1%	0.5
Ethanol 96% (v/v)	2x2
Ethanol 100% (v/v)	2x2
Roti®-Histol	2x2

2.2.5 Alcian blue staining

For more accurate characterization of bronchial epithelial equivalents and determination of possibly increased; thus, pathophysiological, amounts of mucus secretion, alcian blue staining was performed. After de-waxing micro-sectioned slides, these were placed into petri dishes and covered with 500 µl alcian blue reagent for 30 min. Subsequently to several washing steps (2 min tap water, 30 s dH_2O, 30 s ddH_2O), a counterstaining of nuclei with α-nuclear red was performed the same way

in a petri dish for 5 min. After another washing step (1 min tap water) and several dehydration steps (2x2 min 96% ethanol, 2x2 min 100% ethanol, 2x2 min Roti®-Histol), slides were air-dried, mounted with 1 drop of Roti®-Histokitt, and covered with a cover slip before visualization with a wide-field fluorescence microscope (Keyence, Neu-Isenburg, GER).

2.2.6 Immunofluorescence staining

Immunofluorescence staining was performed to observe and localize protein expression in equivalents, hence visualizing phenotypic changes in differentiation and inflammation. After fixing cryo-sections (4% PFA, 5 min) or de-waxing micro-sections (2.2.2), slides were washed with PBS (5 min) and permeabilized with 0.5% (v/v) triton-X in PBS. Subsequent to washing (5 min PBS, 5 min PBS/BSA/Tween), a blocking step with goat serum (1/20 in PBS) was performed for 30 min in order to block unspecific binding sides. Afterwards, primary antibody was diluted (**Tab. 4**) in PBS/BSA/Tween and drops of 400 µl were placed onto Parafilm inlaid into steel boxes. Slides were then placed upside-down onto these 400 µl of antibody dilution and kept at 4°C for overnight incubation. For secondary antibody only control, PBS/BSA/Tween was used for incubation.

The next day, slides were washed in PBS/BSA/Tween (3x5 min) and incubated upside-down with corresponding secondary antibody (1/400 dilution) for 1 h (RT, dark) on Parafilm. After several washing steps (3x5 min PBS/BSA/Tween, 2x5 min PBS, 1x30 s ddH_2O), slides were air-dried, mounted with one drop of Anti-fading Mounting Medium DAPI, covered with a cover slip, and stored at 4°C for up to 2 days before visualizing protein expression with a wide-field fluorescence microscope (Keyence, Neu-Isenburg, GER).

Table 4. List of primary and secondary antibodies for immunofluorescence (IF) and western blot (WB):

Antibody	Dilution IF	Dilution WB	Specimen	Clone	Isotype
α-acetylated tubulin	1:2,000		mouse	monoclonal (6-11B-1)	IgG2b
α-smooth muscle actin	1:200	1:1,000	rabbit	polyclonal	IgG
β-actin		1:10,000	mouse	monoclonal (15G5A11/E2)	IgG1
Cytokeratin 10	1:200		mouse	monoclonal (DE-K10)	IgG1
Cytokeratin 14	1:200		mouse	monoclonal (LL002)	IgG3
E-Cadherin	1:100		mouse	monoclonal (67A4.2.1)	IgG1
Filaggrin	1:1,000	1:1,000	mouse	polyclonal	IgG
GAPDH		1:1,000	rabbit	monoclonal (14C10)	IgG
Ki-67	1:200		mouse	monoclonal (Ki-67P)	IgG
Loricrin	1:500	1:1,000	rabbit	polyclonal	IgG
MUC5AC	1:600	1:100	mouse	monoclonal (CLH2)	IgG1
PAR-2	1:200	1:200	rabbit	monoclonal (SAM11)	IgG2A
SCCA-1	1:125	1:1,000	rabbit	monoclonal (886524)	IgG2B
sPLA-2	1:400	1:1,000	rabbit	polyclonal	IgG
Tenascin C	1:200	1:1,000	mouse	monoclonal (EB2)	IgG1
TSLP	1:400	1:1,000	rabbit	polyclonal	IgG
Uteroglobin		1:1000	rabbit	polyclonal	IgG
Vimentin	1:100		mouse	monoclonal (V9)	IgG1
ZO-1	1:200	1:1,000	rabbit	polyclonal	IgG
Anti-rabbit, HRP-conjugated		1:1,000	goat	polyclonal	IgG
Anti-mouse, HRP-conjugated		1:1,000	goat	polyclonal	IgG
Alexa Fluor® 488	1:400		goat (anti-rabbit or anti-mouse)	polyclonal	IgG
Alexa Fluor® 594	1:400		goat (anti-rabbit or anti-mouse)	polyclonal	IgG

2.2.7 Quantitative real-time polymerase chain reaction (qRT-PCR)

To analyze changes on mRNA-level, a qRT-PCR was performed. The mRNA from the epidermal layer of skin (disease) equivalents was isolated by gently peeling off epidermis, shock-freezing it in liquid nitrogen, grinding (30 s, 25 Hz) using a TissueLyzer (Qiagen, Hilden, Germany), and resuspending in lysis buffer from innuPREP RNA mini 2.0 Kit (Analytik Jena, Jena, Germany).

In order to analyze changes in mRNA expression in human and mouse T cells, cells were lysed in lysis buffer (2 min, RT) and centrifuged (13,200 rpm, 4°C, 10 min).

RNA purification was performed according to the manufacturer's instructions from innuPREP RNA mini 2.0 Kit (Analytik Jena, Jena, Germany).

Allprep mini DNA/RNA/Protein Isolation Kit (Qiagen) was used for isolation of mRNA and protein from bronchial epithelial equivalents according to manufacturer's instructions.

After isolation of mRNA, iScript™ cDNA Synthesis Kit was immediately used for cDNA synthesis according to manufacturer's instructions. Briefly, 11 µl of sample were mixed with 4 µl of RNase-free water, 4 µl of 5xreaction mix, and 1 µl of reverse transcriptase. A standard program was performed using a Biometra T-Gradient thermocycler (Analytik Jena, Jena, GER) according to manufacturer's instructions (**Tab. 5**). Samples were stored at -80°C until further use.

Table 5. cNDA synthesis program:

Reaction step	Temperatur [°C]	Duration [min]
Priming	25	5
Reverse transcription (RT)	46	20
RT inactivation	95	1
Optional hold	4	—

The subsequent qRT-PCR was performed using SYBR Green I Master Mix (Bio-Rad Laboratories, Munich, Germany). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) for bronchial epithelial equivalents, *YHWAZ* for human CD4+ and CD8+ T cells, and *Hprt* and *Yhwaz* for mouse CD4+ T cells served as housekeeping genes. Primer sequences are listed in 2.1.6. 2 µl of each sample was mixed with 8 µl of a master mix (0.5 µl forward primer, 0.5 µl reverse primer, 2 µl RNAse-free water, 5 µl 2xSYBR Green) and a qRT-PCR was performed on a LightCycler480 (Roche, Mannheim, GER) according to an established protocol (**Tab. 6**).

Table 6. PCR program:

Reaction step		Temperatur [°C]	Duration [s]	Cylces
Preincubation	denaturation	95	300	1
Amplification	denaturation	95	10	45
	annealing	60	10	
	elongation	72	10	
Melting curve	denaturation	95	10	1
	annealing	65	60	
	cool down	40	10	—

2.2.8 Isolation of proteins

In order to relatively quantify protein expression in skin and bronchial epithelial equivalents additionally to immunofluorescence staining, SDS-page and western blot were performed. Therefore, proteins were isolated by gently peeling off epidermis from skin equivalents, placing into RIPA buffer containing 1% (v/v) Protease Inhibitor and grinding (30 s, 25 Hz) using a TissueLyzer (Qiagen, Hilden, Germany). After 30 min incubation on ice and centrifugation (4°C, 30 min, 13,200 rpm), samples were stored at -80° until use.

For protein isolation from bronchial epithelial equivalents, whole equivalent was used and protein was isolated together with mRNA using Allprep mini DNA/RNA/Protein isolation Kit (Qiagen, Hilden, Germany) according to manufacture's instructions.

2.2.9 SDS-PAGE and western blot

Protein amount was quantified using Pierce® BCA Protein Assay Kit (Thermo Scientific, Schwerte, Germany) according to manufacture's instructions. From the measured absorption, protein concentration was calculated for a 15-30 µg/µl concentrated protein solution. Subsequently, the desired amount was mixed 2:1 with laemmli-buffer (10 parts loading buffer (3x) and 1 part DTT (30x)), heated (5 min, 95°C), and centrifuged (11,000 rpm, 5 min, 4°C) in order to denaturate proteins. These samples were pipetted into (bis)-acrylamide gels (**Tab. 7**) — gel concentration depending on protein size of interest — and separated according to their molecular weight. Afterwards, gels were blotted (30 min 100 V, 90 min 150 V) onto previously activated (15 min blotting buffer) nitrocellulose membrane (Bio-Rad, Munich, Germany), washed with TBST (3x5 min), and blocked with 5% (w/v) skimmed milk powder in TBST (1 h, RT). Gels were incubated with primary antibody (**Tab. 4**) over night (4°C, dark), washed with TBST the next day (3x5 min), incubated with secondary antibody (1h, RT, dark), and washed with TBST (3x5 min). Blots were visualized with a PXi/PXi Touch gel imaging system (Syngene, Cambridge, UK) after

developing with SignalFire™ ECL or ECL Elite reagent (Cell Signaling, Frankfurt/ Main, Germany). Intensity of bands was relatively quantified with ImageJ (Version 1.46, Wayne Rusband, National Institute for Health, USA) according to each loading control.

Table 7. Composition of running (r) and stacking (s) gels for SDS-PAGE:

Percent gel	ddH_2O [ml]	(Bis-)Acrylamide [ml]	Gel buffer [ml]	10% (w/v) SDS [ml]
4% (s)	6.1	1.3	2.5	0.1
6% (r)	5.4	2.0	2.5	0.1
10% (r)	4.1	3.3	2.5	0.1

2.2.10 LDH-Assay for cell viability

In order to analyze viability of equivalents after media adaptation and in the co-culture without the need to destroy the equivalent itself, LDH measurement in cell culture media was performed with CytoTox-ONE™ Homogeneous Membrane Integrity Assay (Promega, Madison, USA). Experiments were performed according to manufacturer's protocol. Starting the day before media adaptation or co-culture, and following every second day, media samples were collected and LDH was measured in duplicates for each sample in 96-well plates by mixing media sample with Cytotox reagent (1:1, 10 min, RT, dark) and stopping reaction with half the amount of stop solution.

2.2.11 Enzyme-linked Immunosorbent Assay (ELISA)

To measure secretion of asthma-relevant and inflammatory cytokines from bronchial epithelial and skin (disease) equivalents into cell culture media and to determine levels of cytokines of interest in healthy and patient-derived plasma samples, several sandwich ELISAs were performed. For IL-25 a DuoSet kit (R&D Systems, Abingdon, UK), for IL-1β, -6, -8, -10, -33, TNFα, and TSLP ELISA kits from Thermo Fisher (Schwerte, GER), for C3 a colorimetric ELISA kit from R&D were used according to manufacturer's instructions. Therefore, 96-well plates were pre-coated with target-specific antibody overnight, blocked, and incubated with standards and samples over night at 4°C for increased sensitivity. An HRP-linked secondary antibody was added the next day for specific target protein binding and signal intensity was measured at a wavelength of 450 nm while subtracting values measured at 562 nm. Pre-coated plates were used according to manufacturer's instruction without previous blocking and prolonged overnight incubation with samples.

2.2.12 ECM characterization[5]

Collagen quantification

To quantify the collagen content in ECM, a colorimetric hydroxyproline assay kit was used according to manufacturer's instructions (Abcam, Cambridge, UK). Briefly, the ECM was hydrolyzed with concentrated NaOH at 120°C for 1 h. 10 N concentrated HCl was added to neutralize residual NaOH and the supernatant was collected after centrifugation. Samples hydrolysate was evaporated at 65°C and dissolved in an oxidation reagent mix. Finally, absorbance was measured at 560 nm on a microplate reader.
For desmosine quantification, a kit from My BioSource, San Diego, USA was used according to manufacturer's instruction.

Scanning Electron Microscopy (SEM)

SEM was utilized to visualize the fiber arrangement and structure of ECM produced by AD patient-derived and healthy fibroblasts as well as excised human skin (#CREB H19-03096). First, the ECM was carefully cut under a dissection microscope and subsequently fixed with 2.5% glutaraldehyde (pH 7.4, RT, 10 min). Samples were then dehydrated stepwise by immersing them into increasing concentrations of ethanol. Subsequently, the samples were transferred into a critical point dryer after which they were mounted carefully in lateral view on a metal stub with double-sided carbon adhesive tape that has been previously coated with Au/Pd using a Leica EM MED020 Coating System. Samples were then imaged by a Helios NanoLabTM 650 (FEI, Hillsboro, OR) scanning electron microscope, operated at 2–10 kV.

2.2.13 Proteomics analysis of skin equivalent secretome[6]

In preparation of the secretome analysis, FBS was removed from culture medium of the skin equivalents at day 10 and culture media was then collected at day 12 and 14. Samples were prepared by quantification of protein amount and denaturation prior to separation via SDS-PAGE (2.2.9). Gel was stained with SimplyBlue ™ SafeStain according to manufacturer's instructions (Invitrogen, Carlsbad, CA) and bands of interest were excised from the gel. Further sample preparation and analysis were performed by UBC proteomics core as follows:
The excised bands of interest were cut into 1-2 mm pieces, washed with de-stain buffer (50 mM NH_4HCO_3/100% EtOH – 6:4), and subsequently dehydrated in ethanol. Reduction of disulfide bonds was done by incubation in 10 mM DTT for 45 min at 56°C, followed by alkylation in 55 mM IAA for 30 min at RT in the dark. Gel pieces

[5] These methods were performed and method descriptions were provided by Preety Panwar (Brömme Lab, Department of Oral Biological & Medical Sciences, Faculty of Dentistry, University of British Columbia; Centre for Blood Research, UBC, Vancouver, BC, Canada.

[6] This method description was provided by Jason Rogalski (UBC Proteomics Core Facility, Vancouver, Canada)

were then dehydrated in EtOH, rehydrated in digestion buffer (50 mM NH_4HCO_3 – pH 8), dehydrated again, and then the digestion was done in digestion buffer (overnight, 37°C). Digestion was stopped with 1% TFA and samples were extracted twice with extraction solution (acidified water with acetonitrile – 40% ACN, 0.1% TFA). Samples were then concentrated via vacuum centrifugation. Subsequently, extracted peptide samples were then cleaned up via STAGE-tip purification. In brief, re-solubilized acidified sample was forced through conditioned and equilibrated column with 7 mm of C18 packing, washed with 1% TFA twice, eluted into clean tubes by buffer containing 40% ACN, 0.1% TFA, and dried down.

MS analysis: For MS analysis, the samples were reconstituted in 2% ACN, 0.5% formic acid, and the peptides were analyzed using a quadrupole – time of flight mass spectrometer (Impact II; Bruker Daltonics) on-line coupled to an EasyLC 1000 HPLC (ThermoFisher Scientific) using a Captive spray nanospray ionization source (Bruker Daltonics) including Aurora Series Gen2 (CSI) analytical column (25 cm x 75 µm 1.6 µm FSC C18, with Gen2 nanoZero and CSI fitting; Ion Opticks, Parkville, Victoria, Australia), and a µ-pre-column (300 µm ID x 5 mm, C18 PepMap, 5 µm, 100 A; Thermo Scientific, Waltham, Massachusetts, US). The analytical column was heated to 50°C using tape heater (SRMU020124, Omega.com and in house build microprocessor temperature controller). Buffer A consisted of 0.1% aqueous formic acid and 2% acetonitrile in water, and buffer B consisted of 0.1% formic acid in 90 % acetonitrile. Samples were resuspended in buffer A and loaded with the same buffer. Standard 45 min gradients were run from 5% buffer B to 18% B over 22 min, then increased to 35% B over 23 min, increased to 90% B over 2 min period, held at 90% B for 13 min. For 90 min run, the gradient was from 5% B to 18% B over 45 min, then to 35% B from 45 to 90 min, then to 90% B over 2 min, held at 90% B for 13 min. Before each run the analytical column was conditioned with 4 µL of buffer A and the pre-column was conditioned with 20 µl of buffer A. The LC thermostat temperature was set at 7°C. The analysis was performed at 0.35 µL/min flow rate. Impact II was run with OTOF Control v. 4.1 (Bruker). LC and MS were controlled with HyStar 4.1 (4.1.21.2, Bruker). The Impact II was set to acquire in a data-dependent auto-MS/MS mode with inactive focus fragmenting the 20 most abundant ions (one at the time at 18 Hz rate) after each full-range scan from m/z 200 Th to m/z 2000 Th (at 5 Hz rate). The isolation window for MS/MS was 2 to 3 Th depending on parent ion mass to charge ratio and the collision energy ranged from 23 to 65 eV depending on ion mass and charge. Parent ions were then excluded from MS/MS for the next 0.3 min and reconsidered if their intensity increased more than 5 times. Singly charged ions were excluded, since in ESI mode peptides usually carry multiple charges. Strict active exclusion was applied. Mass accuracy: error of mass measurement is typically within

5 ppm and is not allowed to exceed 10 ppm. The nano ESI source was operated at 1900 V capillary voltage, 0.25 Bar pressure with methanol in the nanoBooster, 3 L/min drying gas, and 150°C drying temperature.

Search: The acquired data was then searched against the Uniprot protein database for Homo sapiens using the Byonic search Algorithm from Protein Metrics Inc, with 20 ppm and 40 ppm mass accuracies for precursor and product ion masses respectively, and a 1% false discovery rate cutoff.

2.2.14 Proteomics analysis of ECM models[7]

In-solution digest protocol: Each extracellular matrix was lysed with 50% TFE protocol (30 uL TFE:30 uL H_2O), sonicated for 5 min, frozen, thawed, sonicated for 10 min, and pH adjusted by 1M Tris pH 8.5. Then taken from 26 ug to 86 ug of each sample for further process. Reduction of disulfide bonds of proteins was done by tris(2-carboxyethyl)phosphine (TCEP) solution (1 ug to every 50 ug protein) and incubated for 20 min at room temperature. Blocking of free sulfhydryl groups was performed by chloroacetamide (CAA) solution (5 ug to every 50 ug protein) and incubated for 10 min at 95°C in the dark. Then samples were diluted by four volumes of 50 mM ammonium bicarbonate (pH ~ 8) for pH correction, followed by adding LysC enzyme (1 ug to every 50 ug protein) and incubated 2 h at 37°C. Lastly, samples were digested with Trypsin (1 ug to every 50 ug protein) for 19 h at 37°C, followed by the second round of Trypsin digestion (1 ug to every 125 ug protein), and incubated 5 h at 37°C.
Trypsin activity was quenched by acidifying samples by adding 1% TFA for a final pH < 2.5 of the samples followed by cleaned up via STAGE-Tip purification, briefly:
Each of the acidified samples was forced through a conditioned and equilibrated column with 8 mm of C18 packing, washed with 1% TFA twice, and eluted into clean tubes by buffer containing 40% ACN, 0.1% TFA, then dried down.

MS analysis: see 2.2.13

Search: Acquired data were then searched by MaxQuant (V1.6.10.43) against the Uniprot protein database for Homo sapiens, LFQ intensities extracted and normalized using the MaxLFQ algorithm (PMID: 24942700), with 20 ppm and 30 ppm mass accuracies for precursor and product ion masses, respectively, and a 1% false discovery rate cut-off.

[7] This method was performed and method description was provided by Jason Rogalski and Renata Moravcova (UBC Proteomics Core Facility, Vancouver, Canada)

Data analysis: Label-free quantitation results were then filtered and tested with statistical software as follow:
Data were loaded into the Perseus computational Proteomics Platform (V1.6.15.0) (PMID: 27348712). First, the data were filtered for common contaminants and false-positive identification, then log-transformed followed by checking for tightness of technical replicates (Pearson correlation > 0.95).

2.2.15 RNA-sequencing of patient-derived fibroblasts[8]

First, bulk RNA was isolated from AD patient-derived fibroblasts and healthy control samples using the Invitrogen PureLink RNA mini Kit (Thermo Scientific, Burnaby, Canada) according to the manufacturer's protocol. Sample quality control was then performed using the Agilent 2100 Bioanalyzer. Qualifying samples were then prepped following the standard protocol for the NEBnext Ultra ii Stranded mRNA (New England Biolabs). Sequencing was performed on the Illumina NextSeq 500 with Paired End 42bp×42bp reads. Sequencing data was de-multiplexed using Illumina's bcl2fastq2.
De-multiplexed read sequences were then aligned to the Homo sapiens/hg19 reference sequence using STAR aligner (Dobin et al. 2013). Assembly and differential expression were estimated using Cufflinks (http://cole-trapnell-lab.github.io/cufflinks/) through bioinformatics apps available on Illumina Sequence Hub. Uniquely mapped reads had a minimum of 13,978,753, median of 17,760,215 and a maximum of 23,957,122. Differential expression (DE) analysis was performed using DESeq2 v1.28.1 (Love, Huber, and Anders 2014) and significance for DE genes was determined using an absolute fold change value of ≥ 1.5 and adjusted p-value ≤ 0.05. Since samples were sequenced in multiple runs, batch was included in the DESeq2 design formula to control for potential batch effects. Pathway enrichment of DE genes was performed using Sigora v3.0.5, with significance defined as a Bonferroni-corrected p-value ≤ 0.001 (Foroushani, Brinkman, and Lynn 2013). Network analysis was done by uploading genes and their respective fold change values to NetworkAnalyst for construction of protein-protein interaction (PPI) networks (Xia, Gill, and Hancock 2015).

2.2.16 *In vivo* experiments in BALB/c mice[9]

In order to verify effects and possibly translate results from *in vitro* to more complex *in vivo* systems, three (SD-4, CD44, TSP-1) out of five identified compounds were tested *in vivo*. Experiments were mainly performed by Dr. Jenny Wilzopolski with support from Dr. Viviane Filor and Pascal Canbolat (Group of Prof. Dr. Wolfgang

[8] Experiment was performed and method description provided by Travis Blimkie (Hancock Lab, Department of Microbiology and Immunology, Centre for Microbial Diseases and Immunity Research, UBC, Vancouver, BC, Canada)

[9] This experiment was mainly performed and method description was provided by Dr. Jenny Wilzopolski (Group of Prof. Dr. Wolfgang Bäumer, Department of Veterinary Medicine, Freie Universität Berlin, Germany)

Bäumer, Department of Veterinary Medicine, Freie Universität Berlin, Germany) at the Institute of Veterinary Pharmacology and Toxicology (Koserstr. 20, 14195 Berlin, Germany).

Experiments were approved by LaGeSo (Berlin, Germany; G0204/20) and mostly go in line with the ARRIVE guidelines. Groups were randomly assigned per cage and people working on implantation of pumps, observation, scoring, organ explantation, evaluation of final data, and statistical analysis were fully blinded until the end. Pumps were loaded by one person and labeled with rising numbers from 101-124. The assignment was unblinded after full data evaluation.

Female and male BALB/c (BALB/cAnNCrl) mice at the age of 5-6 weeks were purchased from Charles River (Sulzfeld, Germany). All mice were kept in groups of three mice per cage (type III makrolon) with a 12 h light/dark cycle at 22°C. Water and standard diet (Altromin, Lage/Lippe, Germany) were provided ad libitum.

Mice were acclimatized to their new housing environments for 14 days prior to the experiments. The back of the mice was depilated (Veet depilation crème sensitive skin, RB Healthcare, Hull, UK) three days prior to the surgical procedure. On day 0 of the experiment, Alzet micro-osmotic pumps 1007D (Charles River, Sulzfeld, Germany) were filled with 100 µl PBS only for control group, 84 ng/100 µl SD-4 in PBS, 21 µg/100 µl TSP-1 in PBS, or 105 µg/100 µl CD44 in PBS directly before implanting. The resulting release was 12 ng/d SD-4, 3 µg/d TSP-1, and 15 µg/d CD44 with a release rate of 0.5 µl/hr. The implantation was performed according to Lu et al. (Lu et al. 2015).

Anesthesia was induced with 3% isoflurane (Isofluran CP®, CP Pharma Handelsgesellschaft mbH, Burgdorf, Germany) in 100% oxygen in an anesthetic chamber with sliding cover (Evonik Plexiglas, 240 x 140 x 120 mm). The chamber was not prefilled to prevent distress. After surgical tolerance began, mice were transferred to a nose cone and anesthesia was maintained with 1.5% isoflurane in 100% oxygen. Mice were placed in an abdominal position on a heating pad (36°C). Eye ointment (Vitagel®, Bausch + Lomb GmbH, Berlin, Germany) was administered to both eyes to prevent the eyes from drying out. 2% lidocaine hydrochloride (bela-pharm GmbH & Co. KG, Vechta Germany) was injected subcutaneously (s. c.) with an insulin syringe (BD Micro-Fine™ + BD Medical – Diabetes Care, Le Pont de Claix Cedex, France) at the incision site. Meloxicam (Metacam 2 mg/ml, Böhringer Ingelheim Vetmedica GmbH, Ingelheim/Rhein, Germany) was administered 1 h prior and 12 and 24 h after the surgical procedure at a concentration of 5 mg/kg (s.c.).

The shaved area was swapped with Braunol® (B. Braun Deutschland GmbH & Co. KG, Melsungen, Germany) followed by three wipes of 70% ethanol (Carl Roth GmbH + Co. KG, Karlsruhe, Deutschland). Behind the shoulder blades, a 0.5-1 cm incision perpendicular to the tail was made with a surgical scalpel. With a hemostat a pouch

for the Alzet pump was created under the skin. A bolus of approximately 0.5-1 ml of sterile 0.9% NaCl (B. Braun Deutschland GmbH & Co. KG, Melsungen, Germany) was applied into the pocket. Afterwards, the Alzet pump was inserted into the pouch. The incision sites were pinched together with two hemostats and the wound was closed with 1-2 wound clips (11 x 2 mm; AESCULAP, B. Braun Deutschland GmbH & Co. KG, Melsungen, Germany). The whole implantation process took about 5 minutes per mouse. Afterwards, mice were placed in a dark cage on a heating pad for the recovery period. On day 4, a 100 µl blood sample was collected from V. facialis with a 5 mm Goldenrod™ Animal Lancet (Medipoint Inc. Mineola, NY, USA). Measurement of the temperature and wound control were performed every day until the mice were sacrificed at day 7. For sample collection, mice were deeply anesthetized with an intraperitoneal injection of ketamine-xylazine-NaCl mix (100 µl/ 10 g bodyweight). The mix contained 2 parts of ketamin 10% 100 mg/ml (bela-pharm GmbH & Co. KG, Vechta Germany), 1 part xylazine 20 mg/ml (Xylariem®, Ecuphar N.V., Oostkamp, Belgium), and 9 parts sterile NaCl. After loss of all reflexes, abdominal skin and cavity were opened and mice were exsanguinated via the V. cava with a 24G needle. Spleen, thymus, lung, and intestines were removed for further investigations.
Simultaneously, the T cells from the mouse spleen were isolated following a modified protocol as described previously (Bäumer et al. 2005). Before isolation, mouse spleens were weighted and size was documented. For isolation, the spleen was squeezed through a cell strainer, which resulted in a single-cell suspension in the filtrate. This single-cell suspension was washed with PBS and centrifuged (1,000 g, 10 min) before incubation in an erythrocyte lysis buffer for 5 min followed another washing step with PBS (1,000 g, 10 min). Subsequently, CD4+ T cells were isolated by negative selection using magnetic-activated cell sorting beads according to the manufacturer's instructions (MACS; Miltenyi-Biotec, Bergisch-Gladbach, Germany) (2.2.1.3). Finally, mRNA from CD4+ T cells was isolated by incubating in lysis buffer for 2 min and processing as already mentioned above (2.2.7).

Histological analysis of mice lungs[10]

Histopathology was performed as previously described (Dietert et al. 2017). Prior to preparation of the lungs, the trachea was ligated to prevent alveolar collapse. After careful removal from the thoracical cavity, lungs were immersion-fixed in 4% buffered formalin (pH 7.0) for 24 h, embedded in paraffin, and cut in 2 µm sections. Slides were de-waxed in xylene, rehydrated in graded ethanol's, and stained with hematoxylin and eosin (H&E). Three evenly distributed sections per lung were

[10] These experiments were performed and method description was provided by Anne Voß and are part of her thesis with focus on the comparison of histological and transcriptome data in different models (Prof. Achim Gruber, Veterinary Pathology at FU Berlin)

microscopically evaluated in a blinded, randomized fashion by a board-certified veterinary pathologist.

2.2.17 Statistical analysis

Statistical analysis was performed with GraphPad Prism 6 (Ver. 6.07) software. Values are expressed as mean ± SEM from at least three independent donors. Statistical analysis was performed for experiments with sample size ≥ 5. For patient-derived plasma samples, a test for independent, non-parametric values was performed (Mann-Whitney). For skin and bronchial epithelial equivalent experiments, the Wilcoxon signed-rank test for dependent, non-parametric values was performed.

3. RESULTS

3.1 Co-cultivation of skin and bronchial epithelial equivalents

3.1.1 Optimization of the co-culture medium

Media adaptation in skin equivalents

The first step on the way of establishing a co-culture between skin and bronchial epithelial equivalent, was to identify a co-culture medium where both equivalents, skin and bronchial epithelium, do not undergo significant changes compared to their usual culture conditions. The main issues here were the complexity of these media, the different culture times of 14 and 21 days, and, particularly for bronchial epithelial equivalents, no knowledge about the exact composition of PneumaCult™-ALI medium (P-ALI), since the company was not willing share any information.

In order to gain a fundamental overview about the influence of tested media, histology and viability were analyzed. Since skin equivalents have been established for quite a long time and are known to be more robust than recently established bronchial epithelial equivalents, different media were tested in skin equivalents first.

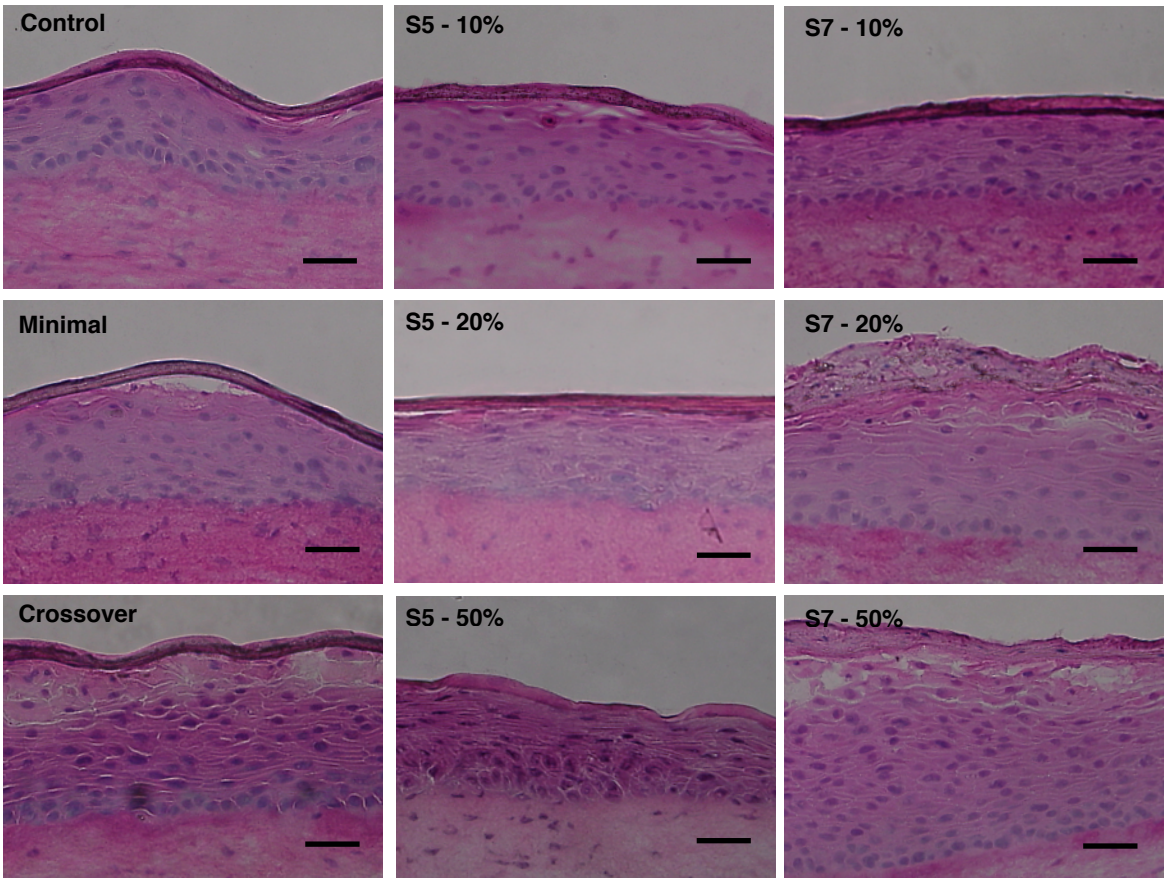
The first approach consisted in simply mixing skin equivalent medium (KDM) and bronchial epithelial medium (P-ALI) in a ratio of 50% each. For skin equivalents, P-ALI was either added directly (50%), was fast (20% per day) or slowly adapted (10% per day) starting at day 5 (S5) or day 7 (S7) until a mixture of 50:50 was achieved.

With the direct (50%) and fast (20%) media adaptation starting at day 5 or day 7, histological staining revealed inconsistent results with high inter-donor variability showing either hyper-proliferation, disorganization of skin layers, or huge variations in thickness of epidermal layer. The uppermost layer, the stratum corneum, did not develop well as shown for S7 - 20% and S7 - 50%. The epidermal thickness of skin equivalents with media adaptation started at day 7 was higher on average with partly high standard deviation, like 119.07% ± 67.07 for S7 - 20% and 116.82% ± 76.59 for S7 - 50%. Within this set, S7 - 10% showed the slightest deviation of epidermal thickness with 112.37% ± 27.68 compared to control and a clearer structure of viable epidermis with a well-developed stratum corneum (**Fig. 8**).

The media adaptation starting at day 5 on the other hand showed a thinner epidermal layer by an average of approximately 20%. For S5 - 20%, even no clear subdivision into epidermal layers (s. basale, s. granulosum, s. spinosum) was observed but a comparably thin and unorganized viable epidermis with a thickness of 83.33% ± 28.35 compared to the conventional culture.

A second approach was to either use a medium containing certain supplements from both media (“crossover“) - as far composition of different media for culturing bronchial epithelial equivalents was available in literature - or reducing the medium to the necessary basic components (“minimal“) in order to ensure a steady-state cultivation with no trigger for further differentiation and proliferation, hence cultivating with the basal medium supplemented with 10% FCS and 1% penicillin/streptomycin. Both these media showed

consistent results for histology with clearer developed viable epidermis and stratum corneum. Whereas “crossover“ medium led to a slightly thicker epidermis (120.83% ± 13.61), “minimal“ media resulted in slightly thinner epidermis (90.40% ± 18.79) (**Fig. 8**).



Epidermis thickness [%]							
Minimal	**Crossover**	**S5 - 10%**	**S5 - 20%**	**S5 - 50%**	**S7 - 10%**	**S7 - 20%**	**S7 - 50%**
90.40 ± 18.79	120.83 ± 13.61	83.33 ± 28.35	77.71 ± 18.45	86.90 ± 43.28	112.37 ± 27.68	119.07 ± 67.07	116.82 ± 76.59

Figure 8. Histology and epidermal thickness of skin equivalents after media adaptation

Representative images of H&E-staining of control equivalents and equivalents supplemented with P-ALI starting at day 5 (S5) or at day 7 (S7) with slow (10%), fast (20%) or direct (50%) media adaptation, and “minimal“ and “crossover“ medium. Scale bar: 50 µm. Epidermal thickness of skin equivalents compared to control model (=100 %) as determined with ImageJ is shown in the table below. For n=3 independent donors. Mean ± SD.

In a next step, a deeper characterization of the most promising media adaptation approaches followed by staining skin equivalents for important barrier proteins such as filaggrin (FLG) and involucrin (IVL) as well as the proliferation marker Ki-67. Whereas Ki-67 was strongly upregulated in S7 -10%, no alteration was observed for “minimal“ and “crossover“ medium compared to control. An increase of IVL was found for “crossover“, whereas “minimal“ and S7 - 10% showed similar expression compared to the control.

Expression of FLG in stratum corneum was slightly increased for S7 - 10%. Whereas no changes were observed for “minimal“ medium, a diffuse expression of FLG not only in stratum corneum, but throughout viable epidermis was observed for “crossover” medium (**Fig. 9**).

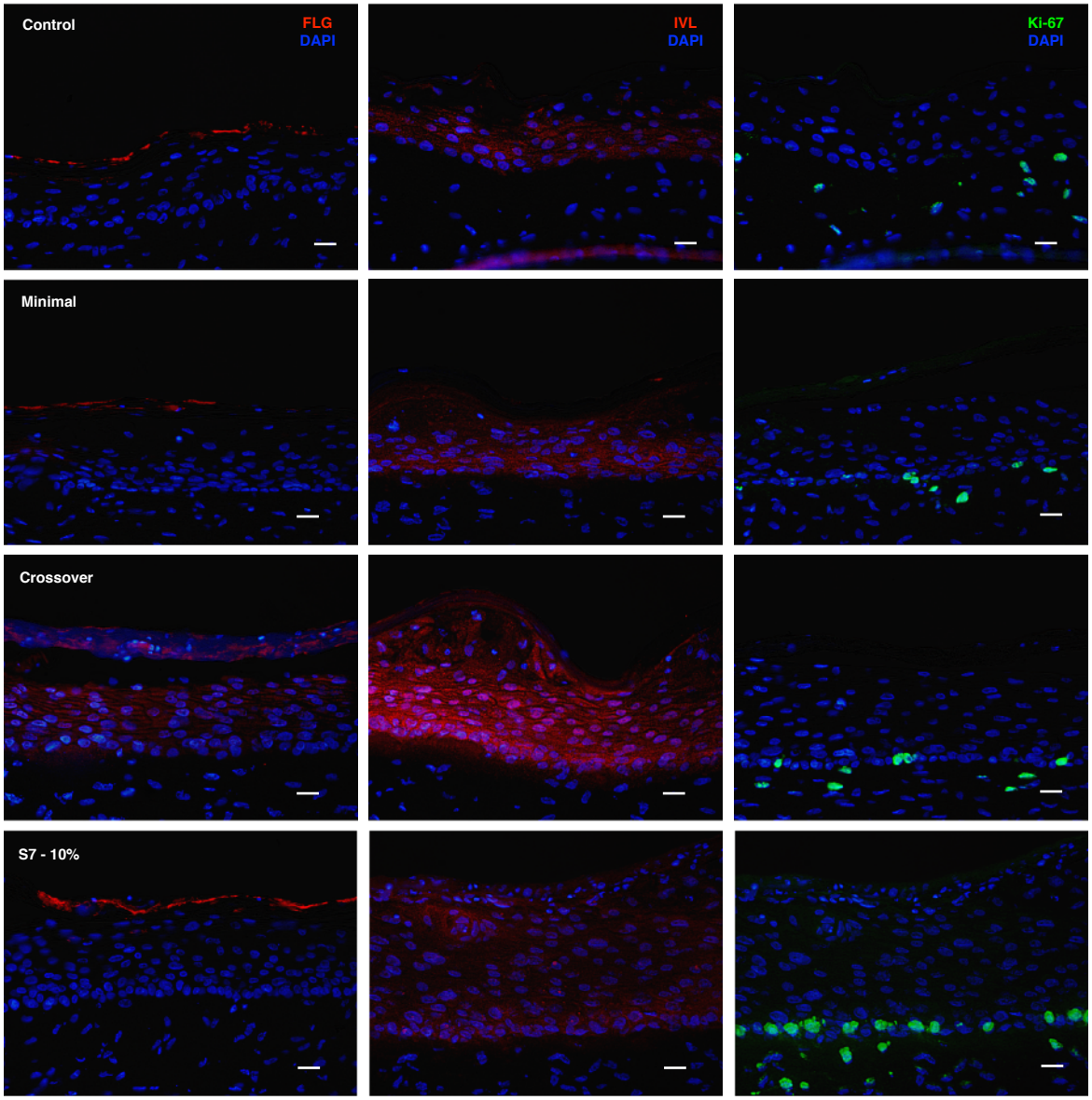


Figure 9. Immunofluorescence staining of skin equivalents after media adaptation
Representative images of immunofluorescence staining against FLG (red), IVL (red), and Ki-67 (green) in skin equivalents cultivated with “minimal“ medium, “crossover“ medium as well as equivalents supplemented with P-ALI medium starting at day 7 (S7) with slow (10% daily) media adaptation counterstained with DAPI compared to conventionally cultured control at day 14. Scale bar: 20 µm. Exposure times: red 1/7 s, green 1/10 s, blue 1/50 s.

In a last step, skin equivalent viability was analyzed by measuring secretion of LDH, a surrogate parameter for cell viability indicating cell damage, into cell media every other day starting 24 h after day one of media adaptation. Although slight differences were observed, none of the tested media showed any relevant increase or decrease of LDH

throughout the last 7-9 days of culture compared to conventionally cultured control equivalent and to the positive control treated with SDS for 24 h (**Fig. 10**). Lower LDH release was observed from skin equivalents cultured with “minimal“ and “crossover“ medium in the first days after adaptation, whereas other media led to slight increase compared to the control equivalent. Although a low variability between the donors and relatively constant LDH levels was seen from day 6 (8) to 12, an increased LDH-release for S5 - 50%, S7 - 50%, and “minimal“ medium was observed at day 14.

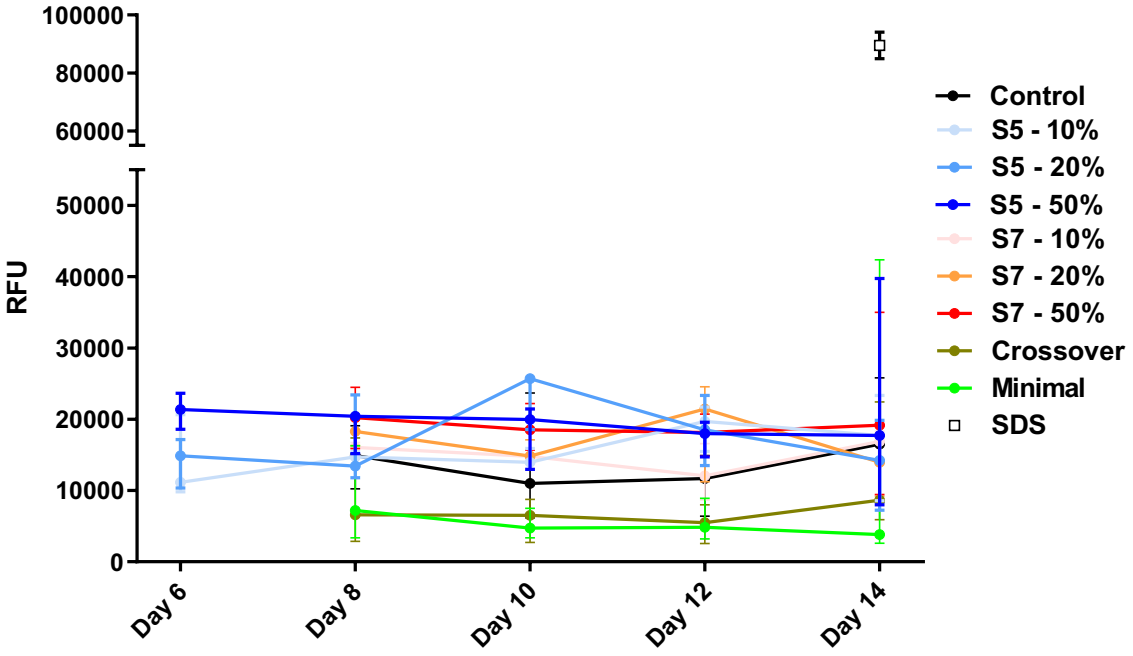


Figure 10. **Viability of skin equivalents after media adaptation**
LDH released into cell culture media from skin equivalents after media adaptation at days 6, 8, 10, 12, and 14 compared to untreated and positive control shown as RFU (relative fluorescence units). For n=4 independent donors. Mean ± SEM.

Media adaptation in bronchial epithelial equivalents

Taken together, “minimal“ medium, “crossover“ medium, and the slow adaption starting at day 7 (S7 - 10%) were chosen as most consistent and most promising for further experiments in bronchial epithelial equivalents. Consequently, these three media were tested for cultivation of bronchial epithelial equivalents for the last week of the three-week conventional culture, hence starting with media adaptation at day 14 and cultivating equivalents until day 21. Here, S7 - 10% for the slow adaptation (10% more P-ALI media per day) starting at day 7 of skin equivalent culture is called S14 - 10%, referring to the slow adaptation (10% KDM more per day) starting at day 14 of bronchial equivalent culture. Analogous to skin equivalents, bronchial epithelial equivalents were analyzed for histological changes, mucus secretion, expression of bronchial epithelial-specific proteins, and viability. Compared to the conventional cultivation, all three media led to slight hyper-proliferation. The formation of cilia was observed for control and “minimal“ medium, with an apparent lack of cilia for “crossover“ medium and S14 - 10%. Whereas the cultivation with “minimal“ medium resulted in less mucus production and secretion compared to the control, “crossover“ medium and S14 - 10% led to the formation of bigger mucus-filled goblet cells with a higher retention of mucus within these cells (**Fig. 11**).

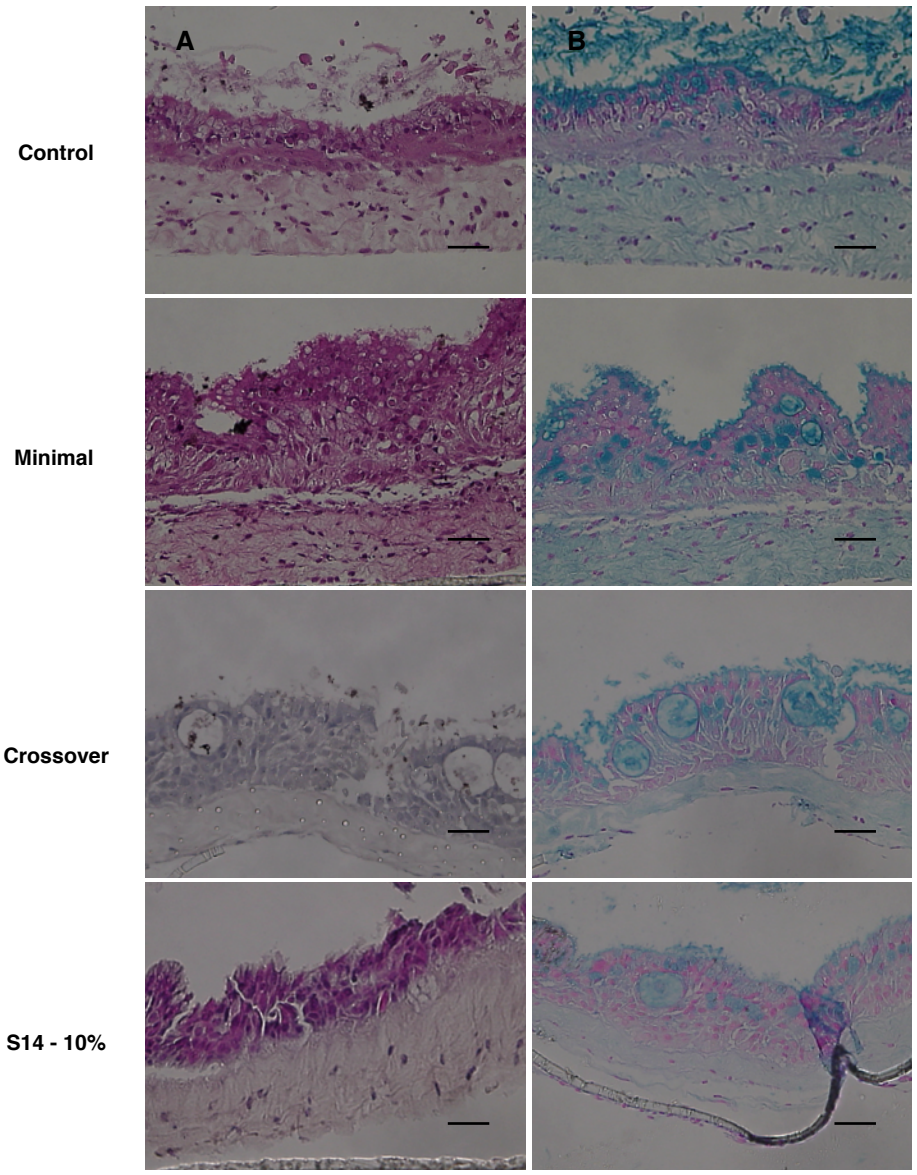


Figure 11. Histology of bronchial epithelial equivalents after media adaptation
Representative images of H&E- (left side) and alcian blue (right side) stainings of bronchial epithelial equivalents cultivated with “minimal“ medium, “crossover“ medium, and S14 - 10% compared to conventionally cultured control harvested at day 21. Scale bar: 50 µm.

For further characterization, the expression of α-acetylated tubulin as marker for cilia, proliferation marker Ki-67, and mucus marker mucin 5AC (MUC5AC) was analyzed in bronchial epithelial equivalents with immunofluorescence staining. There was no alteration in expression of α-acetylated tubulin in bronchial epithelial equivalents cultivated with “minimal“ medium, constantly expressing α-acetylated tubulin throughout the outermost, apical layer of bronchial epithelial. The “crossover“ medium and S14 - 10% meanwhile showed additional expression of α-acetylated tubulin within the goblet cells. Whereas there was no expression of Ki-67 in all models, the expression of MUC5AC was generally low and unaltered (**Fig. 12**).

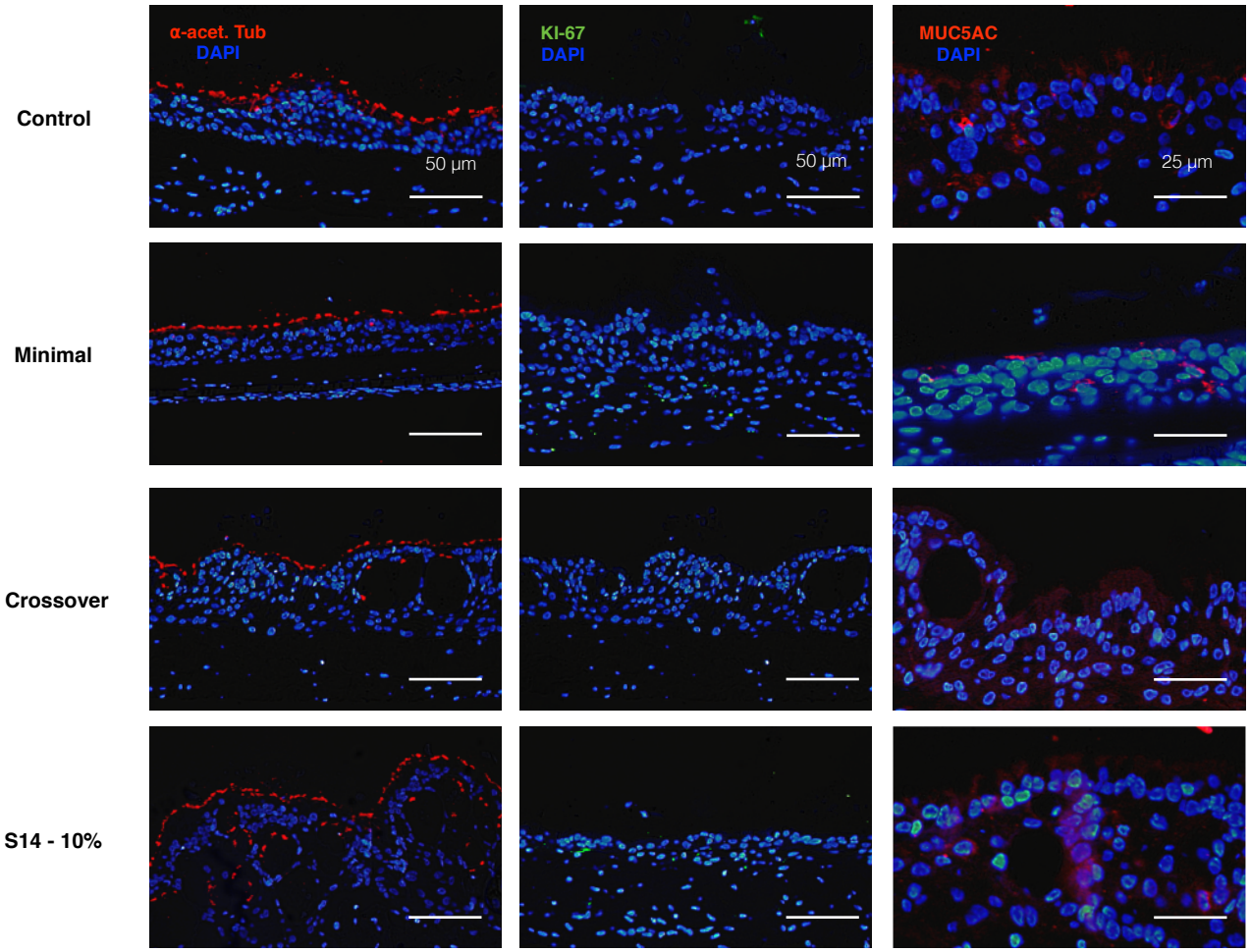


Figure 12. Immunofluorescence staining of bronchial epithelial equivalents after media adaptation

Representative images of immunofluorescence staining of bronchial epithelial equivalents against α-acetylated tubulin, Ki-67 and MUC5AC cultivated with different types of media and counterstained with DAPI. Exposure times: red 1/8 s (10x), 1/2 s (20x); green 1/15 s; blue 1/50 s (20x), 1/12 s (20x). Magnification: 10x and 20x.

The last step of characterization comprised the analysis of cell culture medium for LDH release in order to identify possible toxic effects or changes in metabolism through media adaptation. Compared to the conventionally treated control, LDH release into cell culture media constantly and slightly increased until day 20, with a drop at day 21. Nevertheless, compared to the conventionally cultured equivalent and the positive control, a bronchial epithelial equivalent treated with triton X for 24 h, no relevant increase of LDH, hence no toxic effects, were be observed after media adaptation (**Fig. 13**).

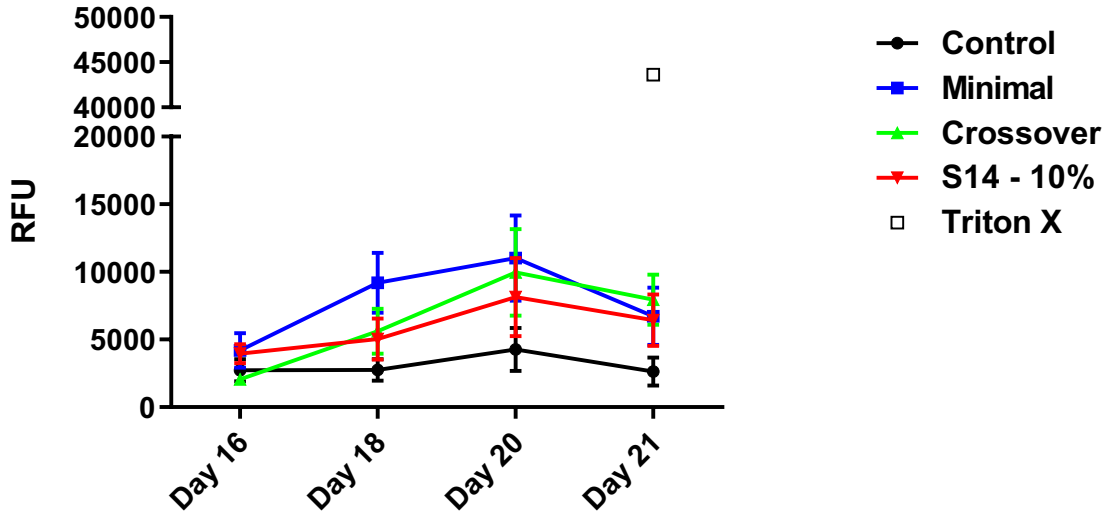


Figure 13. **Viability of bronchial epithelial equivalents after media adaptation**
LDH released into cell culture medium from bronchial epithelial equivalents after media adaptation at days 16, 18, 20, and 21 compared to untreated and positive control shown as RFU (relative fluorescence units). For n=4 independent donors. Mean ± SEM.

3.1.2 Skin disease equivalents induce asthma-like inflammation in bronchial epithelial equivalents after co-culture

Based on the previously shown results, I decided to chose the “minimal“ medium for the co-culture between skin (disease) and healthy bronchial epithelial equivalents for various reasons: (I) The switch from conventional (KDM or P-ALI) medium to “minimal“ medium takes 2 days without a long time period of adaptation. (II) The cultivation of equivalents with “minimal“ medium predominantly led to consistent results concerning histology, mucus secretion, protein expression, and viability with comparable results to the conventional control. (III) The “minimal“ medium has the least amount of supplements, possibly resulting in a steady-state cultivation and leading to the least interference with secreted compounds from skin (disease) and bronchial equivalents.

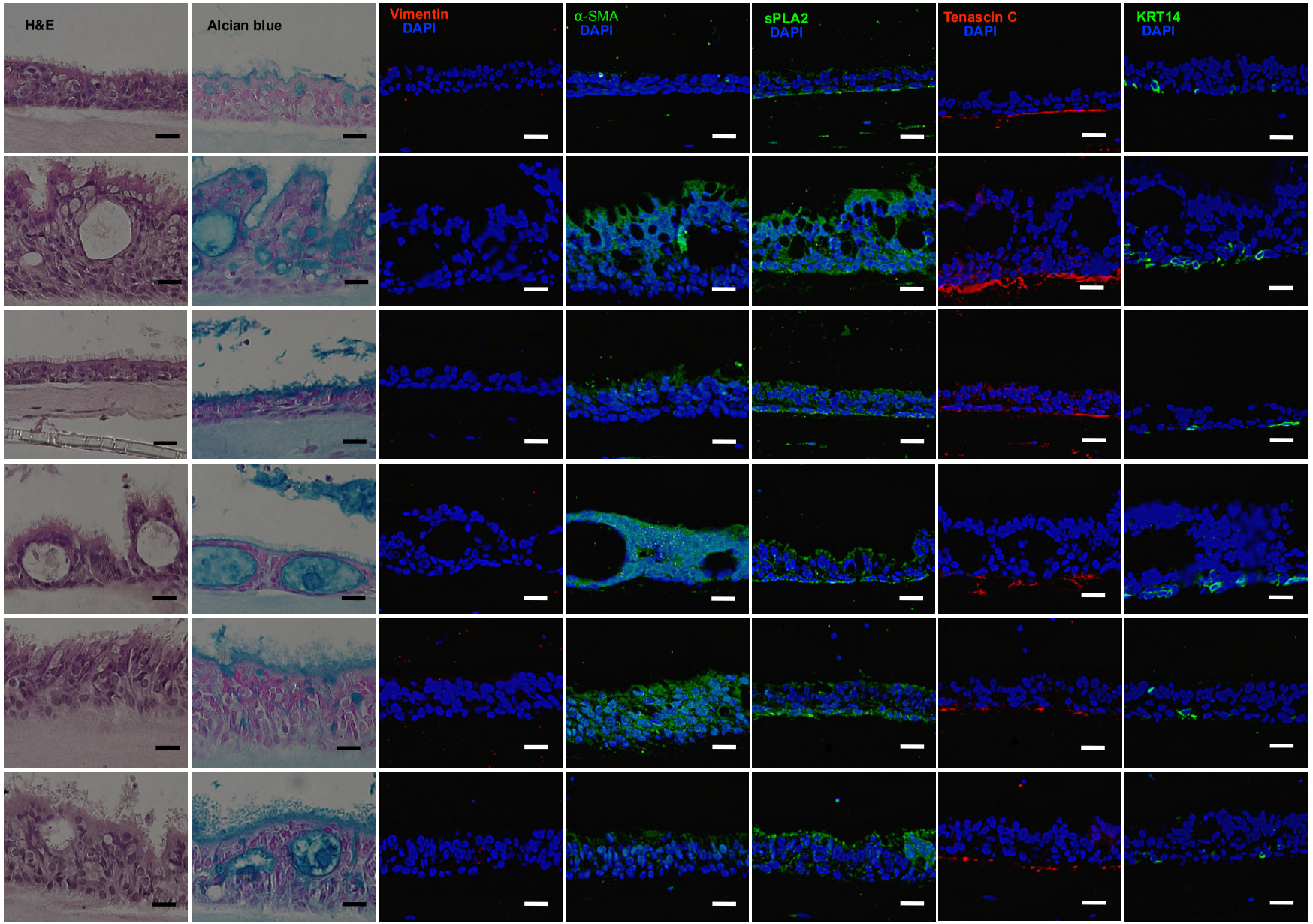
The co-cultivation itself was performed in two different ways. The first way was the so-called “indirect co-culture“ (Ind) via conditioned medium. Therefore, healthy (H) and diseased (*FLG*-$_{IL\text{-}4/\text{-}13}$) (D) skin equivalents were cultured with “minimal“ medium as described above. For diseased skin equivalents with a knockdown in the *FLG* gene, 15 ng/ml IL-4 and 15 ng/ml IL-13 were supplemented for four days. After these four days medium was changed to pure “minimal“ medium for 24 h without IL-4/-13. The “minimal“ medium from healthy skin equivalents was also left for a 24 h conditioning (for details see methods part 2.2.1.8). These conditioned media were mixed 50:50 with fresh “minimal“ medium before adding this mixture to bronchial epithelial equivalents at day 15. The cultivation with conditioned medium was performed until day 21 with medium change every other day.

The second way of co-cultivation was the “direct co-culture“ (Dir). Although the term direct is incorrect, since there was no direct cell-cell-/equivalent-equivalent contact, this term should describe the difference to the indirect co-culture via conditioned medium. For direct co-culture, skin and bronchial epithelial equivalents were cultivated in deep petri dishes for 6 days in pure “minimal“ medium; thus, enabling direct communication via secreted factors between both equivalents. The skin disease equivalents were cultivated with the supplementation of IL-4/-13 until the day of co-culture and moved into deep petri dishes filled with IL-4/-13-free “minimal“ medium together with bronchial epithelial equivalents. Due to the large amount of media in these petri dishes, 50% of the media was refreshed after 3 days of co-culture.

The impact of these two types of co-culture on bronchial epithelial equivalents was analyzed on several different levels such as histology, mucus secretion, viability, mRNA expression, protein expression, and on secretome level. In addition to these co-culture variations, medium from bronchial epithelial equivalents was supplemented with 15 ng/ml IL-4 and 15 ng/ml IL-13, both asthma-relevant Th2 cytokines, from days 15-21 in order to establish an inflammatory bronchial equivalent.

Histological evaluation of co-cultured bronchial equivalents

The co-culture of healthy bronchial epithelial with diseased skin equivalents for 6 days resulted in clear histological alterations of the bronchial epithelium. Comparable to the treatment with IL-4/-13, a hyper-proliferative epithelium, enlarged goblet cells filled with mucus, and a hyper-secretion of mucus were observed. In addition to that, both, the inflammatory (IL-4/-13) and the diseased co-culture equivalents (Ind D, Dir D), showed increased expression of anti-inflammatory uteroglobin (UG), whereas pro-inflammatory TSLP was hardly affected. A marker for epithelial-to-mesenchymal-transition (EMT), alpha smooth muscle actin (α-SMA), was upregulated in Ind D and the inflammatory equivalent, but downregulated in Dir D. Whereas the pro-inflammatory, extracellular form of the enzyme phospholipase A2 (sPLA2), the extracellular matrix glycoprotein tenascin C, and the G-protein coupled receptor protease activated receptor 2 (PAR-2) were exclusively slightly upregulated in inflammatory equivalents, an upregulation of intermediate filament protein cytokeratin 14 (KRT14) and mucus-related protein MUC5AC was observed in inflammatory equivalents and Ind D with no changes in Dir D as compared to their corresponding healthy controls. The expression of tight junction protein zonula occuldens 1 (ZO-1) was exclusively upregulated in Ind D, but downregulated in the inflammatory equivalent and Dir D. Also, the serine protease inhibitor squamous cell carcinoma antigen-1 (SCCA1) was upregulated in the inflammatory equivalent and Ind D compared to respective their controls. Interestingly, tenascin C was increased in the basal layer after treatment with IL-4/-13. The proliferation marker Ki-67 as well as intermediate filament protein vimentin were not detected (**Fig. 14**).


H&E
Alcian blue
Vimentin
DAPI
α-SMA
DAPI
sPLA2
DAPI
Tenascin C
DAPI
KRT14
DAPI
Control
IL-4/-13
IndH
IndD
DirH
DirD
Exp.Time:
Red: 1/3 s
Blue: 1/15 s
Green: 1/3 s
Blue: 1/15 s
Green: 1/3 s
Blue: 1/15 s
Red: 1/3 s
Blue: 1/15 s
Green: 1/3 s
Blue: 1/15 s

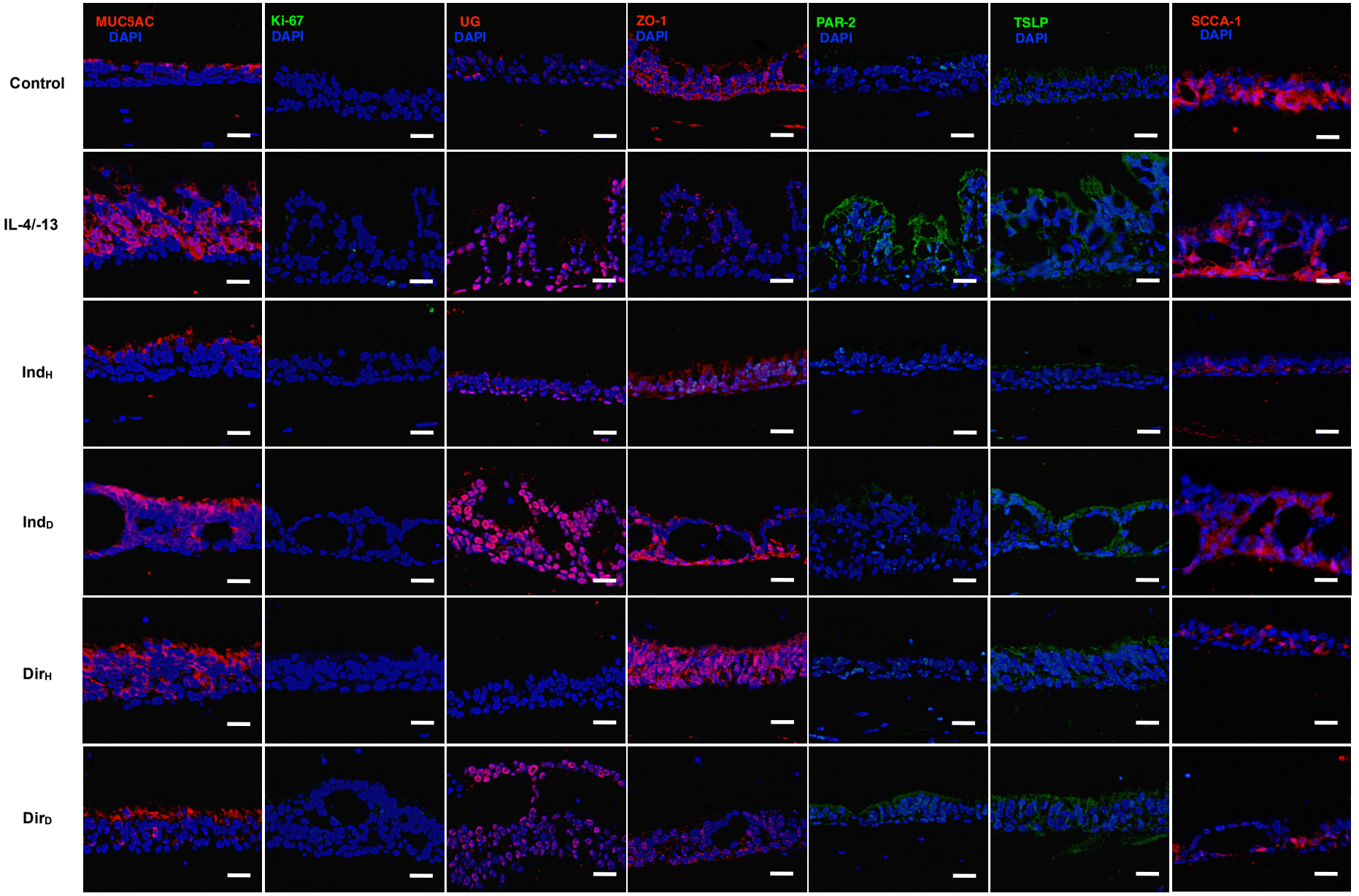

MUC5AC
DAPI
Ki-67
DAPI
UG
DAPI
ZO-1
DAPI
PAR-2
DAPI
TSLP
DAPI
SCCA-1
DAPI
Control
IL-4/-13
IndH
IndD
DirH
DirD
Exp.Time:
Red: 1/3 s
Blue: 1/15 s
Green: 1/3 s
Blue: 1/15 s
Red: 1/3 s
Blue: 1/15 s
Red: 1/3 s
Blue: 1/15 s
Green: 1/3 s
Blue: 1/15 s
Green: 1/3 s
Blue: 1/15 s
Red: 1/5 s
Blue: 1/15 s

Figure 14. Histological, mucus and immunofluorescence staining of bronchial epithelial equivalents after treatment with IL-4/-13 and (in-)direct co-culture
Bronchial epithelial equivalents were either treated with 15 ng/ml IL-4/-13 or co-cultured indirectly (Ind) or directly (Dir) with healthy (H) or diseased (D) skin equivalents for 6 days. Representative images of H&E-, alcian blue (mucus), and immunofluorescence staining against vimentin, alpha smooth muscle actin (α-SMA), secretory phospholipase A2 (sPLA2), tenascin C, cytokeratin 14 (KRT14), mucin 5AC (MUC5AC), Ki-67, uteroglobin (UG), zonula occludens 1 (ZO-1), protease activated receptor 2 (PAR-2), TSLP, and squamous cell carcinoma antigen-1 (SCCA-1) of bronchial epithelial equivalents counterstained with DAPI. Corresponding exposure times are shown. Scale bar: 25 µm.

Protein expression of co-cultured bronchial epithelial equivalents

Subsequently, the expression of proteins was semi-quantified by SDS-PAGE followed by western blot and densitometry analysis. These results confirm the influence of these three methods (supplementation of Th2 cytokines, indirect and direct co-culture) by additionally highlighting their role in pathogenesis and the development of an asthmatic phenotype in bronchial epithelial equivalents.

A slight increase of pro-inflammatory TSLP and EMT-marker α-SMA occurred after both types of co-culture. A 10% increase of TSLP and 25% increase of α-SMA were observed after Ind D, whereas for the Dir D, the increase of TSLP was stronger with up to 25% compared to a less, 15% increase of α-SMA. In the inflammatory equivalent (IL-4/-13), no changes in α-SMA expression but a distinct decrease of TSLP, with approximately 20% less expression, were observed.

Interestingly, the basal marker tenascin C as well as the serine protease inhibitor SCCA-1 were strongly increased in inflammatory equivalents and both types of co-culture. Whereas IL-4/-13 and Dir D had major impact on the expression of SCCA-1 and tenascin C, Ind D resulted in a less prominent increase. The expression of G-protein coupled receptor PPAR-2 is overall neither increased in Ind D, Dir D nor in IL-4/-13-treated equivalents. The tight junction protein ZO-1 was clearly reduced after IL-4/-13 treatment and Dir D. However, Ind D led to a strong increase of ZO-1 (**Fig. 15**).

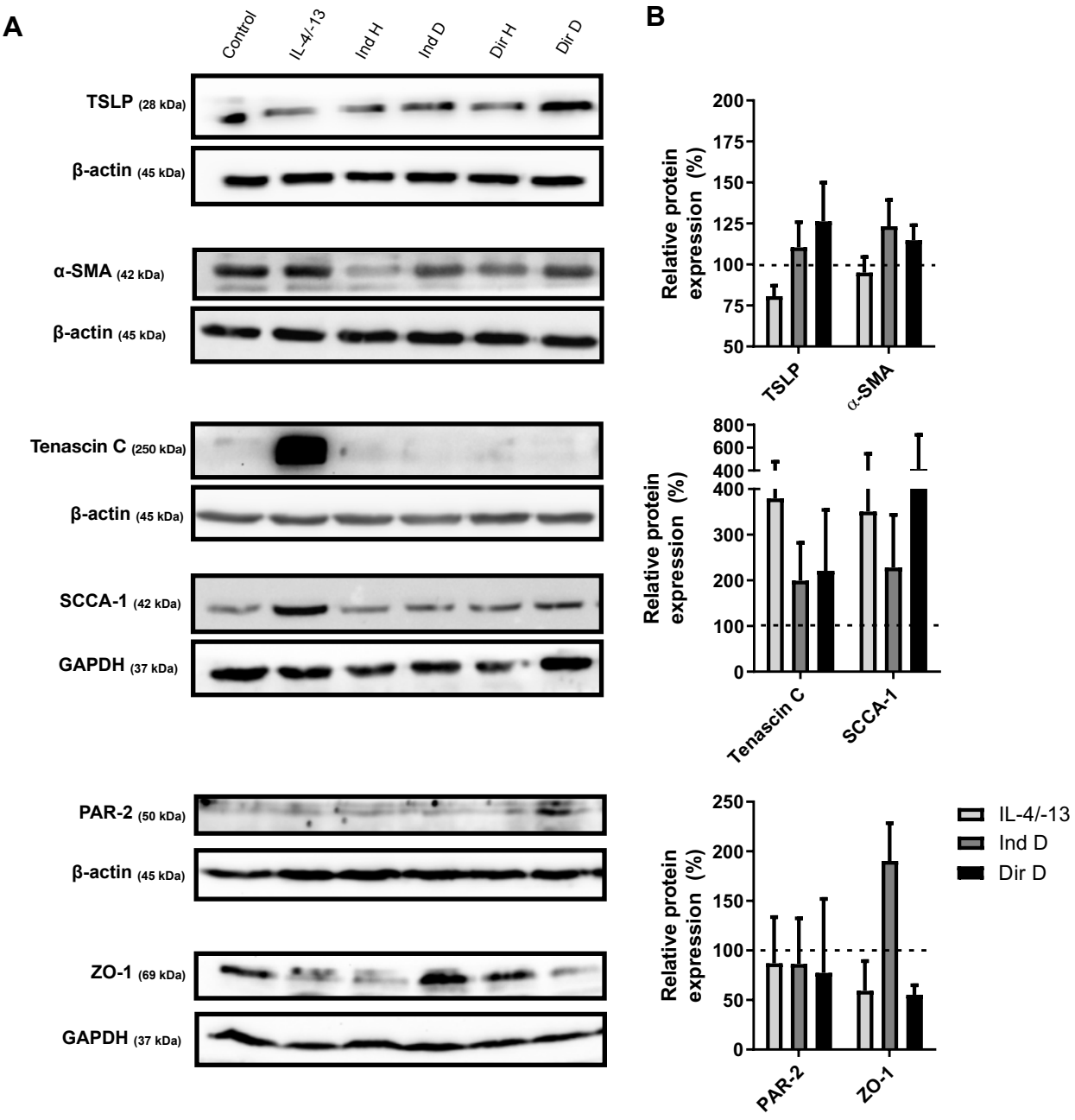


Figure 15. Semi-quantified protein expression in bronchial epithelial equivalents after treatment with IL4/-13 and (in-)direct co-culture

The expression of TSLP, alpha smooth muscle actin (α-SMA), squamous cell carcinoma antigen-1 (SCCA-1), tenascin C, protease activated receptor 2 (PAR-2), and zonula occludens 1 (ZO-1) was semi-quantified by SDS-PAGE with subsequent western blot and densitometry analysis with ImageJ. Representative bands with corresponding loading controls β-actin or GAPDH are shown. For n=3-5 independent donors. Mean ± SEM.

Gene expression of co-cultured bronchial epithelial equivalents

An alteration in the expression of proteins takes a certain amount of time and a 6-day IL-4/-13-treatment or co-culture might simply be too short to see more asthma-relevant changes in bronchial epithelial equivalents on protein level. Hence, in a next step, the respective genes were analyzed with qRT-PCR.

A 6-day treatment with IL-4/-13 led to a distinct gene expression increase of disintegrin and metalloproteinase domain-containing protein 33 (*ADAM33*), *SCCA-1*, and *MUC5AC*. A less prominent increase was observed in gene expression of uteroglobin (*UG*) and *TSLP,* whereas no changes were seen for *ASMA*, tensacin C, *ZO-1*, and *PAR-2* compared to conventionally cultured bronchial epithelial equivalents.

In contrast to that, the Ind D resulted in a slight increase of *UG*, *TSLP*, *SCCA-1,* tenascin C and *ASMA*, a strong increase of *ADAM33*, and minor effects on *MUC5AC, ZO-1,* and *PAR-2* compared to Ind H.

Meanwhile, Dir D generally showed more prominent results on gene level compared to the inflammatory equivalents (IL-4/-13) and Ind D. Here, a >64-fold increase in the gene expression of all analyzed genes was observed (**Fig. 16**).

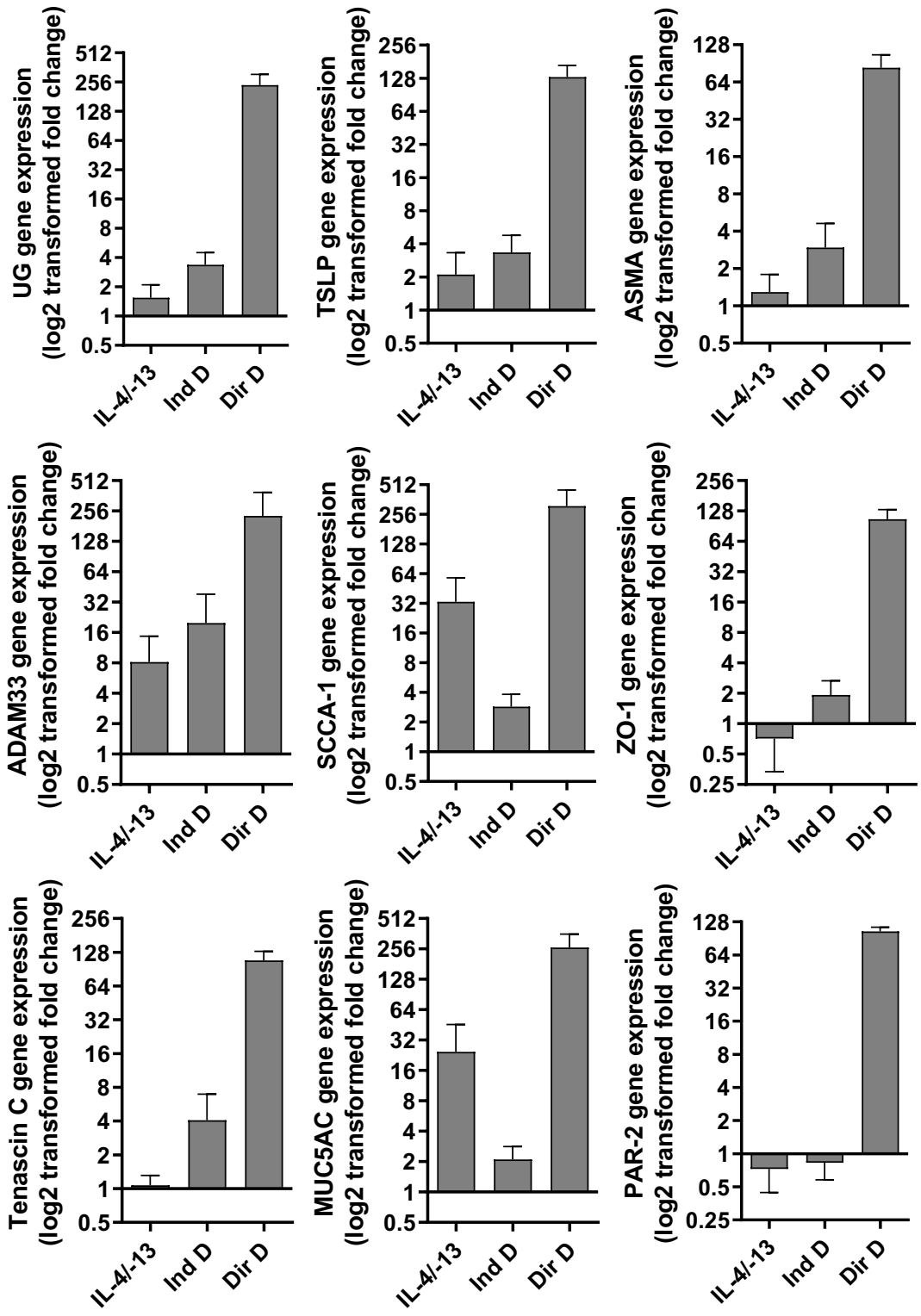


Figure 16. Gene expression in bronchial epithelial equivalents after treatment with IL-4/-13 and (in-)direct co-culture

The expression of uteroglobin (*UG*), *TSLP*, alpha smooth muscle actin (*ASMA*), *ADAM33,* squamous cell carcinoma antigen-1 (*SCCA-1*), zonula occludens 1 (*ZO-1*), tenascin C, *MUC5AC*, and protease activated receptor 2 (*PAR-2*) after either a 6-day treatment with 15 ng/ml IL-4/-13 or indirect (Ind) and direct (Dir) co-culture with diseased (D) skin equivalents as determined with qRT-PCR. Gene expression was normalized to the respective untreated or healthy co-culture controls (=1) and log2 transformed. GAPDH served as housekeeping gene. For n=3 independent donors. Mean ± SEM.

Secretome analysis of co-cultured bronchial epithelial equivalents

In order to complete the whole picture, the cell culture medium was used for final analysis, since an asthmatic phenotype is not only characterized by histological changes and alteration of protein and gene expression within the tissue itself, but also in terms of cytokine secretion. Therefore, the levels of some asthma-relevant cytokines were determined via sandwich ELISA in the cell culture medium at day 21 of (co-)culture.

The supplementation of IL-4/-13 for 6 days resulted in a distinct increase of TSLP and almost 2-fold increase of IL-25 compared to control bronchial epithelial equivalents. Whereas secretion of IL-6 did not change, IL-8 showed a downwards trend.

The indirect cultivation with diseased skin equivalents (Ind D) had significant impact on TSLP levels, whereas levels of IL-25 (~17 pg/ml), IL-6 (~23 ng/ml), and IL-8 (~65 ng/ml) remained unchanged compared to the indirect healthy co-culture (Ind H).

Also, the direct diseased co-culture (Dir D) led to a 3-fold mean increase of TSLP with no effects on the secretion of IL-25 (~12 pg/ml), IL-6 (~10 ng/ml), and IL-8 (~38 ng/ml).

Interestingly, the baseline levels for healthy controls already differed for all the cytokines, e.g., TSLP ranged from ~30-250 pg/ml, IL-25 from 12-20 pg/ml, IL-6 from 4-25 ng/ml, and IL-8 from ~38-65 ng/ml (**Fig. 17**). No IL-33 and TNFα were detected in culture media.

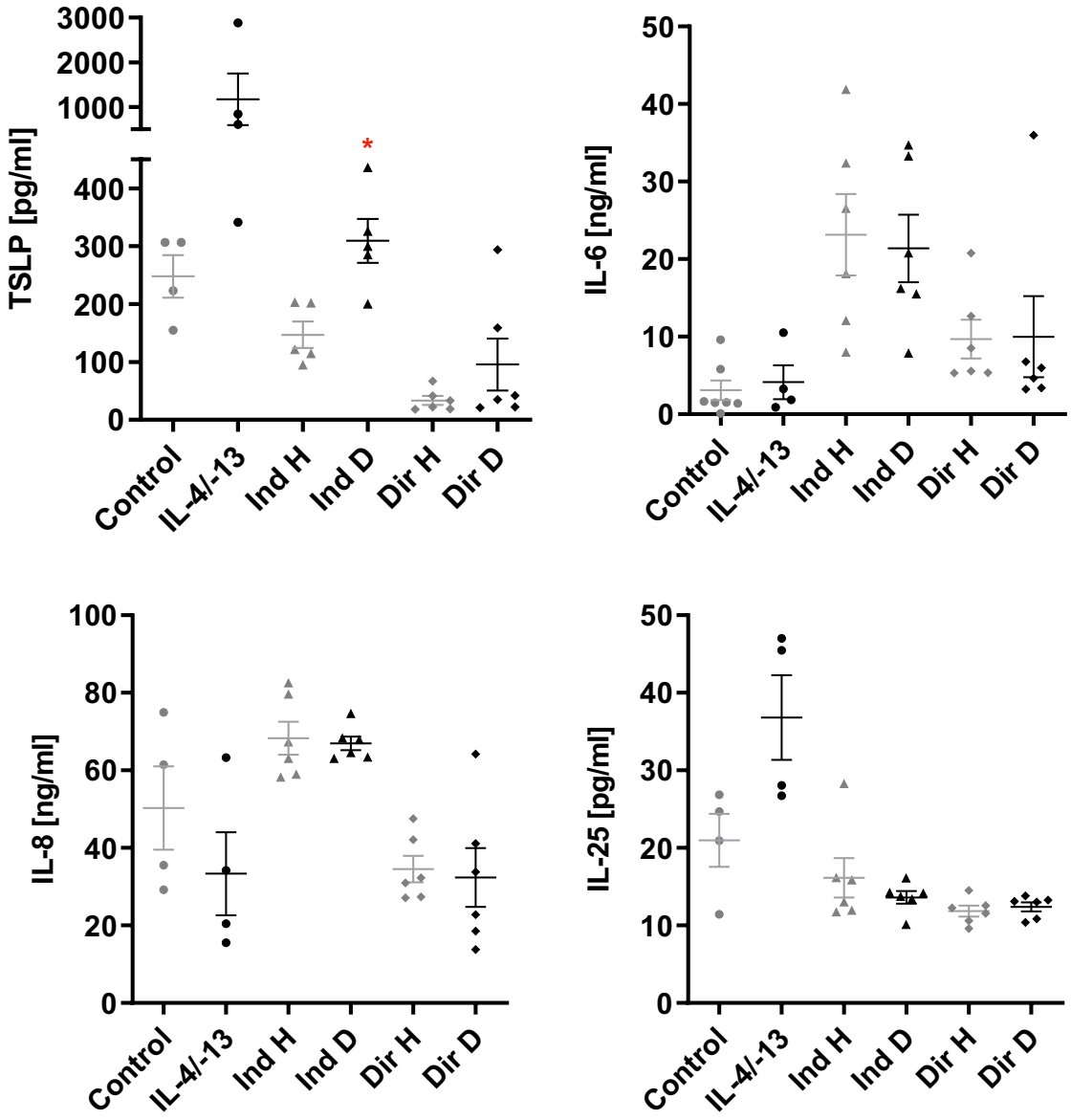


Figure 17. Secretome analysis of bronchial epithelial equivalents after treatment with IL-4/-13 and (in-)direct co-culture

The secretion of TSLP, IL-6, -8, and -25 into cell culture media of bronchial epithelial equivalents after either a 6-day treatment with IL-4/-13 or indirect (Ind) and direct (Dir) co-culture with healthy (H) and diseased (D) skin equivalents was determined via sandwich ELISA. For n=4-6 independent donors. Scatter plot showing mean ± SEM. Wilcoxon signed-rank test for non-parametric, independent samples. *p < 0.05 compared to respective control.

Viability

Finally, LDH levels in the culture media of indirect and direct co-cultures as well as IL-4/-13-treated bronchial epithelial equivalents were analyzed in order to determine possible cytotoxic effects. Whereas LDH levels, especially in Ind H and Ind D, slightly increased, levels in IL-4/-13-treated equivalents decreased towards day 21. Nevertheless, compared to the control and a triton X-treated positive control no distinct toxic effects were observed (**Fig. 18**).

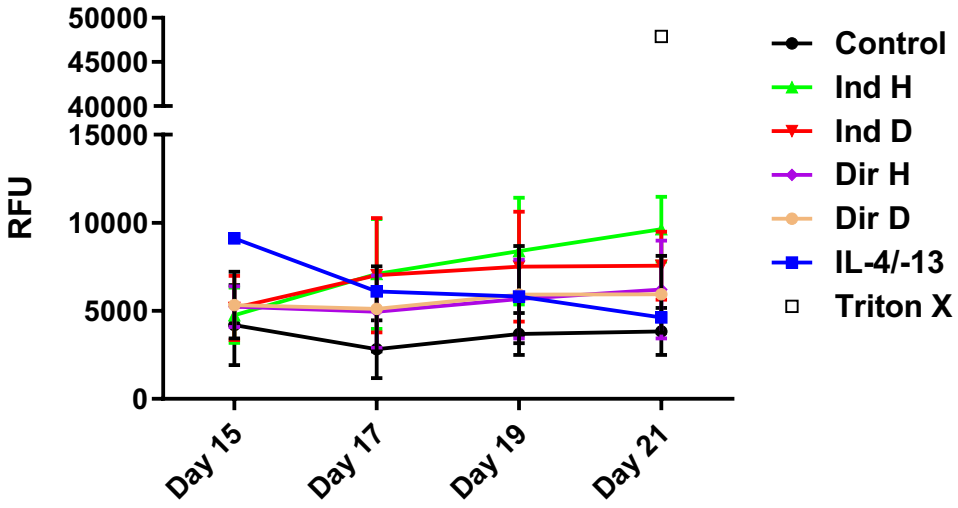


Figure 18. **Viability of bronchial epithelial equivalents after treatment with IL-4/-13- and (in-)direct co-culture**

LDH released into cell culture media of bronchial epithelial equivalents after IL-4/-13 treatment and indirect (Ind) as well as direct (Dir) co-culture with healthy (H) and diseased (D) skin equivalents compared to untreated and positive control shown as RFU (relative fluorescence units). For n=3 independent donors. Mean ± SEM.

3.2 Secretome analysis of diseased skin equivalents

3.2.1 Identification of skin-derived mediators triggering inflammation in bronchial epithelial equivalents

After observing clear changes in histology, protein and gene expression, and in the secretome of bronchial epithelial equivalents pointing towards an asthma-like inflammation after diseased co-cultivation, the next step was to identify possible key players that might trigger those alterations. In a first step, a more basic characterization of skin (disease) equivalent media was performed. Therefore, the cytokines TSLP, TNFα, IL-1β, -6, -8, 10, -25, and 33 were measured via sandwich ELISA. These are cytokines already known to play a role in AD and partly in the atopic march.

The diseased skin equivalents (*FLG*-$_{\text{IL-4/-13}}$) did not secret more IL-6 (~59 ng/ml), IL-8 (25 ng/ml), and IL-25 (~17 pg/ml) than healthy equivalents. The only differences were observed in secretion of TNFα with a mean increase of ~20 pg/ml and a slight increase of TSLP with ~10 pg/ml more in diseased skin equivalents (**Fig. 19**). No IL-1β, -10, and -33 were detected.

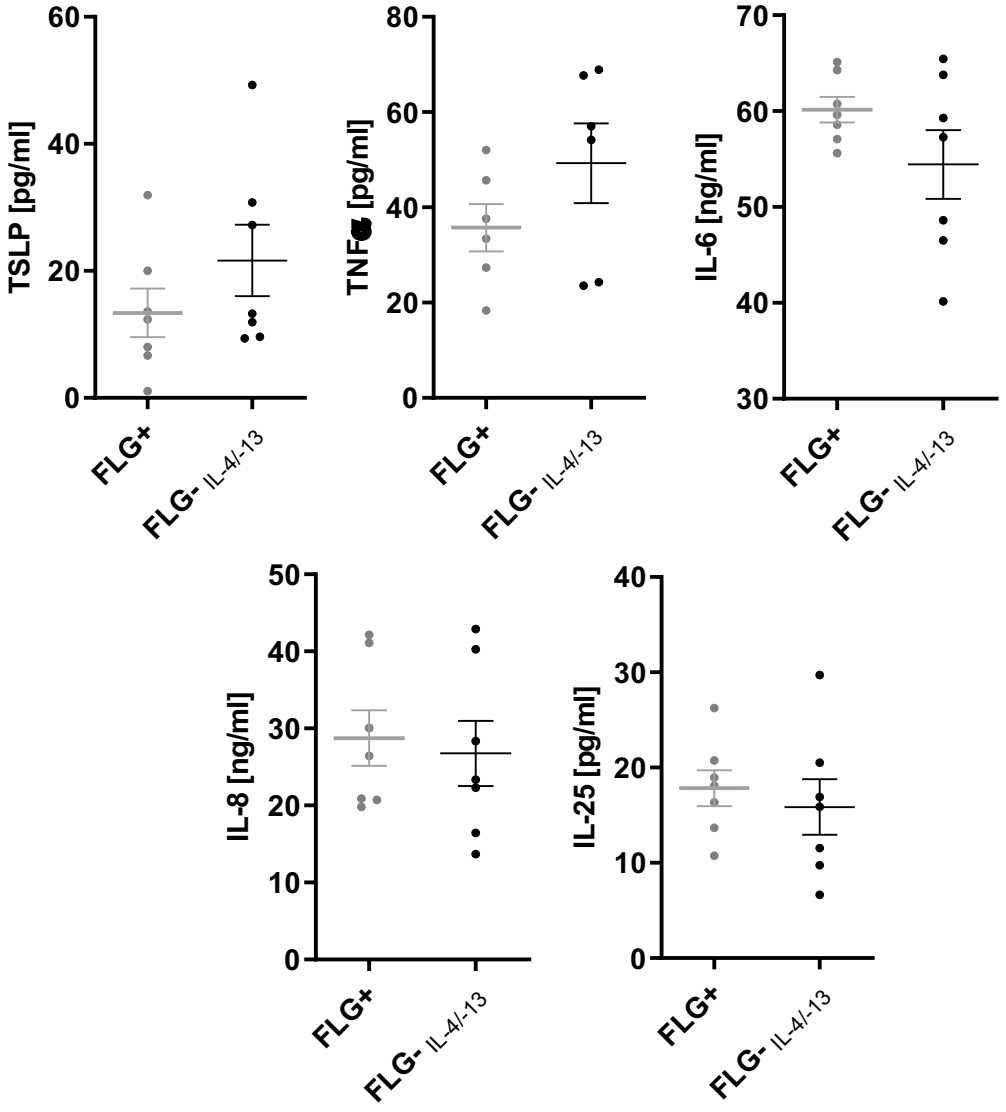


Figure 19. Secretome analysis of skin (disease) equivalents by ELISA
Levels of AD- and atopic march-relevant cytokines TSLP, TNFα, IL-6, -8, and -25 were determined in supernatants of healthy (*FLG*+) and skin disease (*FLG*-$_{\text{IL-4/-13}}$) equivalents by sandwich ELISA. For n=6 independent donors. Scatter plot showing mean ± SEM.

For gaining more detailed information about the secretome skin (disease) equivalents - and in order to identify currently unknown but possible mediators of the atopic march - a proteomics analysis culture media was performed. In a first step, FBS-free culture media of skin (disease) equivalents was collected, prepared for SDS-PAGE, and proteins were separated according to their molecular weight. A subsequent unspecific protein staining with coomassie blue revealed differences in two protein bands. One band >250 kDa and one band at ~150 kDa gave a more intense signal in media of skin disease equivalents indicating an increased secretion into culture media (**Fig. 20**).

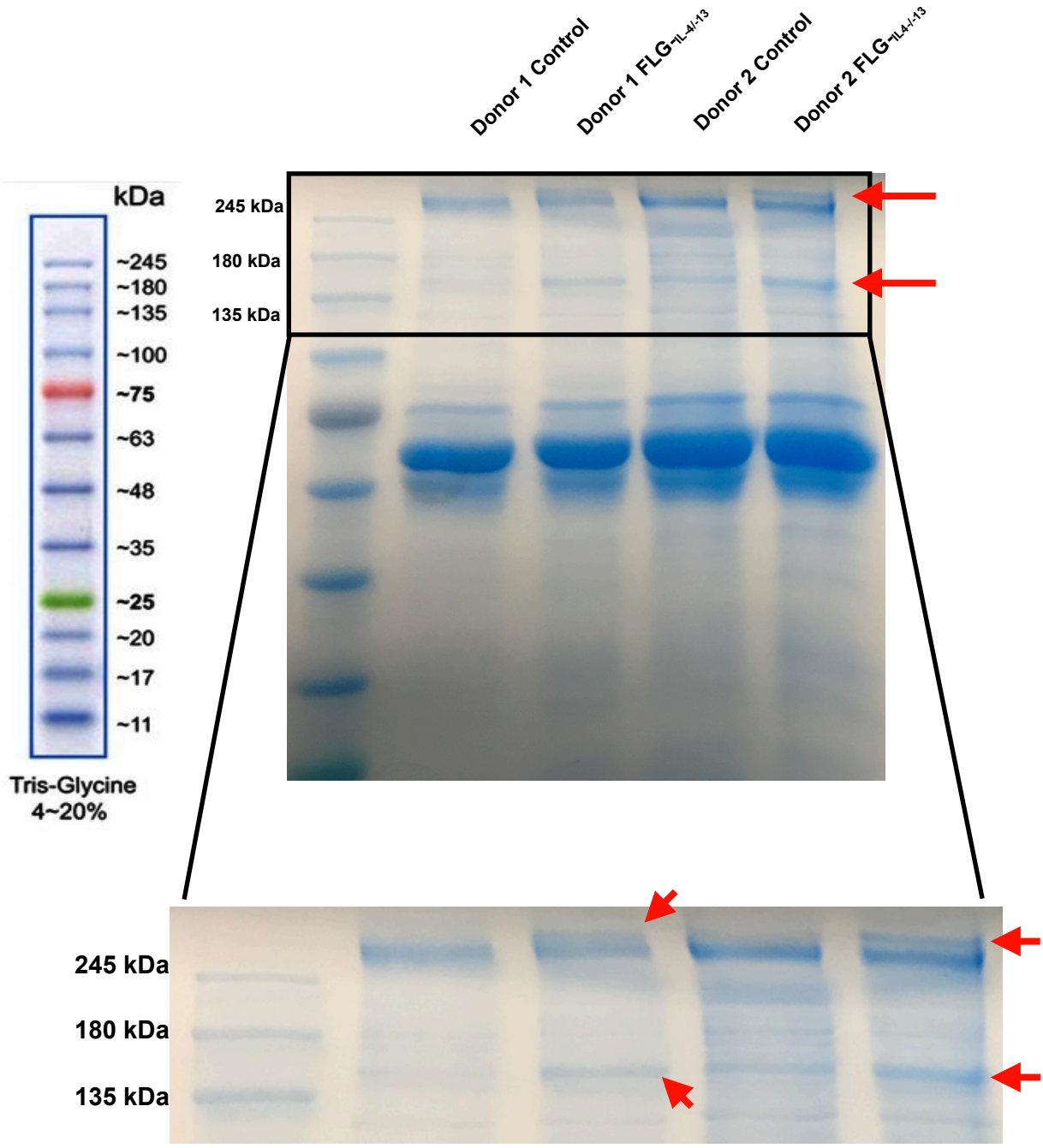


Figure 20. Secretome analysis of skin (disease) equivalents by SDS-PAGE
The components of cell culture media of skin (disease) equivalents were separated via SDS-PAGE and stained with coomassie blue. One representative gel is shown for skin (disease) equivalent media collected at day 14 of culture. Red arrows highlighting bands with increased intensity after staining with coomassie blue compared to healthy control.

These two bands per donor were cut out and a proteomics analysis was performed for a total of three donors by UBC proteomics core facility. A representative list of all hits from one donor (150 kDa band and 250 kDa band) is shown in **table 8** in the appendix.

The proteomics analysis revealed a list of skin-associated proteins such as subtypes of collagen, keratin, and laminin, among others. After extensive literature research, five candidates out of the list (**Tab. 8**) were chosen for further experiments: complement factor C3 (C3) as protein from the innate immune system, ECM glycoprotein fibronectin (FN), shedded plasma membrane proteoglycan syndecan-4 (SD-4), soluble form of cell-surface glycoprotein cluster of differentiation 44 (CD44), and matrix glycoprotein thrombospondin-1 (TSP-1). All five compounds are increased in serum and plasma from AD- and/or asthma-patients with different possible cellular sources according to literature.
To validate their possible relevance, the concentration of these mediators was analyzed in cell culture media of skin (disease) equivalents and patient-derived plasma samples via sandwich ELISA. Therefore, plasma samples were chosen from young healthy and AD donors additionally suffering from EoE at the age of 5-7. These AD patients with the additional clinical diagnosis of EoE represent the clinical picture of the atopic march.
The complement factor C3 was upregulated (2,800 µg/ml vs. 4,300 µg/ml) in patient-derived plasma from AD patients as well as in skin disease equivalent media (3.3 µg/ml vs. 5.3 µg/ml). A similar trend for increased concentration was observed for CD44 (43 ng/ml vs. 51 ng/ml), SD-4 (8 ng/ml vs. 19 ng/ml), and TSP-1 (1,450 µg/ml vs. 1,560 µg/ml) in AD patient-derived plasma samples. Interestingly, a minor increase of SD-4 and a significant increase of CD44 and TSP-1 in skin disease equivalents was found, whereas levels of FN were slightly decreased (80 µg/ml vs. 74 µg/ml) in plasma but slightly increased in media of skin disease equivalents (4.4 µg/ml vs. 5.3 mg/ml) (**Fig. 21**).

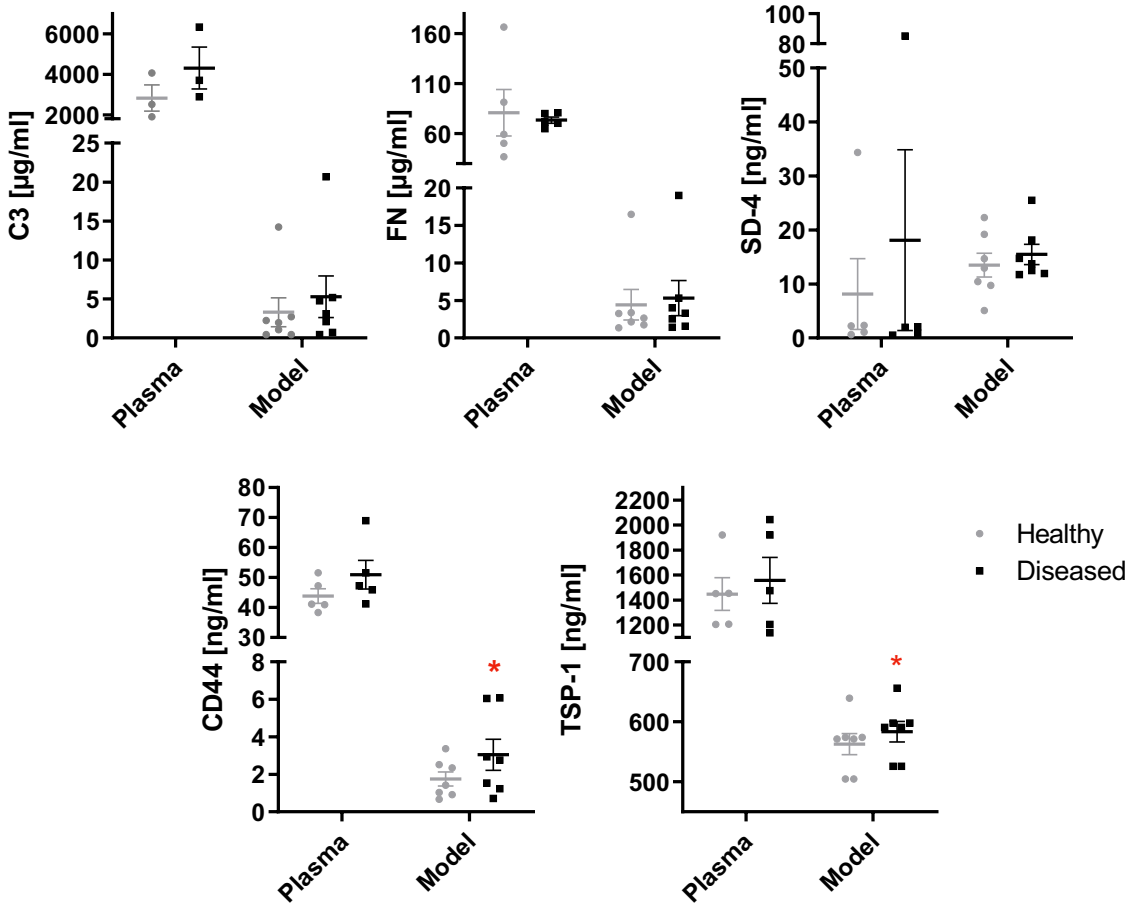


Figure 21. Levels of C3, FN, SD-4, CD44, and TSP-1 in skin (disease) equivalents and patient-derived plasma samples

Levels of complement factor C3 (C3), fibronectin (FN), shedded syndecan-4 (SD-4), soluble cluster of differentiation 44 (CD44), and thrombospondin-1 (TSP-1) were determined in media of skin (disease) equivalents (=model) and patient-derived plasma samples (=plasma) via sandwich ELISA. For n=5-7 independent donors. Scatter plot showing mean ± SEM. Wilcoxon-signed rank test was performed for non-parametric, dependent (paired) skin equivalents. * $p < 0.05$.

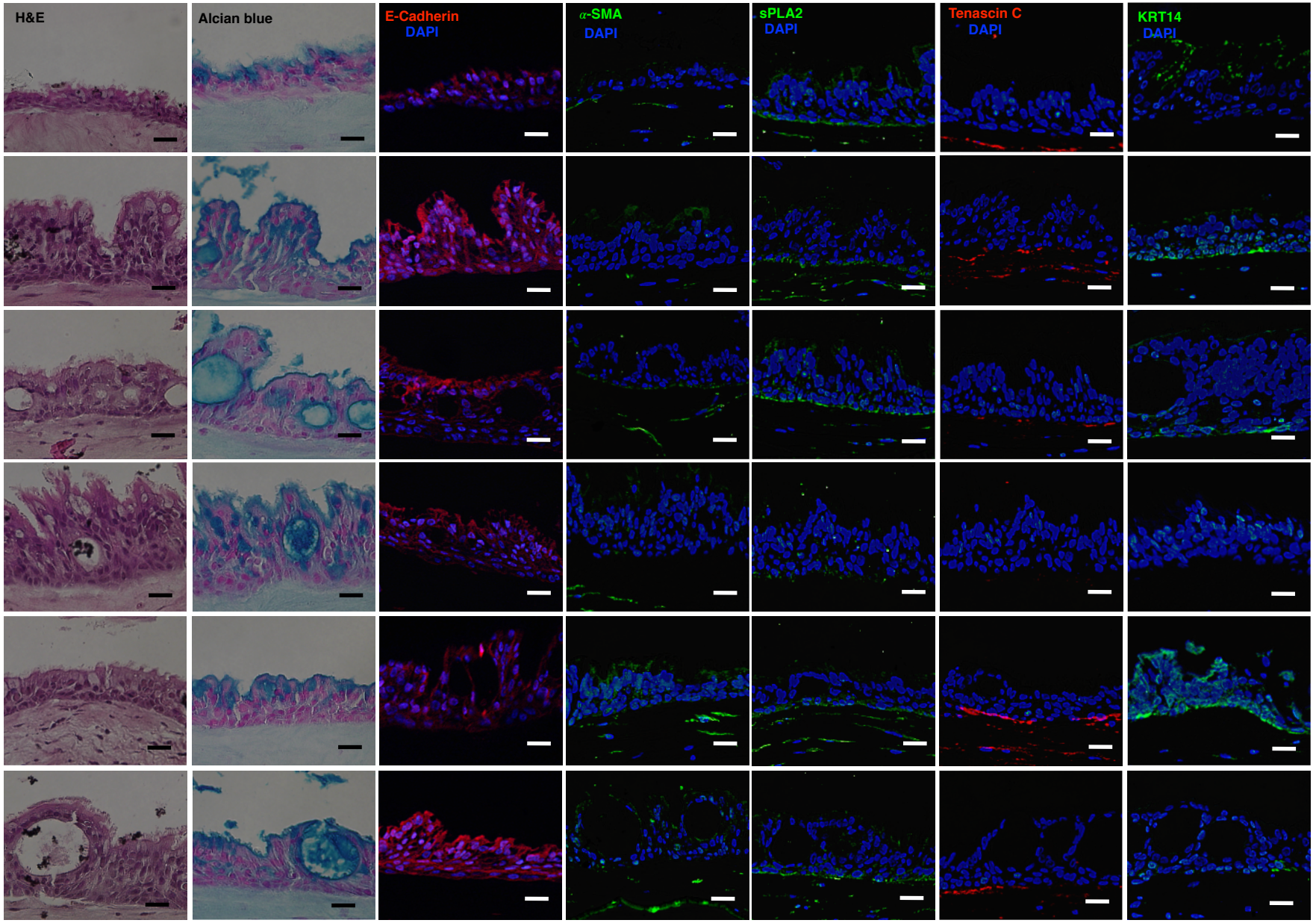
3.3 Role of the skin secretome in the atopic march

3.3.1 Influence of selected skin-derived compounds on bronchial epithelial equivalents

Subsequently, the influence of these five selected compounds on bronchial epithelial equivalents was analyzed. In a first step, two concentrations were chosen based on literature research and out of these two concentrations per compound one effective concentration was determined after several pre-trials. The final concentrations were: 50 ng/ml C3, 1000 ng/ml FN, 5 ng/ml SD-4, 100 ng/ml CD44, and 5 ng/ml TSP-1. Analogous to the co-culture evaluation, the effects of these compounds on bronchial epithelial equivalents were analyzed on histological, protein, gene, and secretome level.

Histological analysis of bronchial epithelial equivalents treated with skin-derived secretome compounds

The treatment of bronchial epithelial equivalents with these compounds showed clear effects concerning histology, mucus secretion, and expression of certain proteins partly resulting in an asthma-like inflammation. Specifically, C3, FN, SD-4, and TSP-1 exhibited a hyperproliferative phenotype with GCH and increased mucus production, whereas no obvious changes for bronchial epithelial equivalents after CD44-treatment were observed. A 6-day treatment of bronchial epithelial equivalents with innate immune factor C3 led to an increased expression of epithelial markers E-cadherin, KRT-14, ZO-1, TSLP, and PAR-2 with no changes in expression of α-SMA, sPLA-2, tenascin C, MUC5AC, and SCCA-1. Treatment with ECM component FN resulted in minor increase of MUC5AC, ZO-1, TSLP, and SCCA-1, whereas SD-4-treated equivalents expressed more UG, ZO-1, TSLP, and PAR-2. A treatment of bronchial epithelial equivalents with CD44 led to an increased expression of KRT14 and ZO-1 throughout the whole epithelial layer as well as elevated levels of TSLP and PAR-2. The treatment with matrix glycoprotein TSP-1 only resulted in an increase of E-cadherin and TSLP (**Fig. 22**). In none of the equivalents any of Ki-67 expression was observed (data not shown).


H&E
Alcian blue
E-Cadherin
DAPI
α-SMA
DAPI
sPLA2
DAPI
Tenascin C
DAPI
KRT14
DAPI
Control
C3
FN
SD-4
CD44
TSP-1
Exp.Time:
Red: 1/5 s
Blue: 1/15 s
Green: 1/4 s
Blue: 1/15 s
Green: 1/4 s
Blue: 1/15 s
Red: 1/4 s
Blue: 1/15 s
Green: 1/3 s
Blue: 1/15 s

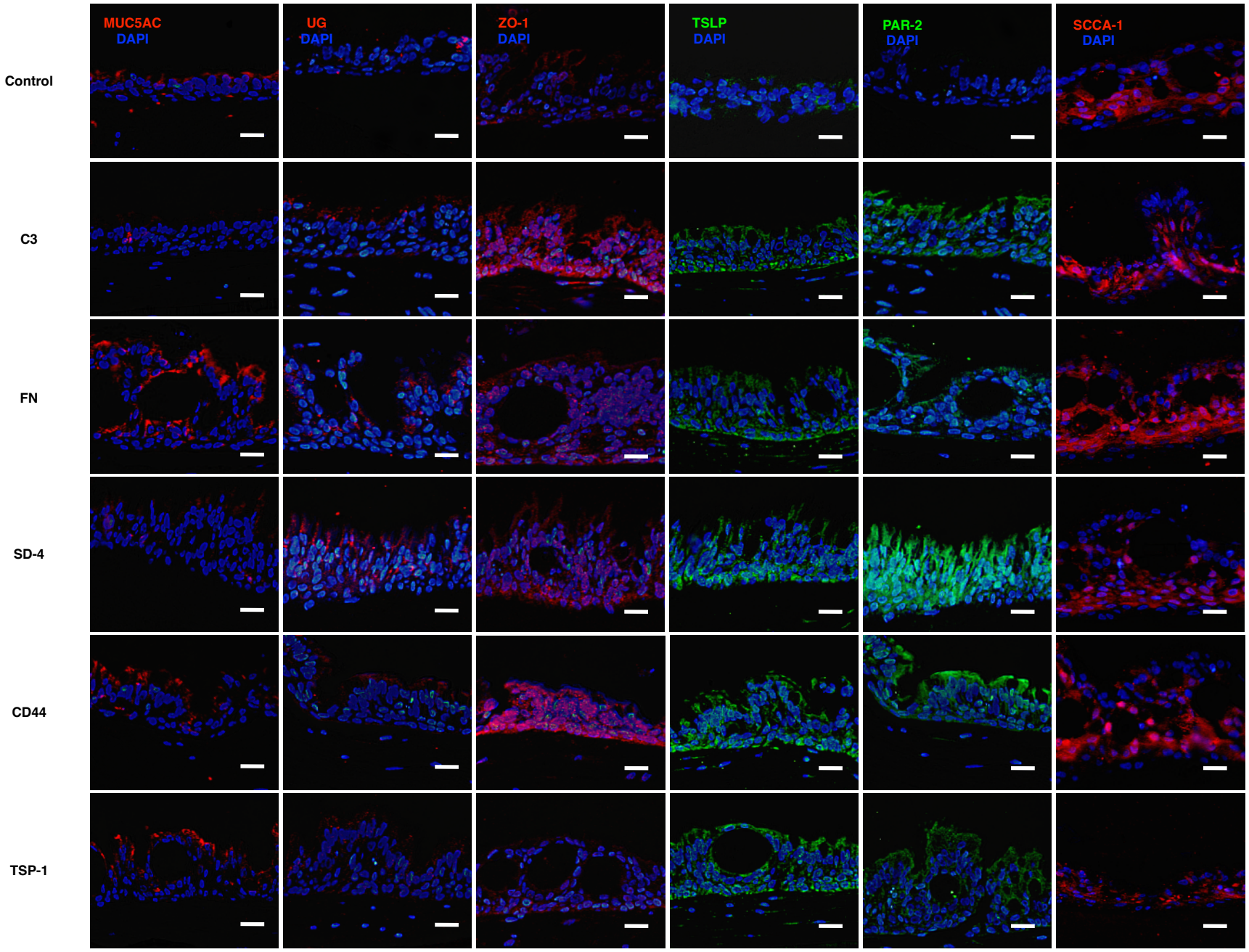

MUC5AC
DAPI
UG
DAPI
ZO-1
DAPI
TSLP
DAPI
PAR-2
DAPI
SCCA-1
DAPI
Control
C3
FN
SD-4
CD44
TSP-1
Exp.Time:
Red: 1/4 s
Blue: 1/15 s
Red: 1/4 s
Blue: 1/15 s
Red: 1/3 s
Blue: 1/15 s
Green: 1/3 s
Blue: 1/15 s
Green: 1/4 s
Blue: 1/15 s
Red: 1/5 s
Blue: 1/15 s

Figure 22. Histological and immunofluorescence staining of bronchial epithelial equivalents after a 6-day treatment with C3, FN, SD-4, CD44, and TSP-1

Bronchial epithelial equivalents were either treated with 50 ng/ml complement factor C3 (C3), 1000 ng/ml fibronectin (FN), 5 ng/ml syndecan-4 (SD-4), 100 ng/ml soluble cluster of differentiation 44 (CD44), or 5 ng/ml thrombospondin-1 (TSP-1). Representative images of H&E-, alcian blue (mucus), and immunofluorescence stainings against E-cadherin, alpha smooth muscle actin (α-SMA), secretory phospholipase A2 (sPLA2), tenascin C, cytokeratin 14 (KRT14), MUC5AC, uteroglobin (UG), zonula occludens 1 (ZO-1), TSLP, protease activated receptor 2 (PAR-2), and squamous cell carcinoma antigen-1 (SCCA-1) of bronchial epithelial equivalents counterstained with DAPI. Corresponding exposure times are shown. Scale bar: 25 µm.

Protein expression of bronchial epithelial equivalents treated with skin secretome compounds

In order to relatively quantify protein expression and confirm results from histological analysis, SDS-PAGE with subsequent western blot analysis was performed for bronchial epithelial equivalents after a 6-day treatment with C3, FN, SD-4, CD44, and TSP-1.

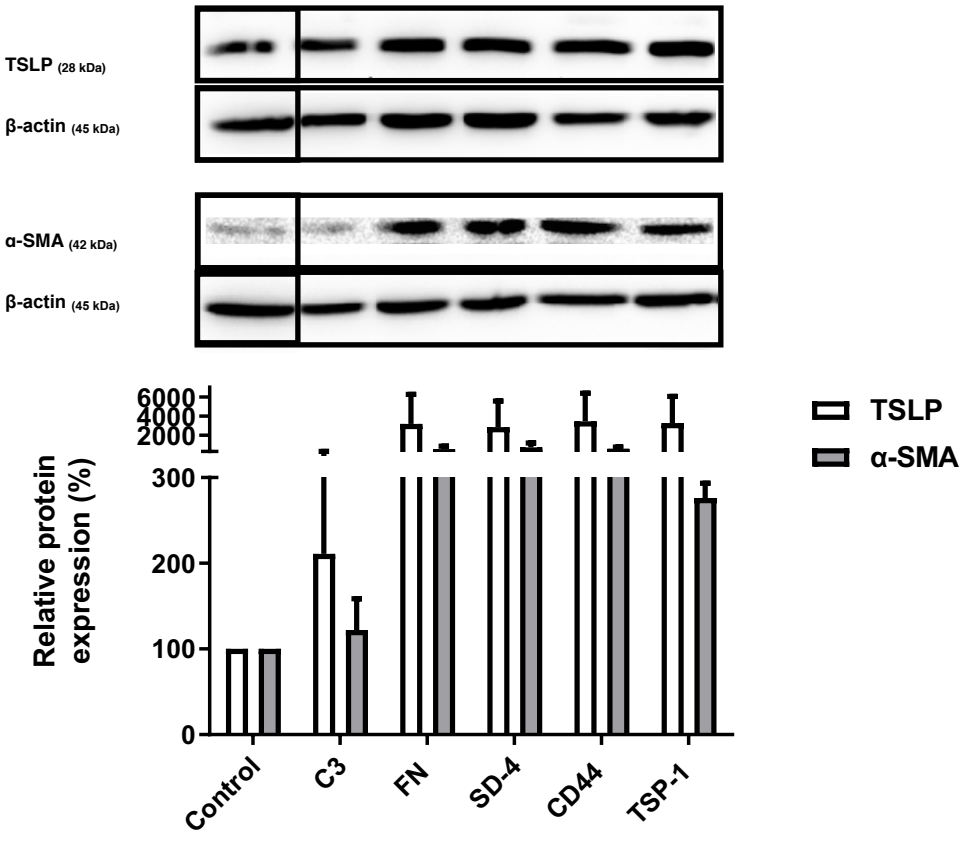
No distinct changes of MUC5AC expression were observed after treatment with these five compounds but only a minor upwards trend occurred through C3, FN, SD-4, and TSP-1.

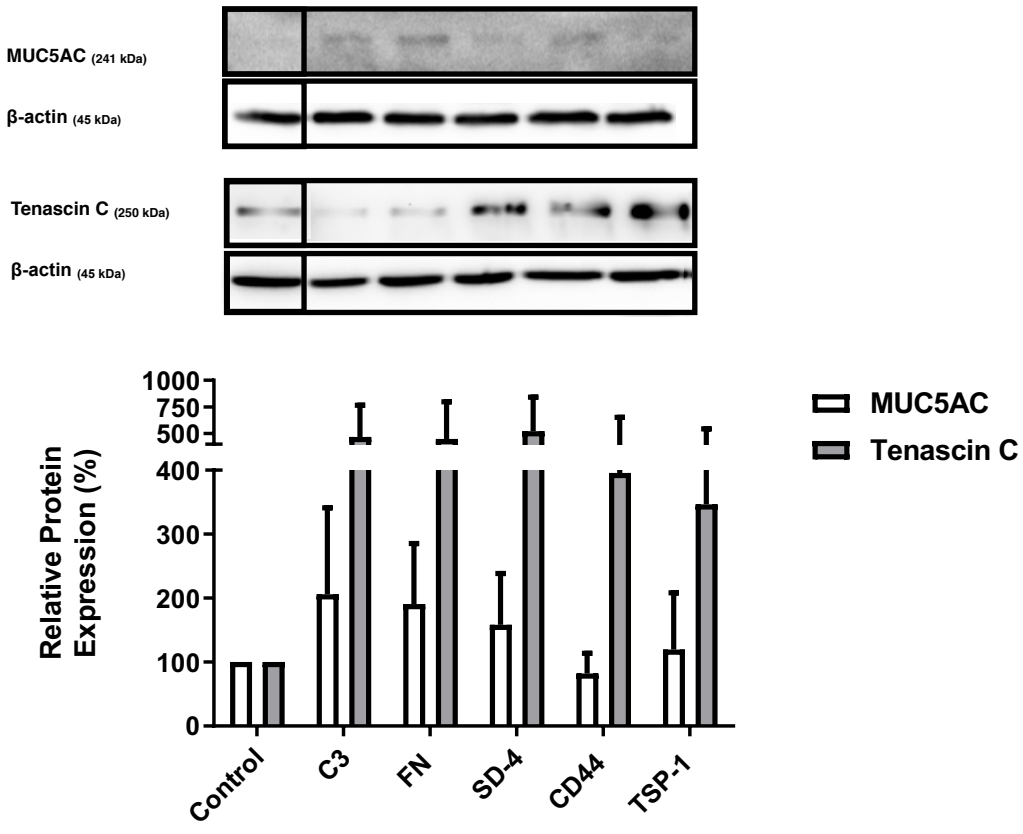
Specifically, a treatment with 50 ng/ml C3 led to a 5-fold increase of tenascin C but no changes in SCCA-1 and PAR-2 expression, whereas 1000 ng/ml FN, in addition to a comparable 5-fold increase of tenascin C expression, resulted in minor increase of SCCA-1 and PAR-2, respectively.

SD-4 treatment led to comparable results concerning tenascin C and PAR-2 with a less prominent increase of SCCA-1, comparable to treatment with CD44. The latter, moreover, led to a minor increase of tenascin C and PAR-2.

The matrix glycoprotein TSP-1 was responsible for an increase of tenascin C, PAR-2, and SCCA-1, comparable to CD44-treated bronchial epithelial equivalents. FN, SD-4, CD44, and TSP-1 moreover led to increased levels of α-SMA

Overall, these five compounds either led to a distinct increase or a trend towards increased levels of the mentioned markers, whereas no decrease was observed (**Fig. 23**).


TSLP (28 kDa)
β-actin (45 kDa)
α-SMA (42 kDa)
β-actin (45 kDa)
Relative protein expression (%)
6000
4000
2000
300
200
100
0
Control
C3
FN
SD-4
CD44
TSP-1
TSLP
α-SMA


MUC5AC (241 kDa)
β-actin (45 kDa)
Tenascin C (250 kDa)
β-actin (45 kDa)
Relative Protein Expression (%)
1000
750
500
400
300
200
100
0
Control
C3
FN
SD-4
CD44
TSP-1
MUC5AC
Tenascin C

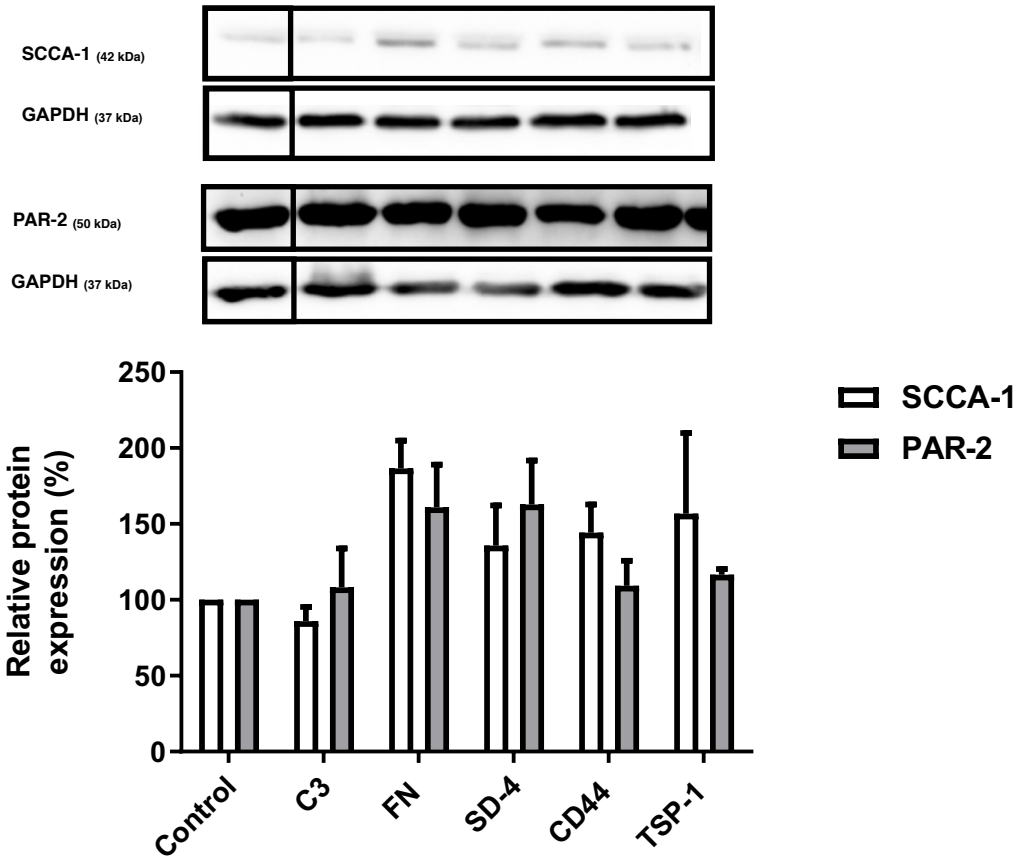


Figure 23. Semi-quantified protein expression in bronchial epithelial equivalents after a 6-day treatment with C3, FN, SD-4, CD44, and TSP-1

The expression of TSLP, alpha smooth muscle actin (α-SMA), MUC5AC, tenascin C, squamous cell carcinoma antigen-1 (SCCA-1), and protease activated receptor 2 (PAR-2) was semi-quantified by SDS-PAGE with subsequent western blot and densitometry analysis with Image J. Representative bands with corresponding loading controls β-actin or GAPDH are shown. For n=3 independent donors. Mean ± SEM.

Gene expression of bronchial epithelial equivalents treated with skin secretome compounds

In a next step, analogous to the co-culture evaluation, the expression of numerous genes was analyzed.

The innate immune factor C3 led to a >6-fold increase of *UG*, *ASMA*, *TSLP*, and *FLG* with a minor (<6-fold), increase of tenascin C and *MUC5AC* gene expression with no changes in *FN*, *LIF*, *PAR-2*, and *ZO-1*.

FN revealed distinct effects on *UG*, *TSLP*, and tenascin C (>8-fold increase), with less prominent effects on *SCCA-1* and *FLG* (>2-fold increase). No changes of *ASMA*, *FN*, *LIF*, *MUC5AC*, *PAR-2*, and *ZO-1* gene expression were observed.

The treatment with SD-4 did not alter gene expression of *ASMA*, *FN*, *LIF*, *PAR-2*, and tenascin C. A more prominent effect was observed for *SSCA-1*, *TSLP*, *FLG, MUC5AC*, and *ZO-1* with the strongest effect on expression of *UG*.

The soluble form of CD44 did not affect *ASMA*, *FN*, *LIF,* and *PAR-2* expression on gene level. Also, a comparably low *ZO-1* but strong *UG*, *SCCA-1*, *TSLP*, and *MUC5AC* increase was observed together with strongest effects on *FLG* and tenascin C gene expression.

TSP-1 treatment did not change expression of *UG*, *ASMA*, *FN*, *LIF*, *PAR-2, and ZO-1*. However, a minor increase of *MUC5AC* and an >8-fold increase of *SCCA-1*, *TSLP,* and *FLG* was observed. (**Fig. 24**).

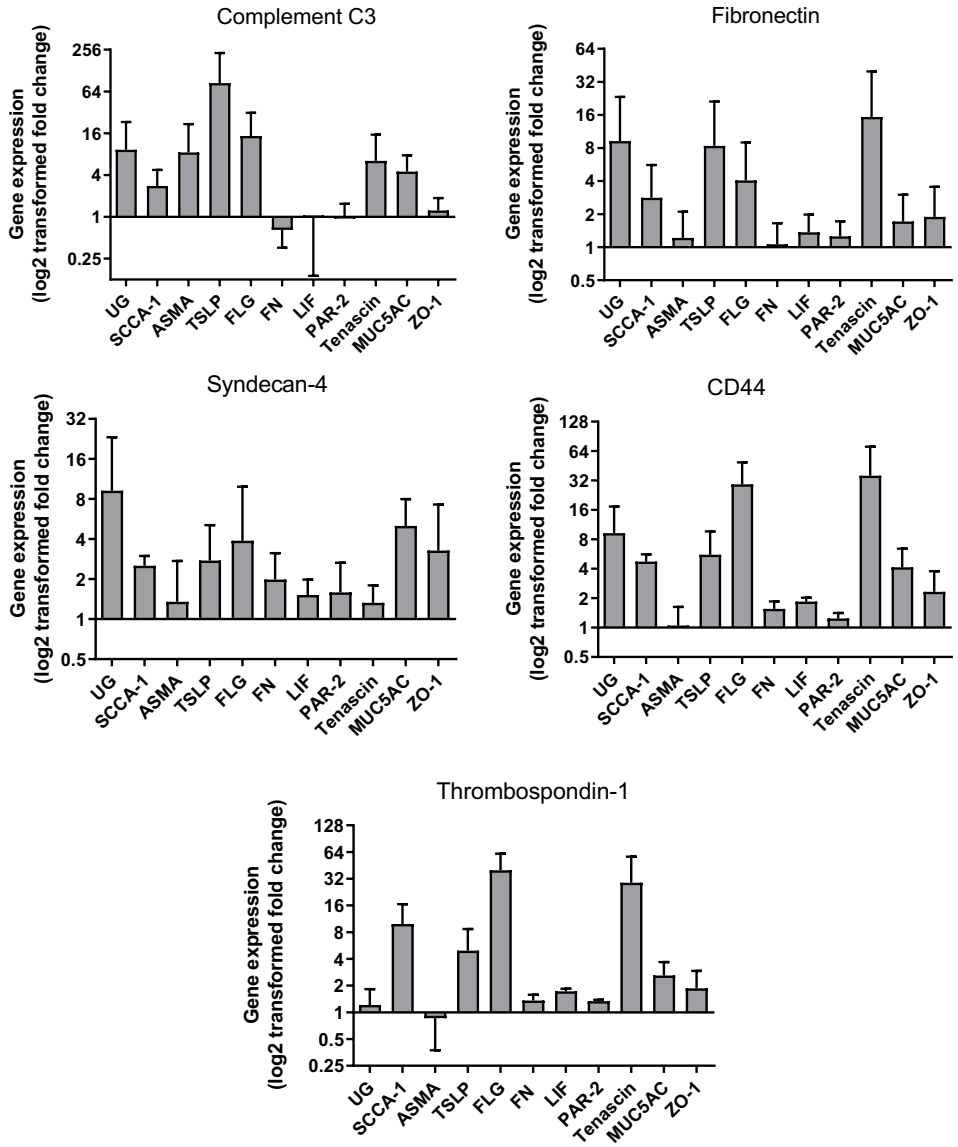


Figure 24. Relative gene expression in bronchial epithelial equivalents after a 6-day treatment with C3, FN, SD-4, CD44, and TSP-1

The expression of uteroglobin (*UG*), squamous cell carcinoma antigen-1 (*SCCA-1*), alpha smooth muscle actin (*ASMA*), *TSLP*, filaggrin (*FLG),* fibronectin (*FN*), leukemia inhibitory factor (*LIF*), protease activated receptor 2 (*PAR-2*), tenascin C, *MUC5AC*, and zonula occludens 1 (*ZO-1*) after either a 6-day treatment with C3, FN, SD-4, CD44, and TSP-1 as determined with qRT-PCR. Gene expression was normalized untreated control (=1) and log2 transformed. GAPDH served as housekeeping gene. For n=3 independent donors. Mean ± SEM.

Secretome analysis of bronchial epithelial equivalents treated with skin secretome compounds

Subsequently, the secretome of bronchial epithelial equivalents was analyzed in order to determine if these compounds are responsible for an increased release of pro-inflammatory cytokines. Interestingly, none of the analyzed cytokines were increased after a 6-day treatment with these five compounds compared to untreated control. The secretion of IL-6 (~2 ng/ml), IL-8 (~25 ng/ml), TSLP (~4 ng/ml), and IL-25 (~9 pg/ml) were at healthy baseline. Solely one donor showed increased IL-6 and IL-25 secretion after treatment with C3. Nevertheless, the overall effect on the secretome is, compared to the co-culture, non-existent **(Fig 25)**. No IL-33 and TNFα were secreted.

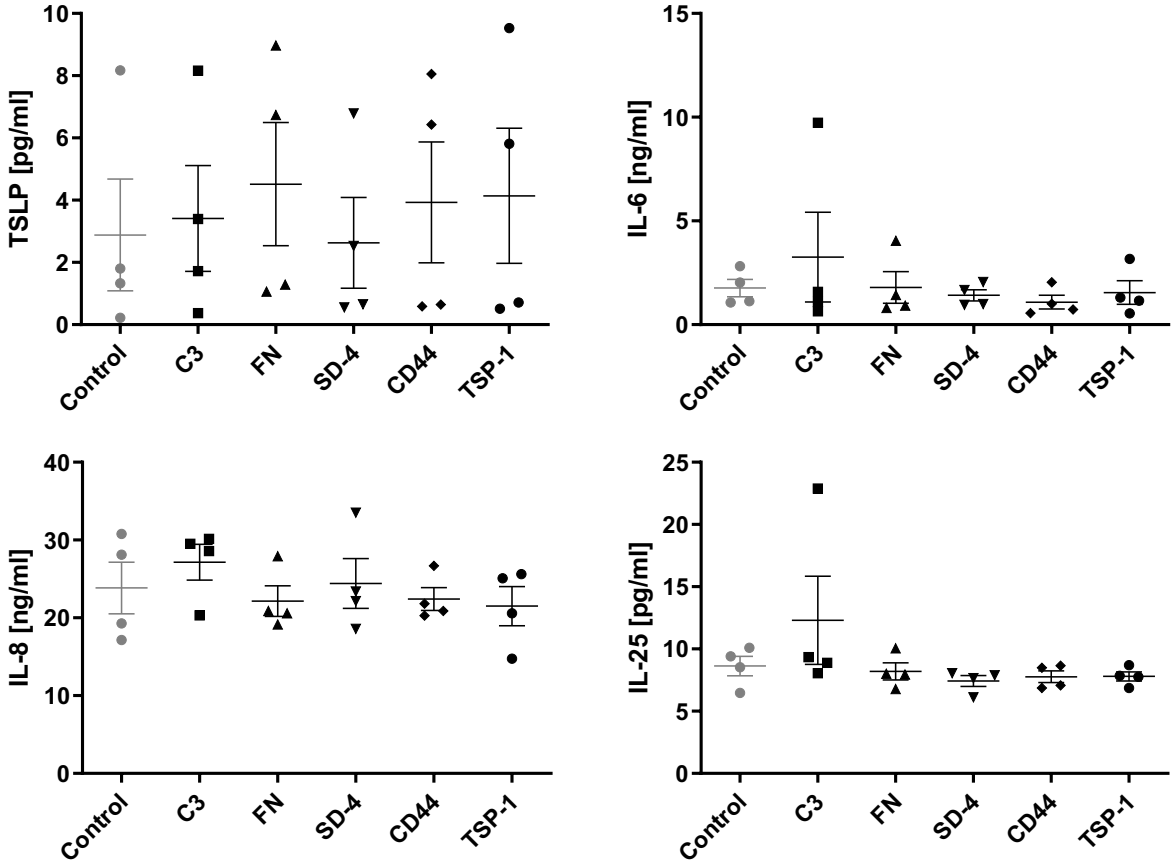


Figure 25. Secretome analysis of bronchial epithelial equivalents after 6-day treatment with C3, FN, SD-4, CD44, and TSP-1

Levels of pro-inflammatory cytokines TSLP, IL-6, -8, and -25 were determined in culture media of bronchial epithelial equivalents after a 6-day treatment with 50 ng/ml C3, 1000 ng/ml FN, 5 ng/ml SD-4, 100 ng/ml CD-44, and 5 ng/ml TSP-1 by sandwich ELISA. For n=4 independent donors. Scatter plot showing mean ± SEM.

Influence of skin secretome compounds on CD4+ and CD8+ T cells

The observed influence of these five compounds on bronchial epithelial equivalents and their direct pro-inflammatory effects without the presence of immune cells are unambiguous. Nevertheless, immune cells are a key factor in the pathogenesis of the atopic diseases. Hence, subsequently the influence of C3, FN, SD-4, CD44, and TSP-1 on naïve CD4+ and CD8+ T cells was analyzed in a next step. These T cell subtypes were isolated from buffy coat preparations from 2 female and 2 male donors, in order to minimize gender-bias.

Therefore, subset markers for Th1/Tc1 (*TBX21*), Th2/Tc2 (*GATA3*), Th17/Tc17 (*RORC*), and Th21/Tc21 (*AHR*) were determined with qRT-PCR. The complement factor C3 led to a strong increase of *TBX21* and *AHR* with minor effects on *GATA3* and *RORC* in activated naïve CD4+ T cells, hence strongly inducing Th1 and Th22 polarization, with less prominent effects towards Th2 and Th17. FN led to similar effects on activated naïve CD4+ T cells by increasing *TBX21*, *RORC* and *AHR* with no mentionable effect on *GATA3*. Consequently, FN induced polarization towards Th1, Th17, and Th22. Interestingly, SD-4 and CD44 showed comparable effects on activated naïve CD4+ T cells.

Whereas SD-4 and TSP-1 strongly increased *TBX21*, *RORC*, and *AHR*, *GATA3* was less affected. CD44 also more prominently increased *TBX21* and *AHR* with minor effects on *GATA3* and *RORC*. Thus, both led to a clear polarization towards Th1 and Th22 with rather minor effects towards Th2 polarization and a stronger influence of SD-4 and TSP-1 towards Th17.

Interestingly, none of these five compounds had hardly any effects on activated naïve CD8+ T cells. Almost all compounds rather decreased gene expression of *TBX21*, *GATA3*, *RORC*, and *AHR* compared to untreated CD8+ T cells (**Fig. 26**).

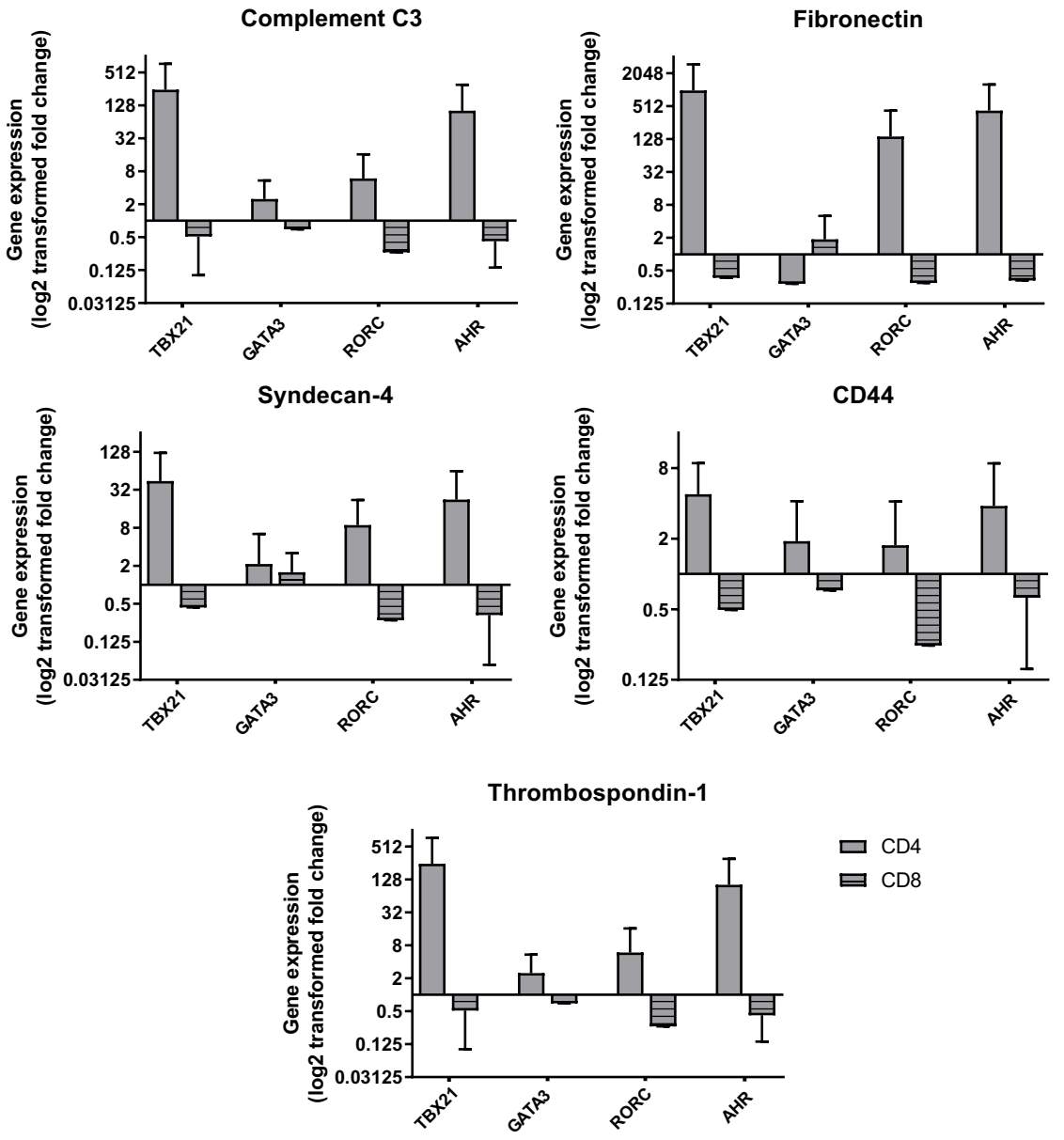


Figure 26. Gene expression of activated human naïve CD4+ and CD8+ T cells after treatment with C3, FN, SD-4, CD44, and TSP-1

After isolation of naïve CD4+ and CD8+ T cells from human buffy coats, CD3/CD28-stimulated T cells were treated with 50 ng/ml C3, 1000 ng/ml FN, 5 ng/ml SD-4, 100 ng/ml CD-44, and 5 ng/ml TSP-1 for 10 days. Normalized log2 transformed gene expression of *TBX21* (Th1/Tc1), *GATA3* (Th2/Tc2), *RORC* (Th17/Tc17), and *AHR* (Th22/Tc22) as determined with qRT-PCR. *YHWAZ* served as housekeeping gene. For n=4 independent donors. Mean ± SEM.

3.4 Characterization of healthy and diseased skin ECM

Altered ECM composition in AD as determined with patient-derived fibroblast

The previous results highlighted the role of just a small fraction of skin-derived secreted compounds. Interestingly, all of these five compounds are linked to the skin ECM, either being part of it or as associated proteins. In order to get deeper inside into changes within the ECM itself and to better understand its role in disease, further analysis are crucial. Therefore, patient-derived AD as well as donor-derived healthy fibroblasts were stimulated to produce ECM. In a first step, an ultrastructural examination of these ECM models after a 3-week cultivation was performed with scanning electron microscopy (SEM). Compared to a reference dermis from an excised human skin from healthy donors and to the healthy ECM model, a clear disorganization with less dense collagen bundles was observed (**Fig. 27A**). In addition, clearly reduced levels of hydroxyproline as well as desmosine were determined in atopic ECM models, indicating reduced collagen and elastin content. (**Fig. 27B**).

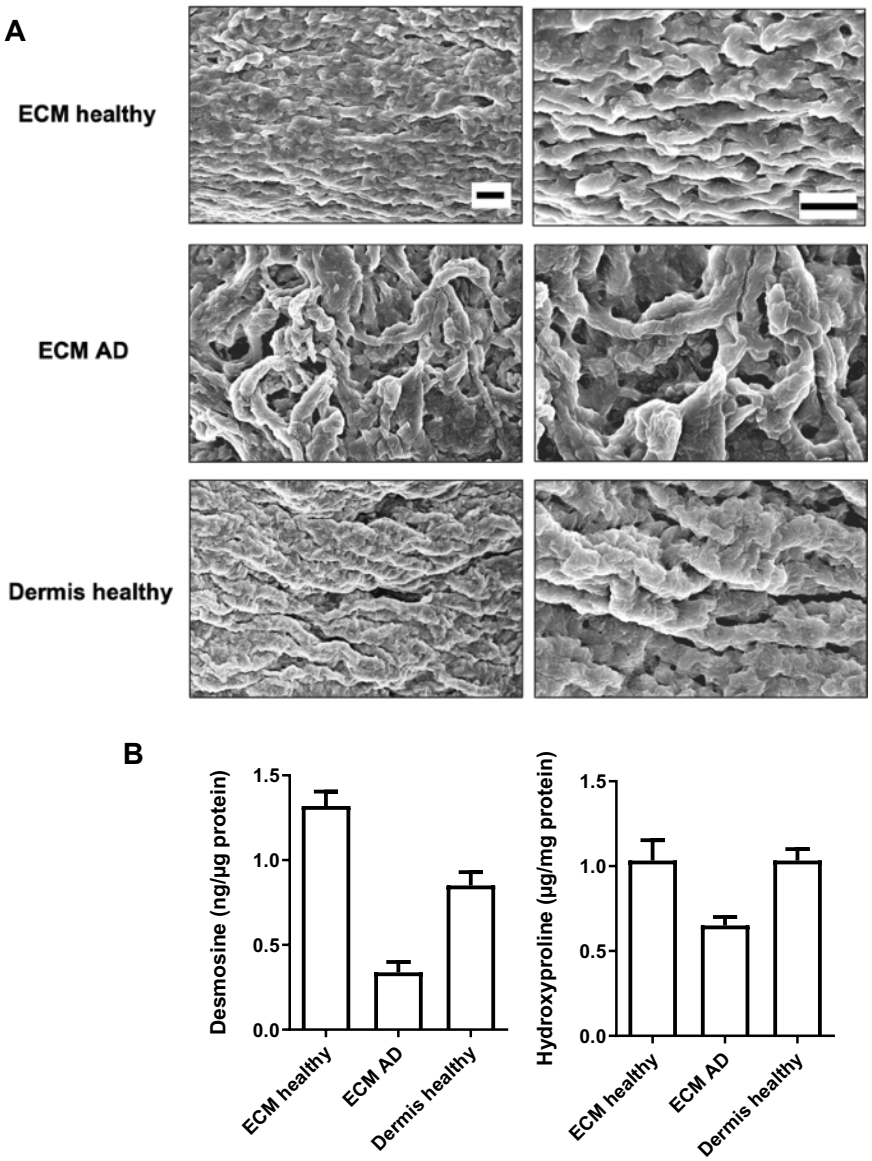


Figure 27. SEM analysis and collagen and elastin quantification of ECM models
Representative SEM images of ECM generated with either healthy- (ECM healthy) or patient-derived AD cells (ECM AD) as well as healthy *ex vivo* skin (Dermis healthy). Scale bar: 5µm. **B** Levels of hydroxyproline and desmosine as indicator for collagen and elastin content of skin ECM. For n=4 independent experiments.[11]

Protein expression is altered in AD ECM

In a next step, a deeper characterization of the ECM proteins via semi-quantitative proteomics analysis was performed on the self-assembled ECM models. This analysis revealed significant differences in the expression of numerous proteins between healthy and AD ECM. A small fraction of 19 proteins with the highest difference is shown in **figure 28**. Within these 19 proteins with the highest difference were for example tenascin (TENA), fibronectin (FINC), serine protease HTRA1, and signal transducer and activator

[11] Self-assembled ECM models were generated by Zheng Tan (Hedtrich Lab, UBC, Vancouver, Canada); ECM-analysis (SEM and collagen/elastin quantification) was performed by Preety Panwar (Brömme Lab, UBC, Vancouver, Canada)

of transcription 1-alpha/beta (STAT1) (**Fig. 28**). Interestingly, several proteins are connected to the ECM and partly known to play a role in atopic disease. A complete list of these 19 proteins + NID1 and extracellular superoxide dismutase (SODE) and their functions can be found in **table 9** in the appendix.

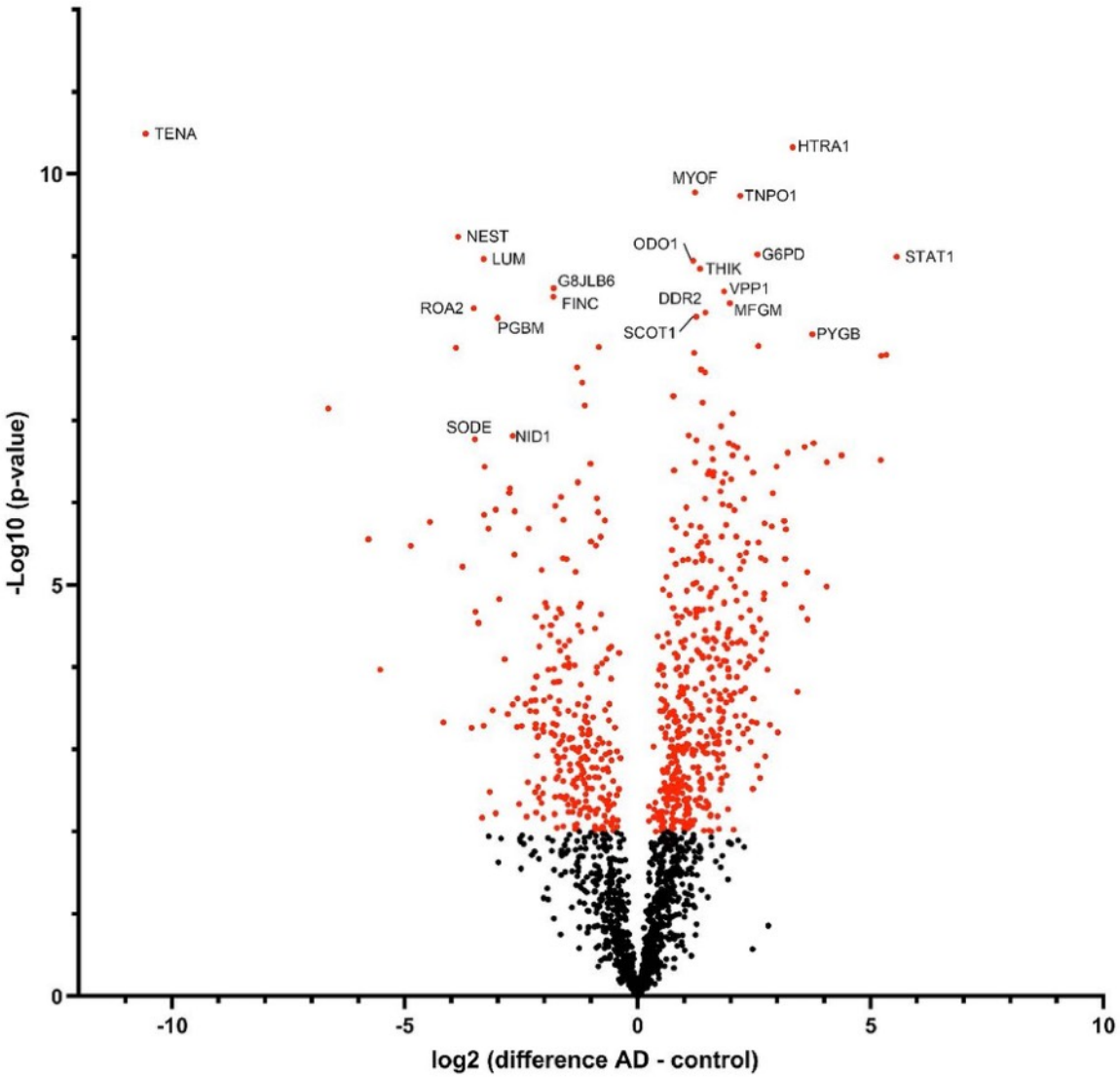


Figure 28. Proteomics analysis of self-assembled ECM models derived from healthy and diseased donors

Volcano plot showing differences in protein expression between donor-derived healthy and AD ECM. Proteins expressed higher in healthy ECM are left from zero, proteins expressed higher in AD ECM are right from zero. The 19 proteins with the highest difference between healthy and AD ECM plus NID1 and SODE are labelled. Red dots indicate significant differences *p≤ 0.01. For n=3 independent donors.[12]

[12] Analysis, bioinformatic evaluation, and data presentation were performed by Jason Rogalski (UBC Proteomics Core, Vancouver, Canada)

AD fibroblasts are characterized by a significantly altered transcriptome

To complete the picture, a transcriptomic analysis was performed of previously used donor-derived healthy and AD fibroblasts. A total of 1,593 significantly differentially expressed (DE) genes as determined. 13 key pathways are shown in **figure 29**. Interestingly, not only genes involved in ECM organization (e.g., fibronectin, tenascin), ECM degradation (e.g., COLs, *MMPs*, *ADAMs*), and ECM interaction are differentially expressed, but also in pathways playing a role in immunological signaling (e.g., *STAT1, HSP90AA1*), cytokine-cytokine receptor interaction (e.g., *ILRs*, *CXCLs*), Wnt signaling pathway (e.g., *LGR5, WNT9A*), and VEGF signaling (*ITGAs,* COLs).

Additionally, a minimum order protein-protein-interaction network was constructed by uploading a list of these 1,593 DE genes to NetworkAnalyst. Out of a highly interconnected network with over 2,000 nodes, subnetworks were created via Reactome enrichment within NetworkAnalyst that represent relevant biological pathways. Subnetworks concerning ECM organization, cytokine signaling and TLR4 signaling are shown in the appendix (**Fig. 30**). These datasets match with the previous results by highlighting the role of fibronectin, *ITGB5 as* a gene coding for a fibronectin receptor, and *ILRs* as well as *MMPs*.

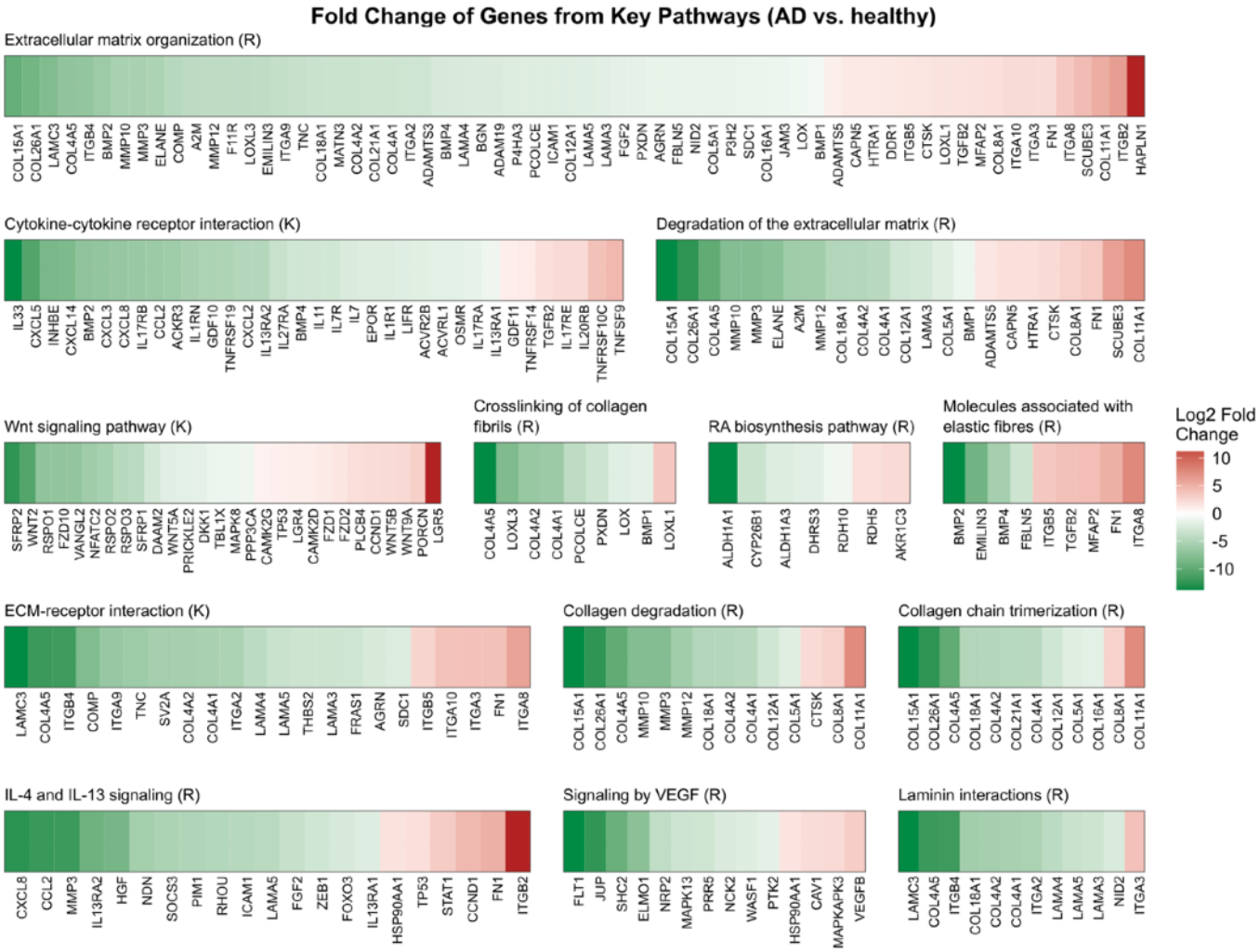


Figure 29. Transcriptome analysis of healthy and AD fibroblasts
A heatmap shows significantly altered genes of AD-relevant key Reactome (R) and KEGG (K) pathways as log2 transformed fold change from pathway enrichment with Sigora.[13]

[13] Analysis, bioinformatic evaluation, and data presentation were performed by Travis Blimkie (Hancock Lab, Department of Microbiology and Immunology, UBC, Vancouver, Canada)

3.5 Impact of SD-4, CD44, and TSP-1 in BALB/c mice

The results from the co-culture and the skin secretome compound testing in bronchial epithelial equivalents as well as CD4+ and CD8+ T cells indeed revealed interesting results confirming a possible role of C3, FN, SD-4, CD44, and TSP-1 in the pathogenesis of the atopic march. Nevertheless, these results are based on separate systems standing for their own. The bronchial epithelial equivalents lack complexity and naïve CD4+ as well as naïve CD8+ T cells are not involved in any other environment. A more complex *in vitro* system is currently not available. Therefore, it was necessary to investigate these compounds in the most complex pre-clinical model that is available. Although far from being optimal, the most used AD asthma models currently are BALB/c mice. In order to compare the suitability of stand-alone *in vitro* models in the context of a translational correlation and to investigate the effects of these compounds under *in vivo* conditions, three out of five selected murine compounds (SD-4, CD44, TSP-1) were tested in BALB/c mice. Therefore, according to serum levels and kinetics data known from literature research, a specific concentration of each compound per day was applied via osmotic pumps for a total of 7 days, a time period almost analogous to the previous cell culture-based experiments. The osmotic pumps were filled with each compound and implanted subcutaneously by Dr. Jenny Wilzopolski with help from Dr. Viviane Filor and Pascal Canbolat (Veterinary Pharmacology, Prof. W. Bäumer, FU Berlin). FN and C3 were not tested due to handling reasons.

After a constant release of these three compounds over seven days, mice were killed and lung tissue, gut tissue, spleen, and blood samples were collected for further analysis.

In a first step, the influence of these three compounds on spleen and CD4+ T cells was analyzed. Therefore, spleen size and weight were determined and CD4+ T cells were isolated.

Whereas the macroscopically measured size of the spleen marginally differed, a trend towards increased spleen weight of mice after the 7-day treatment with SD-4 (158.4 mg ± 9.9), TSP-1 (167.3 mg ± 21.1), and a significant increase after CD44 treatment (183.2 mg ± 31.2) compared to PBS-treated mice (144.5 mg ± 26.4) was observed (**Fig. 31**).

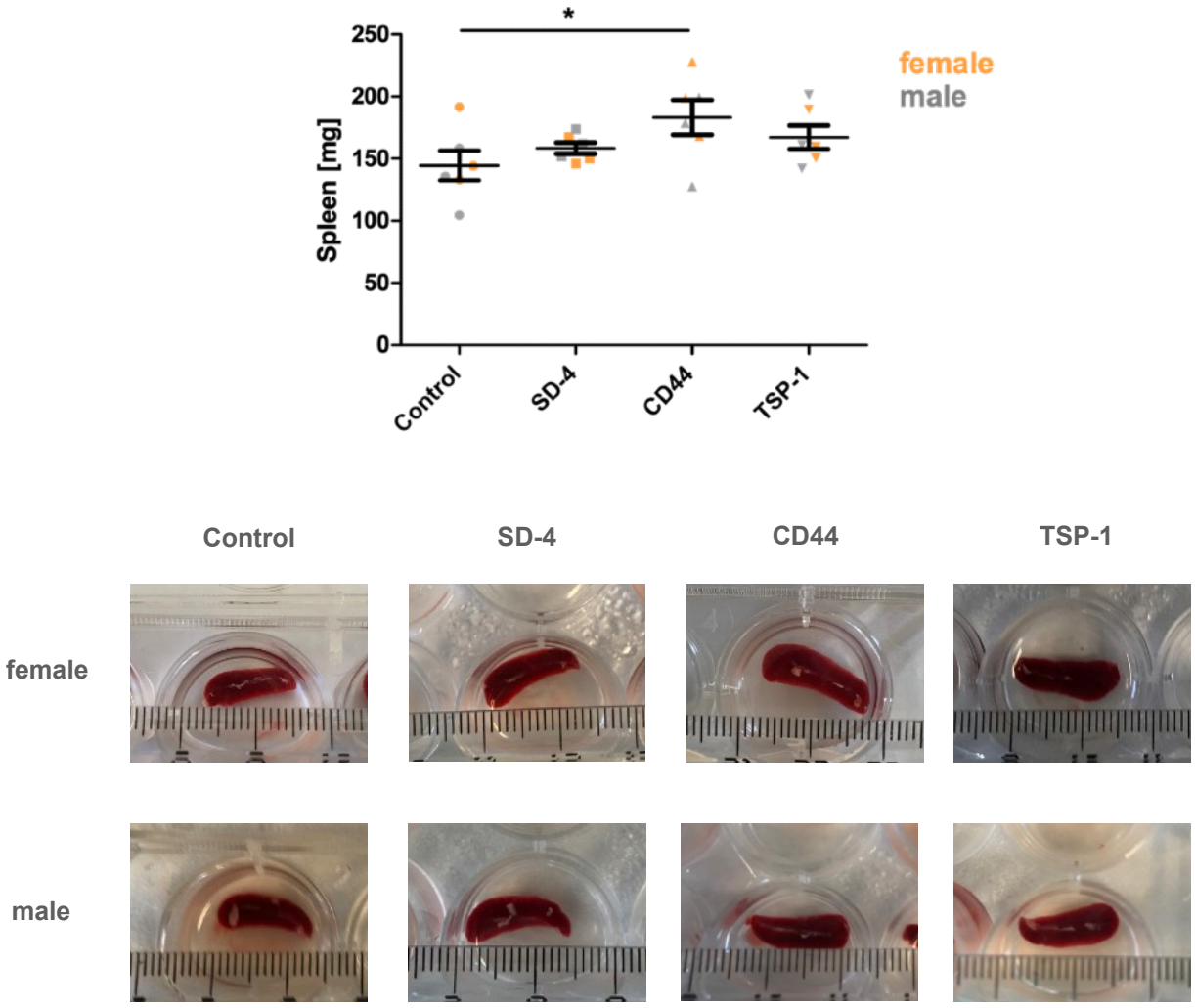


Figure 31. Weight and size of spleen isolated from mice after a 7-day treatment with PBS, SD-4, CD44, and TSP-1

Spleen from BALB/c mice were isolated together with lung, gut, and thymus after a 7-day treatment with PBS (Control), SD-4, CD44, or TSP-1. Weight and size of the spleen were documented. Dot plot shows the weight of spleen from female and male mice in mg. Representative images of female and male spleen are shown below. For n=6 BALB/c mice. Mean ± SEM, * $p < 0.5$. (One-Way-Anova with Dunnett's Multiple Comparison Post-Test for independent parametric values as determined by Shapiro-Wilk Test).

In order to analyze if increased weight of mice spleen after treatment with SD-4, CD44, and TSP-1 goes in line with an effect on Th cells, CD4+ T cells were isolated from spleen, and pooled female and male CD4+ T cells were analyzed for expression of subtype markers as mentioned before. Interestingly, a sex-specific effect was observed on Th-polarization within this small sample size. Whereas SD-4 induced polarization towards Th1 (*Tbx21*) in male mice, it led to polarization towards Th17 (*Rorc*) in female mice. CD44 only affected male CD4+ T cells by polarization towards Th1 (*Tbx21*) and Th22 (*Il-22*), whereas TSP-1 showed distinct effects towards Th1 (*Tbx21*), Th2 (*Gata3*), and Th17 (*Rorc*) in female CD4+ T cells. In male mice, TSP-1 slightly upregulated *Tbx21* and *Il-22*; thus, polarizing towards Th1 and Th22 (**Fig. 31**).

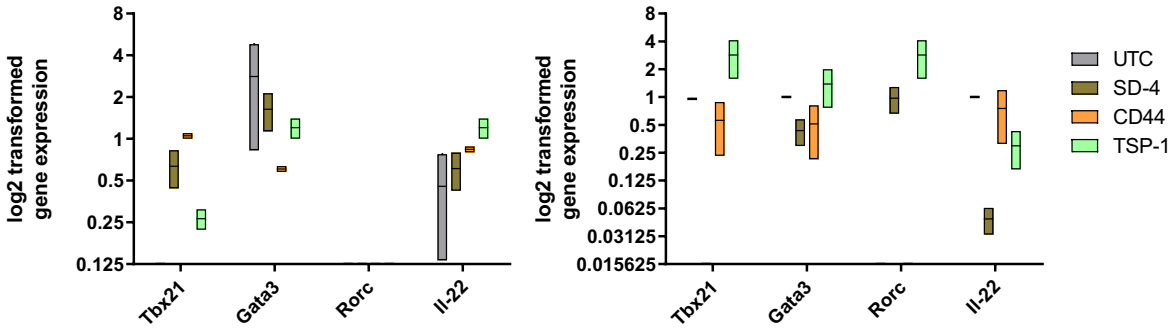
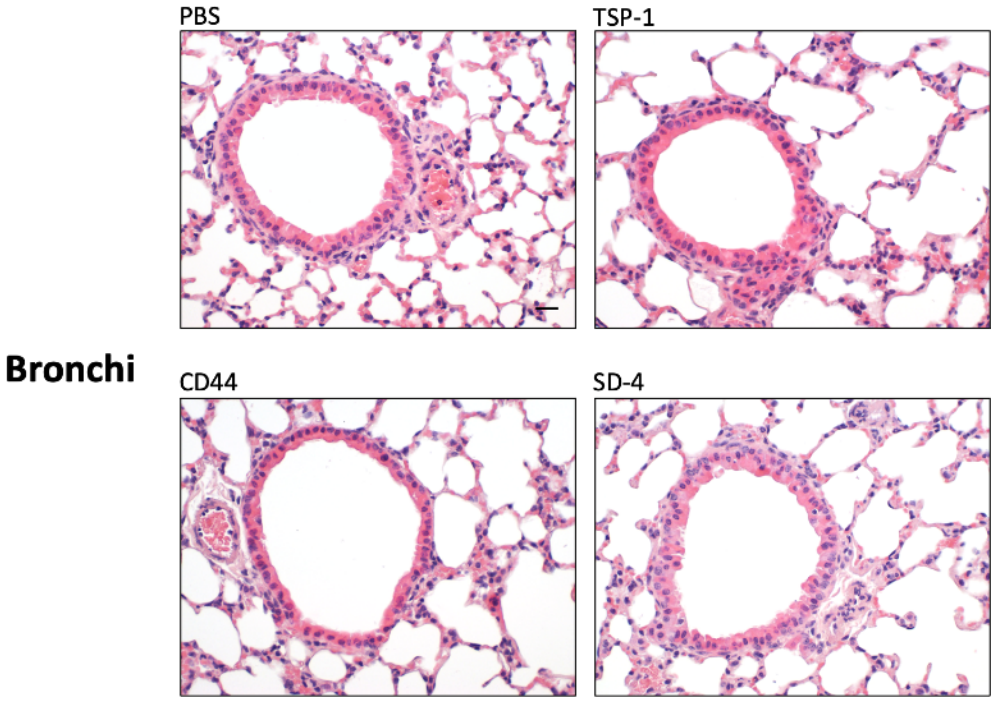


Figure 32. Gene expression of isolated mice CD4+ T cells after a 7-day treatment with PBS, SD-4, CD44, and TSP-1

CD4+ T cells were isolated from mice spleen after a 7-day treatment with PBS (UTC), SD-4, CD44, and TSP-1 and mRNA expression of *Tbx21* (Th1), *Gata3* (Th2), *Rorc* (Th17), and *Il-22* (Th22) of pooled male (left) and female (right) mice was determined via qRT-PCR. Gene expression was normalized to housekeeping genes *Gapdh* and *Hprt*, and log2-transformed. n=3 for each sex. Mean ± SEM.

During the experiment, no macroscopically relevant changes of the skin and no airway symptoms were observed. In addition, histological staining of bronchi and alveoli of one half of the lung were performed and no differences between the groups were determined on histological level (**Fig. 33**).



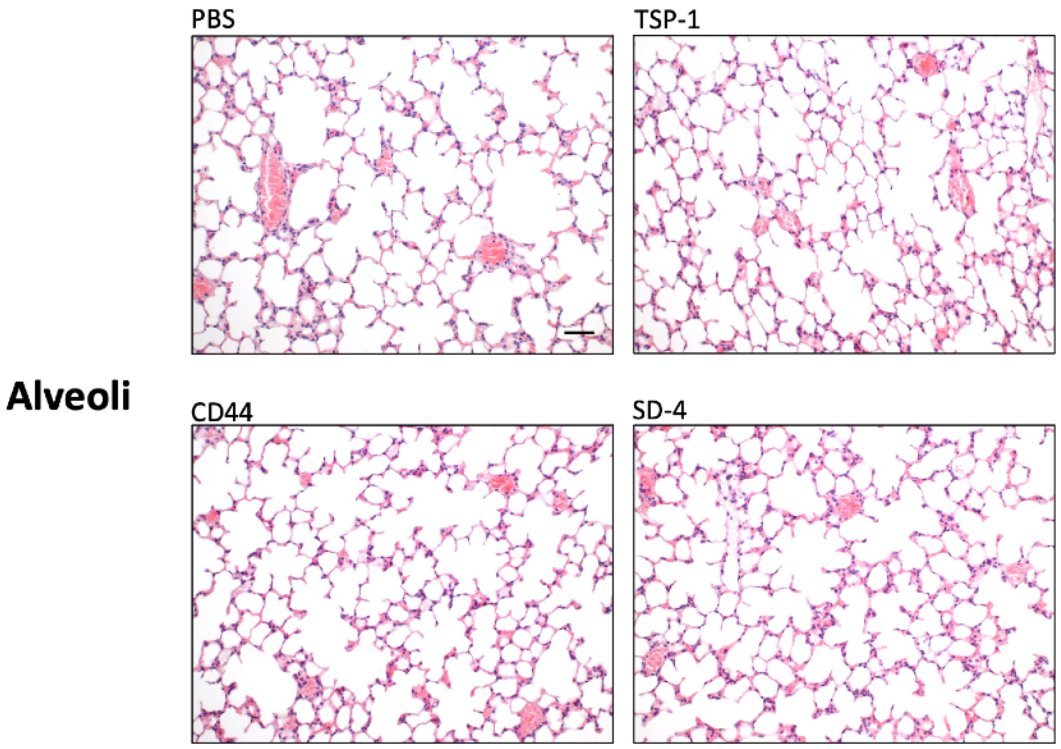


Figure 33. Histology of mice lungs
Representative images of H&E-staining of bronchi and alveoli of control (PBS), TSP-1, CD44, and SD-4 treated mice after 7 days. Scale bars: Bronchi: 20µm; Alveoli: 50µm.[14]

[14] Histological staining, evaluation, and data presentation were performed by Anne Voß (Group of Prof. A. Gruber, Veterinary Pathology, FU Berlin) and are part of her thesis discussed from another point of view

4. DISCUSSION

4.1 Skin disease equivalents induce an asthmatic phenotype in bronchial epithelial equivalents after co-culture

The poor predictability of animal models in pre-clinical trials has already been addressed. Another issue with these models concerns basic research studies for understanding and translating (patho-)physiological mechanisms from animal to human for certain diseases. Indeed, animal models do partly deliver excellent data for the understanding of possible molecular mechanisms and underlying pathophysiology. Also of advantage is the possibility to study a whole biological organism containing all relevant organs, tissues, and mediators such as cytokines, hormones etc. Nevertheless, a one-to-one translation from e.g., mouse data to human is not possible. Therefore, high effort is currently put into the development of reconstructed human-based multi-organ cultures (Low et al. 2021; Schimek et al. 2020; Reiner, Sapir, and Parichha 2021).

The in this study established human-based two-organ co-culture of skin (disease) and healthy bronchial epithelial equivalents was primarily developed to serve as model system for the investigation of the atopic march *in vitro*.

The first step on the way to maintaining two separately established reconstructed models - the skin equivalent and the bronchial epithelial equivalent - within one system, was to find a culture medium in which both equivalents can be cultured for a certain amount of time without significantly changing their phenotype and protein expression. Hence, ideally to keep both equivalents in one system as long as possible. Therefore, a co-culture period of approximately one week was chosen and aimed for due to several reasons: i) according to the in the Hedtrich lab established protocols skin equivalents are cultured for two weeks, bronchial epithelial equivalents for three weeks, and both equivalents need their first days of 3D culture to get used to the co-culture of two cell types (epithelial cells and fibroblasts), to grow, and to differentiate under these new air-liquid-interface conditions separately; ii) additionally to that first few days of growth and differentiation, a certain amount of time is needed for media adaptation; iii) the atopic march as it is known *in vivo* develops over several years. Although a co-culture over several years *in vitro* is not possible, due to the comparably simplified two-organ system a pathophysiological communication between skin and lung within a relatively short amount of time did not seem completely unrealistic.

So, after several different types of media separately tested in skin and bronchial epithelial equivalents, the “minimal” medium led to the most consistent and satisfying results. First, no relevant change was observed for viability of both, skin and bronchial epithelial equivalent (**Fig. 10, 13**). Then, the viable epidermis in skin equivalents and the epithelial layer with its goblet cells, cilia, and mucus in bronchial epithelial equivalents was not distinctly altered compared to the conventionally cultured control equivalents. Other media tested, such as the different mixtures of both differentiation media - KDM and P-ALI - resulted in either poor differentiation, parakeratosis, hyperproliferation, goblet cell

hyperplasia, and increased production of mucus (**Fig. 8, 11**). A possible explanation for these results might be the simplicity of the “minimal” medium. Whereas KDM and P-ALI induce differentiation and proliferation of cells, and the addition of completely new and complex (foreign) culture medium to each of the equivalents might result in an extra stimulus for several cellular processes that might not be clearly assigned to the cell culture supplements, the “minimal” medium solely contains the basic supplements for cells to survive, namely a basal medium and FBS. This, after some days of conventional culture, might be enough to keep the equivalents alive and cultivate them in a kind of steady-state condition in which the equivalents are able to maintain themselves and additionally avoid any unwanted supplements interfering with the co-cultivation and the communication between skin and bronchial epithelial.

In a next step, two different approaches were investigated for the two-organ co-culture of skin (disease) equivalents and healthy bronchial epithelial equivalents. The first type of co-culture is called the “indirect co-culture” (Ind). Here, bronchial epithelial equivalents were supplemented with 50% of conditioned “minimal” medium from health and diseased skin equivalents from day 15-21 of culture. The reason for this mixture of 50% conditioned medium and 50% fresh medium was, on the one hand, not to overload bronchial epithelial equivalents with an unknown amount of unidentified factors secreted by skin (disease) equivalents and, on the other hand, to supply bronchial epithelial equivalents with at least 50% fresh “minimal” medium every other day.

The second approach is called the “direct co-culture” (Dir). Although this term is not fully correct since it implicates a co-culture where both equivalents are in direct contact, it was chosen to better distinguish between those two types of co-culture. Dir was performed in deep petri dishes (**Fig. 7**) with “minimal” medium from day 15-21 of culture with refreshing half of the medium once, since these deep petri dishes had a capacity for 33 ml which was sufficient to supply both equivalents for this period of time.

In order to have a positive control, a third approach was established. Asthma as well as AD are both characterized by a Th2-dominant immune response (Brandt and Sivaprasad 2011; Villani et al. 2021). Therefore, analogous to the inflammatory skin equivalents, bronchial epithelial equivalents were treated with 15 ng/ml IL-4 and -13 from day 15-21 in order to have a valid comparison with the two types of co-culture.

Interestingly, all three approaches - the treatment of bronchial epithelial equivalents with IL-4/-13 and the co-cultures with diseased skin equivalents - resulted in a hyper-proliferative phenotype characterized by clear goblet cell hyperplasia (GCH) and increased mucus production (**Fig. 14**). Asthma itself is characterized by GCH (increased number of goblet cells), goblet cell metaplasia (GCM - development of goblet cells from other epithelial cell types), and mucus hypersecretion (Ordoñez et al. 2001; Alagha et al. 2019). Airway mucus itself has protective function, whereas excessive production of tough mucus as well as airway hyperresponsiveness play an important role in asthma

pathophysiology and contribute to increased morbidity and mortality (Sterk 1993; Grootendorst and Rabe 2004). Interestingly, in a murine mouse model of asthma sensitized and challenged with ovalbumin (OVA), the process of GCM was observed as well (Reader et al. 2003). Here, Reader et al. showed a 25% decrease of ciliated cells and a 75% decrease of club cells with a compensation of these losses by an increase of goblet cells. In order to prove GCM in the diseased co-cultures as well as the IL-4/-13 treatment, a staining for co-expression of markers for goblet as well as ciliated or club cell might be worth following up in the future; in particular a classical immunofluorescence staining for proteins or single molecule fluorescence *in situ* hybridization (smFISH), a method for detection of RNA in single cells (Kwon 2013), are possible options.

However, the clearly observed hyperproliferation in all three (IL-4/-13, Ind D, Ind H) approaches can unexpectedly not be proven by staining of proliferation marker Ki-67 (**Fig. 14**). Although Hackett et al. was able to show a clear increase of Ki-67 in asthmatic compared to healthy airways by IHC staining, in ALI cultures of these same asthmatic and healthy donors, no change in Ki-67 expression was observed (Hackett et al. 2011). Nevertheless, Hackett et al. was able to show increased mucus production in asthmatic airways by PAS-staining but no increase in asthmatic ALI cultures compared to non-asthmatic airways and ALI cultures.

Interestingly, these are the only identical results between these three disease approaches. Moreover, a discrepancy between gene and protein data was partly observed, whereas the results on gene level clearly point towards an asthma-like inflammation, results from protein analysis were less prominent. Generally, it is known that levels of gene expression and expression of the corresponding protein do not necessarily correlate as shown in several studies (Koussounadis et al. 2015; Kosti et al. 2016; Gry et al. 2009; Guo et al. 2008). Although the relationship between gene and protein expression is clear, since mRNA gets translated to peptides and proteins, predicting protein expression from mRNA levels sometimes might mislead. One the one hand, the timing might be a reason since the amount of time between mRNA upregulation and its effects on the upregulation of the corresponding protein might differ depending on translation and post-translational modifications. On the other hand, upregulated protein levels might directly act as negative feedback upon mRNA expression.

The former, the timing issue, might also be a possible explanation here. As mentioned, the process of the atopic march is a long process (>5 years) and involves allergens, the immune system, and genetical factors; thus, being a multifactorial disease picture. The bronchial epithelial equivalent lacks immune cells, allergens, and genetic modifications, and the co-culture itself is performed for just 6 days.

Finally, an explanation for the partly observed differences between indirect and direct co-culture might be that there is a one-way interaction and a re-stimulation with fresh conditioned medium every other day in indirect co-culture, whereas in the direct co-culture

the effects of skin disease equivalents might get attenuated over time due to missing inflammatory (IL4-/-13) stimulation within the co-culture. Additionally, in the direct co-culture there is more than just a one-way communication, since both equivalents might affect and influence each other.

4.2 Role of AD skin in the atopic march

Due to its increasing prevalence, AD has become a significant health problem of the industrialized countries. Currently, up to 25% of children and 1-3% suffer of adults in the western world suffer from AD (Odhiambo et al. 2009). Numerous studies additionally demonstrated that more than 50% of AD patients suffering from a moderate to severe phenotype are sensitized to numerous allergens which during childhood results in the manifestation of other allergies such as FA, AR and/or AA. This disease progression is also called atopic march (Rhodes et al. 2002; Sicherer and Leung 2012). The molecular mechanisms of the atopic march are still largely unknown and represent a large knowledge gap hampering the development of new approaches for the treatment and prevention of atopic diseases. However, a correlation between skin and lung is very likely, since AD and AA interestingly are characterized by similar pathophysiological patterns such as Th2-dominated inflammation and increased IgE levels. In addition, the keratinocyte cytokine thymic stromal lymphopoietin (TSLP), by promoting allergen awareness, leads to the manifestation of AA in the atopic march (Hongwei Han, Roan, and Ziegler 2017; Leyva-Castillo et al. 2013). Despite extensive research activities, TSLP, IL-25, and IL-33 are just some of the few factors known so far to be associated with the development of the atopic march. Over the past few years, the Hedtrich group has developed a human *in vitro* model of atopic skin (Hönzke, Wallmeyer, et al. 2016) that could close this knowledge gap.

As already shown previously, this model of atopic skin can be used for the investigation of pathophysiological communication with other reconstructed tissues such as the bronchial epithelial equivalent. In order to reveal the responsible factors inducing this asthma-like inflammation in the bronchial epithelium *in vitro*, in a first step, the secretion of several cytokines into cell culture medium of healthy and diseased skin equivalents was analyzed. Interestingly, no changes in IL-6 and -8 were found and no signal for IL-1β and IL-10 at all (**Fig. 19**). A possible explanation might be the AD-severity of the skin disease equivalent. As *in vivo* studies show, levels of IL-6 and -8 are highly dependent on the disease severity and were found to be increased in patients' sera suffering from moderate to severe AD, whereas no increase was found in patients suffering from mild AD (Toshitani et al. 1993). An increase of IL-8 and IL-1β in a different kind of skin disease equivalents was found by Hönzke et al. who stimulated skin equivalents with 20 ng/ml TNFα (Hönzke, Gerecke, et al. 2016). However, according to the current knowledge IL-1β, -6, -8, and -10 do not

primarily play a role in the atopic march. These were analyzed for gaining a better basic characterization of the secretome of skin disease equivalents. Also, no increase was found in levels of IL-25 and even no secretion of IL-33 at all (**Fig. 19**). One possible explanation, as already mentioned, could also be that this skin disease equivalents simply represents a mild form of AD, whereas another reason might be the missing immunological component in this model, since AD is a multifactorial disease with clear interplay between epithelial and immune cells. For IL-6 and -8, Wallmeyer et al. did prove that the skin equivalents (*FLG*+ and *FLG*-) secret more IL-8 and even significantly more IL-6 when co-cultivated with activated naïve CD4+ T cells (Wallmeyer et al. 2017).

Nevertheless, one of the key factors playing a role in the atopic march, KC-derived TSLP, is increased in the epithelial layer as shown by Hönzke at al. and Wallmeyer et al. (Hönzke, Wallmeyer, et al. 2016; Wallmeyer et al. 2017), and was found to be increased in the cell culture medium of diseased skin equivalents as well (**Fig. 19**). These findings go in line with TSLP found to be strongly expressed in lesional areas of AD skin as well as the increase in AD-patients' sera (Soumelis, Reche, Kanzler, Yuan, Edward, Homey, Gilliet, Ho, Antonenko, Lauerma, Smith, Gorman, Zurawski, Abrams, Menon, McClanahan, De Waal-Malefyt, et al. 2002). Moreover, TNFα was found to be slightly increased in skin disease equivalents which is also known to be increased *in vivo* (Sumimoto et al. 1992), whereas it is not considered relevant for the atopic march. Another interesting finding from Löwa et al. who used patient-derived AD fibroblasts for cultivation of skin equivalents were significantly increased levels of IL-6, -13, TNFα, and leukemia inhibitory factor (LIF) when co-cultured with activated naïve CD4+ T cells, whereas levels of LIF without CD4+ T cell supplementation were significantly downregulated in equivalents generated with AD fibroblasts (Löwa et al. 2020). The latter, being in line with other studies showing reduced expression of LIF on protein and gene level, hence highlighting the role of fibroblast in dermal-epidermal communication and AD-pathophysiology, since LIF is important for differentiation of normal skin and reduced expression of LIF in dermal fibroblasts leads to impaired epidermal structure (Berroth et al. 2013).

The skin disease equivalents used for these current projects and thesis were generated with healthy NHDF and NHEK inducing a knockdown of the *FLG* gene solely in the NHEK, pointing towards an important, here missing, feature: the role of AD-fibroblasts.

Nevertheless, in order to gain even more insight, the secretome of skin (disease) equivalents — in form of the culture media of healthy and diseased (*FLG*-$_{\text{IL-4-/-13}}$) skin equivalents — was analyzed with separation of proteins by SDS-PAGE and subsequent proteomics analysis of protein bands with obviously increased expression in medium of skin disease equivalents. As shown in **Fig. 20**, two bands, one with the size of approximately 150 kDa, the other one with a size >250 kDa, showed a clearly increased signal after coomassie blue staining of the gel in skin disease equivalents. Out of a large

list of hits (**Tab. 8, appendix**), the following proteins were chosen for further investigation according to literature research and the potential to play a role in the atopic march: complement factor C3 (C3) as protein of the innate immune system, ECM glycoprotein fibronectin (FN), shedded form of plasma membrane proteoglycan syndecan-4 (SD-4), soluble form of cell-surface glycoprotein cluster of differentiation 44 (CD44), and matrix glycoprotein thrombospondin-1 (TSP-1).

Interestingly, all five mediators are part of the skin matrisome. The matrisome is the entirety of skin extracellular matrix (ECM-) associated factors with FN and TSP-1 being part of the core skin matrisome, SD-4 being a matrisome-associated protein, and CD44 a receptor of ECM proteins such as HA, COLs, and MMPs (Ponta, Sherman, and Herrlich 2003). C3 is produced by keratinocytes upon inflammatory stimuli (Pasch et al. 2000) and is bound by ECM (Fernandez-Godino, Bujakowska, and Pierce 2018). Whereas ECM glycoproteins, COLs, and proteoglycans are considered to be the core matrisome, all further ECM-connected proteins, such as regulators of ECM as well as secreted factors are considered matrisome-associated (Naba et al. 2016). The ECM is a complex structure that provides tissue boundaries and biomechanical properties. The role of ECM in disease has been underestimated for quite a long time. Recent data proved that ECM has huge impact on cell survival, proliferation, differentiation (Naba et al. 2016), and inflammatory processes (Shimshoni et al. 2021).

The role of ECM in AD is still not fully understood. Two recent studies started to focus a possible crosstalk between ECM and immune cells in skin inflammation (Pfisterer et al. 2021; Bhattacharjee et al. 2019).

The role of the complement system and its receptors in AA and AD is frequently being discussed. The complement system itself a complex system of not only membrane-bound but also numerous soluble proteins serving as bridge between innate and adaptive immune response (Wills-Karp 2007).

Above all, the cleavage products of the factors C3 and C5, C3a and C5a, play a role in the regulation of the Th2 immune response (X. Zhang and Khl 2010); thus, being important for pathogenesis of AD and AA. The plasma concentration in 60 Egyptian children with asthma (not in the acute asthmatic attack) was 14,060 mg/ml, in healthy people 10,770 mg/ml C3, as determined in a case-controlled study (Fattah et al. 2010). No difference in serum C4 was measured. This goes in line with the increased concentration of C3 measured in plasma of children up to 7 years of age suffering from AD and EoE; thus, going through the atopic march, with 4.5 mg/ml compared to healthy control. Accordingly, the diseased skin equivalents secreted more C3 with mean 5.5 µg/ml than the healthy equivalents with mean 3.5 µg/ml (**Fig. 21**).

In a *C3*$^{-/-}$ mouse model (C57BL/6 mice) with allergen-induced pulmonary allergy, it was also shown that the lack of C3 led to significantly lower bronchial hyperreactivity, reduced

number of macrophages, neutrophils as well as lymphocytes in BALF, and reduced Th2-dominated inflammation in form of IL-4 reduction after allergen contact (Drouin, Corry, et al. 2001). In contrast to that, a lack of C3 interestingly led to a protective effect in the skin (Purwar et al. 2011). Not only C3 itself, but also its activated form, C3a, must be considered. In a C3a receptor-deficient C57BL/6 mouse model of AA, a reduced number of goblet cells and MUC5AC was found compared to WT. Additionally, the authors showed a direct and Th2 cytokine-independent upregulation of MUC5AC by C3a (Dillard, Wetsel, and Drouin 2007). Generally, C3a has been found to be increased in BALF of asthmatic patients (Krug et al. 2001) together with an increased expression of its receptor, C3aR, on bronchial epithelial and smooth muscle cells during airway inflammation (Drouin, Kildsgaard, et al. 2001; Fregonese et al. 2005).

Several of these systemic effects cannot be analyzed within the bronchial epithelial equivalents. Nevertheless, clear and direct effects of a 6-day “systemic“ treatment of bronchial epithelial equivalents with 50 ng/ml rhC3 were observed here on histological, protein, and gene level (**Fig. 22-24**). The clear upregulation of certain asthma-relevant genes indicates that treatment of bronchial epithelial equivalents with C3 might have more relevant effects that could not be observed on protein level within that short time period of 6 days (**Fig. 23, 24**). A 6-day treatment is comparably short and might increasingly affect gene expression, whereas a prolonged treatment might possibly result in clearer effects on protein levels. This might also explain the unaltered levels of secreted pro-inflammatory cytokines (**Fig. 25**). However, C3 seems not only to exhibit its pro-asthmatic effects via immune cells in an allergy-sensitized situation but also has direct influence on bronchial epithelium.

In concordance with the role of the complement system, here in form of C3, as bridge between innate and adaptive immune system, an impact on polarization of naïve activated human Th cells towards Th1 and Th22 was observed (**Fig. 26**).

In the case of fibronectin (FN) it is known that levels are elevated in the plasma of asthmatics and correlate with the severity of AA and AD (Ohke, Tada, Kataoka, et al. 2001; Bazan-Socha et al. 2018).

The effective concentration determined *in vitro* was 1 µg/ml, whereas *in vivo* the plasma concentration in healthy human subjects was 2.06 µg/ml and in asthmatics 4.11 µg/ml (Bazan-Socha et al. 2018).

The levels of FN in plasma of healthy and AD/EoE-patients did not differ, whereas a trend towards increased FN concentration in skin disease equivalents was observed (**Fig. 19**)**.** One explanation might be the severity. It is not known whether the plasma samples tested here are from patients suffering severely, moderately, or mildly from AD and EoE. A possible history of asthma is also not known. Interestingly, FN levels seem to be dependent on the type of asthma, whereas patients suffering from non-atopic asthma

have significantly higher FN than patients suffering from atopic asthma (Ohke, Tada, Kataoka, et al. 2001). Additionally, FN levels are dependent on the current asthmatic state. During an acute asthmatic attack phase, FN is reduced and increases during non-attack phase (Bazan-Socha et al. 2018; Ohke, Tada, Nabe, et al. 2001). This also goes in line with FN concentration in skin disease equivalent media. As already mentioned before, these skin disease equivalents indicate to represent a mild phenotype of AD, resulting in less prominent levels of pro-inflammatory mediators in culture medium.
Although not being the typical pro-inflammatory mediator and just known to be increased in AD and asthmatics plasma, treatment of bronchial epithelial equivalents with FN clearly resulted in a pro-inflammatory asthma-like inflammation. It is worth mentioning that FN has the least broad impact on the bronchial epithelium out of these five tested though in no way being inferior concerning its impact on naïve human Th cells. Here, FN leads to polarization of Th1 and Th22 (**Fig. 22-26**).

The transmembrane protein Syndecan-4 (SD-4) is an ubiquitous cell surface proteoglycan receptor involved in signal transmission during cell adhesion, migration, proliferation, and endocytosis (Elfenbein and Simons 2013). SD-4 is expressed on numerous cell types such as endothelial cells, epithelial cells, T cells, and eosinophils. In wound healing, inflammatory processes, or by mechanical stress the extracellular part of SD-4 is released by sheddases (e.g., MMP-2, -3, -7) and enters the systemic circulation as shedded form (Samarel 2008; Gondelaud and Ricard-Blum 2019)
In AD, the SD-4 serum concentration is significantly increased (1,406 versus 711 pg/ml) (Nakao et al. 2016). This also goes in line with determined SD-4 mean levels in AD-/EoE-patients with 18 ng/ml compared to 9 ng/ml in healthy subjects. A high inter-donor variability that hampers final evaluation has to be mentioned here. In order to confirm these results, more donors need to be analyzed. However, the increased SD-4 concentration in culture media of skin disease equivalents support the proteomics data.
SD-4 is a powerful regulator of fibroblast growth factor (FGF-) receptors 1 and 2 (Elfenbein et al. 2012; Y. Zhang et al. 2003) by either directly activating FGF-receptor signaling or functioning as co-receptor (Elfenbein and Simons 2013). Interestingly, FGF-receptors 1 and 2 in KCs are known to play a role controlling skin homeostasis, keratinocyte proliferation, and cutaneous inflammation (J. Yang et al. 2010). Thus, it might have been of interest to not only investigate the impact of SD-4 on bronchial epithelial equivalents but also on healthy skin equivalents.
It continues to play a role in the migration of DCs and eosinophils in asthma (Polte et al. 2015; Feistritzer et al. 2004). Polte et al. highlighted the role of SD-4 in DC migration and Th2-driven inflammation in AA in SD-4$^{-/-}$ mice. In that study, OVA-sensitization resulted in decreased number of inflammatory cells in the BALF, no increase of lung resistance, and reduced OVA-specific IgE levels compared to WT mice.

The shedded form of SD-4 meanwhile has clear effects on bronchial epithelial equivalents, comparable to those observed by C3 and FN, resulting in a hyper-proliferative phenotype and GCH (**Fig. 22**). Additionally, SD-4 upregulates gene and protein expression of MUC5AC, tenascin C, SCCA-1, and PAR-2; all being relevant in asthma (**Fig. 22-24**). However, SD-4 did not to induce secretion of pro-inflammatory cytokines such as IL-6, -8, -25, -33, TNFα, and TSLP (**Fig. 29**), whereas it cannot be excluded that it induces production and secretion of numerous other pro-inflammatory asthma-relevant cytokines from NHBE or NHLFb. The supplementation of immune cells such as DCs or Th2 cells to the bronchial epithelial equivalents might have been of interest in combination with SD-4, since SD-4 impacts DC migration and Th2 inflammation as mentioned above. Interestingly, SD-4 induces polarization of naïve activated CD4+ T cells towards Th1 and Th22 (**Fig. 26**).

In the case of the hyaluronic acid (HA) receptor CD44, it is known that it is increased in allergic dermatitis as well as asthma. The cell adhesion molecule CD44 is a class I transmembrane glycoprotein — consisting of a carboxylic acid-terminal-cytoplasmic domain, a transmembrane domain as well as an ectodomain with a HA binding site — found on the surface of several cell types and tissues, hence being ubiquitously expressed (Higashikawa et al. 1996; Ponta, Sherman, and Herrlich 2003). Among other things such as the interaction with several ECM components like fibronectin, laminin, collagen, and HA CD44 regulates migration, activation, and proliferation of lymphocytes; thus, playing a role in cell-cell and cell-matrix-adhesion (Borland, Ross, and Guy 1998; Naor, Sionov, and Ish-Shalom 1997; Funaro et al. 1994; Huet et al. 1989). The extensive splicing and post-translational modifications result in great structural heterogeneity of CD44 proteins making it hard to identify clear molecular mechanisms of CD44 (Ponta, Sherman, and Herrlich 2003). In the epidermis, dermis, and on lymphocytes in AD, the expression is increased compared to healthy mice (Lugović-Mihić et al. 2020). An acutely disturbed skin barrier is characterized by an increased expression of CD44 in the epidermis which in turn affects epidermal proliferation and inflammation (Man et al. 2008). In asthma models of the mouse, it was shown that CD44-deficient mice, after allergen contact, express a lower Th2-dominant inflammation as well as a reduced airway hyperresponsiveness (AHR) (Katoh et al. 2011). The administration of anti-CD44 antibodies for example, additionally resulted in reduced accumulation of lymphocytes and eosinophils in the lung (Katoh et al. 2003). These studies focused on the transmembrane CD44. However, about the role of soluble form of CD44 in the atopic march little is known. The soluble CD44 is primarily used as a possible biomarker for different types of cancer (NHL, NSCLC, colon cancer); thus, being discussed as possible indicator for tumor burden and clinico-pathological outcome (Ristamäki et al. 1997; Masson et al. 1999; Takigawa et al. 1997). A possible explanation for increased levels of soluble CD44 in

serum of cancer patients and patients suffering from other inflammatory diseases might be the process of shedding, a proteolytic cleavage by different types of MMPs from the cell surface (Stamenkovic and Yu 2009; Anderegg et al. 2009) like MMP14, ADAM10, and ADAM17 (Nagano and Saya 2004). Interestingly, oligosaccharides of HA, products of HA degradation, a process that occurs extensively in asthmatic inflammation (Ghosh et al. 2015), are also able to trigger the shedding of CD44; thus, releasing soluble CD44 (Sugahara et al. 2008).

Thrombospondin-1 (TSP-1) is a 450 kDa sized homotrimetric glycoprotein playing a role in angiogenesis inhibition, stimulation of endothelial cell (EC) apoptosis as well as inhibition of EC migration and proliferation, in tumor progression via integrins, integrin-associated protein (IAP), low density lipoprotein receptor-related protein (LRP), proteoglycans, sulfatides, and CD36 (Sottile, Selegue, and Mosher 1991; Sargiannidou, Qiu, and Tuszynski 2004; Dawson et al. 1997; T. Huang et al. 2017; H. Chen, Herndon, and Lawler 2000). It is also being discussed in the fields of AD and asthma. Increased TSP-1 plasma concentrations are known in AD dependent on clinical course (before treatment 942 ng/ml, after treatment 122 ng/ml with a combination of topical erythromicin, topical triamcinolonacetonide, and daily anti-histamine intake) (S. W. Huang and Kao 1993). However, for AA the role of TSP-1^{+} B cells is currently being discussed. The TSP-1 serum concentrations measured in asthma in humans are in the range of an average of 20.7 ng/ml (healthy) to 31.0 ng/ml (asthma) (Shi et al. 2018). However, the first *in vitro* tests showed an immediate effect of TSP-1 on bronchial epithelial equivalents already at a concentration of 5 ng/ml.

A direct influence of KC-derived TSP-1 is currently unknown. The role of TSP-1 in this whole process generally is quite ambiguous and contradictory. Interestingly, it is known for TSP-1 to directly induce TGFβ *in vivo* (Crawford et al. 1998). TGFβ meanwhile also has numerous contradictory effects depending on the addressed cell type and the microenvironment (Moses and Serra 1996). It might play a role in tissue repair or inflammation, and it is able to stimulate or inhibit cell growth (McCartney-Francis and Wahl 1994). One interesting function of TGFβ concerning asthma is its impact on human airway smooth muscle cells. Here, the integrin receptor α5β1, a subunit of the fibronectin receptor, is stimulated by TGFβ resulting in increased proliferation of alpha smooth muscle cells and, subsequently, upregulation of fibronectin deposition in human asthmatic airways, a process known to correlate with AHR and reduced lung function (Moir, Burgess, and Black 2008). Also, TGFβ plays an important role in the process of epithelial-to-mesenchymal (EMT) transition (J. Xu, Lamouille, and Derynck 2009). This transition, or transformation, of epithelial cells into other cell types, especially of mesenchymal origin, is known to play an important role in airway remodeling; however, the plasticity of airway epithelium in asthma is still poorly understood (Hackett 2012).

Role of selected skin secretome compounds in T cell polarization

Interestingly, all compounds led to an increase of Th1-polarized T cells. Additionally, all except for TSP-1 also induced Th22 polarization and C3, FN, and SD-4 moreover induce polarization towards Th17 cells (**Fig. 26**).

The dominating subset found in AD lesions meanwhile depends on the specific phase (acute, sub-acute, chronic). AD and AA are known to be characterized by Th2-driven inflammation (Azzawi et al. 1990; Grewe et al. 1995; Nishi et al. 2008; Robinson et al. 1992), a fact that seemed consistent for several years since Th2 cytokines IL-4 and -13 are key regulators of IgM-to-IgE isotype switch (Bacharier and Geha 2000; Wills-Karp et al. 1998), and IL-5, another Th2 cytokine, plays a role in recruitment, differentiation, and activation of eosinophils and B cells (Rosenberg, Phipps, and Foster 2007). IL-13 on the other hand strongly impacts airway smooth muscle cells and airway epithelium, leading to hypersecretion of mucus, induction of AHR, GCM, and sub-epithelial fibrosis (Wills-Karp 2004; Wills-Karp et al. 1998). In the skin, IL-13 promotes skin desquamation and induces TEWL (Kamsteeg et al. 2011). These results were confirmed in several mice models of AA where induction of airway inflammation failed in IL-4, -5, and -13 deficient mice (Brusselle et al. 1994; Foster et al. 1996; Walter et al. 2001). However, several clinical studies addressing these cytokines failed (Borish et al. 1999; S. Wenzel et al. 2007). Ongoing T cell research opens further insight into the complexity of T cell biology in AA and proposes the involvement of other T cell subtypes such as Th1, Th17, and Th22. In the acute phase of AD, Th2 and Th17 cells were found to be increased, whereas the chronic phase is characterized by Th1 and Th22 subsets (C. Su et al. 2017; A. X. Wang and Xu Landén 2015).

The signature cytokine of Th1 cells, IFNγ, upregulates hBD-2/3 and CCL20 expression, activates phagocytic cells which protect against intracellular pathogens, and enhances ceramide synthesis; thus, regulating differentiation of KCs and skin barrier function (Nakayamada et al. 2011; Pernet et al. 2003; Sawada et al. 2012). However, persistently increased levels of IFNγ may result in negative effects such as KC apoptosis and increased responsiveness of KCs towards pro-inflammatory cytokines like TNFα (Johnson-Huang et al. 2012). Mice studies showed that increased Th1 activity stopped the development of AA (Gavett et al. 1995; Hofstra et al. 1998). Although Th1 cells were supposed to function as antagonists of Th2 inflammation, IFNγ was found to play a role in AHR (Sel et al. 2007). Both, Th1 and Th2 cells, are increased in asthmatic airways (Krug et al. 1996). Hence, the role of IL-17 is still not fully understood.

During Th1 differentiation in chronic phase of AD, the differentiation of its antagonistic subset Th17 is inhibited (X. O. Yang et al. 2008). Although Th2 cytokines such as IL-4 and CCL17 might also lead to decreased numbers of Th17 cells (Hayashida, Uchi, Moroi, et al. 2011; Dhingra and Guttman-Yassky 2014), in acute phase of AD Th17 cells are

increased compared to the chronic phase (Szegedi et al. 2012; Dhingra et al. 2013; Gittler et al. 2012). Additionally, the amount of Th17 cells in peripheral blood is dependent on the severity of AD (Koga et al. 2008). The key cytokine, IL-17, is known to downregulate the expression of barrier protein FLG and tight junction proteins ZO-1 and -2 (Danuta Gutowska-Owsiak et al. 2012). Contrary to that, IL-17 protects KCs from IFNγ-induced apoptosis and regulates anti-microbial protein (AMP) production in KCs (Nakagawa et al. 2011). Although Th17 cells are increased in AD patients compared to healthy subjects, numbers of Th17 cells are significantly lower compared to psoriasis, suggesting a minor role in AD but a more dominant in psoriasis (Nograles et al. 2009). In asthmatic patients, increased levels of IL-17 in serum and *IL-17* mRNA in airway tissue were found (Molet et al. 2001). Later, Th17 cells were also found in asthmatic bronchial biopsies, accounting for approximately 20% of all infiltrating lymphocytes in severe asthma (Pène et al. 2008). Additionally, Th17 cells recruit neutrophils, a process resulting in airway neutrophilia (Bullens et al. 2006; Hoshino et al. 2000; Traves and Donnelly 2008), and induce secretion of pro-inflammatory cytokines from NHBEs and bronchial smooth muscle cells such as IL-6, -8, GM-CSF, and TNFα (Jones and Chan 2002; Kawaguchi et al. 2001; Henness et al. 2004; Laan et al. 1999).

As already mentioned, IL-22-secreting Th22 cells are more likely to be found in the chronic phase of AD and its number correlates with disease activity (Nograles et al. 2009; Trifari et al. 2009). Moreover, Th22 cells express skin-associated receptors such as CCR4, CCR6, and CCR10; and ligands for CCR4 and CCR10 like CCL17 and CCL27 are increased in chronic AD lesions (Nograles et al. 2009). IL-22, which is increased in skin and serum of AD patients in chronic phase (Trifari et al. 2009; Hayashida, Uchi, Takeuchi, et al. 2011), downregulates the expression of important barrier proteins such as FLG, IVL, LOR resulting in barrier deficiency (D. Gutowska-Owsiak et al. 2011; Sabat, Ouyang, and Wolk 2014). A finding that supports the role of Th22 in chronic AD is the lack of IL-22 increase in newly AD-diagnosed children (Czarnowicki et al. 2015). Like IL-17, IL-22 regulates AMP production in KCs; thus, also playing a role in mucosal host defense (Sabat, Ouyang, and Wolk 2014). Contrary, Th2-derived cytokines exert enormous inhibitory effects on the production of AMPs, which might explain the susceptibility of AD patients towards bacterial infections (Yamasaki and Gallo 2008).

In serum and sputum of asthmatics, an increase in IL-22 was observed compared to healthy controls even with a significant increase in severe compared to moderate asthma (Besnard et al. 2011). Nevertheless, it is not at all clear if Th22 cells act pro- or anti-inflammatory in asthma. Whereas some studies highlight a pro-inflammatory role of IL-22 with effects on EMT via TGFβ1 (Johnson et al. 2013), leukocyte infiltration of BALF (Besnard et al. 2011), and IL-5 induced angiogenesis and chemotaxis (Hakemi et al. 2014), other studies show contrary effects. OVA-stimulation in IL-22-deficient mice resulted in increased number of eosinophils, increased IL-13, induced AHR, and GCH/

GCM (Fang et al. 2014; Taube et al. 2011). Additionally, *in vitro* studies showed that IL-22 inhibits chemotaxis of T cells, eosinophils, and monocytes through inhibiting IFNγ-dependent activation of CXCL10, CCL5, and CD54 in NHBE (Alkhouri et al. 2014; Pennino et al. 2013; Kalinowski et al. 2014). Interestingly, IL-22-deficient mice suffered from increased inflammation characterized by Th2-driven immune response and infiltration of eosinophils as well as neutrophils upon allergen challenge that diminished after treatment with IL-22 (Greenfeder et al. 2001; Taube et al. 2011; Besnard et al. 2011).
Taken together, the effects of all five mediators on Th polarization are quite ambiguous, since not only pro- and anti-inflammatory Th subsets are induced but also the role of these subsets partly differs between AD and asthma. Although a clear impact of each compound on Th cell polarization can be observed here, these five compounds out of possibly >40 proteins of interest were chosen exemplary as representatives for ECM-associated compounds; thus, a final conclusion cannot be drawn.

Altered gene and protein expression in diseased ECM

Notably, for secretome analysis of skin disease equivalents just two bands of interest from an SDS-PAGE gel were analyzed; thus, these >40 proteins of interest cover secretome data of only a small fraction. Further transcriptomics and proteomics analysis of diseased fibroblast and ECM models revealed a highly complex picture of pathway regulations (**Fig. 27-30**).
Among numerous proteins being differently regulated in healthy and diseased ECM models, tenascin and fibronectin were significantly upregulated in healthy ECM. Both, together with periostin, are important for ECM organization and deposition of fibronectin as well as tenascin into ECM (Kii et al. 2010). A process that seems disturbed in diseased ECM; thus, possibly leading to less deposition into ECM but more release into blood circulation. Moreover, tenascin positively regulates production of COL-1 and reduces MMP-1 expression (Choi et al. 2020). Further, HTRA1 was found upregulated in AD ECM. This serine protease is involved in the fragmentation of fibronectin. These fibronectin fragments regulate cytokine activity, suppress matrix synthesis, and increase MMP activity; thus, resulting in ECM degradation and remodeling (Homandberg 2001; Grau et al. 2006). The combination of both, increased tenascin in healthy and increased HTRA1 in AD ECM - combined with their downstream effects - might partly explain the disorganized AD ECM (**Fig. 27A, B**).
Notably, two more factors, signal transducer and activator of transcription 1-alpha/beta (STAT1) and extracellular superoxide dismutase (SODE) were significantly altered. STAT1, upregulated in AD ECM, is a down-stream mediator of AD- and atopic march relevant TSLP, it is involved in B cell maturation as well as eosinophil activation, and it up-regulates epidermal chemokines as well as pro-angiogenic factors (Bao, Zhang, and Chan 2013; Welsch et al. 2017). Moreover, it plays a role in Th1 immune response, hence

playing a role in chronic AD. SODE, upregulated in healthy ECM, is an anti-oxidant enzyme protecting tissues against reactive oxygen species (ROS). It is well-known that increased ROS; thus, increased oxidative stress, and its downstream activation of pro-inflammatory pathways plays an important role in inflammation and AD (Agrahari et al. 2020; Sah et al. 2018; Prassas et al. 2015).
Furthermore, the alteration of genes playing a role in VEGF signaling needs to be mentioned here. VEGF is pro-angiogenic and it is known to be increased in the stratum corneum as well as plasma of AD patients. Moreover, plasma levels correlate with AD severity (Samochocki et al. 2016; Koczy-Baron, Jochem, and Kasperska-Zajac 2012).
The subsequent transcriptome analysis of AD fibroblasts underlined the role of ECM in AD by showing significant alteration of fibronectin and *STAT1* on gene level as well. In addition to that, further mentionable genes were differently expressed comparing healthy and AD ECM. On the one hand, *HSP90AA1*, a gene coding for a highly conserved stress protein, the heat shock protein, and playing major roles in ECM organization as well as cytokine signaling with a yet unknown role in skin homeostasis, was upregulated in AD ECM. Its role in AD, especially an interaction with immune cells and its impact on KC differentiation, are known so far (Kapitein et al. 2013; Scieglinska et al. 2019). On the other hand, *SHC1* and *CAV1* were upregulated in AD ECM as well. *SHC1* regulates mitochondrial ROS production, whereas caveolin-1 (*CAV1*) is known to be involved in inflammatory skin conditions by interacting with MMPs, HSPs, and TGF (K. G. Su et al. 2012; Kruglikov and Scherer 2020).

Consequently, the importance of ECM in atopic diseases can clearly be shown. Concluding, there is not only a complex interplay between allergens, immune cells, genetics, and different tissues but also a high number of potential pro-inflammatory and protective ECM-associated proteins released into circulation that increases the complexity of this interplay and has to be considered when unraveling mechanisms of the atopic march.

4.3 Translation from *in vitro* to *in vivo*

Analogous to the *in vitro* polarization studies of human CD4+ and CD8+ T cells, the impact of SD-4, CD44, and TSP-1 on CD4+ T cells was analyzed *in vivo*. Therefore, weight and size of spleen were determined and CD4+ T cells were isolated from spleen for Th subset analysis on gene level. Size and weight of spleen were increased in all treated groups compared to untreated mice, which might lead to the suggestion of increased immune cell activity after a 7-day treatment (**Fig. 31**).
To further analyze the impact of SD-4, CD44, and TSP-1 on CD4+ T cells *in vivo*, genetic markers for Th1 (*Tbx21*), Th2 (*Gata3*), Th17 (R*orc*), and Th22 (*Il-22*) were determined via qRT-PCR. Whereas CD44 had no effect. SD-4 induced Th17-, and TSP-1 induced Th1,

Th2, and Th17 polarization in female mice. All mediators led to Th1 and Th22 polarization in male mice with no effect on Th2 and Th17 (**Fig. 32**). Although these effects in this small sample group are marginal and a larger confirmatory study is needed, the importance of sex-specific studies can be highlighted. Due to a heterogeneous genetic and hormonal makeup, both, the innate and the adaptive immune response, vary (Klein and Flanagan 2016). The asthmatic phenotype can also differ between men and women (Zein and Erzurum 2015). Incidence, prevalence, and severity differ depending on the sex. In order to avoid this gender-bias and to generate meaningful data generally, sex-specific studies need to be considered *in vivo* and *in vitro* in order to cover donor- and sex-variability.
In this first low-dose treatment, no differences on histological level (mucus, hyperplasia, immune infiltration) in bronchi and alveoli were observed (**Fig. 33**). This might be due to inter-species related differences or due to short, single-compound treatment without allergen challenge. In the first case, surely these compounds might not affect mice lung *in vivo* as they affect reconstructed human bronchial epithelial equivalents *in vitro* due to the enormous species- (Travaglini et al. 2020; Gillich et al. 2020) as well as experimentally related differences in which a whole biological murine system is compared to a reconstructed human-based two-organ co-culture. The latter reason might concern the experimental design itself. Conventionally, an AD or asthma mouse model is characterized by artificial barrier disruption and a long-term allergen exposure over several weeks before symptoms occur (Leyva-Castillo et al. 2013; Akei et al. 2006).
Another reason might be that the mediators are not stable for 7 days in a pump being permanently exposed to around >32°C on the skin surface of mice. In the *in vitro* experiments, compounds were renewed every other day with a medium change. Here on the other hand, pumps were filled with dissolved compounds and implanted into mice for 7 days. Whereas there is comparably low metabolic activity *in vitro*, compounds may have been rapidly metabolized *in vivo* before affecting any organs.

In summary, this data demonstrates that emerging human-based *in vitro* methods are suitable models for basic research. These comparably simple and economic models can be used for complex pathophysiological research, unraveling pathways and key mediators in a simple and effective way. Strikingly, this study revealed the importance of ECM compounds in the atopic march based on exemplary experiments with only five ECM compounds by simply considering two relevant human-based reconstructed organs, skin and lung. A detailed analysis of patient-derived fibroblasts and ECM models on protein and gene level revealed significant changes in ECM composition; thus, underlining the possible role of ECM in the atopic march. Ultimately, a direct side-by-side comparison of simple, human-based reconstructed *in vitro* model systems and complex *in vivo* animal models revealed that reconstructed human-based *in vitro* models are useful systems for

investigating pathophysiological processes, determining key mediators as well as pathways, and lying foundation for further target and drug discovery studies.

4.4 Outlook

Although this data demonstrates promising results for unraveling the pathological mechanisms of atopic march with the use of human-based approaches, a deeper analysis of all involved factors and the implementation of further possible actors are crucial.

On the one hand, it is necessary to further characterize the bronchial epithelial equivalent. Since Travaglini et. al (Travaglini et al. 2020) it is known that the lung consists of a minimum of 58 cell types, of which 25 are immune and 9 are endothelial cells. Hence, 15 types of epithelial and 9 types of stromal cells exist. The two latter were used to generate a bronchial epithelial equivalent. The development of ciliated, basal, and goblet cells was observed based on (immuno-)histological analysis. A deeper look inside for example through further immunostaining, fluorescence *in situ* hybridization, or a single-cell sequencing approach might reveal further cell types evolving in this model; thus, proving the plasticity of lung cells and their role in the atopic march.

The implementation of endothelial and immune cells (Th cells, DCs, macrophages) is crucial for cell-cell crosstalk and a more complete understanding of physiological and pathophysiological conditions as well. Beside the implementation of further cell types, additional reconstructed organs need to be developed and a multi-organ co-culture of e.g., skin, lung, liver, gut, nose, and pharynx needs to be established in order to analyze the pathophysiological atopic march-relevant tissue crosstalk. Moreover, an improvement of static towards dynamic cultivation is undoubtedly necessary to get closer to physiological conditions.

In addition, it might be worth comparing AA patient-derived bronchial epithelial equivalents with those from the co-culture with a single-cell sequencing approach in order to compare the pathophysiological alterations and determine affected cell types. In line with that, the use of patient-derived NHDF and NHEK to generate atopic skin equivalents for a co-culture with bronchial epithelial equivalents will be of interest.

Another drawback of this system that needs to be overcome is the relatively short culturing period. A prolonged culture time of reconstructed models with the help of stem cells or even the use of tissue-specific organoids, whereas the latter ideally can be cultured for several months, are an option to study longterm tissue crosstalk; thus, being ideal for a long pathophysiological process like the atopic march. Organoids, due to their size and culture conditions, also allow live-cell imaging. This method might further reveal pathophysiological sub-cellular changes during the atopic march. Nevertheless, organoids are limited due to missing air-liquid-interface cultivation.

The improvement of the skin disease equivalent concerning severity of AD might also be of interest, since AD severity correlates with the development of the atopic march.

Additionally, a deeper characterization of patient-derived samples (e.g., blood, biopsies) with known mild, moderate, and severe AD as well as patients suffering from the atopic march is necessary.
Moreover, a treatment of bronchial epithelial models with epithelial cytokines TSLP, IL-25, and IL-33 might be interesting in order to compare those with the effects of ECM compounds and a Th2-type of inflammation (IL-4/-13), since the atopic march seems to be a highly complex interplay of immune cells, epithelial cells, and ECM compounds.
With the implementation of immune cells, another decisive factor, the allergen, needs to be considered. In case of a successful establishment of an immunocompetent co-culture, the topical application of allergens might complement this pathophysiological process even further.
Furthermore, it will be necessary to investigate the molecular mechanisms of each compound in order to understand their interaction with bronchial epithelium and immune cells. Finally, it is of interest to address skin ECM-associated factors as possible pharmacological targets.
Taken together, a successful establishment of a dynamic, multi-organ, immuno-competent, long-term cultivation of an allergen-sensitized human-based system and a comparison with *in vivo* data needs to be performed in order to prove the validity and reproducibility of these models as alternatives to animal testing within the framework of the 3R principle. This improvement might lead to further understanding of complex pathophysiological processes and help finding options for prevention of the atopic march.

4.5 Limitations

Although the in this thesis used methods are well-established and the generated results are impressive, several limitations need to be addressed.
Most *in vitro* studies were designed as explorative studies; thus, have a small sample size. Statistical analysis has only been performed for sample size ≥5, hence these results need to be interpreted cautiously. Inter-donor variability is relatively high resulting in high standard deviation. For a robust statistical analysis, an increased number of donors is crucial to verify these results in a follow-up confirmatory study.
Moreover, the *in vitro* equivalents lack several AD- and AA-relevant cell types such as endothelial as well as immune cells, with the unphysiological microenvironment (shear stress, allergen sensitization, etc.) as already discussed above. Moreover, unphysiological bovine collagen was used as matrix for the generation of skin and bronchial equivalents. In the future, this needs be replaced by either a human-based matrix or by stimulation of fibroblasts to produce their own ECM.
Another point worth mentioning is the induction of an AD-like phenotype in healthy cells for the generation of skin equivalents used for co-culture experiments. This method and

the resulting skin disease equivalents only represent a small part of atopic skin. The use of patient-derived NHDF and NHEK will be crucial for further (confirmatory) studies.

Next, although the mouse study was mostly planned and performed according to the ARRIVE guidelines, randomization and blinding were semi-optimal. Ideally, treatment should have been assigned per mouse, not per cage and labeling of pumps should have been with random codes rather than rising numbers. Both factors would have also increased blinding. The minimal number of mice per group were chosen for animal welfare reasons and for a robust explorative study, whereas a higher number of mice per group might have shown statistically clearer results.

Another important limitation is the short treatment time *in vitro* and *in vivo*. The atopic march is a process developing over years, not one week. Due to limitations in available models, I nevertheless choose a 6- and 7-day treatment, respectively.

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LIST OF FIGURES

LIST OF PUBLICATIONS AND CONTRIBUTIONS

ORIGINAL ARTICLES

P. Graff, J. Wilzpolski, O. Kershaw, T. Blimkie, P. Panwar, D. Brömme, T.Hackett, L. Loyal, A.Thiel, B. Hancock, A. Gruber, W. Bäumer, S. Hedtrich. *Extracellular Matrix Remodeling in Atopic Dermatitis Harnesses the Onset of an Asthmatic Lung Phenotype and is a Potential Driver of the Atopic March.* Manuscript currently in preparation

P. Graff§, S. Hönzke§, A. A. Joshi, G. Yealland, E. Fleige, M. Unbehauen, M. Schäfer-Korting, A. Hocke, R. Haag[3], S. Hedtrich. *Preclinical Testing of Dendritic Core-Multishell Nanoparticles in Inflammatory Skin Equivalents.* Submitted to Mol Pharm 2021. § shared first-authors

A. Gruber, A. A. Joshi, **P. Graff**, J. L. Cuéllar-Camacho, S. Hedtrich, D. Klinger. *The influence of nanogel amphiphilicity on dermal delivery: Balancing surface hydrophobicity and network rigidity***.** Submitted to Biomacromolecules 2021

R. Charbaji, M. Kar, LE. Theune, J. Bergueiro, A. Eichhorst, L. Navarro, **P. Graff**, F. Stumpff, M. Calderón, S. Hedtrich. *Design and Testing of Efficient Mucus-Penetrating Nanogels - Pitfalls of Preclinical Testing and Lessons Learned.* Small 2021

I. Dolz-Pérez, MA. Sallam, E. Masiá, D. Morelló-Bolumar, MD. Pérez Del Caz, **P. Graff**, D. Abdelmonsif, S. Hedtrich, VJ. Nebot, MJ. Vicent. *Polypeptide-Corticosteroid Conjugates as a Topical Treatment Approach to Psoriasis.* J Control Release 2020

A. Löwa, **P. Graff**, S. Kaessmeyer, S. Hedtrich. *Fibroblasts from Atopic Dermatitis Patients Trigger Inflammatory Processes and Hyperproliferation in Human Skin Equivalents*. J Eur Acad Dermatol Venerol 2020

F. Zabihi, **P. Graff**, F. Schumacher, B. Kleuser, S.Hedtrich, R. Haag. *Synthesis of Poly(lactide-glycerol) as a Biodegradable and Biocompatible Polymer with High Loading Capacity for Dermal Tacrolimus Delivery.* Nanoscale 2018

R. Plank, G. Yealland, E. Miceli, D. L. Cunha, **P. Graff**, ..., H. C. Hennies, S. Hedtrich. *Transglutaminase 1 Replacement Therapy Successfully Mitigates the Autosomal Recessive Congenital Ichtyosis Phenotype in Full-Thickness Skin Disease Equivalents*. J Invest Dermatol 2018

CONFERENCE PROCEEDINGS

Perspectives in Percutaneous Penetration Conference 04/2018 (Poster) **P. Graff**, F. Zahibi, L. Verheyen, S. Hönzke, R. Haag, S. Hedtrich. Development of Inflammatory and Immunocompetent Skin Disease Models as Preclincal Test Systems

SCHOLARSHIPS

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CURRICULUM VITAE

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

APPENDIX

TABLES

Table 8. List of hits from proteomics analysis of skin disease equivalent secretome: Results from one donor for 150 kDa and 250kDa bands are shown. Data generated by UBC proteomics core facility.

150 kDa	|Log Prob|	Best |Log Prob|	Best score	Total Intensity
HUMAN Collagen alpha-1(VI)	61,42	6,00	601,90	11421776,0
HUMAN Keratin, type II cytoskeletal 1	47,35	8,76	755,00	7090654,0
HUMAN Keratin, type I cytoskeletal 9	30,73	10,63	884,80	4002394,0
HUMAN Collagen alpha-2(VI)	24,60	3,60	394,20	2683508,0
HUMAN Complement C3	20,47	4,33	530,80	2317328,0
HUMAN Laminin subunit beta-3	20,16	3,76	477,20	1855918,0
HUMAN Keratin, type II cytoskeletal 2 epidermal	17,87	3,67	507,70	2158950,0
HUMAN Collagen alpha-2(I)	16,43	6,20	604,30	1368398,0
HUMAN Collagen alpha-1(I)	16,30	5,75	592,80	1144412,0
HUMAN Laminin subunit gamma-2	11,57	6,75	635,40	696136,0
HUMAN Keratin, type I cytoskeletal 10	8,91	5,25	506,20	816910,0
HUMAN Thrombospondin-1	8,63	3,23	400,50	743528,0
HUMAN Keratin, type II cytoskeletal 6A	6,45	2,88	349,20	657466,0
HUMAN Keratin, type I cytoskeletal 16	6,05	3,17	437,80	396112,0
HUMAN Keratin, type II cytoskeletal 5	5,89	2,96	312,60	311670,0
HUMAN Hornerin OS=Homo sapiens	5,86	2,48	330,00	791874,0
HUMAN Decorin OS=Homo sapiens	5,59	3,80	411,30	473636,0
HUMAN Keratin, type I cytoskeletal 14	3,18	3,18	459,70	240328,0
HUMAN CD44 antigen	2,69	2,69	263,90	177028,0
HUMAN Uncharacterized protein C1orf122	2,61	2,61	242,70	303350,0

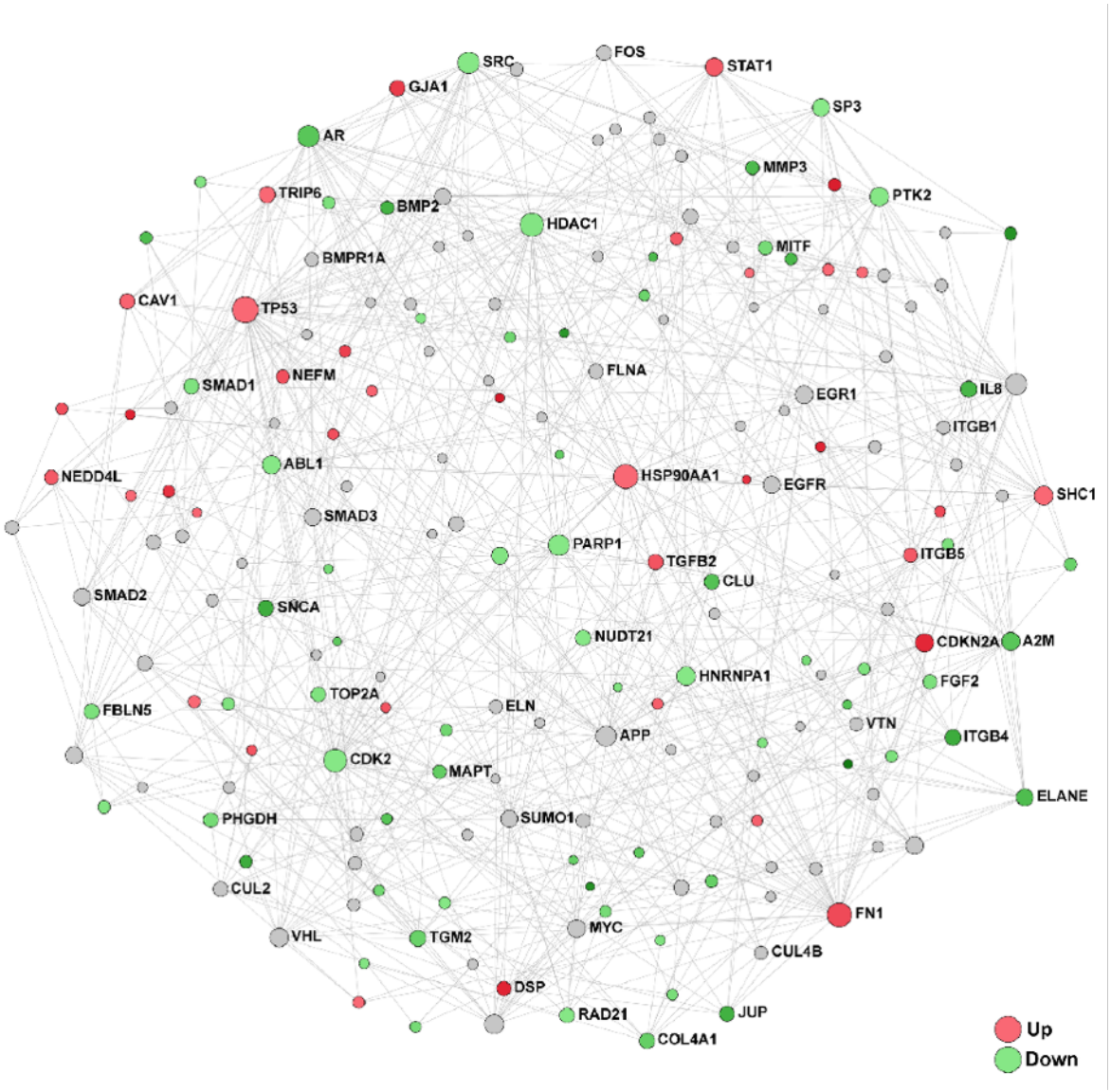
250 kDa	|Log Prob|	Best |Log Prob|	Best score	Total Intensity
HUMAN Collagen alpha-3(VI)	82,94	3,89	574,10	12374792,0
HUMAN Fibronectin	53,32	6,74	657,00	9615468,0
HUMAN Tenascin	42,92	3,88	518,40	7625964,0
HUMAN Keratin, type II cytoskeletal 1	34,00	7,24	729,40	4681734,0
HUMAN Keratin, type I cytoskeletal 9	20,67	7,29	775,90	2347358,0
HUMAN Keratin, type I cytoskeletal 10	20,63	3,76	534,90	3263792,0
HUMAN Keratin, type II cytoskeletal 2 epidermal	19,49	3,05	355,30	2230018,0
HUMAN Syndecan-4	4,60	4,60	503,20	302304,0
HUMAN Collagen alpha-3(VI)	3,69	3,70	517,10	267622,0
HUMAN Collagen alpha-1(XII)	3,64	2,66	353,20	501730,0
HUMAN Keratin, type II cytoskeletal 5	2,99	2,99	292,50	217878,0
HUMAN Adenylyl cyclase-associated protein 1	2,98	2,99	445,00	183684,0
HUMAN Laminin subunit gamma-2	2,79	1,64	295,20	416460,0
HUMAN Fibrillin-1	2,66	1,96	210,50	444986,0
HUMAN Collagen alpha-1(VII)	2,66	2,66	210,00	136208,0
HUMAN Collagen alpha-2(I)	2,53	2,53	393,90	176700,0
HUMAN Hornerin	2,39	2,34	205,80	389766,0
HUMAN Glypican-1	2,29	2,29	292,10	246724,0

Table 9. Proteomics analysis of healthy and AD ECM models: Functions of proteins found significantly increased in healthy or diseased ECM models (**see Fig. 28**)

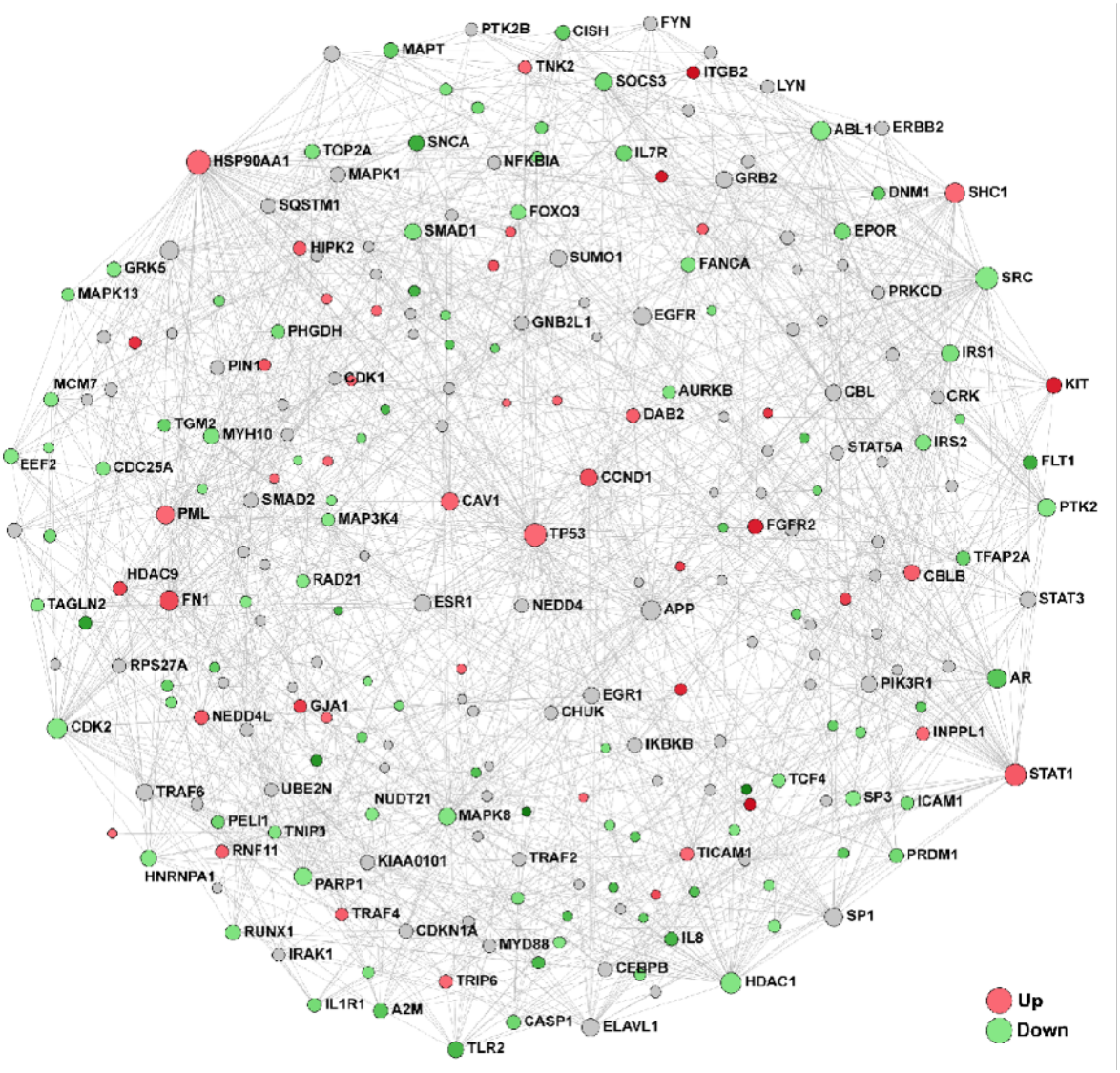
Increased in healthy ECM		
		Function
TENA	**Tenascin**	ECM Protein, anchor protein for FINC
SODE	**Extracellular superoxide dismutase**	Converts superoxide radicals into hydrogen peroxide and oxygen
ROA2	**Heterogeneous nuclear ribonucleoproteins A2/B1**	Transcription and processing of pre-mRNA
NEST	**Nestin**	Promotes disassembly of phosphorylated vimentin intermediate filaments during mitosis
LUM	**Lumican**	Organizes collagen fibril and ECM
PGBM	**Basement membrane-specific heparan sulfate proteoglycan core protein**	Attachment substrate for cells playing an essential role in vascularization
NID1	**Nidogen 1**	Organizes ECM and basement membrane
G8JLB6	**Heterogeneous nuclear ribonucleoprotein H**	RNA binding
FINC	**Fibronectin**	Involved in cell adhesion, cell motility, opsonization, wound healing, and maintenance of cell shape

Increased in diseased ECM		
		Function
SCOT1	**Succinyl-CoA:3-ketoacid coenzyme A transferase 1, mitochondrial**	Key enzyme for ketone body catabolism
DDR-2	**Discoidin domain-containing receptor 2**	Regulates remodeling of ECM
ODO1	**2-oxoglutarate dehydrogenase, mitochondrial**	Mediates the decarboxylation of alpha-ketoglutarate
THIK	**3-ketoacyl-CoA thiolase, peroxisomal**	Involved in thiolytic cleavage of straight chain 3-oxoacyl-CoAs
MYOF	**Myoferlin**	Calcium/phospholipid-binding protein
VPP1	**V-type proton ATPase 116 kDa subunit a1**	Involved in ion membrane transport
MFGM	**Lactadherin**	Promotes VEGF-dependent neo-vascularization
PYGB	**Glycogen phosphorylase, brain form**	Regulates glycogen mobilization
STAT1	**Signal transducer and activator of transcription 1-alpha/beta**	Mediates cellular responses to interferons and growth factors
TNPO1	**Transportin 1**	Nuclear transport receptor
G6PD	**Glucose-6-phosphate 1-dehydrogenase**	Provides NADPH and pentose phosphates for fatty acid and nucleic acid synthesis
HTRA1	**Serine protease HTRA**	Protease targeting ECM proteins

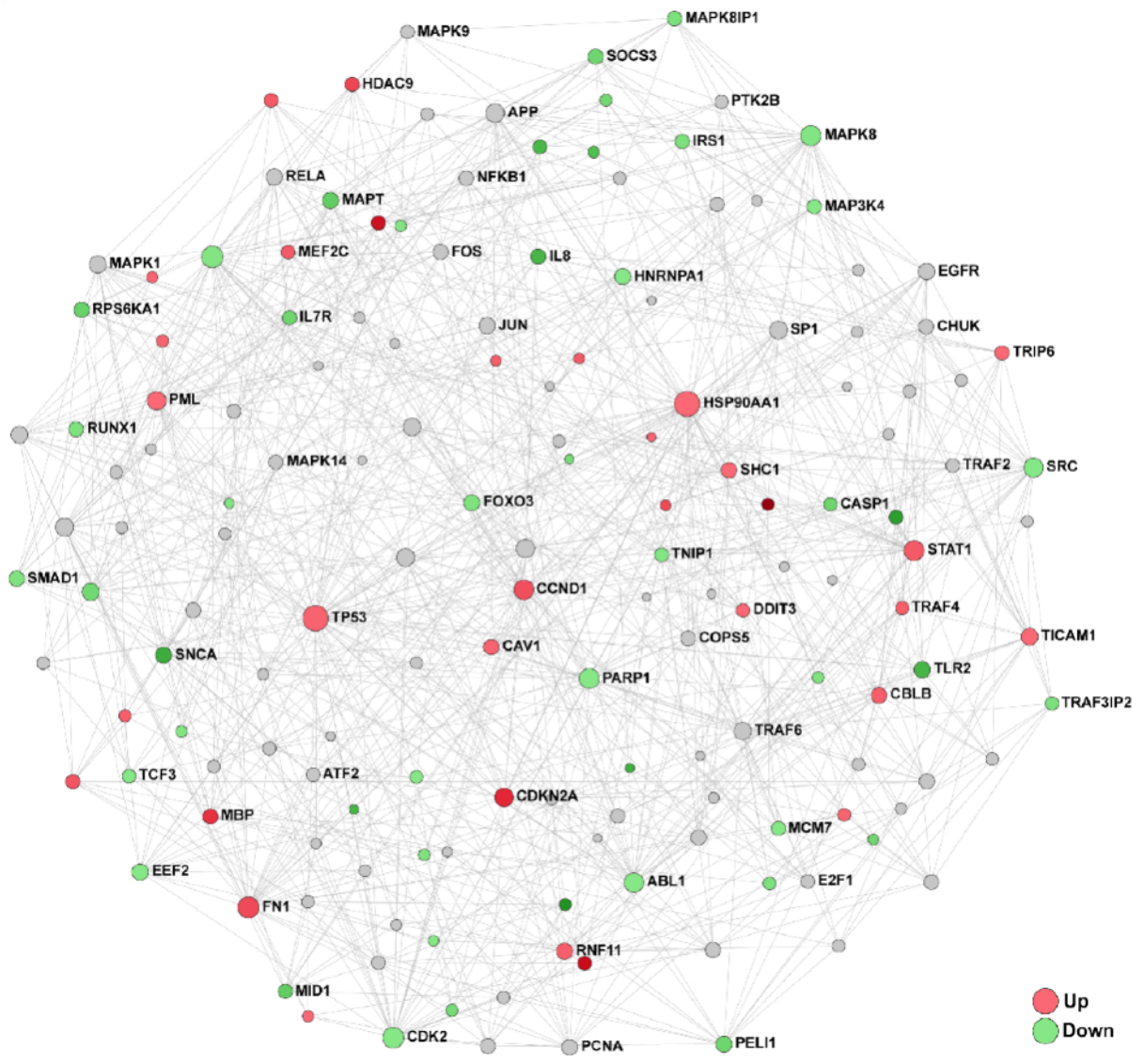
FIGURE



ECM organization



Cytokine signaling



TLR 4 signaling

Figure 30. Network analysis of RNA-sequencing data of healthy and AD fibroblasts Minimum order subnetworks, created in NetworkAnalyst. These were generated by extracting nodes belonging to the indicated pathway from the initial PPI network built with the 1,593 DE genes. Key nodes with biological implications for AD present in one or both networks include HSP90AA1, STAT1, ITGB5, and SHC1. Subnetworks of ECM organization, cytokine and TLR4 signaling.