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Langerhans and inflammatory dendritic epidermal cells in atopic dermatitis tolerate Toll-like receptor 2-specific activation

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Our love is a ghost that the others can’t see

- Agnes Obel

- for Fin

Contents

Abstract

Atopic dermatitis (AD) is a chronic multifactorial inflammatory skin disease. The skin of AD patients shows a significant dysbalance of the microbiome with high colonization of *S. aureus*, which positively correlates with the disease severity. Langerhans cells (LC) are immune system sentinels and reside in the epidermis, where they sense invading pathogens by pattern recognition receptors, such as Toll-like receptors (TLR). LC bridge the innate and the adaptive immune systems, orchestrate primary immune responses to pathogens, allergens, and maintain tolerance. Inflammatory dendritic epidermal cells (IDEC) migrate in the epidermis of inflamed AD skin and contribute to the pro-inflammatory microenvironment of the AD skin.

The aim of this thesis was to investigate the TLR2-specific functional differences between LC from healthy and AD individuals in an *ex vivo* human skin model that is very close to the *in situ* situation of the skin. Therefore, an *ex vivo* skin model was established from which the epidermal cells were isolated and analysed. The synthetic protein Pam3CSK4 (P3C) mimics *S. aureus* structures and P3C was used to activate the TLR2 on immature DC. Furthermore, the migration activity and the ability to activate naïve T cells by TLR2-stimulated DC was analysed.

The results of this thesis show that the maturation status of freshly isolated LC from AD patients was similar to LC from healthy controls, as witnessed by equal expression levels of CD83, CD86, MHC class I and II. The main finding of this thesis is that LC and IDEC from AD skin showed tolerance towards TLR2 ligation. The LC did not further increase their maturation status or alter their cytokine profile in response to TLR2 ligand P3C, as witnessed by no alteration of CD83, CD40, CD80, CD86, MHC class II, TLR2, FcεRIα, IL-1β, hBD2, TSLP, and IL-11 when compared to healthy controls. Interestingly, steroid treatment seemed to slightly correct the tolerant behaviour and change the phenotype towards a healthy-like status, as witnessed by P3C induced up-regulation of CD83, CD80,

a down-regulation of TLR2, and a slightly higher MHC class II expression. The co-culture of epidermal cells with naïve T cells showed that P3C-stimulated cells from healthy skin increased the T cell proliferation and the IL-17 level in the supernatant, whereas this was not the case for AD skin, which supported the tolerant behaviour of epidermal cells from AD skin. The analysis of receptors that allow the LC to remain in the epidermis showed that Integrin α3 (CD49c) and CCR6 were down-regulated in LC from AD patients, which indicated, that the LC migrated spontaneously from the tissue to the lymph nodes. Moreover, CCR7 that regulates the specific migration was slightly up-regulated in LC from AD donors. IL-18 and TNF-α induce migration out of the epidermis and both were elevated in the supernatant of cultured *ex vivo* skin from AD, which underlined the strong spontaneous migration. However, P3C ligation did not increase the specific migration rate of LC from AD patients, while it was up-regulated in healthy controls. Taken together, the TLR2-induced maturation of LC, the capability of LC to induce T cell activation, and the migration rate was different in AD skin when compared to healthy controls. The results of this thesis show that LC from AD skin were tolerant towards TLR2-mediated activation, which may be due to prolonged exposure of *S. aureus*, which can influence the TLR2 pathway and lead to a desensitisation of LC from AD patients. The TLR2 tolerance may contribute to the immune deviation in AD and the lack of *S. aureus* clearance, where *S. aureus* changes the local cytokine environment and directly or indirectly inhibits the TLR2 signalling.

1 Introduction

1.1 The human skin

The skin is part of the interface between the human being and the surrounding environment and therefore it is continuously exposed to a variety of stimuli or pathogens, such as viruses, fungi, bacteria, chemicals or ultraviolet light. Thus, it has an important barrier function to protect the body. It has additional functions such as sensory recognition, water protection, thermoregulation, and therefore it can maintain the homoeostasis and the local microenvironment. A simplified area calculation of the skin results in a surface area of 2 m^2, but when the area around each hair follicle is taken into account it results in a skin surface area of 25-30 m^2 [1, 2]. This makes the skin the largest epithelial surface for interaction with microbes next to the gut with an estimated area of 30 m^2 [3].

The human skin can be divided into three layers: the epidermis, the dermis and the subcutaneous tissue or the hypodermis, depicted in figure 1.1. The epidermis has an average thickness of about 0.05 to 0.1 mm and it consists mainly of keratinocytes (KC). They belong to the innate immune system since they produce pro-inflammatory mediators which attract monocytes, T cells, and dendritic cells (DC) after detection of pathogen-associated molecular patterns [4]. The *stratum basale* consists of a single layer of KC which continuously proliferate and differentiate while moving upwards. Melanocytes reside in the basal layer and produce melanin which is transported to nearby KC to provide pigmentation and protect the skin against UV radiation. Furthermore, nerve-ending Merkel cells can be found in this layer which are essential for light-touch sensation and discrimination of shapes and texture [5, 6]. In the spinous layer (*stratum spinosum*), KC typically express keratin 1 and 10 and are connected by intercellular junctions called desmosomes. Further differentiation of KC leads to the formation the *stratum granulosum* where the cells have increased protein synthesis and lipogenesis and appear to be more

flattened and elongated while expressing filaggrin, loricrin and involucrin. The additional layer *stratum lucidium* can only be found in the palms and soles. In the *stratum corneum*, the KC undergo terminal differentiation to corneocytes or dead cells. While these cells lose their nuclei and organelles, they secrete lamellar bodies and form a cornified cell envelope, and eventually shed from the skin surface by the process of desquamation [6–10].

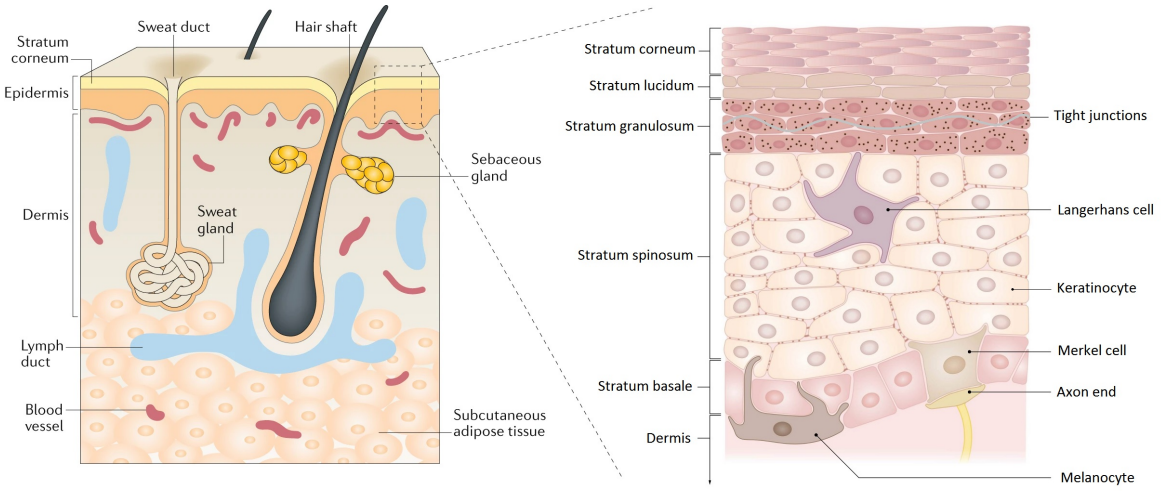

Figure 1.1. The anatomy of human skin.
The figure provides a basic overview of the three main skin layers: the epidermis, the dermis and the subcutaneous tissue. In the zoomed-in section on the right, the histology of the epidermis shows tight junctions in the *stratum granulosum*. Langerhans cells, merkel cells, and melanocytes reside between keratinocyte. The figure was modified from "The immunological anatomy of the skin" [11].

The dermis is separated from the epidermis by the basal membrane and it is characterized by an abundant extracellular matrix, comprised of collagen and elastin fibres that fill the extracellular spaces, and therefore provide essential structure, strength and elasticity to the skin [6, 11, 12]. Furthermore, the dermis harbours additional cell types and structures such as sebaceous glands, sweat glands, lymphatic vessels as well as tissue-resident DC, fibroblasts, mast cells, and macrophages [13, 14]. Additionally, the dermis contains neurons and nerve endings that mediate touch and heat recognition, and blood vessels that provide nutrients and recruit immune cells to the skin [6, 15].

The subcutaneous tissue under the dermis is called hypodermis. It is composed of connective tissue and lobules of fat, accounting for approximately 80 % of fat in human body and it serves as an energy reservoir and as thermo-regulator [16]. The major cells of the hypodermis are adipocytes, pre-adipocytes, fibroblasts, T cells, macrophages,

granulocytes, mast cells and DC [17]. Consequently, the skin has a central role in host defence, both through the barrier function and the located immune cells. A link to a *Nature* video providing a summary of the immunology and structure of the skin can be found in the appendix section (figure 7.1). Defects in the skin structures or skin immune system can have severe consequences, such as chronic inflammatory disorders for instance atopic dermatitis (AD) or psoriasis.

1.2 Dendritic cells

DC are the bridge between the innate and adaptive immune system since they are professional antigen-presenting cells that regulate primary immune responses to pathogens and maintain tolerance to self-antigens [18–20]. They are a heterogeneous population that resides in tissue and secondary lymphoid organs. DC recognize pathogen-associated molecular patterns with a broad range of pattern-recognition receptors, such as TLR, C-type lectin receptors, Nod-like receptors and RIG-I-like receptors [21–24]. Immature DC have a high phagocytosis activity and their pathogen uptake induces maturation processes and reduces the activity of phagocytosis [25]. This maturation process changes the surface receptor expression profile which enables the cells to migrate to the skin-draining lymph nodes. There, the DC can present parts of the pathogen on their major histocompatibility complex (MHC) II to activate naïve T cells. The activation of T cells (figure 1.2) is dependent on co-receptors expressed by mature DC, such as CD40, CD80, CD86, and CD83, which are barely expressed on immature DC.

The T cell polarization is dependent on the stimulus, the cellular metabolism of the DC, and the surrounding cytokine milieu (figure 1.3) [27]. The Th subsets are identified by their signature cytokines and fulfil several different functions *e.g.* as the defence against external pathogens or viral infections.

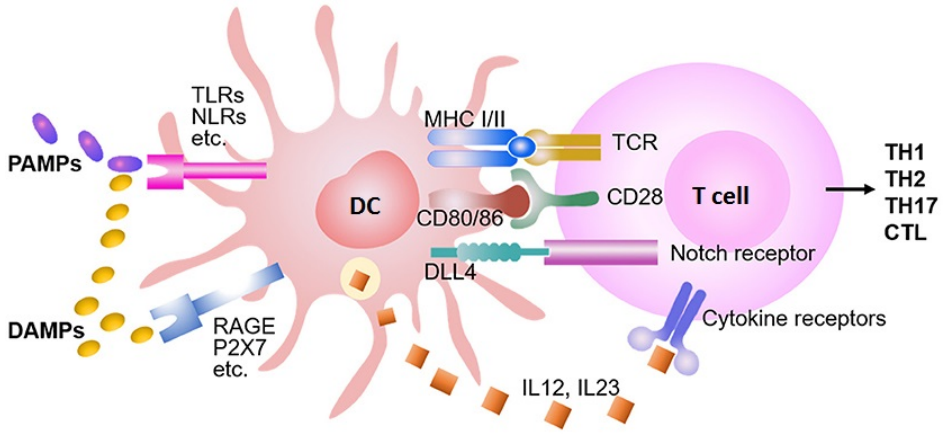

Figure 1.2. DC activate T cells.
Pathogen- and damage-associated molecular pattern (PAMP and DAMP) activate DC through pattern-recognition receptors. Afterwards, they activate T cells with co-stimulatory molecules such as CD80/CD86 and present antigens through MHC class I or II. The co-stimulatory molecules CD28 and the cytokines IL-12 and IL-23 synergize with the T-cell receptor (TCR) signalling and promote proliferation and expansion of antigen-activated T cells. The figure was adapted from "Dendritic Cell Regulation of Graft-Vs.-Host Disease: Immunostimulation and Tolerance" [26].

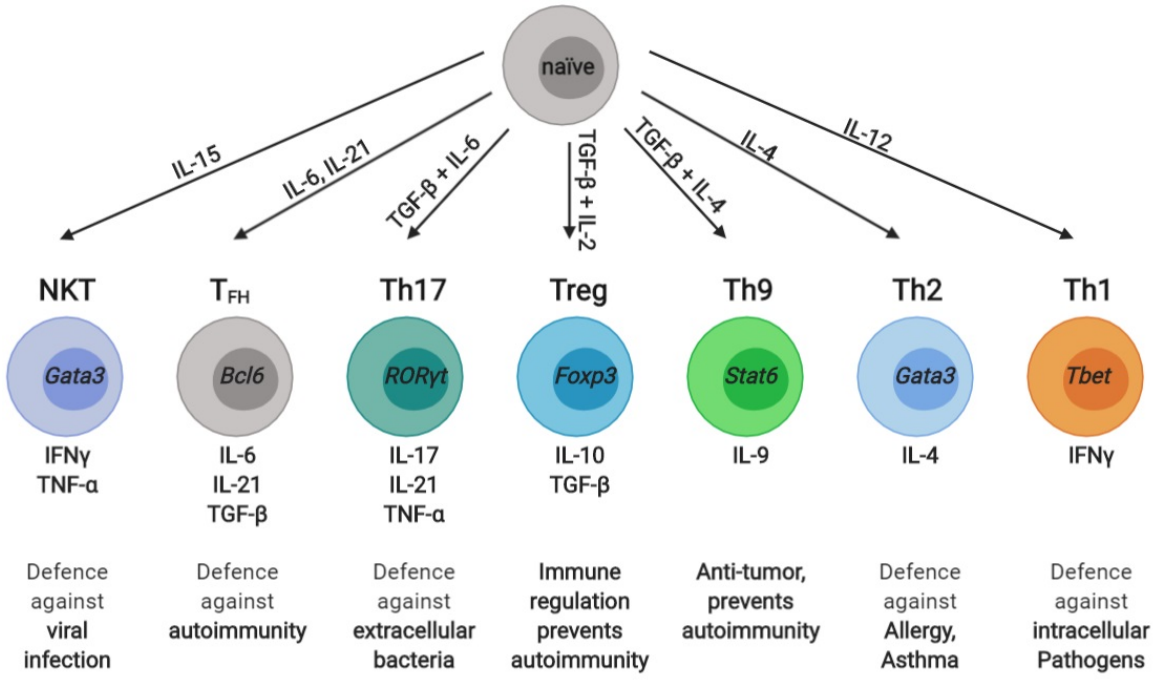

Figure 1.3. Diversity of T helper cells and their induction.
The signature cytokines produced by respective Th cells are shown along with their immunomodulatory properties in the boxes. Abbreviations: Signal transducer and activator of transcription (STAT), RAR-related orphan receptor gamma (RORγ), Forkhead box P3 (Foxp3), B-cell lymphoma (BCL6). The figure was modified from "Immunoporosis: Immunology of Osteoporosis - Role of T Cells" [28].

1.2.1 Langerhans cells

In healthy skin, specialised DC that reside in the epidermis are called Langerhans cells (LC) and serve as immune system sentinels. They were discovered by Paul Langerhans,

who described a dendrite-shaped cell in the human epidermis [29]. LC express the characteristic tennis racket shaped Birbeck granules, the type II transmembrane molecule Langerin (CD207) that is involved in ligand internalization and other proteins such as CD11c, CD205, MHC class I and II [30–32]. Langerin is not exclusive for LC since Langerin is also expressed on the XCR1$^+$/CD103$^+$ dermal DC and on XCR1$^+$ CD8$^+$ DC in the dermis and lymph nodes of mice and is inducible on CD1c$^+$ DC in humans [32–36]. LC are the centrepiece in the epidermis and orchestrate the immune response in different pathologies (figure 1.4).

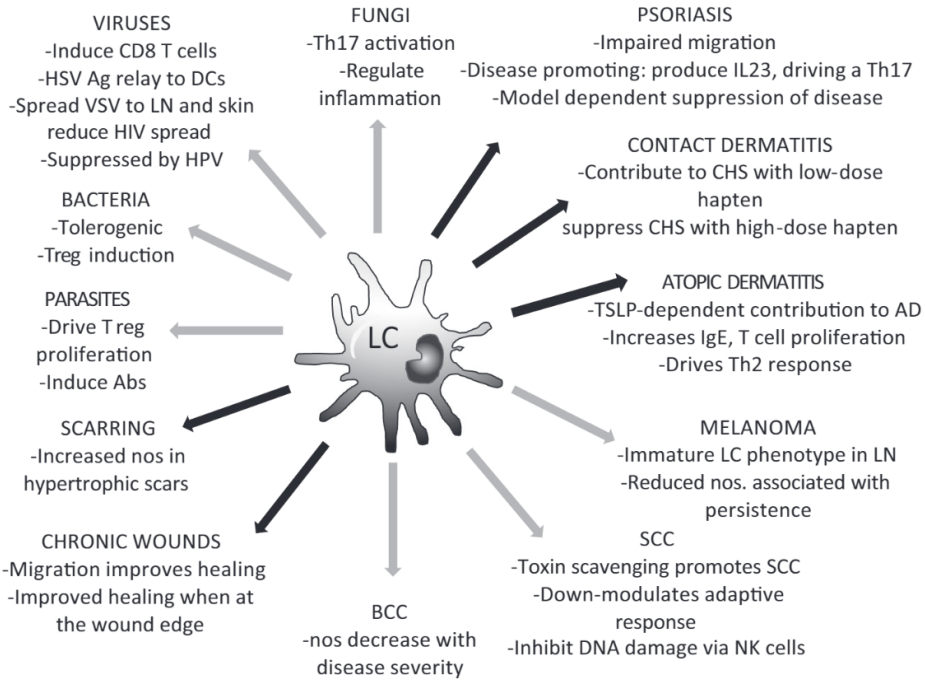

Figure 1.4. Overview of LC effect in different pathologies of the skin.
Abbreviations: herpes simplex virus (HSV), varicella-zoster virus (VZV), human papillomavirus (HPV), antigen (Ag), basal cell carcinoma (BCC), squamous cell carcinoma (SCC), contact hypersensitivity (CHS), lymph node (LN). The figure was adapted from "The role of Langerhans cells in pathologies of the skin" [32].

Unlike classical DC, which arise from bone marrow precursors and require the FMS-like tyrosine kinase 3 ligands for their differentiation, LC appear to be from embryonic origin [37]. It has been observed that LC, that seed the skin before birth, derive from the myeloid cell progenitors from the fetal liver and additionally from the yolk sac [32, 38, 39]. Due to their migratory capabilities, they were considered as DC, but recent studies with mice show that they may originate from the macrophage lineage of the adult fetal liver

progenitor [40]. This indicates that LC are the only resident tissue-specific macrophages that can migrate into the lymph node [41]. However, the origin of LC is still under debate.

The amount of LC in the epidermis is around 2 to 5 % of total nucleated cells where they protrude their dendrites via tight junctions toward the *stratum corneum* to sense antigens [42]. During steady-state, LC replenish themselves by proliferation *in situ* to replace dying and emigrating cells, since LC have an estimated half-life of 2 months, in contrast to DC that are maintained by a circulating pool of bone marrow-derived precursors [43–45]. Moreover, mice experiments showed that LC differentiation is dependent on TGF-β and its associated transcription factors PU.1, Id2, and RUNX3 as well as engagement of the colony-stimulating factor 1 receptor [46–49]. In the epidermis, KC provide TGF-β in a paracrine fashion to LC [50]. The LC-KC cross talk is important during inflammation since the KC provide essential pro-inflammatory cytokines, such as IL-1, IL-18, granulocyte macrophage colony-stimulating factor (GM-CSF), and tumor necrosis factor (TNF) which support and enhance the migration activity of LC [42, 51, 52].

During LC maturation, the receptors connected to migration activity are altered, such as the reduction of E-cadherin which is an essential step that allows LC to disassociate from the surrounding KC [52, 53]. Moreover, CCR6 is reduced in a TNF-α dependent fashion to support the emigration of LC, whereas KC produce the chemokine C-C motif ligand 20 (CCL20) attracting CCR6 expressing LC [54, 55]. The LC migration from the epidermis to the dermis is dependent on the chemokine receptor type 4 (CXCR-4) and its ligand C-X-C motif chemokine (CXCL) 12 that is constitutively released by dermal fibroblasts and further enhanced through various factors [56–58]. The chemokine receptor (CCR) 7 is needed for further migration to the lymph nodes where the concentration of CCL19 and CCL21 is increased to attract LC [55, 59]. There, they present the captured antigens to T cells and activate the adaptive immune system. A link to a *Nature* video providing a small peek at the complexity of cell migration can be found in appendix section (figure 7.2).

1.2.2 Inflammatory dendritic epidermal cells

The inflamed skin of AD patients harbours LC-like cells and they are called inflammatory dendritic epidermal cells (IDEC) [60]. These cells are not present in normal or healthy skin. IDEC reside between LC and KC in the epidermis and express MHC class II, CD1a, CD36, CD11b, FcϵRI and CD206, but lack Langerin and Birbeck granules [60, 61]. IDEC contribute to the pro-inflammatory environment by amplifying the allergic immune response after FcϵRI binding [62]. In particular, IDEC can produce CCL3, IL-1, IL-16, IL-18 and IL-12p70 after FcϵRI ligation [20]. Epicutaneous allergen exposure of AD patients induces eczematous reactions within 72 h [63]. Additionally, they can be generated *in vitro* from AD monocytes under reducing conditions. These cells produce high amounts of pro-inflammatory cytokines and chemokines similar to the cytokines found in lesional skin of the AD patients [64].

1.3 Toll-like receptors

Toll-like receptors (TLR) are part of the innate immune system and are required for the detection of conserved pathogen-associated molecular patterns from microbes to initiate an immune response [65]. These receptors can be found among species from mice to men and are expressed on a variety of cells and tissues that are important sites of host/pathogen interaction, such as the epithelia of skin and the airways [66].

They are type I transmembrane receptors and consist of an extracellular domain with leucine-rich repeats that mediate pathogen recognition, a transmembrane domain, and a cytoplasmic Toll/IL-1 receptor domain which initiates downstream signalling [65]. To date, 10 functional TLR have been identified in humans. Some are bound to the cellular surface (TLR1, 2, 4, 5 and 6) while others are expressed intracellularly (TLR3, 7, 8 and 9) [65]. After pathogen binding, the Toll/IL-1 receptor domain recruits adaptor proteins, such as MyD88 or TRIF which initiate signal transduction [68]. This subsequently induces the mitogen-activated protein kinase, NF-κB, and other transcription factors which lead to the transcription of pro-inflammatory cytokines, chemokines, and type I interferons [69].

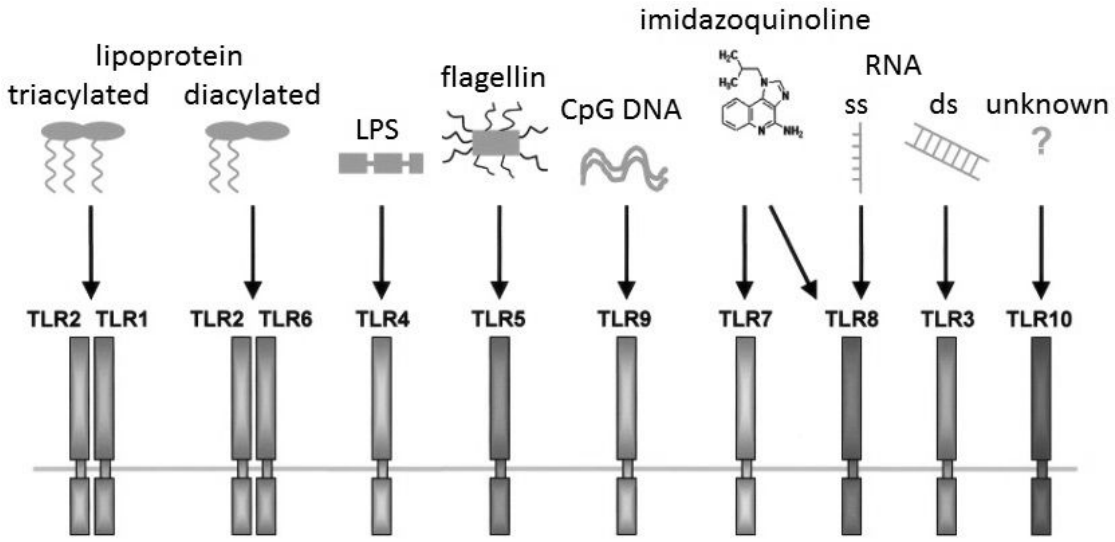

Figure 1.5. TLR and their ligands.
The figure was modified from "TLR signaling pathways" [67].

Figure 1.5 shows the different pathogens that bind to the TLR in the human system. TLR2 has a special role since it detects a variety of structures from Gram$^+$ bacteria and it can form heterodimers with TLR1 or TLR6. In this thesis, the synthetic triacylated lipopeptide Pam3CSK4 (P3C) was used to specifically trigger the TLR1/2 pathway and to simulate the presence or influence of *S. aureus*, since *S. aureus* is strongly associated with flares and severity of AD [70].

1.4 Atopic dermatitis (AD)

AD, also known as atopic eczema, is the most common inflammatory skin disorder in the developed world and affects 10-25 % of the children and 3-10 % of the adults in industrial countries [71–74]. The tendency is still increasing in developed countries, indicating that AD is a disease of modern times [75, 76]. An estimation of the total generated costs by AD in the United States was around \$5.3x10^9 in 2015 [77]. Furthermore, AD patients pay about 930 € per year for additional care or treatment, which is not covered by insurance [78]. This emphasises that this disease has a dramatic impact on each individual and their families and causes high health-care costs worldwide. The impact of AD on quality of life and medical care costs has led to an explosion of basic science and clinical research [79]. AD is an extremely heterogeneous disease and is at least in part related

to age and ethnicity [80]. It is marked by epidermal barrier dysfunction, dry skin, and inflammation which leads to pruritus, redness, and lesions that can occur anywhere with a typically age-related distribution [81]. Moreover, AD is associated with atopic comorbidities such as asthma, allergic rhinitis, food allergies, and non-atopic comorbidities such as depression, anxiety or cardiovascular issues [82–86]. The development of AD usually starts in infancy and progresses to IgE-mediated food allergies, allergic rhinitis, and asthma in later stages [62, 87]. This progression is called the atopic march. The most common severity evaluation tools of AD are the Eczema Area and Severity Index (EASI) and scoringAD (SCORAD). Both severity scoring systems consider erythema, infiltration (swelling), excoriations and lichenification as well as the extent of the area affected to assess clinical signs, where the SCORAD additionally considers oozing and crusting [83].

To date, specific risk triggers for the induction of AD are not known but some risk factors are associated with AD [88–90]. Interestingly, early-life exposure with tobacco smoke may increase the risk of adult-onset AD [91]. An additional environmental risk factor is a western diet (fast-food, low fruit intake) and broad-spectrum antibiotic exposure in early life [92]. Some studies indicate that air pollution, maternal psychiatric symptoms and alcohol intake during pregnancy are linked with an elevated risk of AD [92–94]. Furthermore, AD may be connected to obesity in the Asian- and American population, but not in the European population [95–97]. Several genetic indicators allow a prediction since genetics is most likely involved in the development of AD.

Genome-wide association studies led to the identification of almost 40 risk loci for AD [98, 99]. Not surprisingly, these loci are related to the development and function of the epidermal skin barrier and the innate as well as the adapted immune system [99]. One prominent and well-described risk factor is a mutation in the *filaggrin* gene. This mutation is known to influence the skin barrier function in AD where the KC connection and the water retention is very poor, which subsequently may lead to a change of the skin microbiome of AD patients [100–102]. Additionally, other proteins supporting the skin barrier and tight junctions display a malfunction or impaired expression in AD patients, such as FLG2, hornerin, cornulin, claudin 1, claudin 23 and many more [103–110].

Furthermore, colonization with *Staphylococcus aureus* (*S. aureus*) at the age of three months is associated with the development of AD later in life [111].

A plethora of bacteria, viruses and fungi build the skin microbiome which colonizes the human epidermis [112]. Additional information about the microbiome can be found through the QR code in figure 7.3 in the appendix section. The skin of AD patients is heavily colonized by *S. aureus* accompanied by reduced diversity of commensal bacteria on the skin [113, 114]. *S. aureus* is a gram-positive bacterium and present in 20-30 % of healthy subjects and it can produce α-toxins that induce KC damage and worsen the skin barrier function [115–118]. *S. aureus* binds to the extracellular matrix with surface components recognizing adhesive matrix molecules, such as a clumping factor. Furthermore, *S. aureus* can reside near the sebaceous gland, sweat gland or hair follicle in the dermis, where direct interaction with dermal cells can occur [119, 120]. The microbial composition of the dermis differs from that of the skin surface [121, 122]. Staphylococcal enterotoxins and protein A are produced by *S. aureus* and they directly modulate the immune system. These products act as superantigens that directly influence T cells which subsequently proliferate and release pro-inflammatory cytokines [114, 123, 124]. Additionally, lipoteichoic acid from *S. aureus* acts directly on DC via Toll-like receptors (TLR), which in turn enhance T helper (Th) 1/Th17 cell priming, indicating that *S. aureus* pushes AD to a more chronic Th1-driven state [125]. *S. aureus* strain-specific differences in induction of skin inflammation were shown in a cutaneous colonization model but the exact role of the skin microbiome in the development of AD remains poorly understood [126].

Prominent features of AD are defects in skin barrier function, cutaneous inflammation (figure 1.6), and colonization of *S. aureus*. AD is considered as a primarily Th2 cell-driven disease, where the T cells express an increased amount of adhesion molecules, enabling homing and recruitment into the skin [127]. These skin resident T cells show increased activation in AD patients [127]. The number of type 2 innate lymphoid cells (ILC2) is increased in AD lesions and they enhance the Th2 response since they are a potent source of IL-5 and IL-13 [128–130].

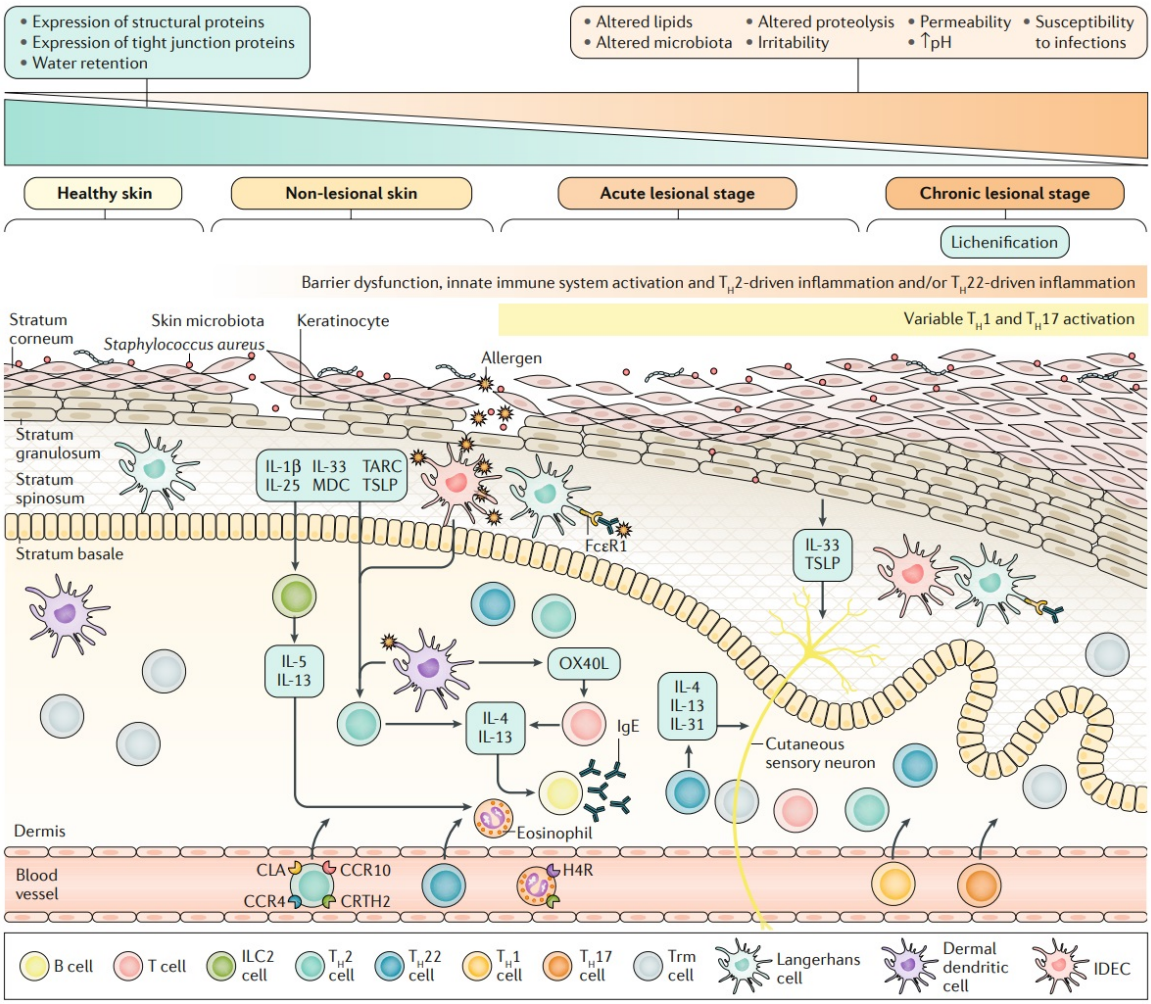

Figure 1.6. Stage-based pathogenesis and main mechanisms of atopic dermatitis.
Disruption of the epidermal barrier stimulates KC to express chemokines and cytokines. These activate skin-resident Th2 and group 2 innate lymphoid cell (ILC2) mediated immune responses, which produce high amounts of IL-5 and IL-13. Uptake of antigens and self-antigens by inflammatory dendritic epidermal cells (IDEC) and dermal DC further promotes type 2 immunity. T cells infiltrate the dermis with various skin-homing adhesion molecules. Antigen-primed T cells remain as resident memory T cells (T_{rm}) in the skin to provide a fast immune response. In chronic stages, the skin thickens and loses skin integrity which results in poor water retention and high susceptibility towards *S. aureus* infection. The figure was adapted from [83].

The Th2 induced inflammation is the hallmark of acute and chronic AD but recently the Th22 and Th17 axis gained attention. Due to the imbalance of the different Th axes, the cytokine profile of AD patients changes and differs from healthy controls, where Th2 (IL-4, IL-10, and IL-13) and Th22 (IL-22) specific cytokines were up-regulated in AD patients [131]. Both Th2 and Th22 axes contribute to the acute and the chronic phase of the disease, whereas Th1 only plays an essential role in chronic AD and Th17 may be involved in the Asian cohort and pediatric AD [132–134]. The disruption of the skin and the increased inflammation promotes the release of damage-associated molecular

pattern molecules so-called alarmins, such as IL-1β, IL-25, IL-33 and thymic stromal lymphopoietin (TSLP), which subsequently activate DC which initiate the Th2 axis or act directly on the cutaneous sensory neurons to exacerbate pruritus [83, 135–138]. TSLP activates the DC production of OX40 ligand which binds to T cells and subsequently induces the expression of Th2 specific cytokines such as IL-4, IL-5 and IL-13 [139]. As a consequence, IL-4 and IL-13 stimulate B cells to induce an immunoglobulin G (IgG) class switch to immunoglobulin E (IgE) and strongly reduce the expression of filaggrin causing skin barrier dysfunction [130, 140, 141].

IgE is the least abundant immunoglobulin in the serum and has a half-life of a few days. It triggers immediate allergic reactions by allergen recognition and subsequent binding to both CD23 and the high-affinity IgE receptor (FcϵRI) [133, 142–144]. In contrast to healthy skin, the skin of AD patients displays elevated numbers of DC subtypes expressing the FcϵR [145]. These DC present the allergens which consequently activate T cells and increase local inflammation [146, 147]. Interestingly, even though most patients with AD have high to extremely high serum levels of IgE, 20 % of the patients display normal IgE serum concentrations [148–152]. Patients with low or normal IgE serum concentration can have allergies [148]. The Th2 axis activity seems to be equal in both groups, while the Th17 and Th22 axis may be increased in patients with normal IgE levels [153]. Moreover, elevated IgE values at the age of 6 to 18 months without AD have been linked with AD in later childhood [154]. However, neutralization of IgE with the recombinant antibody Omalizumab has only shown limited efficacy in the treatment of AD [155].

1.4.1 Treatment options of AD

To treat mild to moderate forms of AD, moisturizers including non-aqueous emollients to soften the skin, occlusive agents to support the physical barrier and humectants to attract water are used daily [156]. The continued use of moisturizers reduces the number of flare-ups, provides essential skin hydration and reduces xerosis, pruritus and the need for topical corticosteroids [157, 158].

Topical corticosteroids are the centrepiece of the AD treatment. They have anti-

inflammatory activity, reduce *S. aureus* levels and are used in the acute phase, flaring, exacerbation and prophylaxis [159, 160]. The intermittent and correct use of topical corticosteroids may have little risk but improper usage may cause adverse effects such as purpura, rosacea, acne, hypopigmentation, hypertrichosis or glaucoma [145]. Systemic adverse effects are very rare but include risk of growth suppression, cataracts, hypothalamic–pituitary–adrenal, and diabetes type 2 in adult patients [161, 162]. A list of topical corticosteroids and their potency can be found in the appendix table 7.2.

Topical calcineurin inhibitors (tacrolimus and pimecrolimus) can be used as an alternative for topical corticosteroids as a first-line treatment but usually are used as second-line therapy. These are a unique class of topical anti-inflammatory agents, which are efficient in both acute flares and maintenance therapy [150, 163–166]. These inhibitors act mainly on T cells where they inhibit Th1 and Th2 activation and proliferation by decreasing the activation of different transcription factors including activated protein (AP)-1 complex, nuclear factor of activated T cells (NFAT) and nuclear factor (NFκB) [167–169]. As a consequence, the inflammatory cytokines such as IL-2, IL-4, IL-5, IFN-γ, IL-31 and TNF-α are reduced [170, 171]. However, the most common adverse effects are burning, pruritus, and during the first treatment cycle, the prevalence of viral infections is increased [172].

Short-term phototherapy (usually 4 to 8 weeks) is a complementary therapy option for AD, where patients are exposed to a UVB light with a wavelength of 311 nm, which is efficient in reducing inflammation and bears only low risks [173].

Systemic immunosuppressants are non-specific and include cyclosporin A, azathioprine, methotrexate and mycophenolate mofetil. Common adverse effects are hypertension, gastrointestinal issues, liver abnormalities, myelosuppression, nausea [174]. Systemic corticosteroids are not advised for long-term management of AD [80]. A specific systemic drug with good efficiency and safety profile is the biological Dupilumab, which is a fully monoclonal humanized antibody [175, 176]. Dupilumab binds to the IL-4 receptor and inhibits the signalling of IL-4 and IL-13.

1.5 Aims and objectives

AD is the most common inflammatory skin disorder and its prevalence is increasing strongly in industrialized countries. AD is considered to be a primarily Th2 cell-driven disease. AD influences the quality of life and pushes the medical care costs, which led to an explosion of basic science and clinical research. Major research was performed in the area of genetics, epidemiology and pathophysiology and lead to a better understanding of AD. However, AD is a complex and heterogeneous disease, and for this reason, further research is necessary to develop new therapeutic strategies or to prevent the outbreak. The skin of AD patients presents a significant disbalance of the microbiome with high colonization of *S. aureus*, which positively correlates with the severity of the disease. *S. aureus* can reside in the skin and directly influence immune cells. In the AD epidermis, LC and IDEC play an essential role in the pathogen detection and are shaping the immune responses. To date, it is not clear whether *S. aureus* or skin barrier defects in combination with impaired LC function may induce the chronic inflammation loop. Previous work has demonstrated that skin DC play a pivotal role in the course and the severity of TLR-induced skin inflammation [177]. This thesis aimed to investigate the influence of *S. aureus* products towards the TLR2 pathway of epidermal LC from AD patients, in order to understand the link between the innate and the adaptive immune systems in AD. Therefore, experiments were performed with *ex vivo* skin from AD patients and healthy donors to address the following objectives:

1. establish an *ex vivo* skin model to analyse immature LC
2. define the phenotype of LC and IDEC in the *ex vivo* human skin system
3. investigate the influence of *S. aureus* towards TLR2 activation with a focus on
    a) maturation processes
    b) cytokine production from the split thickness-skin
    c) the capability of epidermal cells to activate naïve $CD4^+$ T cells
    d) the migration activity of LC

2 Materials

All antibodies, chemicals, reagents, material, software and devices were received from, Abcam (Cambridge, UK), Bandelin electronic GmbH & Co. KG (Berlin, DE), Beckman Coulter Inc. (Brea, US), Becton Dickinson (Franklin Lakes, US), Biochrom (Berlin, DE), BioLegend (San Diego, US), BioTek (Vermont, US), Biozym (Oldendorf, DE), Bio-Rad AbD Serotec (Oxford, UK), Boehringer (Mannheim, DE), Corning GmbH HQ (Wiesbaden, DE), eBioscience (San Diego, US), Eppendorf (Hamburg, DE), EMC microcollections (Tübingen, DE), Fisher Scientific GmbH (Schwerte, DE), Gibco (Eggenstein), Greiner (Solingen, DE), GraphPad Software (San Diego, US), IKA (Staufen, DE), Imgenex (San Diego, US), Immunotools (Friesoythe, DE), Invitrogen (San Diego, US), Jackson ImmunoResearch Laboratories (West Grove, US), LaboGene (Allerød, DK), Labortechnik AG (Oberschleißheim, DE), Merck (Darmstadt, DE), Microsoft (Redmond, US), Millipore (Eschborn, DE), Miltenyi Biotec GmbH (Bergisch Gladbach, DE), MWG-Biotech (Martinsried, DE), Nikon (Tokio, JP), Nunc (Biberich, DE), Phoenix Instrument GmbH (Garbsen, DE), Phoenix Pharmaceuticals (Burlingame, US), Progen Biotechnik GmbH (Heidelberg, DE), R&D Systems (Minneapolis, US), r-project.org, RStudio Team (Boston, US), RayBiotech (Norcross, US), Roche Molecular Biochemicals (Mannheim, DE), Roth (Karlsruhe, DE), Sartorius (Göttingen, DE), Sigma-Aldrich (Taufkirchen, DE), Spherotech (Lake Forest, US), Thermo Fisher Scientic (Waltham, US), VWR (Radnor, US), Zeiss (Oberkochen, DE), and Ziegra Eismaschinen (Isernhagen, DE).

2.1 Chemicals and consumables

Buffers and stock solutions were prepared according to standard procedures using deionized water (dH_2O) or PBS. All media were sterilized by autoclaving or filtration.

Table 2.1. Chemicals and Consumables.

Component	Manufacturer
Compensation beads	Becton Dickinson
BD Perm/Wash™	Becton Dickinson
BD Cytofix/Cytoperm™	Becton Dickinson
β-ME	Sigma-Aldrich
7-AAD	Sigma-Aldrich
BSA	Sigma-Aldrich
DMSO	Sigma-Aldrich
Trypsin	Sigma-Aldrich
DNase I	Sigma-Aldrich
Tween 20	Sigma-Aldrich
Fetal calf serum	Thermo Fisher Scientific
Lymphoprep™	Progen Biotechnik
Ficoll	Biochrom
Normal mouse serum	Jackson ImmunoResearch Laboratories
Pam3CSK4	EMC microcollections
SPHERO™calibration beads	Spherotech
Antibiotic-antimycotic (100x)	Gibco®

Table 2.2. Buffers.

Buffer	Composition	Amount
FACS buffer	NaCl	137 mM
	Na_2HPO_4	75 mM
	NaH_2PO_4	32.5 mM
	FCS	10 % (v/v)
	NaN_3	155 mM
PBS	NaCl	137 mM
	KCl	2.7 mM
	NaH_2PO_4	8.5 mM
	KH_2PO_4	1.47 mM
MACS buffer	NaCl	137 mM
	KCl	2.7 mM
	NaH_2PO_4	8.5 mM
	KH_2PO_4	1.47 mM
	BSA	0.5 % (w/v)
	EDTA (0.5 M stock)	1 % (v/v)
AnnexinV binding buffer	HEPES/NaOH (pH 7.4)	10 mM
	NaCl	140 mM
	$CaCl_2$	2.5 mM
Wash buffer (ELISA)	PBS	500 ml
	Tween 20	0.05 % (v/v)

Table 2.3. Media.

Media	Composition	Amount
Skin media	RPMI 1640 VLE	500 ml
	FCS	10 % (v/v)
	Ab/Am	1 % (v/v)
MLR media	RPMI w/o L-Glutamine	500 ml
	FCS	10 % (v/v)
	Ab/AM	1 % (v/v)
	L-Glutamine	1 % (v/v)
	β-ME (5 mM)	1 % (v/v)
Freezing medium	FCS	90 % (v/v)
	Dimethyl sulfoxide	10 % (v/v)

2.2 Antibodies

Antibodies were used for analysing the expression level of surface receptors on human cells using flow cytometry. A list of used antibodies can be found in table 2.4.

Table 2.4. Antibodies.
*: antibodies against MHC class I and MHC class II were kindly provided by Dr. G. Moldenhauer (Heidelberg, Germany).

Target	Conjugate	Clone	Manufacturer
CD1a	APC	HI149	Becton Dickinson
CD4	APC	RPA-T4	Becton Dickinson
CD25	PE	M-A251	Becton Dickinson
CD80		L307.4	Becton Dickinson
CD86		IT2.2	Becton Dickinson
CD206		19.2	Becton Dickinson
CD40		5C3	Becton Dickinson
CCR6		11A9	Becton Dickinson
CD49c		P1B5	BioLegend
CD207	PE	4C7	BioLegend
CD207	PE	DCGM4	Beckman Coulter
CD36		FA6.152	Beckman Coulter
CD83		HB15a	Beckman Coulter
CD83		HB15a	Santa Cruz Biotechnology
TLR1		GD2.F4	Santa Cruz Biotechnology
TLR2		1030A5.138	IMGENEX
TLR6		86B1153.2	Novus Biologicals
FcϵRIα		AER-37 (CRA1)	eBioscience
CCR7		150503	R&D Systems
MHC I		W6/32	provided*
MHC II		L243	provided*
GoatαMouse	FITC	polyclonal	Jackson ImmunoResearch
IgG1		MOPC-21	Becton Dickinson
IgG1	PE	MOPC-21	Becton Dickinson
IgG1	APC	MOPC-21	Becton Dickinson
IgG2a		UPC-10	Sigma-Aldrich
IgG2a	PE	MOPC-173	BioLegend
IgG2b		MOPC-141	Sigma-Aldrich

2.3 Equipment

A list of used equipment and software can be found in table 2.5.

Table 2.5. Equipment.

Device	Model
ELISA washer	BioTek™ ELx50™ (Fisher Scientific GmbH)
ELISA reader	Synergy HT (BioTek Instruments)
Shaker & mixing	PMS-1000i (VWR), RS-TR05 (Phoenix Instrument GmbH), MS2 Minishaker (IKA)
Scales	Secura 6102-1S, BP211D, BP110 (Sartorius)
Centrifuges	5424R, 5417R (Eppendorf), Avanti J-15R, Allegra X-15R (Beckman Coulter)
Water purification system	Milli-Q® Reference (Merk)
Ice machine	105507 (Ziegra)
Hood	Mars class 2 (LaboGene)
Incubator	Heracell™ 150 (Thermo Fisher Scientic)
Microscope	Nikon eclipse TS100 (Nikon)
Sonication	SONOREX SUPER RK 100 H (Bandelin)
Cell separator	AutoMACS Pro® Separator (Miltenyi Biotec GmbH)
Flow cytometer	FACSCanto™ (Becton Dickinson)
Software	FlowJo v10.6.1 (Becton Dickinson), FACS Diva (Becton Dickinson), MS Office (Microsoft), SPSS 24 (IBM), Legendplex 7 (BioLegend), Gen5 (BioTek Instruments)
Open source	RStudio (RStudio Team [178]), R-project, LaTeX, Texmaker 5.0.4, JabRef 5.0.2, Manjaro 18.1.5

3 Methods

3.1 Skin of AD and healthy donors

The healthy control skin was obtained from patients undergoing plastic surgery at the Department of Plastic and Aesthetic Surgery of the University Hospital Bonn and kindly provided by Dr. Walgenbach. Patients with moderate to severe AD were diagnosed according to the criteria of Hanifin and Rajka [179]. The total serum IgE levels were measured by the core facility of the University Hospital Bonn using the ImmunoCAP system. AD skin was provided by medical doctors from the Department of Dermatology and Allergy of the University Hospital Bonn. The willingness of AD patients to donate skin was limited and dependent on the medical doctor promoting this study. Therefore, the biggest limiting factor was the amount of AD skin since the area which can be collected from the patients is relatively small compared to the amount of healthy skin. Patients who received a local or systemic steroid-based treatment were grouped in the treated AD (tAD).This study was approved by the local ethics committee of the University of Bonn and performed in accordance with the declaration of Helsinki. Informed consent was obtained from all of the participants in this study.

3.1.1 Preparation of *ex vivo* skin and epidermal cell suspensions

After obtaining skin from healthy donors, a wash step was performed with a sterile gauze soaked in PBS to remove excess blood. Afterwards, the split thickness-skin (0.4 mm) was immediately prepared using a dermatome. Biopsies of lesional AD skin were prepared by shave biopsy after local anaesthesia. In both situations, the epidermal cell suspension was generated with freshly prepared trypsinization solution (PBS supplemented with 0.5 % trypsin and 1 % Ab/Am). The floating split thickness-skin was incubated in the trypsin solution for 1 h at 37 °C, whereby the dermal side was in contact with the trypsin

solution. Afterwards, the dermis was removed and the epidermis was added to skin medium supplemented with 1 % DNase to remove DNA and to avoid cell clumping. Next, the epidermal cell suspension was generated by several steps of pipetting up and down or by vortexing until the media became cloudy, indicating that the cells were in suspension. The cells were counted with a Neubauer counting chamber, centrifuged (400 × g, 8 min, 4 °C) and the supernatant was discarded. The cell pellet was resuspended in medium or FACS buffer, depending on further usage.

3.1.2 Cell counting

Cell numbers were determined using the Neubauer counting chamber. Therefore, a cell sample was taken and mixed with trypan blue to exclude dead cells and 10 µl of the mixture was added to the counting chamber. The amount of trypan blue defined the dilution factor (DF). The cells were counted in the four big squares and the number of cells was calculated as follows:

$$N = \bar{x} * DF * V * 10^4 \left[\frac{10^6\ cells}{ml}\right]$$

3.1.3 Culture and stimulation of *ex vivo* skin

To increase the quality and reproducibility of the experimental setup, the examined skin samples were adjusted to a standardized area with a 6 mm punch biopsy for all culturing experiments. The floating split thickness-skin was cultivated for 24 h in standard cell culture condition (37 °C, 5 % CO_2) whereby the dermal side was in contact with the medium. The skin was divided into several punches and they were distributed equally between the two conditions (P3C treatment and control). One punch was incubated in 1 ml skin media with or without 10 µg/ml P3C in a 24 well plate. Afterwards, the skin was briefly washed with PBS and the epidermal cell suspension was generated as described above. The epidermal cell suspension was analysed using flow cytometry.

3.1.4 Migration experiments of *ex vivo* skin

The floating split thickness-skin was cultivated for 24 h or 48 h as described above. Due to the standardized area with a 6 mm punch biopsy (r=3 mm), the migration experiments were related to the skin size. Afterwards, the split thickness-skin was removed and the supernatant containing the migrated cells was collected. The cells were counted, dead cells were excluded by trypan blue staining. Next, the cell suspension was washed and stained with CD1a-APC. BD Cytofix/Cytoperm™ was used for fixation and permeabilization of the emigrated cells according to the manufacturer's instructions. The fixed cells were stained with CD207-PE, washed and submitted to flow cytometry analysis. This staining enables the detection of migrated LC since Langerin is internalized in LC during migration to the lymph nodes. Finally, the number of migrated LC was calculated as follows:

$$N = \frac{LC_{pos\%} - LC_{iso\%}}{100} * \frac{CD1a\%}{100} * \frac{counts/punch}{\pi * r^2}$$

3.2 Preparation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated from whole blood by Ficoll density-gradient centrifugation (800 x g, 30 min, 22 °C, acceleration 5, deceleration 0). This step allows for the separation of erythrocytes and granulocytes in the bottom layer, a Ficoll layer, a buffy coat layer, and an upper layer with plasma and thrombocytes. The buffy coat consists of a concentrated suspension of lymphocytes. Before centrifugation, whole blood was diluted 1:2 with PBS. 15 ml of the Ficoll solution (ρ=1.077 g/ml, Biochrom GmbH) was covered carefully with 30 ml blood/PBS solution and further separated by centrifugation. After centrifugation, the upper plasma phase was removed. The interphase consisting of PBMC was carefully collected and transferred into a fresh 50 ml reaction tube. Finally, $CD4^+$ cells were isolated using magnetic-activated cell sorting.

3.3 Isolation of naïve CD4 positive T cells

The cell isolation with the autoMACS® Pro Separator (Miltenyi Biotec GmbH) provides fast and gentle isolation of virtually any cell type and was used to enrich T cells from the PBMC fraction by magnetic labelling of the cells. For magnetic enrichment of $CD4^+$ T cells, PBMC were incubated with a cocktail of biotinylated CD45RO, CD8, CD14, CD15, CD16, CD19, CD25, CD34, CD36, CD56, CD123, anti-TCRγ/δ, anti-HLA-DR, and CD235a antibodies for at least 5 min on ice. These cells were subsequently labelled magnetically with anti-Biotin MicroBeads for at least 10 min on ice. The MicroBeads were retarded in the magnetic field and naïve $CD4^+$ T cells were enriched. Finally, the cells were stained with CD25-PE and CD4-APC to determine the purity of the enriched cells using flow cytometry.

3.3.1 Cryopreservation of naïve CD4 positive T cells

Freshly isolated naïve T cells were stored in liquid nitrogen for an extended time to use them for further experiments at later time points. Naïve $CD4^+$ T cells were centrifuged (300 × g, 5 min, 4 °C), counted with a Neubauer counting chamber, resuspended in freezing medium (10^7 cells/ml), and transferred into a cryogenic tube. For slow freezing conditions, cells were placed into an isopropanol freezing container (cooling speed -1 °C/min) and were stored overnight at -80 °C before they were transferred into liquid nitrogen for long term storage. At later time points, the T cells were thawed by the addition of 5 ml warm MLR medium to dilute the DMSO as fast as possible. After a washing step (300 × g, 5 min, 23 °C), the cells were resuspended in MLR medium, counted and adjusted to 10^6 cells/ml and immediately used for the MLR experiments.

3.4 Mixed lymphocyte reaction (MLR)

Allogeneic naïve $CD4^+$ T cells were isolated from PBMC of healthy donors as described above. *Ex vivo* skin was cultured with or without 10 µg/ml P3C for 24 h. Afterwards, the

epidermal cell suspension was prepared from *ex vivo* skin. Epidermal cell suspension (10^5 cells/well) was co-cultured with naïve $CD4^+$ T cells (10^5 cells/well) in 200 µl/well T cell culture medium in 96 well round bottom plates for 7 days. The supernatant was collected and analysed for cytokine production. For the proliferation assay, 1 µCi/well ^{3}H-thymidine was added to each well after 6 days of co-culture for additional 24 h. The cell mixture was harvested and analysed for ^{3}H-thymidine incorporation. These experiments were performed in triplicates and the mean was used for the statistical analyses. All procedures were performed according to local radiation safety guidelines.

3.5 Flow cytometry analysis

Flow cytometry was used as a tool for simultaneous multiparametric analysis of the epidermal cell suspension characteristics. Besides the analysis of the shape, size and granularity, flow cytometry was used for detection of the surface markers distribution among different experimental groups. Surface and intracellular staining were employed to analyse epidermal cell suspension and $CD4^+$ T cells. Antibodies used in this thesis are summarized in table 2.4. Viability of the cells was examined by 7-AAD and annexin V staining. Fluorescence minus one and isotype controls were used to define the gates. Intracellular staining was performed according to the BD Cytofix/Cytoperm™ kit protocol (Beckton Dickinson GmbH). For epidermal cell suspension analysis, 300.000 cells in 100 µl FACS buffer mixed with skin media (1:2) were submitted to one staining tube. The cell suspension was incubated with the antibodies (30 min, 4 °C, dark), which were removed by a washing step (400 × g, 4 °C, 5 min). Cells were measured and analysed utilizing a FACSCanto™ I and FACSDiva™ and FlowJo 10.6.1 software (all from Beckton Dickinson GmbH). The analysis of the mean and geometric mean is strongly influenced by outliers, and therefore unreliable for analysis of markers. However, both are good parameters to describe a population that is defined by a single marker. The median is a good parameter to qualitatively describe the properties of a given population, and therefore was used for the analysis of markers. For statistics, the median fluorescence

intensity was recorded and the relative fluorescence index (rFI) was calculated as follows:

$$rFI = \frac{MFI_{target}}{MFI_{control}}$$

3.6 Cytokine and chemokine detection

3.6.1 ELISA

Enzyme-linked immunosorbent assays (ELISA) were performed for protein quantification using paired antibodies or kits. The culture supernatants from *ex vivo* skin culture and MLR were collected and frozen at -80 °C. The ELISA was performed according to the manufacturer's instructions. Cytokines that were secreted into the supernatant were analysed by ELISA kits for CCL20 (R&D Systems), IL-6, IL-10, IFN-γ (Invitrogen), IL-4, IL-13 (Immunotools), IL-1β (R&D Systems), IL-17 (RayBiotech), IL-22 (eBioscience) and hBD2 (Phoenix Pharmaceuticals). Briefly, the surface of the plates was coated with capture antibody in appropriate coating buffer and incubated overnight. Plates were washed three times with ELISA wash buffer and then incubated with assay diluent buffer for 1 h at 37 °C as a blocking step. Following three washes, samples and standards were added. After incubation for 2 h at room temperature, plates were washed five times before the detection antibody was applied. After 1 h at room temperature, the plates were washed three times and streptavidin horseradish peroxidase conjugate was added. After 30 min, the plates were washed three times and the TMB substrate was added. Plates were incubated in the dark for up to 30 min or until sufficient blue colour had developed. Afterwards, the reaction was stopped with sulfuric acid stop solution, which results in a colour change from blue to yellow. Absorbance was read immediately at 450 nm and for correction of optical imperfections at 540 nm on the Synergy HT (BioTek Instruments). The values were subtracted (450 nm - 540 nm) and analysed with Gen5 software (BioTek Instruments).

3.6.2 Flow cytometry-based cytokine analysis

Cytokines were additionally analysed by flow cytometry. BioLegend's LEGENDplex™ assays are bead-based immunoassays using the same basic principle as sandwich immunoassays. The human Th cytokine panel (IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IL-17A, IL-17F, IL-21, IL-22, IFN-γ and TNF-α) and the human cytokine panel 2 (TSLP, IL-1α, IL-1β, GM-CSF, IFN-α2, IL-23, IL-12p40, IL-12p70, IL-15, IL-18, IL-11, IL-27, and IL-33) were used to analyse the supernatant of skin culture or MLR. Beads are differentiated by size and internal fluorescence intensities. Each bead set is conjugated with a specific antibody on its surface and serves as the capture beads for that particular analyte. When a selected panel of capture beads is mixed and incubated with a sample containing target analytes specific to the capture antibodies, each analyte will bind to its specific capture beads. After washing, a biotinylated detection antibody cocktail was added, and each detection antibody in the cocktail will bind to its specific analyte bound on the capture beads, thus forming capture bead-analyte-detection antibody sandwiches. Subsequently, streptavidin labeled with phycoerythrin (PE) was added, which binds to the biotinylated detection antibodies, providing fluorescent signal intensities in proportion to the amount of bound analytes. Since the beads are differentiated by size and internal fluorescence intensity on a flow cytometer, analyte-specific populations can be segregated and PE fluorescent signal quantified. The concentration of a particular analyte is determined using a standard curve generated in the same assay. The assay uses two sets of beads. Each set has a unique size that can be identified based on its forward scatter and side scatter profiles. Each bead set can be further resolved based on their internal allophycocyanin (APC) intensities. The smaller beads A consists of 6 bead populations and the larger beads B consists of 7 bead populations. Each analyte is associated with a particular bead set. Consequently, the concentration of each cytokine could be calculated using LEGENDplex™ 7.0 data analysis software provided by BioLegend.

4 Results

This thesis aimed to investigate the impact of TLR2-mediated activation on human LC in healthy and AD skin. P3C, a TLR1/2 specific synthetic protein, was used to mimic *S. aureus* to trigger the TLR pathway in the human epidermis, especially in DC. To achieve a suitable strategy for targeting naïve LC, an *ex vivo* model was needed which avoids artificial side effects and is very close to the *in vivo* situation. Figure 4.1 displays the full flow of the experimental work.

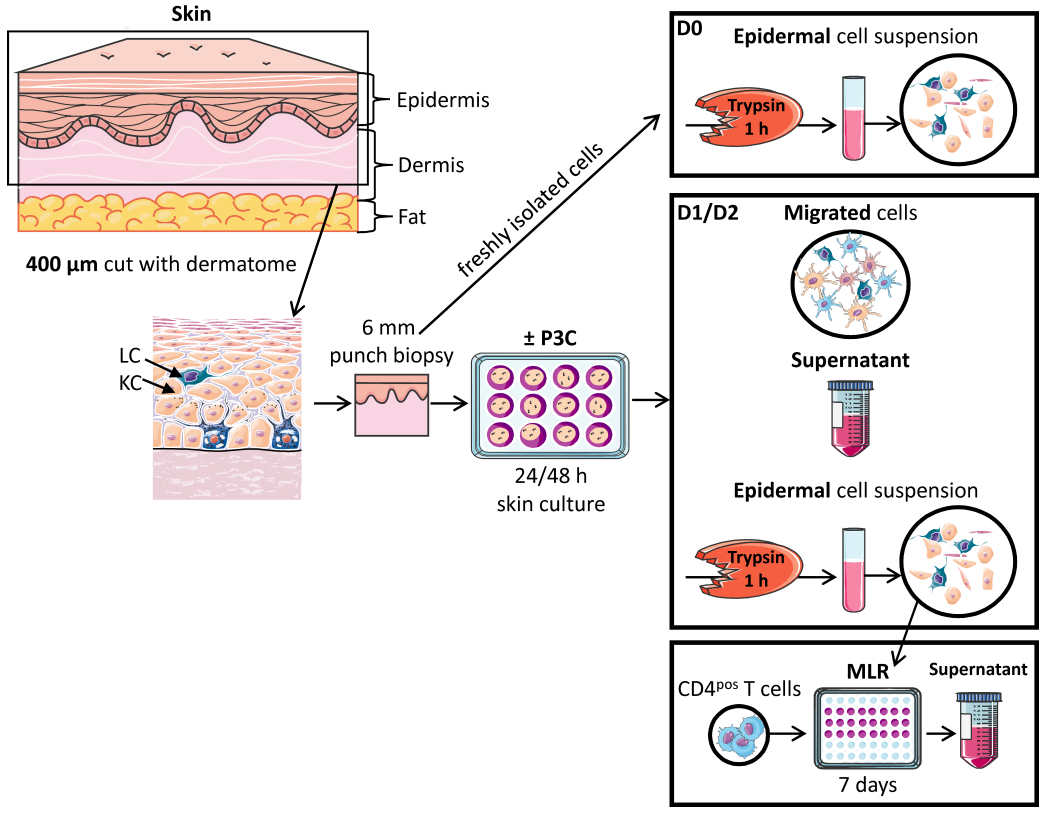

Figure 4.1. Overview of the full experimental setup.
Healthy split thickness-skin was obtained from patients immediately after plastic surgery using a dermatome with a depth of 400 µm. AD skin was obtained by shave biopsies after local anaesthesia. For analysis of day (D) 0 phenotype, the epidermal cell suspension was generated after 1 h trypsinization at 37 °C and the freshly isolated cells were analysed by flow cytometry. For P3C treatment, the size of split thickness-skin was standardized with a 6 mm punch biopsy and cultured for 24 h (D1) with or without P3C, followed by epidermal cell suspension generation and subsequent flow cytometry analysis. The migrated cells and the supernatant were collected and analysed by flow cytometry and ELISA at D1 and D2. For MLR experiments, the $CD4^+$ T cells were mixed in equal parts with the epidermal cell suspension and were co-cultured for 7 days. Afterwards, the supernatant was collected and analysed by flow cytometry or ELISA.

The result section is divided into three main parts:

- Part I: Phenotyping and validation of the *ex vivo* human skin system
- Part II: P3C stimulation of epidermal DC and analysis of their ability to activate T cells
- Part III: TLR2-mediated migration of epidermal DC

4.1 Part I: Phenotyping and validation of the *ex vivo* human skin system

4.1.1 Flow cytometry gating strategy

LC are characterized by Langerin and CD1a expression and are the main DC in the healthy human epidermis. In contrast to healthy, AD epidermis harbours an additional $CD1a^+$ cell population, which is Langerin negative and they are called IDEC [60]. Langerin is the marker of choice to distinguish IDEC from LC in AD skin. To analyse LC or IDEC, an epidermal cell suspension was generated and measured by flow cytometry (methods 3.1.1 and 3.5). The experimental flow for analysis of freshly isolated cells (D0) is presented in figure 4.2.

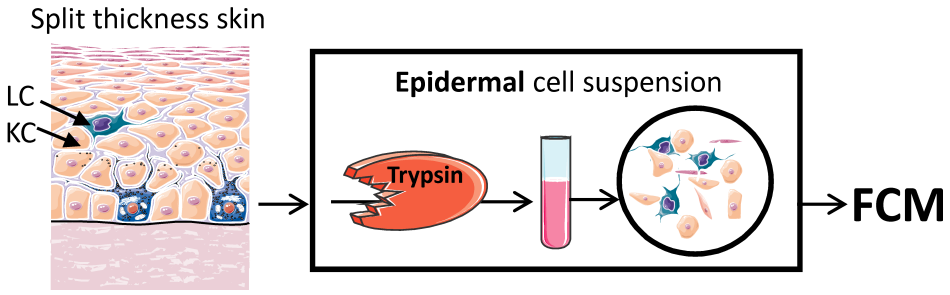

Figure 4.2. Overview of the analyses of freshly isolated cells (D0).
The skin was obtained from patients with AD and healthy donors. Epidermal cell suspension was generated after 1 h trypsinization at 37 °C and analysed afterwards by flow cytometry (FCM).

The overall flow cytometry gating strategy is depicted in figure 4.3 and contains a flow control, doublet discrimination, dead/live discrimination using 7-AAD and finally a Langerin background control to discriminate LC from IDEC in AD epidermis. CD1a negative cells

were considered as KC since at least 95 % of epidermal cells are KC [180]. This setup is suitable for measuring receptor expression on KC, LC, and IDEC and gives a good resolution of human epidermis.

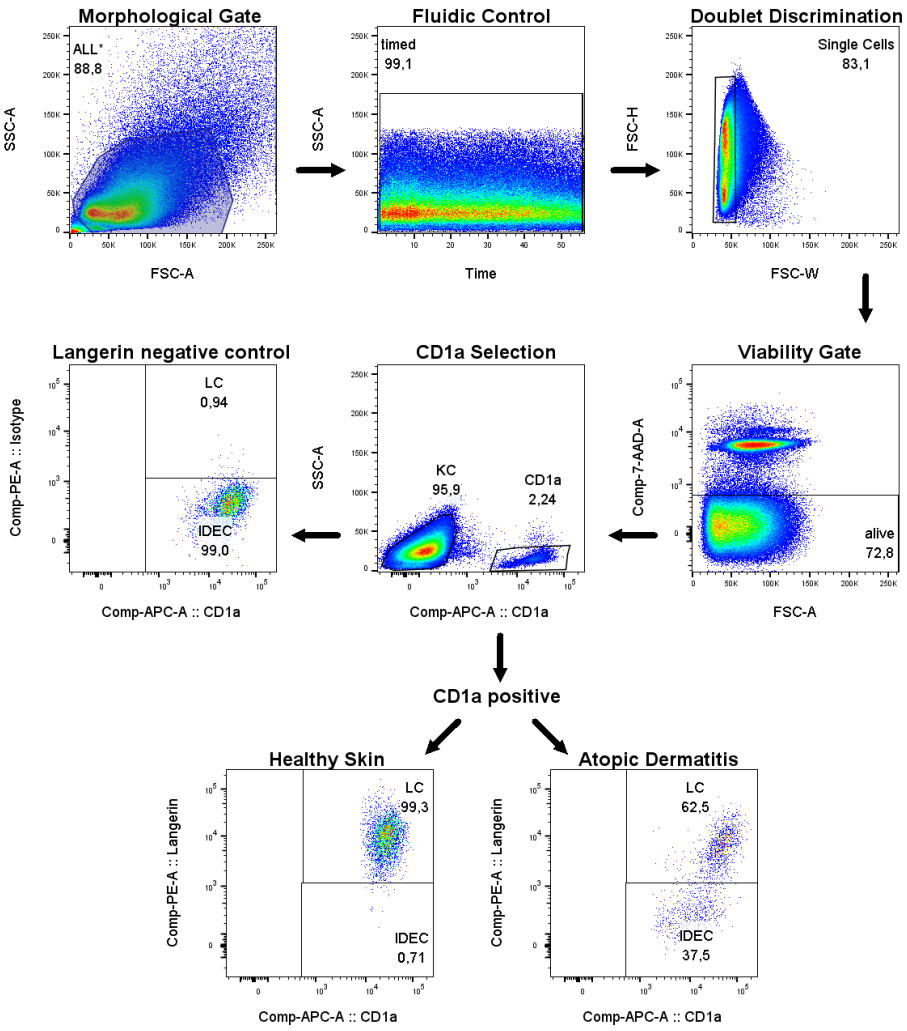

Figure 4.3. Flow cytometry gating strategy of the epidermal cell suspension.
AD skin was obtained by shave biopsies after local anaesthesia. Healthy skin was obtained from patients immediately after plastic surgery using a dermatome. The epidermal cell suspension was generated after 1 h trypsinization at 37 °C and was analysed by flow cytometry. 7-AAD was used for dead/alive discrimination. AD skin harbours an additional $CD1a^+$ and Langerin negative cell population, the inflammatory dendritic epidermal cells (IDEC).

4.1.2 The LC phenotype of healthy skin is different from AD

AD is a well-described disease and some markers (CD36, CD206) are already known to be expressed higher in IDEC from AD skin [60, 61]. Freshly isolated cells (D0) from AD and steroid treated AD (tAD) did not show any difference in their CD36, CD206 and

FcεRIα expression and for this reason, AD and tAD were analysed in one group in this section of the thesis. CD1a is one prominent marker to define DC in the epidermis and thus a good tool to discriminate between KC and DC. The overall amount of $CD1a^+$ cells was equal in healthy and AD epidermis (figure 4.4A), while some AD donors showed indeed elevated $CD1a^+$ cell populations. The CD1a population in AD epidermis consisted of LC and IDEC, while the IDEC expressed less CD1a on their surface when compared to LC in AD (figure 4.4B)

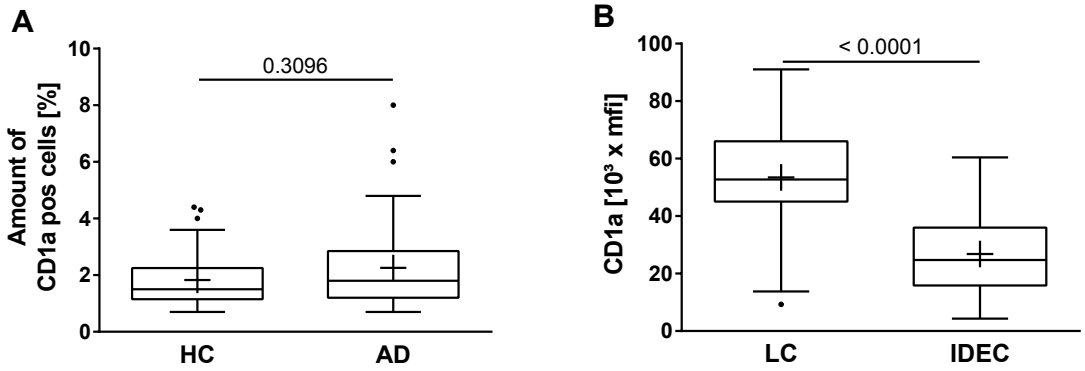

Figure 4.4. Equal distribution of $CD1a^+$ cells in healthy controls (HC) and AD skin, while IDEC expressed fewer CD1a.
The epidermal cell suspension was generated immediately from split thickness-skin and shave biopsies after 1 h trypsinization at 37 °C and was analysed by flow cytometry. The mean is marked by a + and the median by a vertical line inside the box. Outliers are marked as a black dot. **A)** shows the proportion of $CD1a^+$ cells (n=HC (41), AD (45)) in healthy and AD skin. **B)** shows the median fluorescence intensity (mfi) of LC and IDEC (n=31) of AD skin. The Wilcoxon matched-pairs signed-rank test was used for connected and the Mann-Whitney U test for independent samples.

Furthermore, CD206 and CD36 were used to analyse the phenotype of epidermal DC from AD skin. IDEC are known to express high levels of CD36 and CD206. Figure 4.5 displays CD206 and CD36 expression of epidermal DC from *ex vivo* skin. As expected, the CD206 and CD36 expression was increased in LC and to a greater extend in IDEC from AD patients when compared to healthy controls.

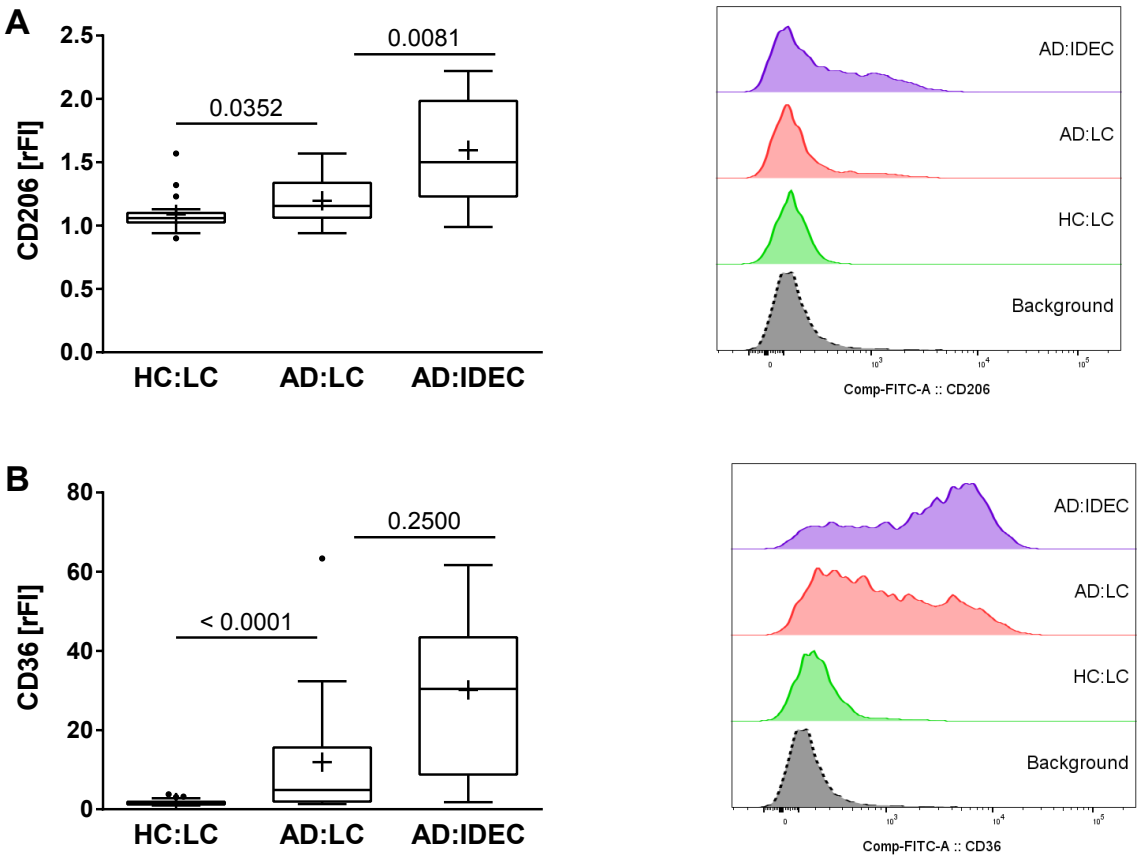

Figure 4.5. CD206 and CD36 are expressed highly IDEC from AD patients.
The epidermal cell suspension was generated immediately from split thickness-skin and shave biopsies after 1 h trypsinization at 37 ℃. Epidermal LC (Langerin$^+$, CD1a$^+$) and IDEC (Langerin$^-$, CD1a$^+$) were analysed by flow cytometry. The mean is marked by a + and the median by a vertical line inside the box. Outliers are marked as a black dot. A) shows the **CD206** expression (n=HC:LC (21), AD:LC (20), AD:IDEC (13)). B) shows the **CD36** expression (n=HC:LC (42), AD:LC (17), AD:IDEC (8)). The Wilcoxon matched-pairs signed-rank test was used for connected and the Mann-Whitney U test for independent samples. One representative experiment of each cell population was chosen for the histograms of CD206 and CD36.

The high-affinity receptor for IgE (FcϵRI) is expressed highly in AD and for this reason its extracellular α-chain is used as a classical marker of AD [62, 181]. The role of FcϵRIα in TLR2-mediated inflammation during AD flares and beyond is currently not clear, and therefore it is worth to investigate it, including the allergy status. The surface expression of FcϵRIα was analysed by flow cytometry, and the IgE serum levels were provided by the medical department. Healthy subjects expressed very low levels of FcϵRIα, even if they had an atopic background (*e.g.* grass pollen allergy). FcϵRIα was expressed highly in LC from AD skin, while IDEC expressed the receptor to an even greater extent (figure 4.6). Nevertheless, FcϵRIα showed a heterogeneous, donor-dependent surface expression.

One representative example for each population is shown in the figure next to the box plot, which underlines the distribution of the receptor in AD.

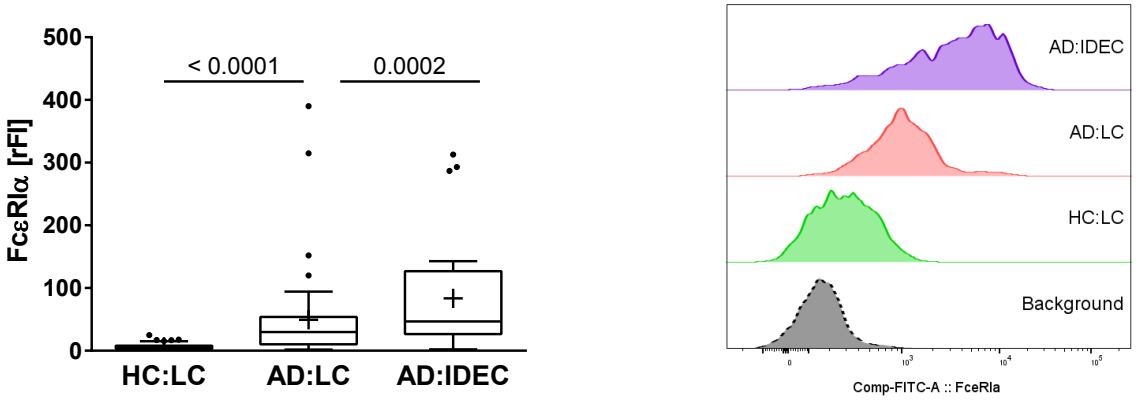

Figure 4.6. FcεRIα is higher expressed in LC and IDEC from AD skin compared to LC from healthy skin.
The epidermal cell suspension was generated immediately from split thickness-skin and shave biopsies after 1 h trypsinization at 37 °C. The mean is marked by a + and the median by a vertical line inside the box. Outliers are marked as a black dot. FcεRIα (n=HC:LC (72), AD:LC (44), AD:IDEC (26)) was analysed by flow cytometry. The Wilcoxon matched-pairs signed-rank test was used for connected and the Mann-Whitney U test for independent samples. One representative experiment of each cell population was chosen for the histograms of FcεRIα.

An additional hallmark of AD is the allergy status, usually witnessed by multiple allergies against house dust mite, grass pollen, birch pollen, cat hair, nuts and many more. This results in very high IgE levels in the blood of AD patients. These high titers of serum IgE can be found in about 80 % of the patients, while the others show normal IgE levels [182]. IgE levels are considered normal below or around 100 IU/ml, while some AD patients can reach up to 40.000 IU/ml. The FcεRIα expression on monocytes correlates with the IgE serum titer of AD patients [183]. This could be confirmed in the present dataset (figure 4.7), where donors with a high FcεRIα expression on LC did indeed show elevated serum levels of IgE. Nevertheless, some patients did express the receptor at high levels despite having medium IgE titers. Linear regression was performed and the coefficient of determination was calculated.

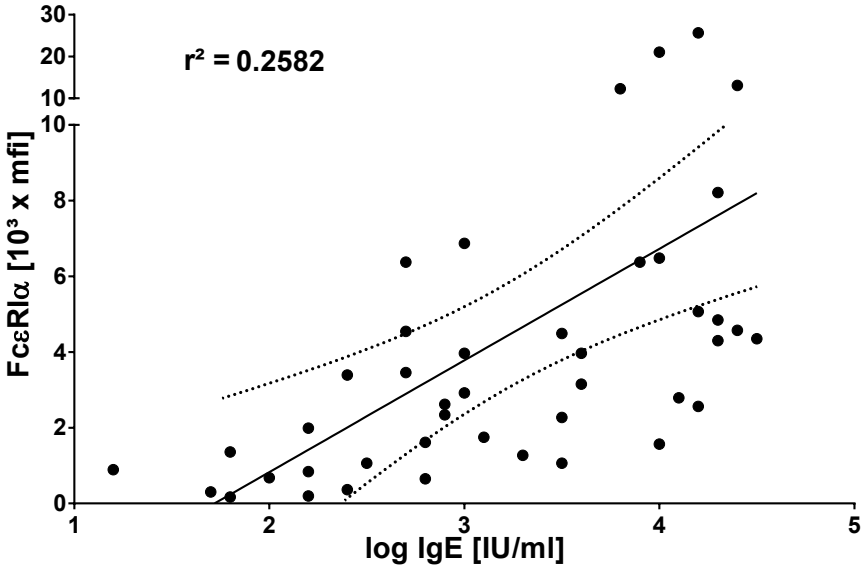

Figure 4.7. FcεRIα on LC correlates with IgE serum titers of AD individuals.
The epidermal cell suspension was generated immediately from shave biopsies after 1 h trypsinization at 37 °C. FcεRIα (n=43) was analysed by flow cytometry and the median fluorescence intensity (mfi) was plotted on the y-axis. IgE serum level was analysed by the medical department and was provided for each patient. Log IgE (IU/ml) were plotted on the x-axis. A linear regression was performed (straight line) and the 95 % confidence band of the regression was plotted (dotted line).

4.1.3 LC from AD and healthy skin have a similar maturation status

The skin is continuously exposed to environmental factors including the skin microbiome, which changes when AD patients have a flare [184]. Moreover, AD patients usually visit the clinic when they suffer from a flare which the patients can not handle by themselves. To determine the impact of the microbiome, it is necessary to monitor several maturation markers since they might already be activated *in situ*. Here, the co-stimulatory molecules CD83, CD40, CD80 and CD86 of epidermal DC were measured (figure 4.8). Flow cytometry analysis revealed that CD83 (figure 4.8A) was expressed constitutively and was not elevated in epidermal DC of AD patients when compared to healthy controls. Interestingly, some of the healthy controls expressed CD83 at higher levels. Moreover, LC from tAD patients expressed significantly less CD83 on their cell surface, compared to control skin. The IDEC population in AD as well as tAD skin tended to have more CD83 compared to LC from AD subjects.

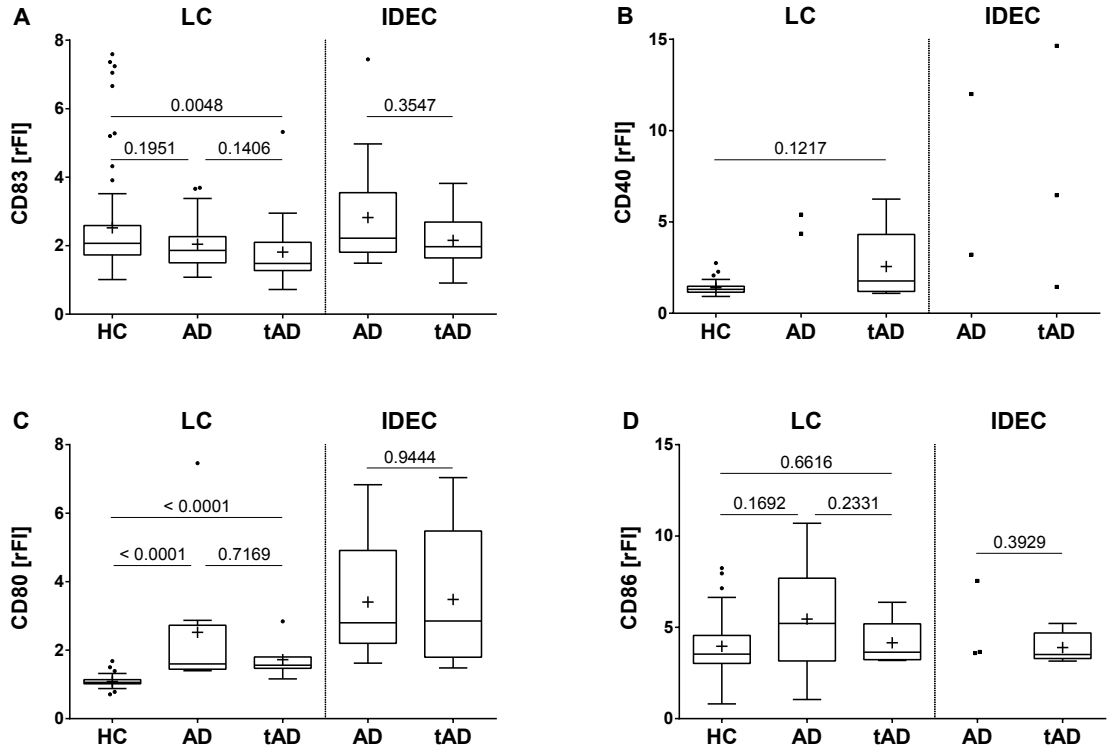

Figure 4.8. Equal maturation status of epidermal LC from healthy and AD skin.
The epidermal cell suspension was generated from split thickness-skin and shave biopsies after 1 h trypsinization at 37 °C. The mean is marked by a + and the median by a vertical line inside the box. Outliers are marked as a black dot. Datasets with less than 4 samples are presented as individual values in black squares. The maturation markers (A) **CD83** (n_{LC} = HC:71, AD:21, tAD:21; n_{IDEC} = AD:15, tAD:9), (B) **CD40** (n_{LC} = HC:44, AD:2, tAD:8; n_{IDEC} = AD:2, tAD:3), (C) **CD80** (n_{LC} = HC:57, AD:8, tAD:7; n_{IDEC} = AD:5, tAD:5), and (D) **CD86** (n_{LC} = HC:57, AD:6, tAD:7; n_{IDEC} = AD:3, tAD:5) were analysed using flow cytometry. The Mann-Whitney U test was used for independent samples.

Activated DC increase their CD40 expression to stimulate T cells [185]. LC from healthy skin expressed less CD40 when compared to AD and tAD (figure 4.8B). CD80 is one classical T cell co-stimulatory molecule, which can be found on activated DC. The CD80 expression (figure 4.8C) was low on LC from healthy donors, while all other epidermal DC showed significantly elevated levels compared to healthy controls. The CD80 expression tended to be increased in IDEC compared to LC, but this was not significant. CD86 was analysed since it is an additional T cell activating molecule. CD86 is related to CD80 since they can work together to modulate T cells [186]. In contrast to CD80, CD86 (figure 4.8D) was expressed continuously, and analysis thereof revealed that AD LC did not express more CD86 compared to control skin.

Next, the expression levels of MHC I and II were analysed since they were suitable for determining the maturation status of DC. The MHC class I is ubiquitously expressed [187].

MHC class I (figure 4.9A) was expressed equally between all epidermal DC. KC expressed the MHC I at higher levels when compared to LC (appendix, figure 7.5D). Interestingly, KC of tAD patients showed slightly higher MHC class I expression when compared to healthy controls. MHC class II (figure 4.9B) is usually unique for antigen-presenting cells, such as DC. Flow cytometry analysis revealed a heterogenic expression in all groups, while no significant difference could be detected between LC. The Wilcoxon matched-pairs signed-rank test revealed that IDEC from AD patients expressed significantly more MHC class II than the LC from the same donor (p=0.0391, n=10, figure 4.9B), while no significant difference could be found for LC and IDEC from tAD patients (p=0.4648, n=11, figure 4.9B).

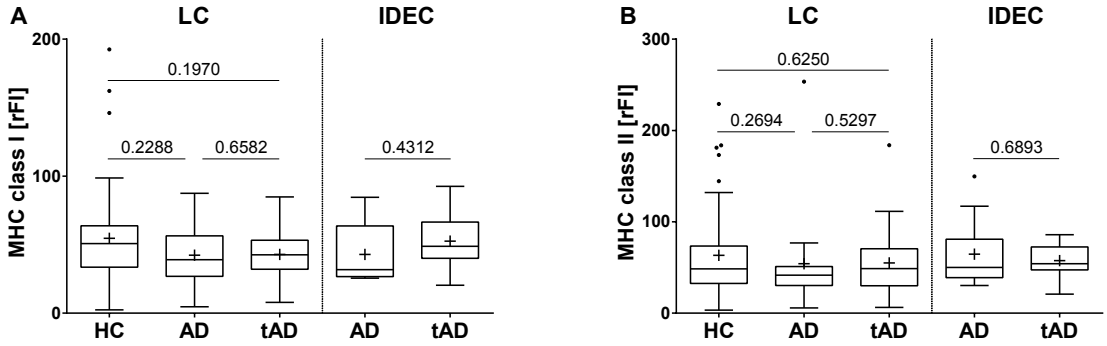

Figure 4.9. Major histocompatibility complexes are not altered in AD.
The epidermal cell suspension was generated immediately from split thickness-skin and shave biopsies after 1 h trypsinization at 37 °C. The mean is marked by a + and the median by a vertical line inside the box. Outliers are marked as a black dot. The maturation markers (A) **MHC class I** (n_{LC} = HC:63, AD:10, tAD:17; n_{IDEC} = AD:6, tAD:7) and (B) **MHC class II** (n_{LC} = HC:63, AD:14, tAD:21; n_{IDEC} = AD:10, tAD:11) were analysed using flow cytometry. The Mann-Whitney U test was used for independent samples.

4.1.4 TLR are expressed equally on LC from AD and healthy skin

Pattern recognition receptors, such as the TLR, are expressed on LC and belong to the innate immune system. TLR2 is of special interest since it detects various pathogens and forms heterodimers with either TLR1 or TLR6 which detect triacylated (TLR2/1) or diacylated (TLR2/6) lipopeptides [69, 188, 189]. In the next step, the expression of TLR1, TLR2 and TLR6 (figures 4.10) on freshly isolated cells (D0) was analysed by flow cytometry. This was crucial information since it allowed the judgement of the LC and IDEC activation status before the TLR engagement, which will most likely influence the

TLR expression. There was no significant difference in the TLR2 expression between LC nor IDEC. However, AD patients tended to have a lower TLR2 surface expression when compared to healthy subjects, though this was not significant but may have biological relevance. The expression of TLR1 and TLR6 was low and could not be analysed for tAD due to limited sample material from tAD.

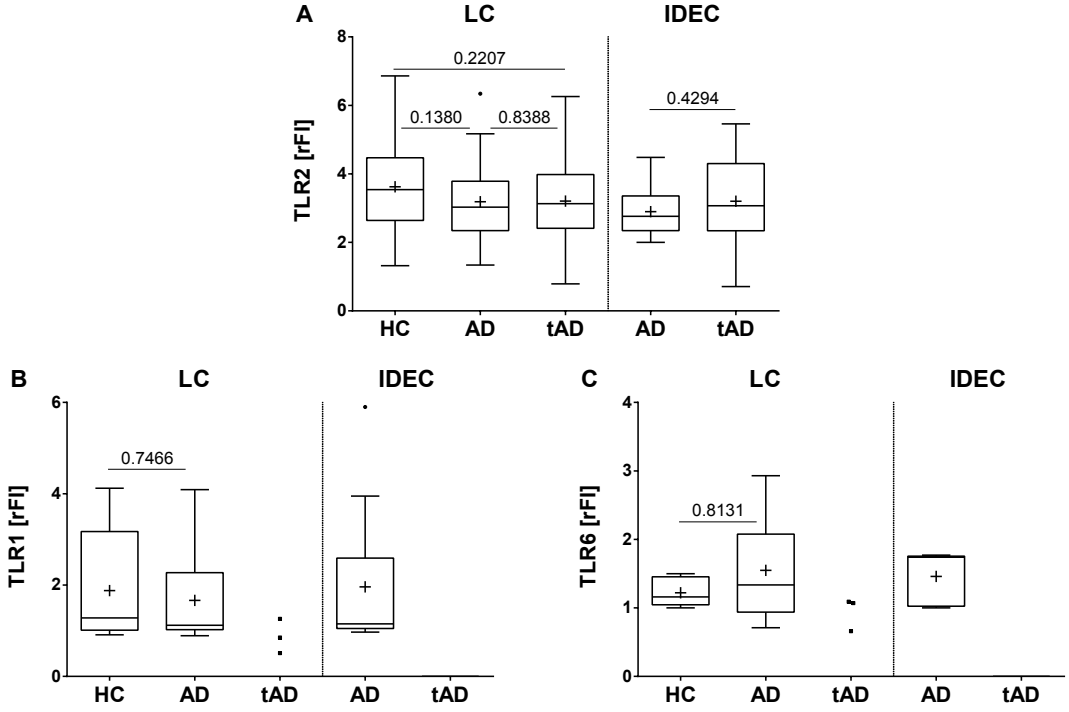

Figure 4.10. The TLR are expressed similarly in freshly isolated cells from healthy or AD skin. The epidermal cell suspension was generated immediately from split thickness-skin and shave biopsies after 1 h trypsinization at 37 °C. The mean is marked by a + and the median by a vertical line inside the box. Outliers are marked as a black dot. Datasets with less than 4 samples are presented as individual values in black squares. The receptors of the innate immune system were analysed, (A) **TLR2** (n_{LC} = HC:71, AD:21, tAD:23; n_{IDEC} = AD:16, tAD:11), (B) **TLR1** (n_{LC} = HC:18, AD:12, tAD:3; n_{IDEC} = AD:9, tAD:0), and (C) **TLR6** (n_{LC} = HC:6, AD:10, tAD:3; n_{IDEC} = AD:5, tAD:0) were analysed using flow cytometry. The Mann-Whitney U test was used for independent samples.

4.1.5 Receptors connected to migratory activity show decreased expression in AD skin

In the next approach, the basal expressions of surface receptors connected to migration were analysed. Here, the C-C motif chemokine receptor 6 (CCR6), the C-C motif chemokine receptor 7 (CCR7) and the cell adhesion molecule Integrin α3 (CD49c) were measured in freshly isolated epidermal cells (D0). Typically, immature DC express CCR6

(figure 4.11A), which is connected with the recruitment of DC to the tissue [190]. Only one AD patient showed an increased CCR6 expression, which was comparable to healthy controls (figure 4.11A). The rest of the AD individuals did express CCR6 at much lower levels. Nevertheless, the difference is not statistically significant but most likely biologically relevant. This becomes clear when analysing the tAD group, which tended to express more CCR6 on LC. Interestingly, the IDEC in both AD and tAD patients showed low expression profiles of CCR6, which was significantly different from the LC in case of tAD (p=0.0039, n=9, figure 4.11A).

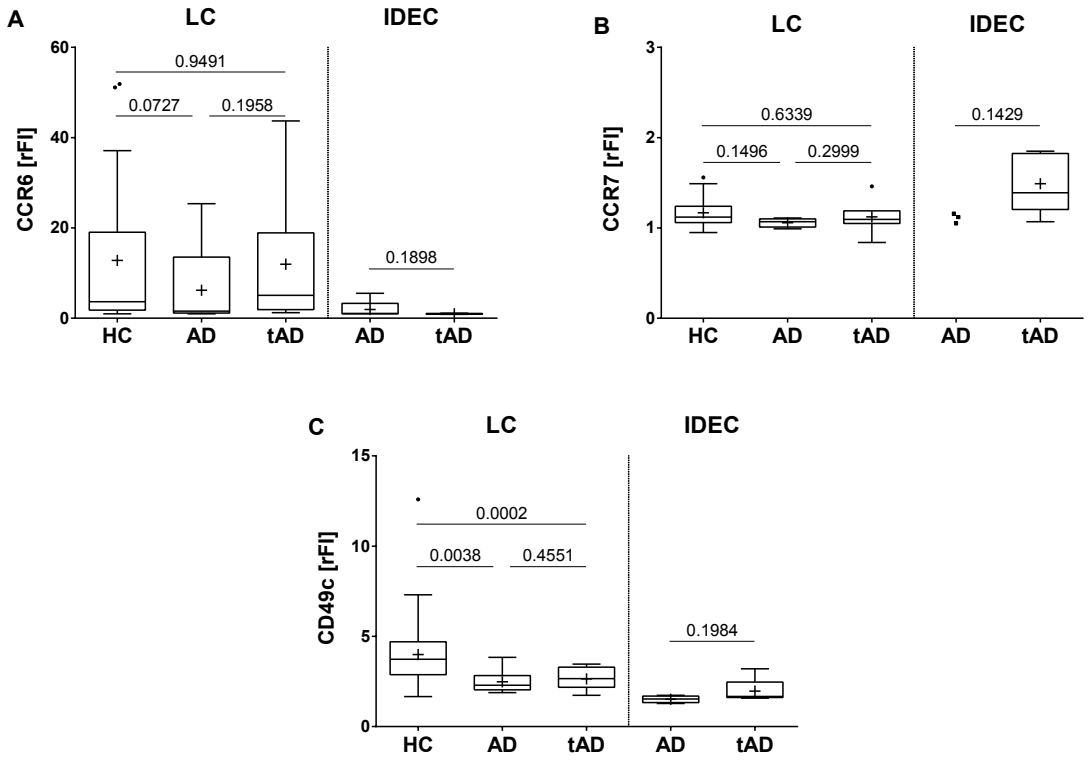

Figure 4.11. Receptors connected to migration are impaired in freshly isolated cells from AD patients.
The epidermal cell suspension was generated immediately from split thickness-skin and shave biopsies after 1 h trypsinization at 37 °C. The mean is marked by a + and the median by a vertical line inside the box. Outliers are marked as a black dot. Datasets with less than 4 samples are presented as individual values in black squares. (A) **CCR6** (n_{LC} = HC:16, AD:5, tAD:10; n_{IDEC} = AD:5, tAD:9), (B) **CCR7** (n_{LC} = HC:23, AD:5, tAD:8; n_{IDEC} = AD:3, tAD:5), and (C) **CD49c** (n_{LC} = HC:52, AD:6, tAD:14; n_{IDEC} = AD:4, tAD:5) were analysed using flow cytometry. The Mann-Whitney U test was used for independent samples.

On the other hand, CCR7 is involved in promoting migration to the lymph nodes and is usually expressed on activated cells [191]. CCR7 expression was comparably low on all LC, while only IDEC from tAD patients seemed to express the receptors at higher levels compared to their LC counterpart from tAD (p=0.0625, n=5, figure 4.11B).

CD49c is a transmembrane glycoprotein, which mediates cell adhesion and migration [192, 193]. It forms a heterodimer with integrin $\beta 1$ and is expressed on epidermal DC and to a greater extent on KC. Interestingly, CD49c was inversely expressed on LC and KC in healthy (low in KC, high in LC) and AD skin (high in KC, low in LC) as depicted in figure 4.11C and appendix figure 7.4C. Moreover, IDEC from AD (p=0.125, n=4, figure 4.11C) and tAD (p=0.0625, n=5, figure 4.11C) donors tend to express less CD49c compared to the LC population.

4.1.6 Culturing of split thickness-skin show low spontaneous maturation of LC

To analyse the impact of any external stimulus, the skin must be cultured over time. Therefore, different culturing approaches were compared to determine the best model for the analysis of isolated cells from *ex vivo* skin. These experiments were performed with skin from healthy donors since the disease-related bias could be neglected. Split thickness-skin was obtained from patients immediately after plastic surgery using a dermatome. Epidermal cell suspension, epidermis only and split thickness-skin were cultured without stimulation for 24 h. CD83 (figure 4.12A) and FcϵRIα (figure 4.12B) were analysed to determine the maturation status of each system [177]. LC from the epidermal cell suspension and epidermis only showed a strong maturation status in terms of high CD83 levels, while they were not able to maintain FcϵRIα expression after 24 h of culture. This was a strong indicator that those models were not suitable for analysing untouched LC. LC in the split thickness-skin did not show that degree of maturation. Furthermore, these LC maintained FcϵRIα after culture, while the CD83 expression was comparable to the background control. Therefore, this model was used for all further experiments since it had more advantages compared to the other models. Moreover, it reflects reality the most since the dermal compartment was a part of the skin and disruption thereof would cause the activation of maturation processes.

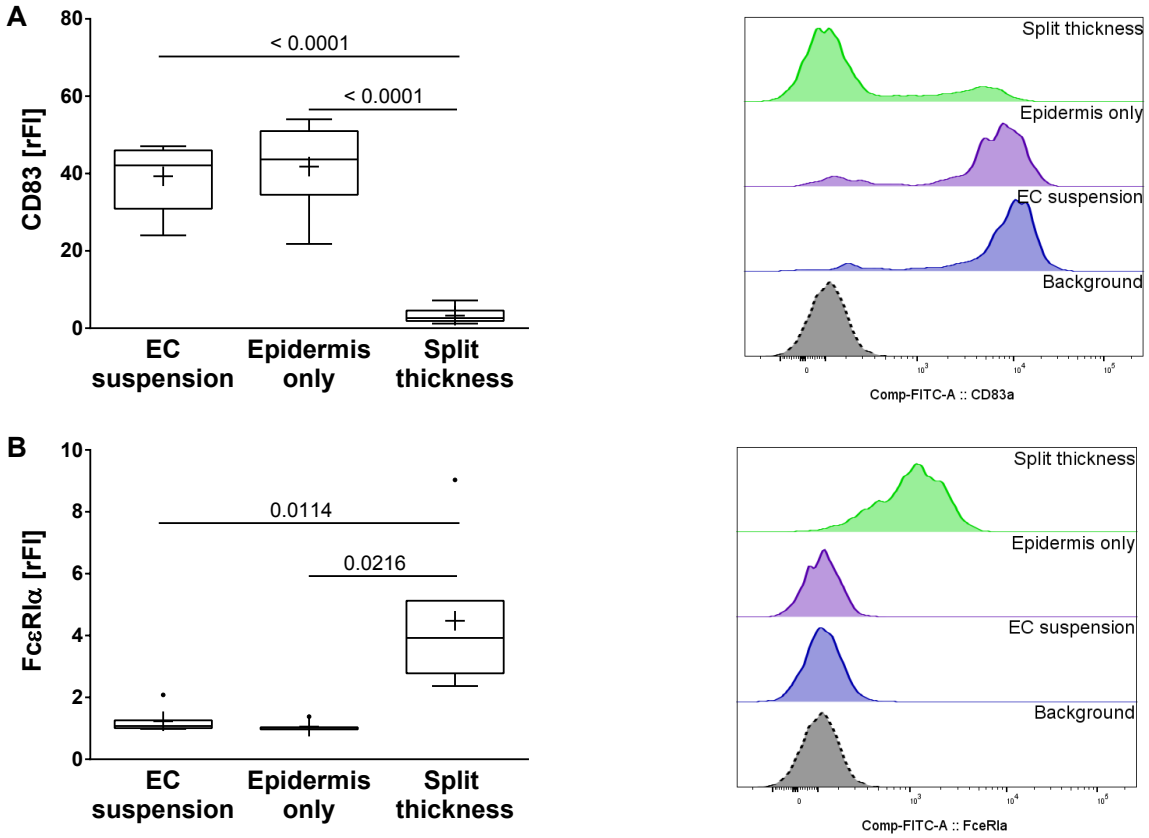

Figure 4.12. The *ex vivo* skin culture system shows the lowest spontaneous maturation of LC. Epidermal cell (EC) suspensions (10^6 cells/ml), epidermis only and split thickness-skin from healthy *ex vivo* material were cultured for 24 h without stimulation. The mean is marked by a + and the median by a vertical line inside the box. Outliers are marked as a black dot. After culture, the maturation markers (A) **CD83** (n=8) and (B) **FcεRIα** (n=4) of LC were compared between three different culture systems. The significant difference between these three groups was analysed by a one-way ANOVA followed by Bonferroni's multiple comparisons test. One representative experiment of each system was chosen for the histograms of CD83 and FcεRIα.

4.1.7 Summary of part I: phenotyping and validation of the *ex vivo* human skin system

Taken together, LC could be distinguished from IDEC using antibodies against CD1a and Langerin. IDEC expressed typical surface markers like CD36, CD206 and FcεRIα to a higher extent compared to LC from healthy donors. CD80 is a maturation marker which was significantly higher expressed in LC from AD skin compared to LC from healthy controls. Very important is the equally low expression of CD83, CD86, MHC class I and MHC class II on LC from AD skin compared to LC from healthy controls. This indicated that LC from AD and healthy epidermis had a similar maturation status. Interestingly,

surface TLR expression is heterogeneous but no significant difference could be observed between the groups, though LC from AD tended to express less TLR2. Furthermore, CCR6 and CD49c showed reduced expression levels in LC from AD skin. Of great importance was the establishment of an *ex vivo* culturing system where the culturing of split thickness-skin was superior compared to the other methods.

Table 4.1. Summary of the receptor expression of freshly isolated epidermal cells.
Symbols used in the table: +/- expression level.

Receptor	healthy LC	AD LC	AD IDEC
Langerin	+++	+++	-
CD1a	+++	+++	++
CD206	(+)	++	+++
CD36	-	++	+++
FcεRIα	+	++	+++
CD83	+	+	++
CD80	+	++	+++
CD86	+	+	+
CD40	+	+	+
MHC class I	+	+	+
MHC class II	+	+	+
TLR1	+	+	+
TLR2	+	(+)	+
TLR6	+	+	+
CCR6	+++	++	+
CCR7	(+)	(+)	+
CD49c	+++	+	+

4.2 Part II: LC and IDEC in AD are tolerized towards TLR2 activation

AD skin is heavily colonized by *S. aureus* and for this reason, its influence is of particular interest. The phenotype of LC from AD and healthy donors was similar and independent from steroid treatment. However, steroid treatment may influence the responsiveness of TLR2 ligation in the tAD group and for this reason, this subgroup was analysed separately. P3C, a synthetic analogue of the triacylated N-terminal part of bacterial lipoproteins, was used to mimic the ability of *S. aureus* to trigger the TLR1/2 pathway. The successfully established split thickness-skin culture system was used to analyse epidermal cells after P3C treatment. The experimental flow for this setting is depicted in figure 4.13.

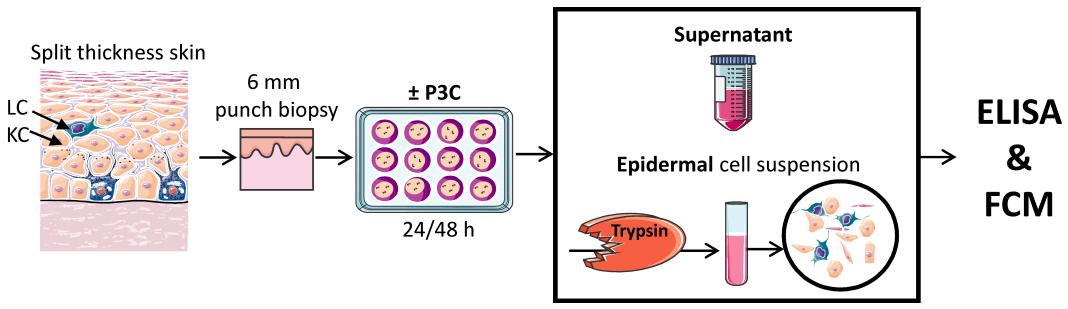

Figure 4.13. Experimental flow of P3C treated skin.
Healthy split thickness-skin was obtained from patients immediately after plastic surgery using a dermatome with a depth of 400 µm. AD skin was obtained by shave biopsies after local anaesthesia. The diameter of the punch biopsy was standardized to 6 mm. One punch biopsy was added to one well containing 1 ml medium with or without 10 µg/ml P3C. After 24 h culturing, the supernatant was collected, the epidermal cell suspension was generated, and analysed by flow cytometry (FCM) or ELISA.

4.2.1 TLR2-mediated maturation is impaired in LC and IDEC of AD *ex vivo* skin

The *ex vivo* skin was cultured for 24 h with or without P3C as described previously in chapter 3.1.3. Next, the epidermal cell suspension was prepared and stained with specific antibodies according to the respective protocol (chapter 3.5). Firstly, the influence of P3C on maturation processes and reactivity was addressed. For this purpose, the expression levels of CD83, TLR2, FcεRIα and CD40 were analysed at the cell surface.

Figure 4.14A shows the CD83 expression on healthy and AD skin. The corresponding histogram of one representative experiment is depicted below. LC from healthy and tAD skin responded to P3C with an up-regulation of CD83. In contrast, LC and IDEC from AD donors could not up-regulate the maturation marker compared to the unstimulated control. However, unstimulated LC from AD expressed the receptor at higher levels compared to healthy controls ($p=0.0002$, n: HC=51 and AD=21, figure 4.14A), while no difference could be observed after P3C treatment ($p=0.3380$, n: HC=51 and AD=21, figure 4.14A).

The TLR2 expression (figure 4.14B) was slightly reduced in unstimulated LC from AD compared to healthy controls, though this was not significant. Stimulation with P3C significantly reduced the TLR2 expression in LC from healthy and tAD skin, indicating that healthy and tAD were able to react towards TLR2 ligation. This is in contrast to LC from AD subjects which did not respond with a down-regulation of TLR2 by P3C stimulation. Moreover, IDEC showed a significantly reduced expression of TLR2 compared to LC in AD skin ($p=0.0059$, n=11, figure 4.14B), while IDEC from tAD did not show this trend ($p=0.1875$, n=5, figure 4.14B). Culturing (D0 vs. D1) reduced the TLR2 expression in unstimulated LC from healthy skin compared to freshly isolated cells ($p<0.0001$, n=37), while this was not the case for LC from AD ($p=0.8311$, n=15) nor tAD ($p=0.2500$, n=9). The culturing-mediated reduction of TLR2 expression in healthy LC further underlines the unresponsiveness towards TLR2 in AD patients. Furthermore, P3C ligation did not influence TLR2 expression on KC in any given group (appendix, figure 7.5A).

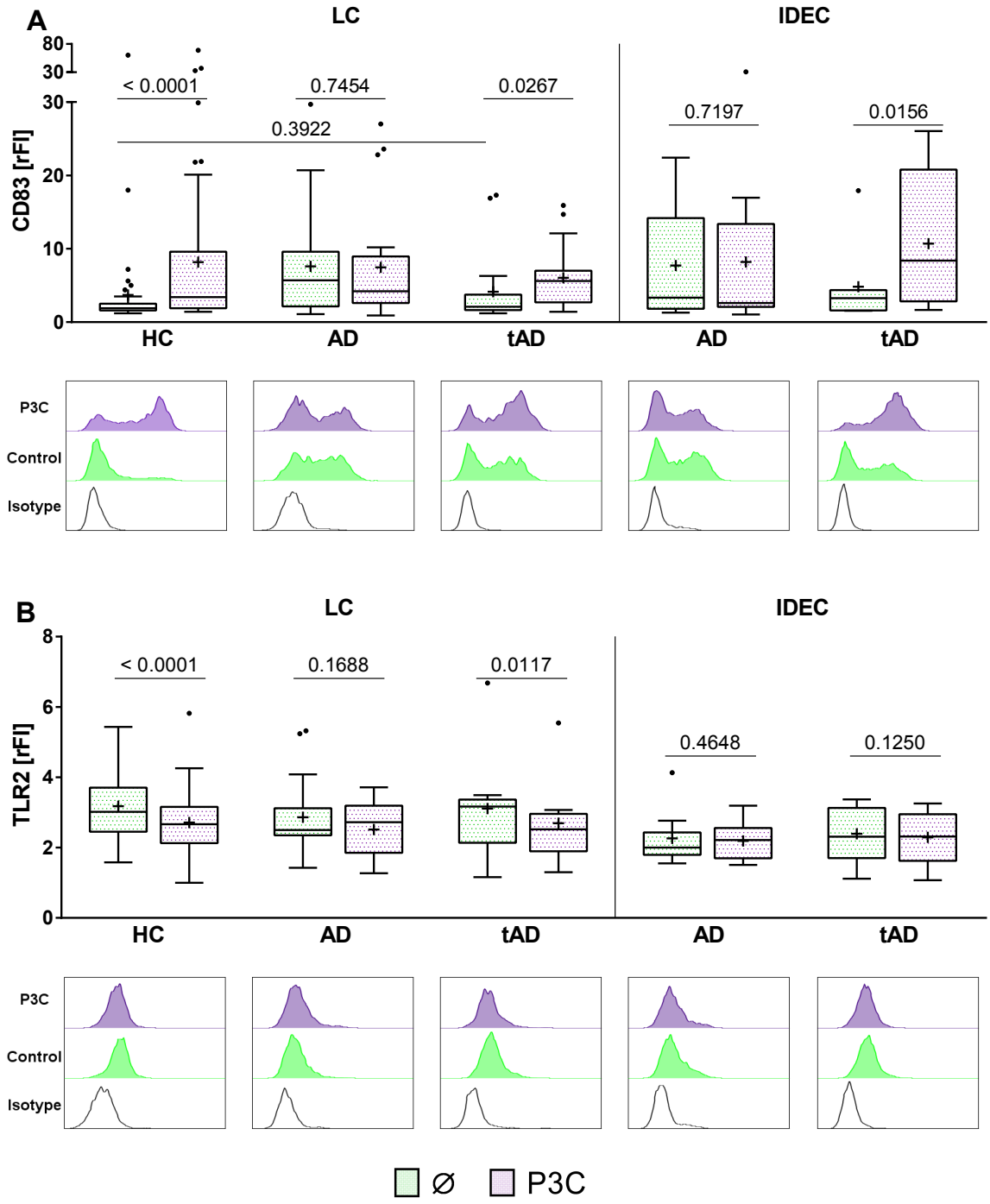

Figure 4.14. LC from HC and tAD, but not AD directly respond to TLR2 ligation.
Epidermal LC (Langerin$^+$, CD1a$^+$) and IDEC (Langerin$^-$, CD1a$^+$) were analysed by flow cytometry. (A) **CD83** expression (n_{LC} = HC:51, AD:21, tAD:17; n_{IDEC} = AD:15, tAD:7) and (B) **TLR2** expression (n_{LC} = HC:37, AD:15, tAD:9; n_{IDEC} = AD:11, tAD:5) were presented as a box plot. The mean is marked by a + and the median by a vertical line inside the box. Outliers are marked as a black dot. Control experiments (∅) are indicated in green and P3C in purple. One representative histogram was chosen for each population and is depicted below the corresponding box plot. The Wilcoxon matched-pairs signed-rank test was used for connected samples.

The response to P3C can be measured additionally by the change of the FcεRIα and CD40 receptors. FcεRIα (figure 4.15A) is expressed highly in unstimulated LC of AD

compared to healthy donors (p=0.0015, n: HC=45 and AD=19), while no significant difference between unstimulated LC from tAD and healthy skin (p=0.9716, n: HC=45 and tAD=11) could be observed with the Mann-Whitney U test. Despite the low FcεRIα expression on LC from healthy skin, P3C could significantly down-regulate the expression thereof. This was not the case for AD nor tAD skin where FcεRIα was expressed at a much higher degree. Surprisingly, the FcεRIα expression in unstimulated LC (D1, figure 4.15A) of healthy donors was up-regulated significantly (p=0.0075, n=45) compared to freshly isolated cells (D0, figure 4.6). LC from tAD (p=0.0005, n=11) and AD (p=0.0006, n=19) skin showed exactly the opposite tendency by reducing the FcεRIα expression during the culture when compared to freshly isolated cells (D0, figure 4.6).

The maturation marker CD40 (figure 4.15B) was enhanced significantly by P3C in LC from healthy donors. The inability to react with increased maturation processes by TLR2 ligation (as shown for CD83, figure 4.14B) was confirmed despite limited sample power for AD (figure 4.15B), witnessed by constant levels of CD40 expression. Furthermore, KC from healthy donors expressed CD40 at a low level and the Wilcoxon matched-pairs signed-rank test revealed a significant up-regulation (p=0.0034, n=17, appendix: figure 7.5B) with P3C. KC are known for their immunomodulatory ability to shape the microenvironment and influence the DC migration [194]. This may play an important role since CD40 is slightly higher expressed in freshly isolated KC (D0) from AD skin (appendix, figure 7.4B).

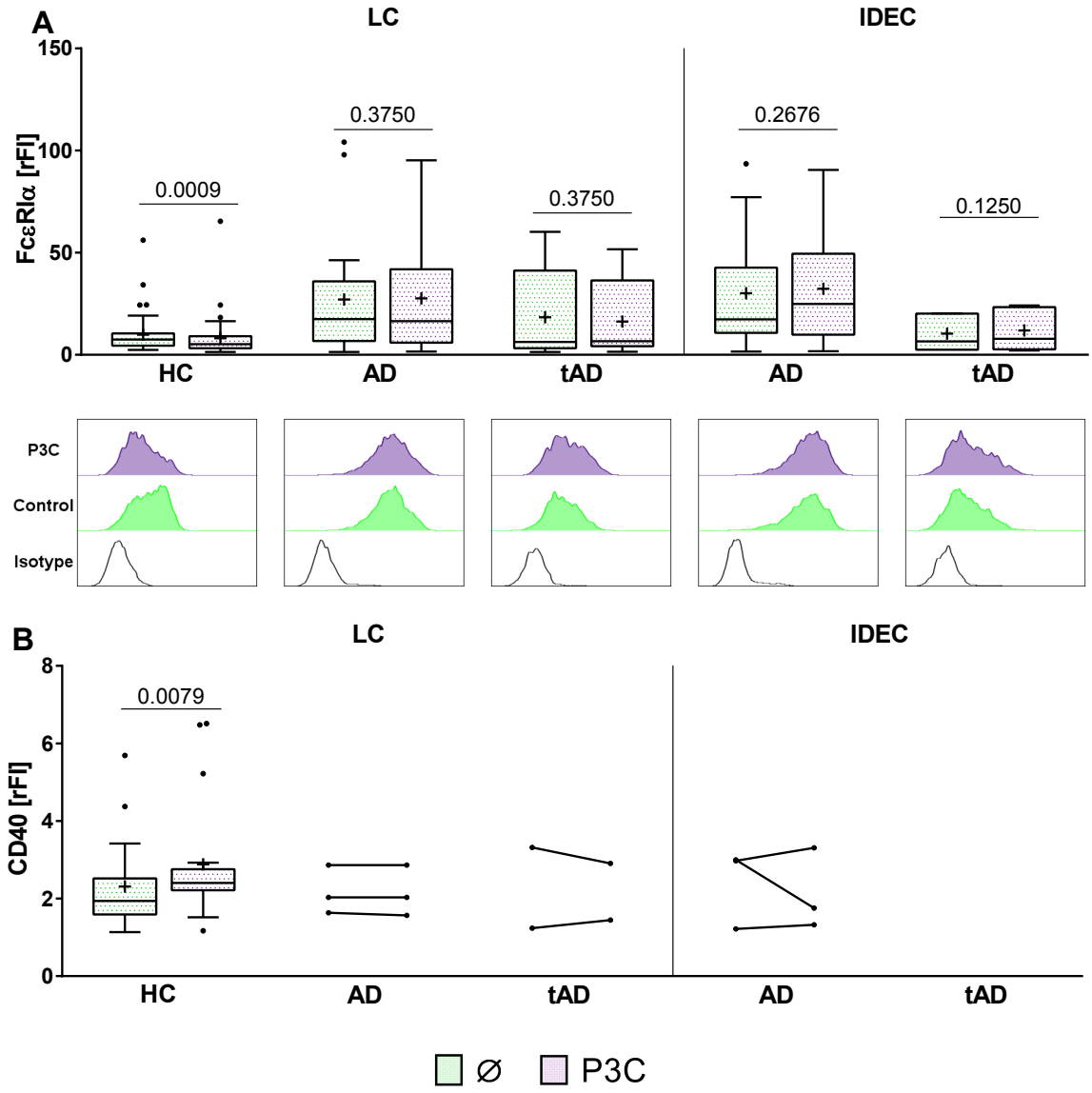

Figure 4.15. FcεRIα and CD40 expression are altered by TLR2 engagement in LC from healthy but not from AD skin.
Epidermal LC (Langerin$^+$, CD1a$^+$) and IDEC (Langerin$^-$, CD1a$^+$) were analysed by flow cytometry. (A) **FcεRIα** expression (n_{LC} = HC:45, AD:19, tAD:11; n_{IDEC} = AD:14, tAD:5) and (B) **CD40** expression (n_{LC} = HC:17, AD:3, tAD:2; n_{IDEC} = AD:3, tAD:0) are presented as a box plot. The mean is marked by a + and the median by a vertical line inside the box. Outliers are marked as a black dot. Control experiments (∅) are indicated in green and P3C in purple. Datasets with less than 4 samples are presented as connected lines from ∅ to P3C. One representative histogram was chosen for each population and is depicted below the corresponding box plot. The Wilcoxon matched-pairs signed-rank test was used for connected samples.

4.2.2 TLR2-mediated cytokine induction of split thickness-skin is impaired in AD subjects

The missing P3C-mediated increase of maturation markers in LC from AD donors lead to the question if the cytokine production in the supernatant was modified by P3C. For this reason, the cytokine production of the split thickness-skin from healthy and AD

donors was analysed after 24 h in the cell culture supernatant with or without P3C. Therefore, cytokines that are known to be altered by inflammation processes or TLR2-mediated activation, such as IL-1, hBD2, TSLP, IL-10, IL-11, TGF-β, and IL-12p40 were analysed in the supernatant. Unfortunately, it was not possible to determine the exact cellular source of each cytokine, since epidermal and dermal cells contributed to the microenvironment. However, this was an advantage since the cytokine microenvironment of the split thickness-skin is most likely very similar *in vivo*. The following cytokine concentrations were below the detection limit of the assay and could not be analysed: IL-2, IL-4, IL-5, IL-9, IL-12p70, IL-13, IL-15, IL-17A, IL-17F, IL-21, IL-22, IL-27, IL-33, IFN-α2, and IFN-γ. The standard curves are depicted in the appendix section (figure 7.6 and 7.7). The majority of cells in the cultivated split thickness-skin are KC and therefore the driving element. Figure 4.16 displays the analysed cytokines in the supernatant from split thickness-skin. The IL-1α expression (figure 4.16A) was not influenced by P3C, while no difference could be observed for the basal expression between the groups. This was not the case for IL-1β (figure 4.16B), where P3C increased the production significantly in healthy but not AD nor tAD supernatant. Here, the tAD showed a slight but not significant tendency of increased IL-1β expression after P3C treatment. Moreover, IL-1β expression was about 30-times higher in AD *ex vivo* skin supernatant compared to healthy controls. Interestingly, unstimulated tAD expressed IL-1β at an equal level compared to healthy controls (p=0.1281, n: HC=21 and tAD=11, figure 4.16B).

The human β-defensin-2 (hBD2, figure 4.16C) was up-regulated in healthy *ex vivo* skin by P3C but not in AD nor tAD. The hBD2 concentration of P3C treated healthy skin was similarly high as the level of AD, independent from the applied stimulus. However, this was a strong indicator that AD and even tAD produce hBD2 at a much higher constitutive level than healthy controls. The production of TSLP (figure 4.16D) was increased by P3C in healthy but not in AD or tAD *ex vivo* skin. Some donors of the tAD group indeed respond to P3C treatment by inducing TSLP. Surprisingly, the TSLP concentration was equally low in AD or tAD supernatant, indicating that TSLP may not have the important role in this disease as thought previously. [138, 195, 196].

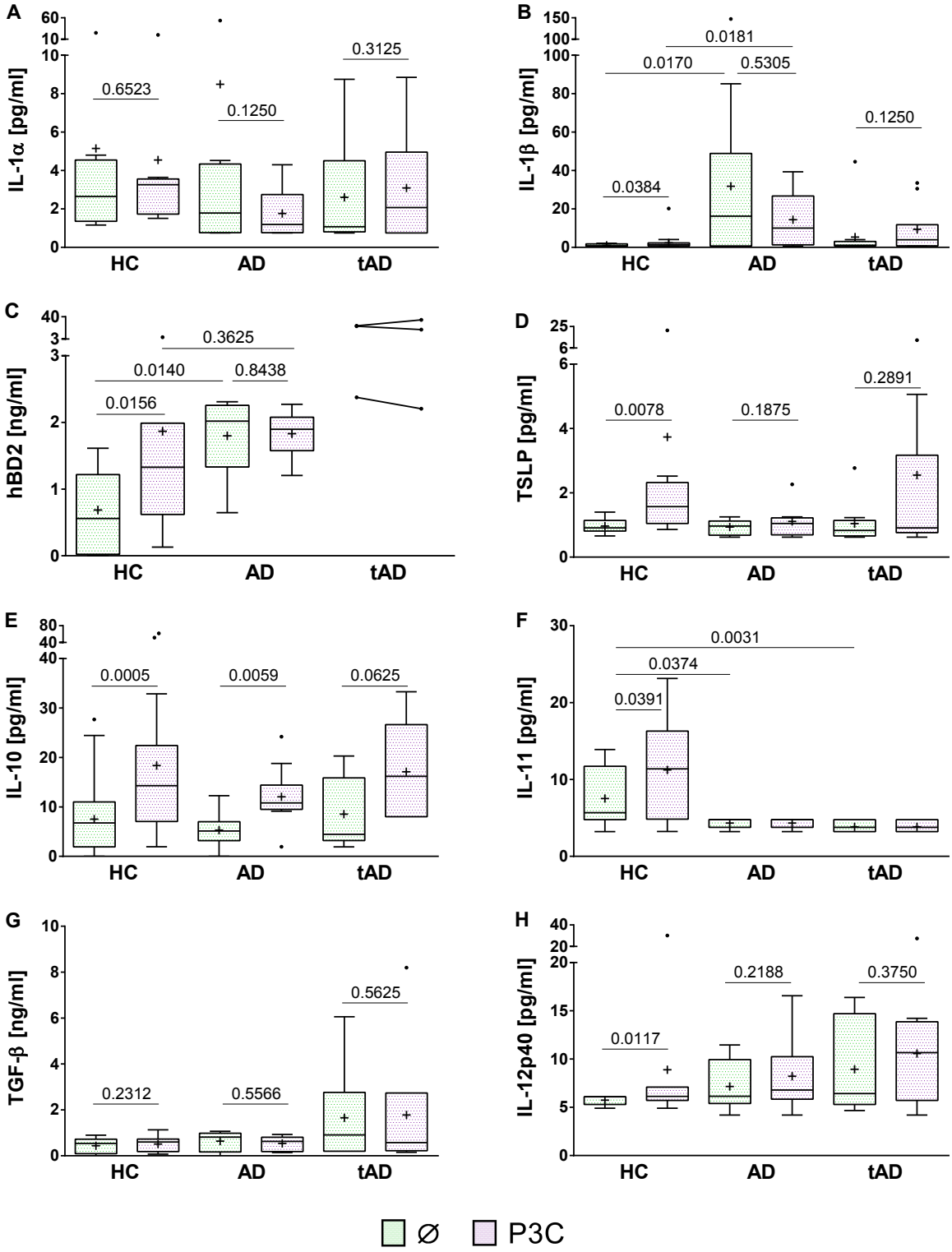

Figure 4.16. Cytokine production is impaired in AD.
Ex vivo human skin was cultured with or without P3C for 24 h. The cytokine expression of (A) **IL-1α** (n=HC:9, AD:8, tAD:11), (B) **IL-1β** (n=HC:21, AD:14, tAD:11), (C) **TSLP** (n=HC:9, AD:8, tAD:11), (D) **hBD2** (n=HC:7, AD:6, tAD:3), (E) **IL-10** (n=HC:19, AD:10, tAD:5), (F) **IL-11** (n=HC:9, AD:8, tAD:11), (G) **TGF-β** (n=HC:16, AD:10, tAD:6), and (H) **IL-12p40** (n=HC:9, AD:8, tAD:11) were analysed in the supernatant by ELISA or flow cytometry. The mean is marked by a + and the median by a vertical line inside the box. Outliers are marked as a black dot. Control experiments (∅) are indicated in green and P3C in purple. Datasets with less than 4 samples are presented as connected lines from ∅ to P3C. The Wilcoxon matched-pairs signed-rank test was used for connected and the Mann-Whitney U for independent samples.

IL-10 (figure 4.16E) was significantly up-regulated by P3C in healthy and AD *ex vivo* skin, whereas the tAD showed the same trend (p=0.0625, n=5). Healthy skin tended to express more IL-10 despite the stimulus in comparison to AD. IL-11 (figure 4.16F) was up-regulated by P3C in healthy donors and not in AD nor tAD. The finding that AD and tAD expressed much lower levels of IL-11 compared to healthy skin was striking. The TGF-β (figure 4.16G) concentration was not influenced by P3C treatment in any group. Moreover, the expression was similar in all groups. Like TGF-β, IL-12p40 (figure 4.16H) was not affected by P3C treatment but the expression was slightly increased in AD, though this was not significant.

4.2.3 AD *ex vivo* skin fail to activate CD4 positive T cells upon TLR2 ligation

As a next step, the ability of epidermal DC to induce and activate T cells was analysed. Therefore, the co-stimulatory molecules CD80 and CD86 (figure 4.17), which are known for their important role in T cell activation, were analysed using flow cytometry. P3C increased the CD80 expression in LC from healthy and tAD donors. Furthermore, the CD80 expression was significantly higher in untreated LC of AD and tAD skin compared to LC from healthy donors. This reflects the status of freshly isolated cells from AD and tAD where CD80 was higher expressed (figure 4.8C). However, the expression of CD80 was low in freshly isolated (D0) healthy LC, while it clearly rose during culturing, confirming it was a sensitive marker for maturation processes. The co-receptor CD86 (figure 4.17B) showed a similar behaviour like CD80, where only LC from healthy skin were able to increase CD86 during P3C engagement. LC and IDEC from tAD tend to increase the CD86 expression after P3C application. Indicating that the unresponsiveness towards P3C ligation is true for CD80 and CD86 in AD. Unlike CD80, CD86 expression was not significantly higher in untreated LC of AD or tAD skin when compared to LC from healthy donors. However, both populations express the co-receptors for T cell activation, therefore they should *per se* be able to induce any T cell activation.

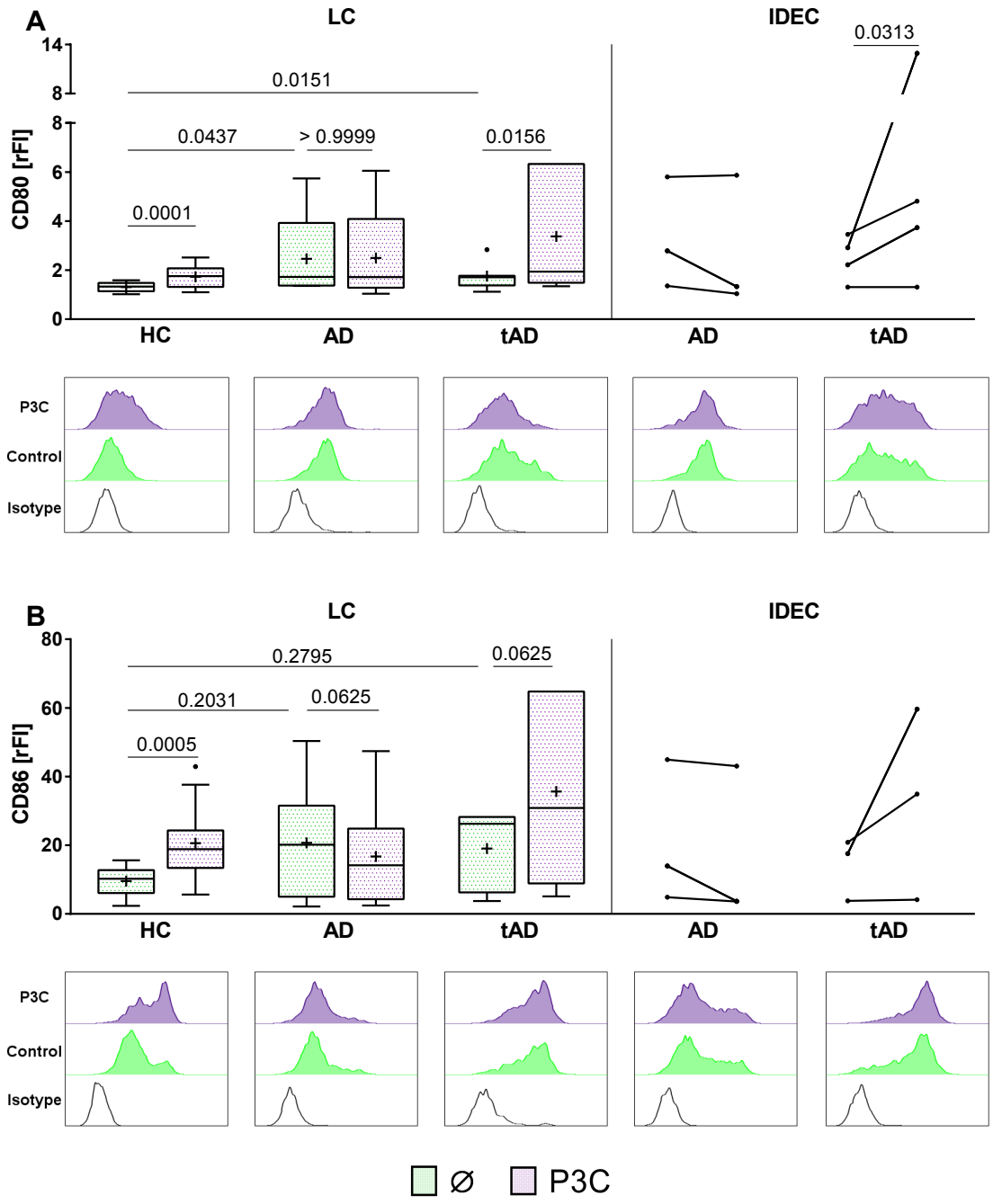

Figure 4.17. Co-receptors responsible for T cell activation are impaired in LC from AD patients. Epidermal LC (Langerin$^+$, CD1a$^+$) and IDEC (Langerin$^-$, CD1a$^+$) were analysed by flow cytometry. (A) **CD80** expression (n_{LC} = HC:14, AD:5, tAD:5; n_{IDEC} = AD:3, tAD:4) and (B) **CD86** expression (n_{LC} = HC:13, AD:5, tAD:4; n_{IDEC} = AD:3, tAD:3) are presented as a box plot. The mean is marked by a + and the median by a vertical line inside the box. Outliers are marked as a black dot. Control experiments (∅) are indicated in green and P3C in purple. Datasets with less than 4 samples are presented as connected lines from ∅ to P3C. One representative histogram was chosen for each population and is depicted below the corresponding box plot. The Wilcoxon matched-pairs signed-rank test was used for connected samples.

To address the issue regarding the T cell activation, receptors known to affect the adaptive immune system in different ways were analysed by flow cytometry. MHC class I and MHC class II are special receptors which induce different T cell responses, namely

cytotoxic CD8$^+$ T cells or CD4$^+$ T helper cells. MHC class I is expressed on all cells with a nucleus and plays a significant role during viral infection.

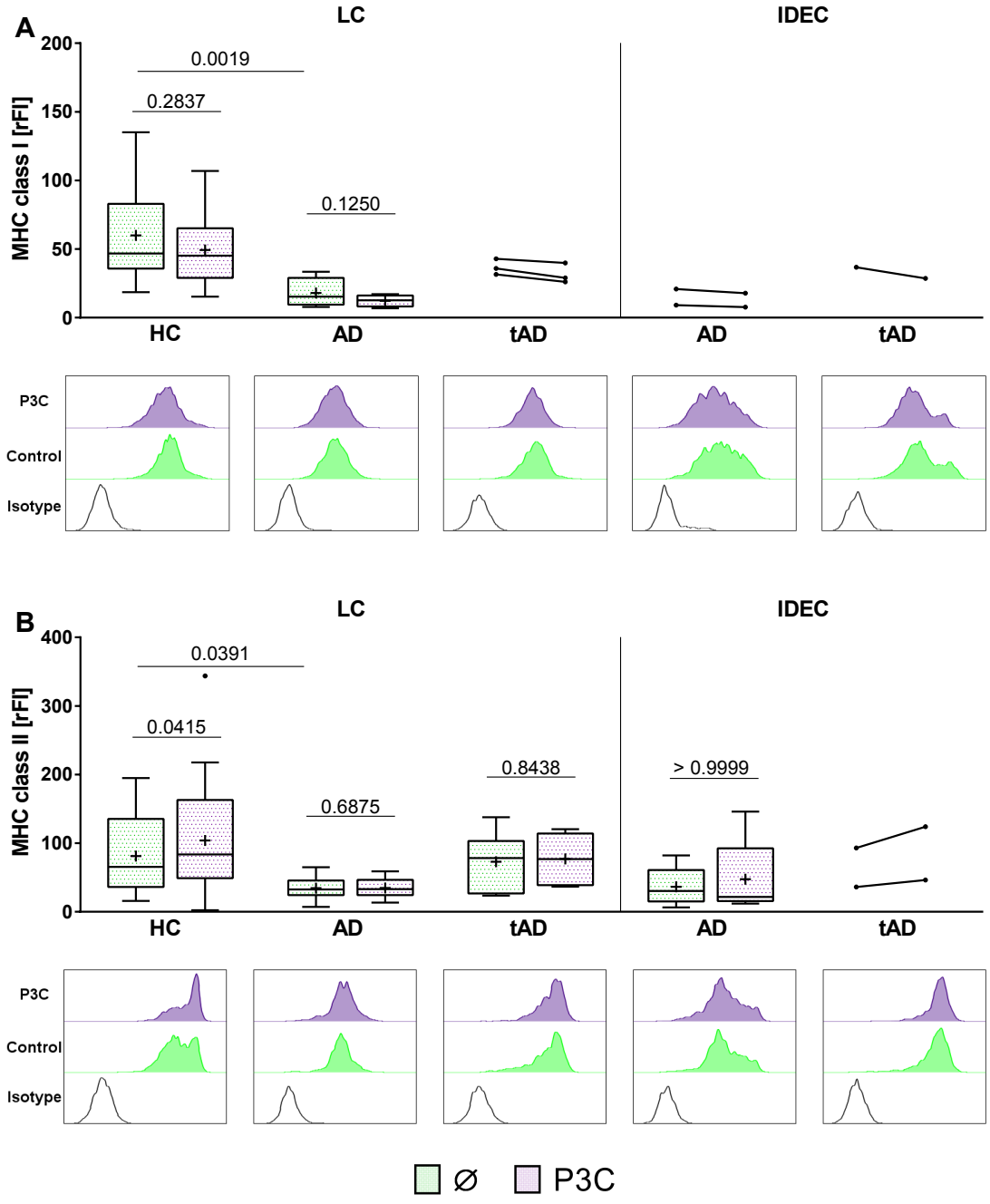

Figure 4.18. MHC expression is not influenced by TLR2 ligation on LC form AD donors. Epidermal LC (Langerin$^+$, CD1a$^+$) and IDEC (Langerin$^-$, CD1a$^+$) were analysed by flow cytometry. (A) **MHC class I** expression (n_{LC} = HC:18, AD:4, tAD:3; n_{IDEC} = AD:2, tAD:1) and (B) **MHC class II** expression (n_{LC} = HC:23, AD:6, tAD:6; n_{IDEC} = AD:5, tAD:2) are presented as a box plot. The mean is marked by a + and the median by a vertical line inside the box. Outliers are marked as a black dot. Control experiments (∅) are indicated in green and P3C in purple. Datasets with less than 4 samples are presented as connected lines from ∅ to P3C. One representative histogram was chosen for each population and is depicted below the corresponding box plot. The Wilcoxon matched-pairs signed-rank test was used for connected samples.

On the other side, MHC class II is mainly expressed on professional antigen-presenting cells. MHC class I (figure 4.18A) expression was not influenced by P3C on LC or KC (appendix, figure 7.5C) in any group. Additionally, LC from AD skin expressed the receptor at a significantly lower level than LC from healthy skin after 24 h of culturing. P3C ligation leads to an upregulation of MHC class II (figure 4.18B) in LC from healthy but not from AD or tAD skin. Surprisingly, LC from AD skin expressed MHC class II at a much lower level when compared to healthy controls, while the comparison of healthy vs. tAD (p=0.8226, n: HC=23 and tAD=6) or AD vs. tAD (p=0.2381, n: AD=6 and tAD=6) did not show any difference. This is a interesting observation, pointing to possible influence on the T cell activation despite the expression of CD80 or CD86 (figure 4.17). Moreover, the slightly higher MHC class II expression on LC from tAD indicated that the tAD group changed their LC phenotype towards a more healthy-like direction 4.18.

As a consequence of impaired receptor expression responsible for T cell activation, a mixed lymphocyte reaction (MLR) was implemented in the experimental flow. This should clarify whether LC from AD can induce any T cell activation. Naïve $CD4^+$ T cells were isolated (see chapter 3.3) and the purity of the $CD4^+$ T cells was > 95 % as determined by flow cytometry (data not shown). Human *ex vivo* skin was cultured for 24 h with or without P3C and afterwards an epidermal cell suspension was prepared. Figure 4.19 shows the generic workflow of this experimental setup. The T cell activation was analysed by a sensitive proliferation assay where 1 μCi ^{3}H-thymidine was added after six days of co-culture for additional 24 h (see chapter 3.4). Afterwards, the incorporation of ^{3}H-thymidine was measured in the harvested cells. T cells without any addition of cells or stimuli were used as a background proliferation control where ^{3}H-thymidine incorporation revealed significantly fewer counts per minute indicating a low proliferation (data not shown).

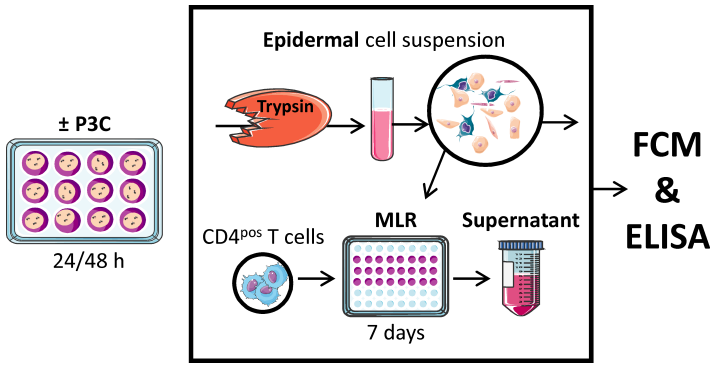

Figure 4.19. Experimental flow: analyses of MLR.
Healthy split thickness-skin was obtained from patients immediately after plastic surgery using a dermatome with a depth of 400 µm. AD skin was obtained by shave biopsies after local anaesthesia. One 6 mm punch biopsy was added to one well containing 1 ml medium with or without 10 µg/ml P3C. After 24 h culturing, the epidermal cell suspension was generated, which was mixed in equal parts with naïve $CD4^+$ cells. They were co-cultured for 7 days and afterwards the supernatant was analysed by flow cytometry or ELISA.

The executed MLR was an alloreaction since the T cells were isolated from peripheral blood mononuclear cells from different donors. P3C treated *ex vivo* epidermal cells were able to significantly increase the proliferation in healthy but not in AD MLR (figure 4.20). This indicated that only P3C treated healthy LC increased the proliferation rate beyond the background of the alloreaction when compared to AD. Nevertheless, the background induced by the alloreaction was similarly high in healthy and AD, indicating a defect in the epidermal cells from AD donors rather than in the T cells.

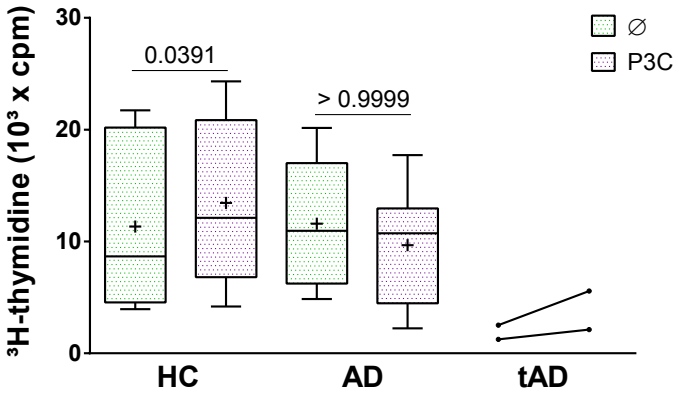

Figure 4.20. TLR2 stimulated epidermal DC from AD skin fail to induce T cell proliferation.
Ex vivo human skin was cultured with or without P3C for 24 h. An epidermal cell suspension was generated which was mixed 1:1 with naïve $CD4^+$ T cells and cultured for 6 days. Afterwards, 1 µCi ^{3}H-thymidine was added for 24 h and its incorporation (n= HC:8, AD:6, tAD:2) was measured. The mean is marked by a + and the median by a vertical line inside the box. Outliers are marked as a black dot. Control experiments (∅) are indicated in green and P3C in purple. Datasets with less than 4 samples are presented as connected lines from ∅ to P3C. The Wilcoxon matched-pairs signed-rank test was used for connected samples.

As a next step, the capability of epidermal DC to polarize naïve $CD4^+$ T cells was

investigated (figure 4.21). The supernatant was collected after seven days of co-culture and the Th specific cytokine expressions were analysed by flow cytometry using the Legendplex™ kit and ELISA. The standard curves of each analysed Th cytokine are depicted in the appendix section (figure 7.7). As an additional background control, T cells were cultured without any addition of cells or stimuli. The cytokine concentration thereof was always below the detection limit (data not shown). This observation was in line with the low ^{3}H-thymidine incorporation of same control in the proliferation assay.

IL-2 is produced by activated T cells and known for its proliferation-inducing activity [197]. This is especially interesting since P3C treated *ex vivo* skin produced more IL-2 in healthy and to a much lesser degree in AD MLR, though this was not significant. This could be linked to the proliferation assay (figure 4.20) where healthy P3C treated skin induced specific proliferation compared to the allogenic basal proliferation in AD. Moreover, IL-2 was higher expressed in the MLR supernatant of healthy compared to AD, which may be an additional reason as to why AD skin failed to induce T cells at all in this *in vitro* system. Surprisingly, IL-4 and IL-5 were not influenced by P3C but they were significantly higher expressed in the MLR supernatant of healthy compared to AD donors. However, both are IL-2 dependent, which was lower expressed in AD. IL-6 was not changed by P3C but it was highly expressed in the MLR supernatant of AD. In contrast, IFN-γ was barely detectable in AD but much higher expressed in the MLR supernatant of healthy controls. IFN-γ was slightly up-regulated by P3C treatment, though this was not significant. Interestingly, IL-9 expression was up-regulated by P3C treatment in healthy but not AD MLR supernatant, though some of the AD skins were able to respond to P3C by an increase of IL-9. However, the concentration did not differ significantly between the groups. The expression of IL-17A was influenced strongly by P3C in healthy but not in AD MLR supernatant. IL-17A was expressed lower in AD when compared to healthy MLR supernatant though this was not significant.

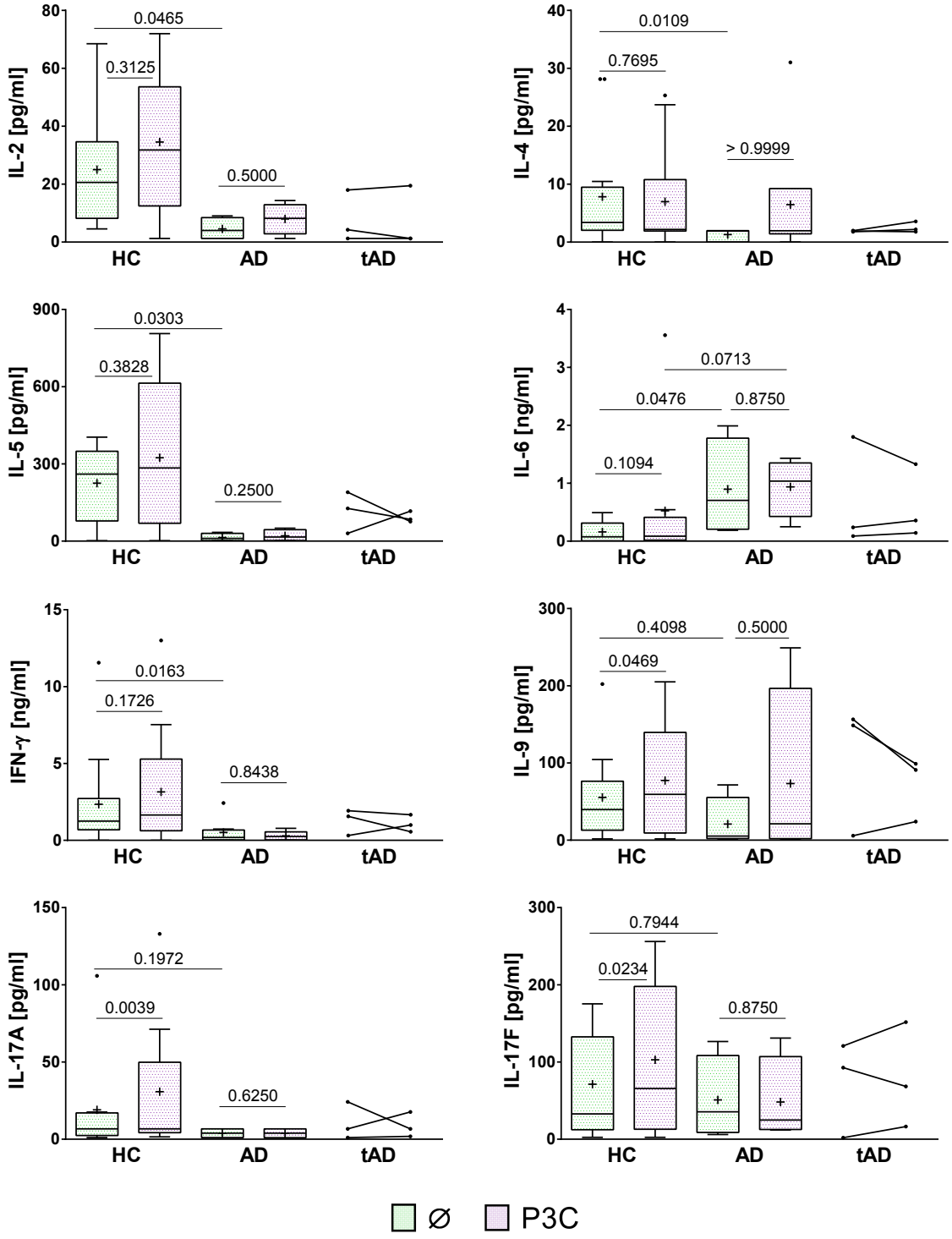

Figure 4.21. Cytokines produced by Th cells are reduced in AD.
Ex vivo human skin was cultured with or without P3C for 24 h. Subsequently, an epidermal cell suspension was generated which was mixed 1:1 with allogenic naïve $CD4^+$ cells co-cultured for seven days. The supernatant was harvested and the cytokine expression of **IL-2** (n=HC:9, AD:4, tAD:3), **IL-4** (n=HC:13, AD:6, tAD:3), **IL-5** (n=HC:9, AD:4, tAD:3), **IL-6** (n=HC:9, AD:4, tAD:3), **IFN-γ** (n=HC:14, AD:8, tAD:4) **IL-9** (n=HC:9, AD:4, tAD:3), **IL-17A** (n=HC:9, AD:4, tAD:3), and **IL-17F** (n=HC:9, AD:4, tAD:3) was analysed by ELISA or flow cytometry. The mean is marked by a + and the median by a vertical line inside the box. Outliers are marked as a black dot. Control experiments are indicated in green and P3C in purple. Datasets with less than 4 samples are presented as connected lines from ∅ to P3C. The Wilcoxon matched-pairs signed-rank test was used for connected and the Mann-Whitney U for independent samples.

An additional ELISA specific for IL-17A was performed with the help of Kazumasa Iwamoto and a similar tendency was observed (appendix figure 7.9) underlining the incapability of epidermal DC from AD skin to induce Th17 cells. P3C up-regulated the IL-17F expression in healthy but not in AD MLR, while the concentration was equal in untreated MLR supernatant from all groups. The expression levels of IL-13, IL-22, TNF-α and IL-10 cytokines were expressed similarly in the MLR supernatant from healthy and AD (appendix figure 7.8). Interestingly, only IL-10 from healthy donors was up-regulated by P3C (appendix figure 7.8).

4.2.4 Summary of Part II: LC and IDEC in AD are tolerized towards TLR2 activation

Taken together, P3C treatment induced a significant change in healthy LC, while LC from AD skin failed to react comparably. TLR2 ligation induced maturation processes, witnessed by induction of CD83, CD40, CD80, CD86 and MHC class II while TLR2 and FcεRIα were reduced in healthy but not in AD skin. It seems that steroid treatment shifts the LC reactivity from tAD to a more healthy-like direction, witnessed by TLR2, CD83, CD80, and CD86 (figure 4.14 and 4.17), but the evidence is limited. Moreover, only healthy *ex vivo* skin was able to induce the expression of IL-1β, hBD2, TSLP, and IL-11 by P3C treatment. AD skin expressed more IL-1β and hBD2 and much less IL-11 than the healthy skin, indicating that desensitization, tolerance or intrinsic alterations of epidermal LC are key in AD. Additionally, P3C-stimulated epidermal cells from healthy donors increased the proliferation rate above the alloreaction background of the MLR when compared to AD skin. Further evidence for tolerance is the lack of T cell-specific cytokine production in the MLR. Healthy MLR supernatant revealed a much higher concentration of IL-2, IL-4, IL-5, IFN-γ and lower IL-6 concentrations compared to AD, suggesting that AD skin is not able to induce T cell responses. Striking was the inability of AD epidermal cells to induce Th17 cells, witnessed by missing IL-17 induction compared to healthy donors.

Table 4.2. Summary of the P3C induced alteration of (i) receptors on LC and (ii) cytokines in the supernatant from healthy and AD skin.
The table shows a summary of the receptor and cytokine expression after P3C application, when compared to the unstimulated control. Symbols used in the table: +: increased expression, -: decreased expression, ∅: no alteration, (): tends to a direction.

Receptor alteration on LC	healthy	AD	tAD
CD83	+++	∅	++
CD80	+++	∅	+
CD86	+++	∅	(+)
CD40	+++	∅	∅
MHC class I	(-)	(-)	(-)
MHC class II	+	∅	∅
TLR2	- -	∅	-
FcεRIα	- -	∅	∅
Cytokine alteration in the supernatant	**healthy**	**AD**	**tAD**
IL-1α	∅	∅	∅
IL-1β	+	∅	(+)
hBD2	+	∅	∅
TSLP	++	∅	(+)
IL-10	+	+	+
IL-11	+	-	-
TGF-β	∅	∅	∅
IL-12p40	+	∅	(+)

4.3 Part III: LC from AD patients exhibit a TLR2 independent high spontaneous migration rate

During the experimental flow of this thesis, culturing of healthy and AD *ex vivo* skin and its stimulation with P3C was an essential part. One observation was obvious, the amount of migrated cells in experiments with AD skin was much higher when evaluated under the microscope. To address this issue, a migration assay was performed where the size of the skin was standardized and migrated cells were collected, counted and stained with antibodies specific for CD1a and Langerin to analyse the quality and quantity of migrated cells. The flow of the experiment is depicted in figure 4.22.

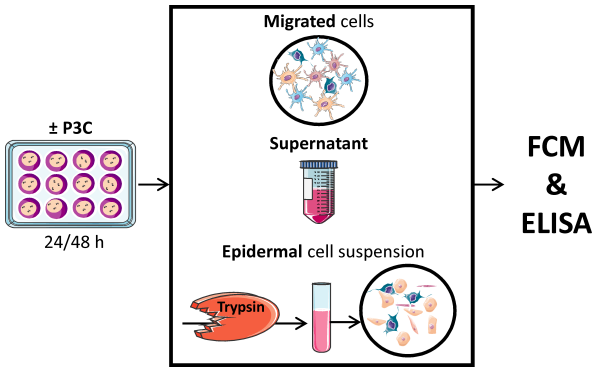

Figure 4.22. Experimental flow: analysis of the migration activity.
Healthy split thickness-skin was obtained from patients immediately after plastic surgery using a dermatome with a depth of 400 μm. AD skin was obtained by shave biopsies after local anaesthesia. One 6 mm punch biopsy was added to one well containing 1 ml medium with or without 10 μg/ml P3C. After 24 h culturing, the supernatant was collected and the epidermal cell suspension was generated, which were analysed by flow cytometry (FCM) or ELISA. For the migration assay, the emigrated cells were collected after 24 h and 48 h and analysed by FCM.

It was clear that LC from AD split thickness-skin had a high spontaneous migration rate after 24 h, in contrast to few migrated cells from healthy skin (figure 4.23). To understand the migration induction in healthy and AD *ex vivo* skin, the experiment was redesigned and a new condition was introduced where the *ex vivo* split thickness-skin was cultured for 48 h with or without P3C. While spontaneous migration of unstimulated LC from healthy skin was hardly detectable, the addition of P3C led to a significant induction of LC migration after 48 h (figure 4.23). In contrast, LC from AD patients showed a strong spontaneous migratory activity, which was even further increased after 48 h. There was no evidence for a further impact on the migration rate of LC from AD skin in response to P3C. $CD1a^{+}$/$Langerin^{-}$ cells, such as dermal DC, macrophages, and IDEC (only in AD skin), showed a similar migratory activity when compared to LC from healthy and AD donors (data not shown).

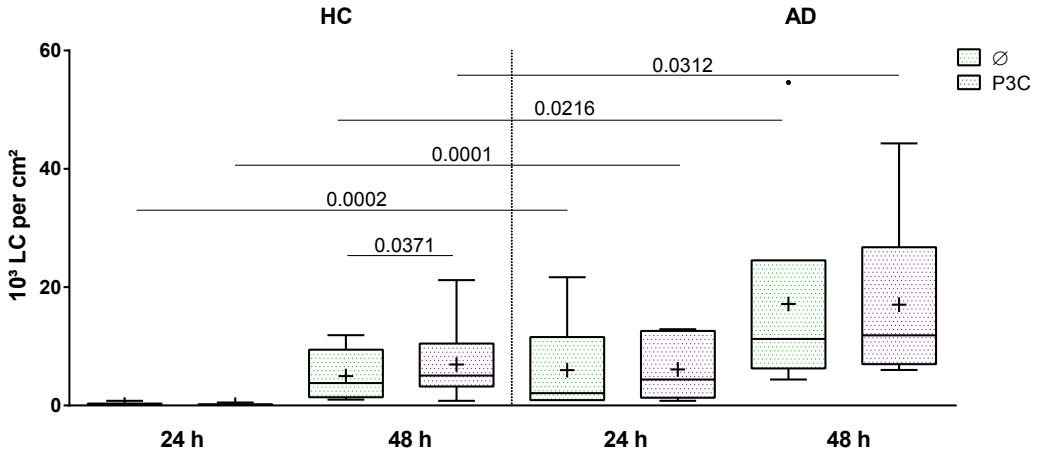

Figure 4.23. TLR2 ligation enhances the emigration of LC from healthy but not from AD skin.
Ex vivo human skin was cultured with or without P3C for 24 h and 48 h, respectively. Emigrated cells were counted after the indicated time points and dead cells were excluded by trypan blue staining. The number of migrated LC was calculated based on flow cytometry analysis of CD1a and Langerin expression of healthy controls (n=10) and AD (n=6). The mean is marked by a + and the median by a vertical line inside the box. Outliers are marked as a black dot. Control experiments (∅) are indicated in green and P3C in purple. The Wilcoxon matched-pairs signed-rank test was used for connected and the Mann-Whitney U test for independent samples.

To understand the cause for the increased emigration rate, migration-influencing cytokines were analysed. For this purpose, split thickness-skin was treated with or without P3C as described in chapter 3.1.3 and cultured for 24 h. Next, the supernatant was collected and frozen at -80 °C and the expression of cytokines was analysed at a later time point. The standard curves of the cytokine analyses are attached in the appendix section (figure 7.6). The correlation coefficient r^2 of the standard curves was always between 0.99 and 1. The amount of IL-6 was significantly enhanced by TLR2 ligation in healthy but not in AD skin, whereas unstimulated AD skin produced 15 times less IL-6 than the level of healthy skin and the concentration was not further enhanced upon TLR2 ligation (figure 4.24A). Interestingly, this is in contrast to analysed MLR supernatant where more IL-6 could be detected in the MLR of AD (figure 4.21). IL-18 was considerably higher expressed in the supernatant of AD and tAD compared to healthy skin, while TLR2 ligation did not alter the IL-18 expression significantly in any group (figure 4.24B). TNF-α (figure 4.24C) was much higher expressed in the supernatant of AD compared to healthy controls, while P3C up-regulated TNF-α expression in healthy but not in AD skin. Furthermore, the chemoattractant CCL20 (figure 4.24D) was significantly elevated in unstimulated AD skin compared to healthy controls. This may explain the low CCR6

expression in freshly isolated (D0) LC and IDEC from AD skin (figure 4.11) since binding of CCL20 to CCR6 leads to an internalization of CCR6 [198]. Nevertheless, P3C up-regulated the expression of CCL20 significantly in healthy and AD skin.

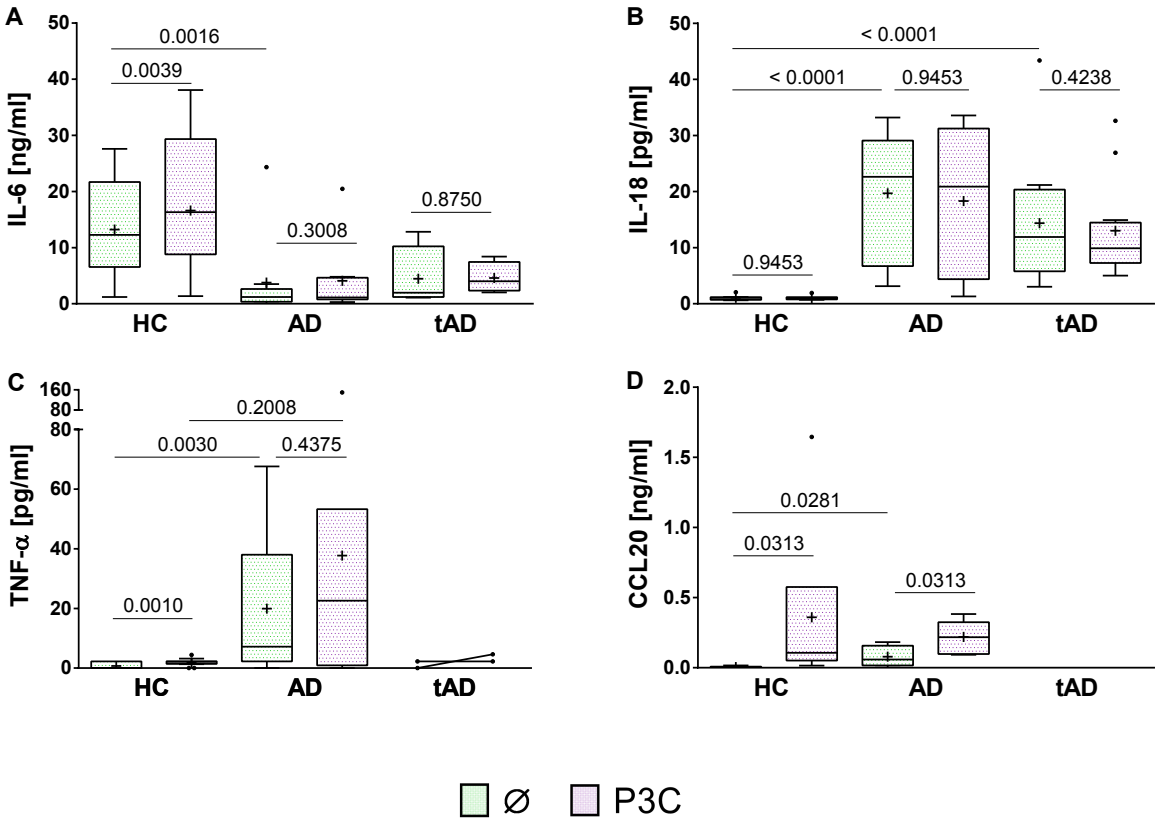

Figure 4.24. Cytokine production linked to migration is impaired in AD *ex vivo* skin.
Ex vivo human skin was cultured with or without P3C for 24 h. The cytokine expression of (A) **IL-6** (n=HC:19, AD:9, tAD:4), (B) **IL-18** (n=HC:9, AD:8, tAD:11), (C) **TNF-α** (n=HC:13, AD:7, tAD:2), and (D) **CCL20** (n=HC:6, AD:6, tAD:0) was analysed in the supernatant by ELISA or flow cytometry. The mean is marked by a + and the median by a vertical line inside the box. Outliers are marked as a black dot. Control experiments (∅) are indicated in green and P3C in purple. Datasets with less than 4 samples are presented as connected lines from ∅ to P3C. The Wilcoxon matched-pairs signed-rank test was used for connected and the Mann-Whitney U for independent samples.

The assay revealed a major difference in the basal cytokine expression (especially of IL-6 and IL-18). The question rose if receptors connected to migration are impaired in AD skin by P3C treatment. The expression of CD49c was significantly reduced due to culturing (D1) in unstimulated LC (p=0.0009, n=15) and KC (p=0.0015, n=15) compared to freshly isolated cells (D0, figure 4.11C) from healthy donors. Interestingly, CD49c was slightly down-regulated by P3C (figure 4.25A) in LC from healthy skin. In contrast, P3C seemed to increase CD49c in LC from AD patients, though this was not significant.

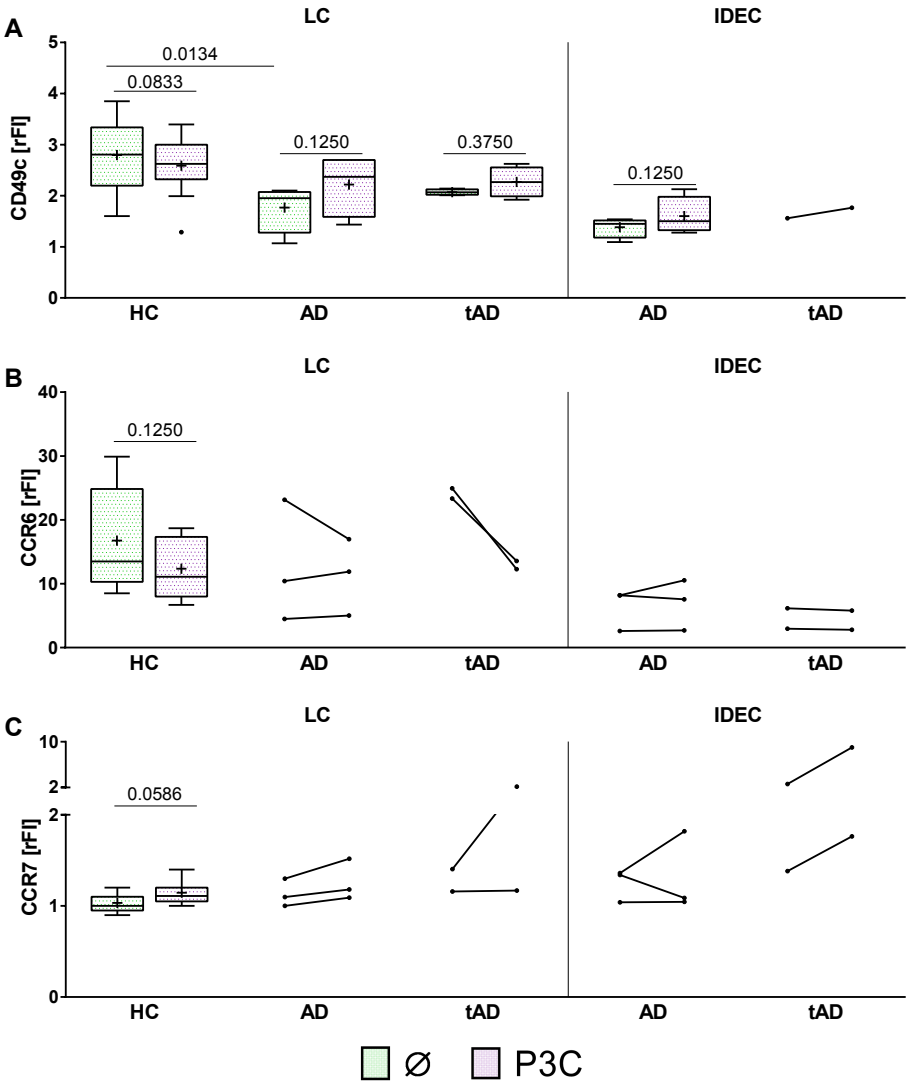

Figure 4.25. CD49c and CCR6 tend to be down-regulated and CCR7 up-regulated by P3C in LC from healthy controls.
Epidermal LC (Langerin$^+$, CD1a$^+$) and IDEC (Langerin$^-$, CD1a$^+$) were analysed by flow cytometry. (A) **CD49c** expression (n_{LC} = HC:15, AD:4, tAD:4; n_{IDEC} = AD:4, tAD:1) (B) **CCR6** expression (n_{LC} = HC:5, AD:3, tAD:2; n_{IDEC} = AD:3, tAD:2) (C) **CCR7** expression (n_{LC} = HC:9, AD:3, tAD:2; n_{IDEC} = AD:3, tAD:2) is presented as a box plot. The mean is marked by a + and the median by a vertical line inside the box. Outliers are marked as a black dot. Control experiments are indicated in green and P3C in purple. Datasets with less than 4 samples are presented as connected lines from ∅ to P3C. The Wilcoxon matched-pairs signed-rank test was used for connected samples.

As mentioned above, CCR6 is associated with cell maintenance in the tissue. Freshly isolated (D0) LC from AD patients expressed the receptor to a lesser degree when compared to LC from healthy skin (figure 4.11A). This trend was confirmed in the 24 h culturing where LC and IDEC from two AD patients expressed less CCR6 (figure 4.25B). This may support the increased migration rate (figure 4.23) or favours an environment where LC or IDEC are not likely to stay in the epidermis due to low CCR6 expression.

P3C down-regulated the CCR6 expression in healthy controls, which underlines the migratory induction and may be related to CCL20 (figure 4.24). Moreover, LC from tAD down-regulated CCR6 by P3C, whereas the IDEC from tAD did not. They showed a much lower CCR6 expression when compared to their LC counterpart. However, the LC and IDEC from two AD donors showed lower CCR6 expression which was not influenced by P3C.

On the other hand, CCR7 is associated with the migration out of the tissue. Interestingly, the CCR7 expression of the two AD and tAD donors seemed to be higher when compared to healthy controls. However, P3C seemed to induce CCR7 expression in healthy and possibly in AD skin, too. This preliminary data is in line with the increased spontaneous migration of AD donors and the P3C induced migration in healthy skin.

4.3.1 Summary of Part III: LC from AD patients exhibit a TLR2 independent high spontaneous migration rate

To sum up, LC of AD skin had a high spontaneous migratory activity after 24 h, which was enhanced by longer cultivation of split thickness-skin. The LC migration was not influenced by P3C in AD but in healthy controls. Next, cytokines influencing the migratory activity were expressed inverse in AD when compared to healthy controls, as IL-6 was lower and IL-18 and TNF-α were higher expressed, which further increased the spontaneous migration activity to the lymph nodes. Moreover, these cytokines were influenced by P3C in healthy but not in AD skin, supporting the unresponsiveness behaviour of AD cells. Additionally, CCL20 was up-regulated by P3C in healthy and AD skin, whereas the baseline of CCL20 was higher in AD skin compared to healthy controls. Preliminary data suggest that the expression of receptors connected to migration was impaired in AD since CD49c, as well as CCR6, was lower, while CCR7 was higher expressed in AD. Taken together, the results suggest that the migration activity was damaged at different levels in AD patients, leading to an enhanced migration rate of LC.

5 Discussion

It is well known that individuals with AD have an increased susceptibility to cutaneous colonization and infection with bacteria, fungi, and viruses [199–202]. LC are important cells of the immune system, which are present in the outer layer of the skin [203]. They are involved in all stages of wound healing such as inflammation, new tissue formation, and remodelling underlining their importance. LC activate T cells after migration to the lymph node while they mediate inflammation or tolerance in the epidermis [204–206]. This makes LC an interesting target in the field of dermatology since their role in inflammation and infection is still not fully understood, especially in AD. Next to LC, a second professional antigen-presenting cell line is present in the epidermis, especially in AD skin under inflammatory conditions, these cells are called IDEC [60]. Interestingly, IDEC are prominent in the lower compartment of the epidermis, suggesting a different role compared to that of LC [207]. LC capture antigens in the upper layer of the epidermis due to their ability to extend their dendrites through the tight junction barrier [208], in contrast to the horizontally extended dendrites of IDEC [207]. In this thesis, the impact of TLR2 ligation was investigated in AD skin compared to healthy controls. P3C was used to mimic *S. aureus* colonization in a human *ex vivo* skin model. First, the phenotype of AD and healthy epidermal cells was analysed followed by the optimisation of the *ex vivo* skin model for culturing purposes. Next, the maturation status, the cytokine production, and the migration activity were investigated under the influence of *S. aureus* structures in the form of the TLR2 specific ligand P3C. Finally, the capability of healthy and AD epidermal cells to activate naïve T cells after P3C challenge was analysed in an MLR.

5.1 The *ex vivo* skin model is suitable to analyse immature epidermal DC

LC are sensitive in detecting external pathogens. Especially when the skin is damaged and pathogens can pass through the physical barrier, the *stratum corneum*, they start to mature and activate the innate immune system [209]. Therefore, a suitable isolation method was needed to analyse the immature LC, which should reflect the phenotype in the skin [210]. First, the quality of the isolation method was verified to compare epidermal cells from healthy and AD skin. Flow cytometry and histological analysis show that more $CD1a^+$ cells are present in the epidermis of AD skin when compared to healthy controls [211, 212]. In the experiments reported herein, slightly more $CD1a^+$ cells were present in the epidermis of AD patients, though this was not significant. Furthermore, the results showed that IDEC expressed less CD1a compared to LC from AD skin (figure 4.4), and confirmed the initial reports [60].

To analyse the phenotype of IDEC, the expression of AD specific markers, such as CD206 and CD36 (figure 4.5), was investigated. Both markers were expressed in LC from AD skin and even to a greater extent in IDEC when compared to healthy controls, as reported previously [60, 61, 213]. FcεRI was expressed highly by LC and IDEC from AD patients (figure 4.6), as shown by others [214]. In the present dataset, the FcεRI on epidermal LC correlated with the IgE serum level in AD patients (figure 4.7). A similar observation was described, where the FcεRI expression on monocytes from AD patients correlates with the IgE serum titer [183]. The development of IgE-mediated food allergies, allergic rhinitis, and asthma in later stages of AD is called the atopic march [62, 87]. This underlines that AD is to be considered as a systemic disease rather than only inflammation of the skin. To sum up, the isolation method and analysis was successful, since the phenotypic hallmarks of AD were clearly detectable, as described above. Therefore, I could reliably distinguish between epidermal LC from healthy and AD donors.

This leads to the next step, where the impact of *S. aureus* structures towards the

TLR2 pathway could be analysed in healthy and AD epidermal cells. Three different approaches to culture the *ex vivo* human skin were used. I tested the culture of epidermal cell suspension, epidermis only (separated from the dermis), and the split thickness-skin, which consisted of the epidermis attached to a part of the dermis. However, culturing of the epidermal cell suspension or the epidermis alone caused the LC to strongly mature, witnessed by the loss their FcϵRI and an up-regulation of CD83 during 24 h culturing (figure 4.12), as reported earlier [215, 216]. Additionally, culturing of isolated human epidermal LC results in a slightly down-regulation of MHC class I, a moderate up-regulation of MHC class II, and strong induction of CD80, CD86 and CD40 [217]. For this reason, I decided to use the split thickness-skin which contained the epidermis attached to the upper part of the dermis, since the LC kept an immature phenotype. Nevertheless, culturing of split thickness-skin was more challenging since the binding of P3C to TLR2 is dependent on the penetration of the skin and the ability of LC to catch P3C with their dendrites. This was indeed the case since LC specifically reacted to P3C by the up-regulation of different maturation markers. The experiment proofed that the method could be used to analyse the influence of *S. aureus* structures on immature LC.

5.2 Equal maturation level of LC from healthy and AD

AD patients suffer from an imbalance of the skin microbiome, which may modulate the TLR2 expression and signalling due to desensitization by prolonged exposure to its ligands [218]. *S. epidermidis* is the predominant *Staphylococcus* species in healthy skin and is able to indirectly inhibit *S. aureus* colonization by a lipopeptide. This lipopeptide activates the TLR2/6 and CD36 pathway on keratinocytes, which subsequently produce more hBD2 and hBD3 which increase the antibacterial defence [219, 220]. In contrast, *S. aureus* can activate the TLR1/2 pathway and secrete virulence factors such as hemolysin, leukotoxin, exfoliative toxin, enterotoxin, PSMα, and toxic shock syndrome toxin-1 which increases tissue penetration and subsequently inflammation [220–222]. The skin microbiome is changing during an AD flare, where colonization with *S. aureus* and *S. epidermidis* is

increased and the positive effect of *S. epidermidis* is vanished [184]. In this PhD-thesis, the impact of TLR2 ligation was investigated in AD skin, since *S. aureus* plays a significant role in inflammation of AD skin. To analyse the influence of *S. aureus*, the maturation status needs to be analysed before TLR engagement experiments. The analysis of the maturation receptor expression of freshly isolated LC (D0) showed that LC from AD skin were not increased significantly *in situ*, despite the *S. aureus* colonization and skin barrier defects. The maturation markers CD83, CD86, MHC class I, and MHC class II were expressed equally in LC from healthy and AD epidermis (figure 4.8 and 4.9). Interestingly, IDEC showed elevated CD83 levels, as reported recently [223], which indicated that IDEC were activated and matured, and therefore possibly drive the inflammation in the AD skin.

Different studies observe that LC from AD patients had a similar maturation receptor expression when compared to healthy controls. They show that monocytes from AD donors express similar levels of CD83, CD80, and CD86 when compared to healthy controls [224]. DC generated from peripheral monocytes from AD are considered as IDEC and express higher levels of maturation receptors, when compared to freshly isolated monocytes. However, these studys show that monocyte derived DC from AD or atopic patients express equally high levels of CD83, CD80, CD86, CD40, MHC class I, and MHC class II when compared to healthy controls or non-atopics [224–226]. The expression of the listed maturation receptors on monocyte derived DC clearly increased over 8 days culturing from healthy and AD donors [224]. It is difficult to compare cell culture conditions to the *in situ* maturation status. Dermal myeloid DC from lesional AD skin express equal levels of CD83, CD80, CD86, and CD40 when compared to healthy skin [181]. Confocal microscopy experiments of mixed groups consisting of AD and healthy donors indicate that more CD83 positive cells, which were all Langerin positive, are present in the epidermis from six donors carrying the filaggrin null mutation when compared to 8 donors with the wild type genotype [227]. Filaggrin null mutations are seen in less than a third of the total population with AD, while 45.7 % to 56.6 % of patients with moderate-to-severe AD and 15 % of the patients with mild-to-moderate AD carry one or more filaggrin null mutations [228–231]. The prevalence of filaggrin null mutations in the

healthy population is around 8 % [231, 232]. In this PhD-thesis, 10 % of healthy donors (n=7) indeed showed elevated CD83 levels when compared to the rest of the group, which may be linked to the filaggrin mutation, which was not analysed herein. However, the filaggrin null mutation should be more common in AD subset, and therefore more LC should be CD83 positive, which was not the case. This is most likely due to the described tolerance towards *S. aureus* products in this thesis.

The comparable TLR2 expression of LC and KC from healthy and AD patients (figure 4.10 and 7.5) is an additional supportive element for the similar maturation status since TLR2 can be down-regulated by activation [233–235]. However, the TLR2 expression is differentially expressed based on the cell type, as reported for macrophages (less TLR2 [233]), KC (equal TLR2 [236]), and monocytes (more TLR2 [237, 238]) when compared to healthy controls. Interestingly, immunohistochemical analyses of human AD skin revealed that a three-week treatment of 0.1 % tacrolimus increased the TLR2 expression in AD skin [239]. In this PhD-thesis, no difference in the TLR2 expression was observed between treated or non-treated AD patients. This could be related to patients who treat themselves with a non-steroid cream, which may influence skin barrier function or *S. aureus* colonization and therefore TLR2 expression. Those patients could be part of the non-treated group and influence the outcome of the statistical test.

The elevated CD80 expression of LC (figure 4.8C) from AD patients was in direct contrast to the equal maturation receptor expression of LC from healthy and AD individuals (equal expression of TLR2, CD83, CD86, MHC class I and II). Others showed an increased maturation status of epidermal LC, epidermal IDEC, epidermal $CD1a^+$ cells, and B cells from AD patients, witnessed by elevated levels of CD80, CD86, and MHC class II when compared to healthy controls [211, 213, 240–243]. However, other studies showed that myeloid DC and B cells from AD donors expressed equal levels of CD80 [181, 242]. T cell activation is mediated by CD28 (activator) and CD152 (inhibitor), which are expressed on the T cell and can bind both CD80 and CD86 (reviewed in [244, 245]). CD80 is probably a more potent ligand for CD152, based on its higher affinity and avidity. There is evidence that CD80 is preferentially expressed to CD86, especially in culture models in which

DC and LC are generated from precursors into an immature state [246–252]. These immature DC can have tolerogenic rather than immunogenic effects and therefore, before DC maturation, CD80-CD152 interactions could promote tolerance to self-antigens [246, 253]. These reports support the equal maturation status of LC from AD donors, when compared to healthy controls. However, it is difficult to compare *in vitro* experiments to *in situ* data of AD patients but it could be a reason why CD80 is elevated in LC from AD donors.

Taken together, depending on the analysed tissue and cell-type, reports in the literature support both observations that LC from AD patients are (i) activated *in situ* and display increased maturation receptors or (ii) express similar levels of maturation markers, which resembles Schrödinger's cat [254, 255]. This is most likely because AD is a very heterogeneous disease which affects not only the skin but rather the whole immune system. Only one marker (CD80) was not in line with the equal maturation status of the LC from AD patients since it was higher than in the LC from healthy controls, whereas the expression of TLR2, CD83, CD86, MHC class I, and MHC class II showed the equal maturation status of LC from AD when compared to healthy skin. These results lead to the conclusion that LC from AD patients have a similar *in situ* maturation status when compared to healthy controls and may already be tolerant towards bacterial products. However, this thesis allows a small snapshot at the wide spectrum of the AD immunology.

5.3 LC from AD skin are tolerized towards TLR2 ligation

In this thesis, P3C was able to induce the TLR2 dependent maturation of LC from healthy skin, while LC and IDEC from AD patients lacked maturation as witnessed by constant levels of TLR2, CD83, FcϵRI, and CD40 upon TLR2 ligation. LC from some AD donors showed a reduced TLR2 expression though when compared to healthy controls (figure 4.14B). LC from healthy donors with similar low TLR2 levels showed a robust maturation after P3C application, as shown for *in vitro* generated LC from CD34 stem cells [177]. This underlines that the failed responsiveness towards TLR2 ligation is not mediated

by TLR2 level but rather by impaired signalling, which leads to an altered or missing response in AD individuals. This observation is supported by different studies that show that the cytokine production after TLR2 ligation was impaired in monocytes, macrophages, and primary KC from AD patients when compared to healthy controls [233, 256, 257]. Furthermore, others showed that TLR2 or MyD88 deficient mice are highly susceptible to *S. aureus* infection [258]. This connects the high *S. aureus* colonization in AD patients to the results in this thesis, which show that LC from AD are desensitized towards TLR2 ligation. Moreover, TLR2 seems to be linked with the repair and enhancement of tight junction functions since the recovery of the skin barrier function was delayed and incomplete in a TLR2 knockout mouse [259]. This could be connected to AD, where a failed TLR2 signalling in KC may lead to an impairment of tight junctions in AD.

The inability of LC from AD donors to respond to TLR2 ligation is one of the main finding in this thesis (results section: 4.2.1). One reason for the missing activation may be the TOLLIP protein which negatively influences the TLR2 signalling since it associates directly with TLR2 [260, 261]. One study analysed a *TOLLIP* polymorphism in an AD cohort [262], but unfortunately, they did not link the polymorphism to the TLR2 signalling activity in the AD cohort. They concluded that TOLLIP modifications may play a role in the AD development, but they also admitted that more studies need to be done since the polymorphism was detected in healthy controls. The TLR2 polymorphism R753Q is associated with a severe phenotype and higher IgE levels in AD patients [263]. Macrophages from mice bearing the TLR2 polymorphism R753Q show an altered MyD88 signalling [264]. However, the influence and the frequency of the TLR2 polymorphism R753Q is still under debate since the frequency of R753Q was higher in AD children, while others showed a similar distribution in healthy children and concluded that the association between AD and R753Q is lacking [265, 266]. Nevertheless, it is unlikely that the polymorphism is playing a major role in the present dataset, as the prevalence of the R753Q polymorphism may be around 10 % in AD [267].

Desensitization by prolonged exposure to *S. aureus* ligands may be one additional reason why epidermal DC from AD fail to react to TLR2 ligation [218, 268]. It has been

shown that continuous exposure of TLR specific ligands induces tolerance in several cell lines and cell types, such as monocytes, DC, THP-1, MonoMac-6, macrophages, and mouse macrophages, where the cells showed an impaired maturation status or cytokine profile after a final TLR challenge [269–275]. A similar study showed that monocytes from adult AD patients did not respond towards staphylococcal enterotoxin B or LPS treatment, while monocytes from children with AD increased the IL-12 levels and MHC class II expression [276]. This indicates that the *S. aureus* exposure may be higher in the adult group and subsequently induce a tolerant status. Interestingly, treatment of P3C in combination with the *S. aureus* virulence factor PSMα induced a tolerant status in bone marrow-derived DC from mice, where the DC produced less IL-12, IL-6, TNF-α and more IL-10 [277]. This showed that *S. aureus* and its products can induce tolerance or altered responsiveness to stimulation rather than simply hyporesponsiveness [275, 278]. Moreover, unpublished internal data from Dr. Nicole Leib showed that continuous stimulation with low dosages of P3C induced a tolerant status towards the TLR ligands in LC generated from $CD34^+$ stem cells. These tolerant cells did not respond to higher concentrations of P3C when compared to unprimed $CD34^+$ LC. Treatment with topical corticosteroids and calcineurin antagonists reduced the staphylococcal burden of individuals with AD [201, 279, 280]. This is of special interest since tAD patients indeed showed healthy-like reactivity to P3C, in terms of down-regulation of TLR2, up-regulation of CD83, and CD80 when compared to healthy controls (figure 4.14 and 4.17). This indicated that steroid treatment could recover their reactivity and probably shift the phenotype to a more healthy-like status. Despite the beneficial treatment, not all AD patients do respond to the steroid treatment and therefore, the tAD group may be a mixture of responders and non-responders leading to a difficult judgement of the data.

In this PhD-thesis, epidermal DC from AD patients showed a high FcϵRI expression, which was not influenced by P3C treatment. Healthy controls down-regulated FcϵRI by P3C and they expressed the receptor at much lower levels compared to LC from AD individuals (figure 4.15A). This TLR2-mediated reduction of FcϵRI was recently shown for

CD34 derived LC [177]. The missing FcϵRI down-regulation of LC from AD patients is an additional evidence that the TLR signalling is impaired in AD patients.

However, even though the TLR2 unresponsiveness is detectable on LC from AD patients, it seems that not only LC are impaired in AD skin. Various skin cells including dermal DC, LC, KC, and fibroblasts shape the skin microenvironment and can activate immune cells [281, 282]. In this thesis, cells from healthy *ex vivo* skin produced more IL-1β, hBD2, TSLP, IL-11, and IL-12p40 after TLR2 ligation, whereas AD *ex vivo* skin did not (figure 4.16). This shows on a functional level that AD skin does not respond to TLR2 ligation. Recent studies suggest that IL-1β is involved in allergy-related diseases such as bronchial asthma, contact hypersensitivity, and AD [283–285]. Moreover, cytokine analysis in the *stratum corneum* of infants with AD revealed elevated IL-1β concentrations compared to healthy controls [286]. In the present work, the IL-1β concentration was not influenced by P3C in the supernatant of AD individuals, while the total amount was higher in AD compared to healthy controls (figure 4.16A). The high IL-1β level may be an additional reason why AD fail to respond to TLR2 ligation since IL-1β uses the same downstream pathway as TLR2 [287]. Therefore, IL-1β can occupy MyD88 and subsequently NF-κB, which inhibits the TLR2 signalling. Interestingly, the same is true for IL-18 (figure 4.24) and IL-33 [288–290], which is known to be elevated in the epidermis of AD patients [286, 291, 292]. This suggests that an imbalance of the cytokine microenvironment additionally inhibits the TLR2 signalling.

Next, hBD2 was analysed which is involved in antimicrobial defence and is up-regulated in AD, when compared to healthy controls [293, 294]. TLR2 ligation induced hBD2 expression in the supernatant of healthy *ex vivo* skin (figure 4.16C), as shown in human airway epithelial cells [295, 296]. The level of hBD2 was higher in AD (figure 4.16C), which is in line with immunostainings that revealed that hBD2 is expressed higher in chronic and acute AD skin when compared to healthy controls [297]. A possible explanation for the high level may be the fact that TNF-α can induce hBD2 expression [298, 299]. Surprisingly, TNF-α was expressed highly in the supernatant of AD *ex vivo* skin (figure 4.24C), which subsequently may have led to higher hBD2 levels. However, it seems that the increased

hBD2 level is not able to control the *S. aureus* population on the AD skin. An explanation can be the sweat of AD patients since the containing salt negatively influences the hBD2 activity [300–302]. Furthermore, metabolomic analyses revealed higher levels of glucose in the sweat of AD patients which correlates with disease severity and may change the microbiome on the skin [300, 303]. This shows that more parameters, not only at a molecular level, influence the microbiome and therefore disease severity. TSLP promotes itching and it is regulated by TNF-α in combination with Th2 cytokines [299, 304] and TSLP is produced mainly by epithelial cells in the skin, gut, and lung [196, 305]. TSLP was surprisingly low and not influenced by P3C in the supernatant of *ex vivo* AD skin, while healthy skin responded to P3C by an up-regulation of TSLP (figure 4.16D). Others showed that TSLP is expressed higher in lesional tissue of AD patients, while the IL-1β, TNF-α, IL-4, and IL-13 concentrations would support the TSLP expression [306, 307]. In this thesis, the higher IL-1β and TNF-α (figure 4.16 and 4.24) expression in AD supernatant from cultured *ex vivo* skin did not influence the TSLP concentration in AD supernatant.

Another interesting observation was the IL-11 expression in the supernatant of *ex vivo* skin (figure 4.16). IL-11 was up-regulated by P3C in healthy controls but more surprisingly, it was almost not detectable in AD supernatant. One study analysed IL-11 in acute and chronic AD, where only chronic AD expressed more IL-11, which seems to be involved in tissue remodelling [308, 309]. However, IL-11 signalling may be involved in adipogenesis [310], and therefore it may be increased in the control group since the plastic surgery of the abdomen usually yields a fatty tissue compared to the AD skin.

To sum up the discussed results and literature, multiple reasons were possibly responsible for the desensitization of the TLR2 pathway in AD skin. These include desensitization by prolonged exposure to *S. aureus* structures and a failed TLR signal transduction since predominantly expressed cytokines (IL-1β, IL-18 or IL-33) may occupy MyD88 and subsequently NF-κB. Several different alterations in AD favour the *S. aureus* colonization, such as the altered sugar composition of AD sweat, the increased itch-induced sweat that inhibits hBD2 activity with its salt, and the impairment of the tight junctions in AD skin. All

these reports and observations are connected to the imbalance of the skin microbiome in AD, and the increased level of *S. aureus*.

5.4 Epidermal DC from AD skin fail to activate CD4 positive T cells

A further hallmark of this thesis was the finding that epidermal cells from AD skin fail to activate naïve $CD4^+$ T cells. LC and IDEC from AD and tAD patients expressed the co-receptors for T cell activation CD80 and CD86 (figure 4.17) and should per se be capable of activating T cells. However, both receptors were not influenced by P3C in AD, while LC from healthy donors up-regulated the expression of CD80 and CD86 after TLR2 ligation, which is in line with the literature [311].

The MLR experiments need to be judged carefully since the epidermal cell suspension was cultivated with naïve $CD4^+$ T cells isolated from buffy coat of different donors. Firstly, this reaction was an alloreaction where T cells could be activated by other (*allo*) or foreign cells, especially by the mismatched gene variant of the MHC. This is the major reason why transplantations are rejected by a patient who received a new organ or tissue (graft-versus-host disease). Secondly, $CD4^+$ T cells were generated from different buffy coats and used for both healthy and AD experiments. This needs to be taken into account when analysing the data. However, no significant difference could be observed between the outcome of the different donors. Thirdly, in the MLR experiments where the antigen-presenting cells should activate the T cells, equal parts of epidermal cell suspension and $CD4^+$ T cells were mixed. The numbers of $CD1a^+$ cells, and therefore LC, were different in each sample between healthy and AD or within one group. This lead to the fact that the activation of T cells was limited to the amount of LC in the suspension. However, since the experiments contained an unstimulated control which reflects the background of the alloreaction, it is possible to interpret the data, especially when compared to AD.

The proliferation assay revealed that only epidermal cells from healthy subjects were able to increase the T cell proliferation after P3C treatment, while AD could not raise

it beyond the alloreaction activity. A study showed that an autoreaction with epidermal LC from suction blisters and T cells from AD patients induced stronger proliferation when compared to healthy controls [312]. In this thesis, the supernatant of the MLR was collected and the cytokine expression was analysed (figure 4.21). IL-2 is known to influence the proliferation rate of T cells [313–315]. This is in line with the slightly increased IL-2 concentration found in the MLR of healthy donors (figure 4.21) since the proliferation is indeed slightly higher in healthy donors. P3C was not able to rise the IL-2 level beyond the alloreaction background in healthy and AD. IL-2 is a interesting cytokine since it contributes to the induction of Th1, Th2, and Treg cells and inhibits the generation of Th17, though it can maintain Th17 cells [316]. Therefore, it plays an inducer role for IL-4, IL-5, IL-13, and IFN-γ production. The cytokines were not detectable in the supernatant of 24 h cultured *ex vivo* skin from healthy and AD donors (data not shown), and were therefore exclusively produced by T cells in this experimental system and not by LC, as shown for DC [317]. Interestingly, the classical Th2 cytokines IL-4 and IL-5 were in line with the increased IL-2 expression (figure 4.21), but not IL-13 (figure 7.8), which was not expressed differentially in healthy or AD and not influenced by TLR2 ligation.

However, the classical Th1 cytokine IFN-γ was not influenced by P3C in healthy and AD MLR, but AD produced much less when compared to healthy controls. Others investigated the influence of KC on a T cell co-culture, where supernatant of staphylococcal enterotoxin B treated KC yielded more IL-2 and IFN-γ [318]. However, when monocytes were added to the system of KC and T cells, the IFN-γ production was further increased but IL-2 was not. The same study showed that IL-4, IL-5, IL-12p40 were barely affected by the KC. This is a supportive element for the conclusion that the LC in the MLR were the driving force that polarized the T cells, which subsequently produced these cytokines. This indicated that epidermal DC from AD donors had a low T cell activation capability.

Despite the Th17 blocking effect of IL-2, IL-17A and IL-17F were significantly increased by P3C in healthy but not in AD MLR supernatant (figure 4.21). This is in line with P3C treated peripheral blood mononuclear cells from AD patients, which have been shown to produce less IL-17 than their healthy control group [319]. A study showed that co-

cultures of naïve $CD4^+$ T cells and naturally occurring $CD4^+$ $CD25^+$ T cells, which were stimulated with different TLR ligands, induced Th17 cells in the presence of IL-6 and TGF-β [320]. They concluded that IL-6 and TGF-β subverts Th1 and Th2 differentiation for the generation of IL-17-producing T cells [320]. In the same study, the generation of Th17 cells was amplified by IL-1β and TNF-α, and interestingly both cytokines were expressed higher in the supernatant of cultured *ex vivo* skin from AD patients (figure 4.16 and 4.24). However, in this thesis, the experimental system did not include Treg cells, since they were excluded in the first place. Th17 cells need IL-1β, TGF-β, and IL-6 to differentiate, autocrine IL-21 for amplification, and IL-23 that expands and stabilizes previously differentiated Th17 [321–323]. Unfortunately, IL-21 was not detectable and IL-23 was not analysed herein. But the IL-6 concentration was increased significantly in the MLR supernatant of AD when compared to healthy controls (figure 4.24). This showed that AD skin might be able to modify the T cells. Taken together, the evaluation of allogenic MLR and the proliferation assay showed that P3C-treated epidermal cells from AD patients could not raise the proliferation of T cells above the alloreaction background and subsequently did not polarize T cells in this experimental setup when compared to healthy controls. The missing induction of T cells supports the tolerant behaviour of epidermal DC from AD donors, as discussed above.

5.5 High spontaneous migration of LC from AD patients

During steady-state, only a minor part of LC migrate to the skin draining lymph nodes, while TGF-β can inhibit the migration, and therefore controls the homoeostasis of LC in the epidermis [46, 324]. In this work, the TGF-β concentration was expressed equally in the supernatant of *ex vivo* cultured skin from healthy and AD donors (figure 4.16). This indicates that TGF-β had limited influence in healthy and AD experiments. Since LC migrate continuously, they do replenish themselves through a constant low-level proliferation, which is similar to other types of tissue-resident macrophages [43, 44, 325]. Moreover, LC seem to alter their proliferation, maturation and migration rate in

inflammation and this leads to a reduction of epidermal LC. The loss of LC is compensated by recruitment of bone-marrow-derived cells into the epidermis, while some monocytes can acquire an LC-like phenotype [49, 326]. This leads to the assumption that the epidermis harbours two populations during inflammation: resident LC, which originate from the fetal liver and LC-like cells from the bone marrow in lesional skin. The migration activity of epidermal DC can be divided into induced and spontaneous migration. TLR2 ligation induced normal LC maturation and consequently increased the migration activity of LC (figure 4.23) when compared to unstimulated controls, as discussed by others [327]. However, the missing activation of LC by P3C failed to explain the strong migration in AD (figure 4.23), which is of unclear nature.

FcεRI-activated LC seem to release chemotactic signals and show an increased migratory capacity [20]. This could explain the spontaneous migration rate since epidermal DC from AD skin expressed more FcεRI on their surface (figure 4.6). Furthermore, the enhanced migration rate of LC from AD donors could be caused by an elevated level of IL-18 (figure 4.24), which was independent of TLR2 ligation. IL-18 is released by KC and IDEC-like DC and is expressed strongly in the serum of AD patients, while it is known to induce LC migration [286, 328, 329]. IL-18 expression was slightly reduced in tAD patients, which could be connected to the steroid treatment, since a study showed a correlation between the corticotropin-releasing hormone and IL-18 expression [330]. However, the influence of steroid treatment on the spontaneous migration of AD patients remains to be investigated.

TLR2 activation of KC activates the production of pro-inflammatory cytokines such as IL-6 and TNF-α and enhances the tight junction barrier function of the epidermis upon pathogen invasion [331, 332]. IL-6 can influence the LC migration by regulation of the epidermal permeability, while the migration induction potential of IL-6 was shown in different cell lines or cell types [333–337]. IL-6 was expressed barely in the supernatant of cultured *ex vivo* skin from AD patients (figure 4.24), while it may play an active role in the migration of LC from healthy controls, as it is up-regulated in healthy controls upon P3C treatment. IL-6 can reduce the expression of filaggrin and worsen the epidermal

barrier function of AD patients and IL-6 is expressed higher in the serum of children with AD and in an AD mouse model [338–340]. In a mouse model, anti-inflammatory drugs reduced the expression of IL-6 and subsequently reduced the migration of cutaneous dendritic cells [341, 342].

TNF-α was expressed much higher in AD when compared to healthy controls (figure 4.24). This is in line with the literature, where TNF-α is expressed higher in the plasma of AD patients, which correlates with the severity of the disease [343]. Multiple studies show that TNF-α is involved in the migration activity, especially in LC migration, tumour development, and metastasis [344–349]. The elevated TNF-α level is most likely due to the high amounts of IL-1β in AD. IL-1β is referred to as the master cytokine since it can initiate the whole cytokine profile, in particular the TNF-α synthesis by KC [350, 351]. IL-1β and TNF-α reduce the E-cadherin expression and weaken the interaction, and therefore reduce the bonding strength of LC to KC, allowing LC to migrate out of the epidermis. As a consequence, the E-cadherin expression is reduced in AD [352]. These are strong indicators that TNF-α is, next to IL-18, a potent inducer of spontaneous LC migration found in the AD experimental setup.

The current model of TLR2-induced migration includes the following steps, where P3C application induces an alteration of the cytokine and receptor expression on the immune cells. TLR2 ligation down-regulates CCR6 and CD49c, which consequently allows the LC to loosen their connection to the surrounding cells and to start the migration process. Moreover, CCR7 up-regulation enables the LC to specifically migrate towards CCL19 and CCL21, which are mainly produced by stromal cells in the thymus [353–357]. These alterations allow the LC to migrate out of the epidermis, and this was the case for LC from healthy donors, while LC from AD patients showed a much higher spontaneous migration activity.

CCL20 is responsible for the recruitment of CCR6-expressing immature DC [358, 359]. In this thesis, CCL20 was higher expressed in the supernatant of untreated split thickness-skin from AD donors when compared to healthy controls and P3C up-regulated CCL20 in the supernatant of healthy and AD (figure 4.24). This shows that LC from AD donors

might migrate more easily in the untreated condition. The corresponding receptor for CCL20 is CCR6 and it was slightly lower expressed in freshly isolated (D0) LC from AD skin when compared to healthy controls (figure 4.11). This has been confirmed in a study where LC from AD donors indeed express fewer CCR6 at their surface [212]. CCR6 was slightly reduced by P3C in LC from healthy donors (figure 4.25), which underlines the migratory induction. Moreover, the CCR7 expression tends to be increased in AD skin, while healthy controls do express the receptor only after stimulation with P3C (figure 4.25). These results show that LC from healthy donors are more likely to remain in the epidermis, most likely due to higher CCR6 expression and lower CCL20 level in the environment.

Besides, CD49c (integrin $\alpha 3$) may participate in the enhanced migration of LC from AD donors since members of the integrin superfamily mediate cell-matrix and cell-cell adhesion [360]. The complex of $\alpha 3$ and $\beta 1$ integrin is an adhesion receptor for laminin and is linked to intercellular adhesion [361–363]. Here, the CD49c expression was strongly reduced in LC from AD patients (figure 4.11 and 4.25) when compared to LC from healthy controls, indicating that LC from AD are poorly connected to the environment and are already in a state that allows enhanced migration rates. However, CD49c was slightly increased in freshly isolated KC and even to a greater extent after culture (D1). Interestingly, CD49c is associated with wound healing processes and therefore, it may be increased in the KC of AD patients [364–367].

Taken together, P3C induced a specific migration activity of LC from healthy donors, while LC from AD donors showed a high spontaneous migration activity. This might be explained by the reduced expression of CD49c and CCR6, the increased expression of CCR7, and the elevated migration inducing cytokines (IL-18, TNF-α, CCL20). The impaired components in this complex disease maintain the inflammation, whereas the enhanced migration rate of LC is one piece in AD.

6 Conclusion

AD is a chronic multifactorial inflammatory skin disease. The skin of AD patients shows a significant dysbalance of the microbiome with high colonization of *S. aureus*, which positively correlates with the disease severity. LC are immune system sentinels and reside in the epidermis, where they sense invading pathogens by pattern recognition receptors, such as TLR. LC bridge the innate and adaptive immune systems, they orchestrate primary immune responses to pathogens, and maintain tolerance. However, IDEC are present in lesional AD skin and they contribute to the local pro-inflammatory microenvironment of AD skin. The aims of this thesis were (i) the establishment of an *ex vivo* skin model to analyse immature LC, (ii) the definition and phenotype of LC and IDEC in the *ex vivo* human skin, (iii) and the analysis of epidermal DC under the influence of *S. aureus*, exemplified by P3C.

The phenotyping of freshly isolated LC from AD suggests that they had a similar maturation status compared to LC from healthy controls (*e.g.* equal CD83, CD86). The key finding of this thesis was that LC from AD patients showed tolerance towards TLR2 ligation, which include the following results. LC from AD donors did not further increase their maturation status (*e.g.* no change of CD83, CD40) after TLR2 ligation when compared to healthy controls. LC from AD skin fail to down-regulate TLR2 and FcϵRIα expression after P3C application. Steroid treatment seems to compensate the tolerant behaviour and change the phenotype towards a healthy-like status. P3C stimulation up-regulated the cytokine expression of *e.g.* IL-1β and hBD2 of healthy skin to the basal level of AD. However, the cytokine production was not influenced by P3C in AD skin. Naïve $CD4^+$ T cells mixed with P3C-stimulated epidermal cells from healthy skin induced a specific proliferation of the T cells while AD skin did not. Allogenic mixed lymphocyte reactions with naïve $CD4^+$ T cells and epidermal cells suggest that AD skin failed to activate naïve $CD4^+$ T cells.

Another major finding was the high spontaneous and TLR2-independent migration rate of LC from AD skin. The expression of receptors that allow the LC to remain in the epidermis (CCR6 and CD49c) were down-regulated in freshly isolated (Day 0) LC from AD skin, indicating that these LC are poorly connected to the KC-environment and might migrate more easily from the tissue to the lymph nodes. Moreover, CCR7 that regulates the specific migration was slightly increased in LC from AD donors. The cytokines which induce migration out of the epidermis were elevated in the supernatant of cultured split thickness-skin from AD (*e.g.* IL-18 and TNF-α), which underlines the strong spontaneous migration. However, P3C ligation did not increase the migration rate of LC from AD patients, while it was up-regulated in healthy controls.

In conclusion, LC from AD skin did not respond to the applied TLR2 stimulus which may have several reasons. It is most likely that *S. aureus* influences *in vivo* the TLR2 pathway with its presence, cell wall structures, and toxins, which in turn leads to a desensitisation of LC from AD patients. The enhanced *S. aureus* colonization eventually induces a cycle of events that change the cytokine environment and inhibit directly or indirectly the TLR2 signalling, which may consequently result in the tolerance of TLR2 ligation. This is witnessed by missing maturation and T cell activation after TLR2 stimulation. Moreover, the continuous *S. aureus* exposure on the skin of AD patients may induce a high spontaneous migration activity of LC which was detected in *ex vivo* experiments with split thickness-skin. The results in this thesis are key to a better understanding of AD and why AD patients fail to resolve the *S. aureus*-induced inflammation.

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Nomenclature

7-AAD	7-Aminoactinomycin D
S. aureus	*Staphylococcus aureus*
S. epidermidis	*Staphylococcus epidermidis*
AD	Atopic dermatitis
CCR6	C-C motif chemokine receptor 6
CCR7	C-C motif chemokine receptor 7
DC	Dendritic cells
FcϵRI	High affinity IgE receptor
FcϵRII	Low affinity IgE receptor
FCM	Flow cytometry
GM-CSF	Granulocyte macrophage colony stimulating factor
IDEC	Inflammatory dendritic epidermal cells
IFN	Interferon
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IL	Interleukin
KC	Keratinocytes
LC	Langerhans cells
LPS	Lipopolysaccharides
mfi	Median fluorescence intensity
MHC	Major histocompatibility complex
P3C	Pam3CSK4
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cells
rFI	Relative fluorescence index
SCORAD	SCORing AD
tAD	Treated atopic dermatitis
TGF	Tumor growth factor
Th	T helper
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TSLP	Thymic stromal lymphopoietin

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7 Appendix

7.1 Supporting information

Figure 7.1. Immunology in the skin - a video provided by *Nature*.
The video can be found with the following url: `https://youtu.be/_VhcZTGv0CU` or with the QR code above. This provides an overview of the immunology and structure in the skin **(most important 0:30 to 4:30)** with a focus to psoriasis.

Figure 7.2. Migration - a video provided by *Nature*.
The video can be found here: `https://youtu.be/uKid8QmBI0I` or with the QR code above. It provides a life imaging of migrating cells in an experimental setup.

Figure 7.3. A introduction to the microbiota - a video provided by *Nature*.
The video can be found here: `https://youtu.be/c_ZRZkU-FEw` or with the QR code. It provides an introduction to the microbiome and the acquisition of bacteria diversity.

Table 7.1. Classification of moisturizers.
SC, subcutaneous layer; NMF, natural moisturizing factor; TEWL, transepidermal water loss. Table was taken from "A review on the role of moisturizers for atopic dermatitis" [368]

Class	Mode of action	Biological similarity	Some examples
Humectants	Attract and bind water from deeper epidermis to SC	NMF in corneocytes	Glycerin Alpha hydroxy acids Hyaluronic acid Sorbitol Urea
Occlusives	Form a hydrophobic film to retard TEWL of SC	Intercellular lipid bilayers - Cholesterol - Free fatty acids - Ceramide	Carnauba wax Lanolin Mineral oils Olive oil Petrolatum Silicone
Emollients	Smoothens skin by filling the cracks between desquamating corneocytes	Natural lipids found on skin and sebum	Collagen Colloidal oatmeal Elastin Glyceryl stearate Isopropyl palmitate Shea butter Stearic acid

Table 7.2. Classification of topical corticosteroids.
Topical steroids are classified by strength, which ranges from super potent (Class 1), to least potent (Class 7). Table was adapted from `nationaleczema.org/eczema/treatment/topicals/`.

Class	Active substance	Brand	Formulation
1	0.05 % clobetasol propionate	Clobex	Lotion
		Olux E	Foam
		Temovate E	Cream/Ointment
	0.05 % halobetasol propionate	Ultravate	Cream
	0.1 % fluocinonide	Vanos Cream®	Cream
2	0.05 % diflorasone diacetate	ApexiCon E	Cream
	0.05 % halobetasol propionate	Elocon	Ointment
	0.01 % fluocinonide	Halog	Ointment
	0.25 % desoximetasone	Topicort	Cream/Ointment
3	0.05 % fluocinonide	Lidex-E	Cream
	0.05 % desoximetasone	Topicort LP	Cream
4	0.1 % clocortolone pivalate	Cloderm	Cream
	0.1 mometasone furoate	Elocon	Cream
	0.1 % triamcinolone acetonide	Aristocort A	Cream
	0.1 %betamethasone valerate	Valisone	Ointment
	0.025 % fluocinolone acetonide	Synalar®	Cream
	0.05 % desoximetasone	Topicort	Cream/Ointment
5	0.05 % fluticasone propionate	Cutivate	Cream/Lotion
	0.1 % prednicarvate	Dermatop	Cream
	0.1 % hydrocortisone butyrate	Locoid	Cream/Ointment
	0.1 % hydrocortisone probutate	Pandel	Cream
	0.1 % triamcinolone acetonide	Aristocort	Cream
	0.025 % fluocinolone acetonide	Synalar®	Cream
6	0.05 % alclometasone dipropionate	Aclovate	Cream/Ointment
	0.05 % desonide	Verdeso	Foam
		Desonate	Gel
	0.025 % triamcinolone acetonide	Aristocort	Cream
	0.01 % fluocinolone acetonide	Derma-Smoothe	Oil
7	2.5 % hydrocortisone	Nutracort	Lotion
		Synacort	Cream
	0.5 – 1 % hydrocortisone	Cortaid	Cream/Ointment

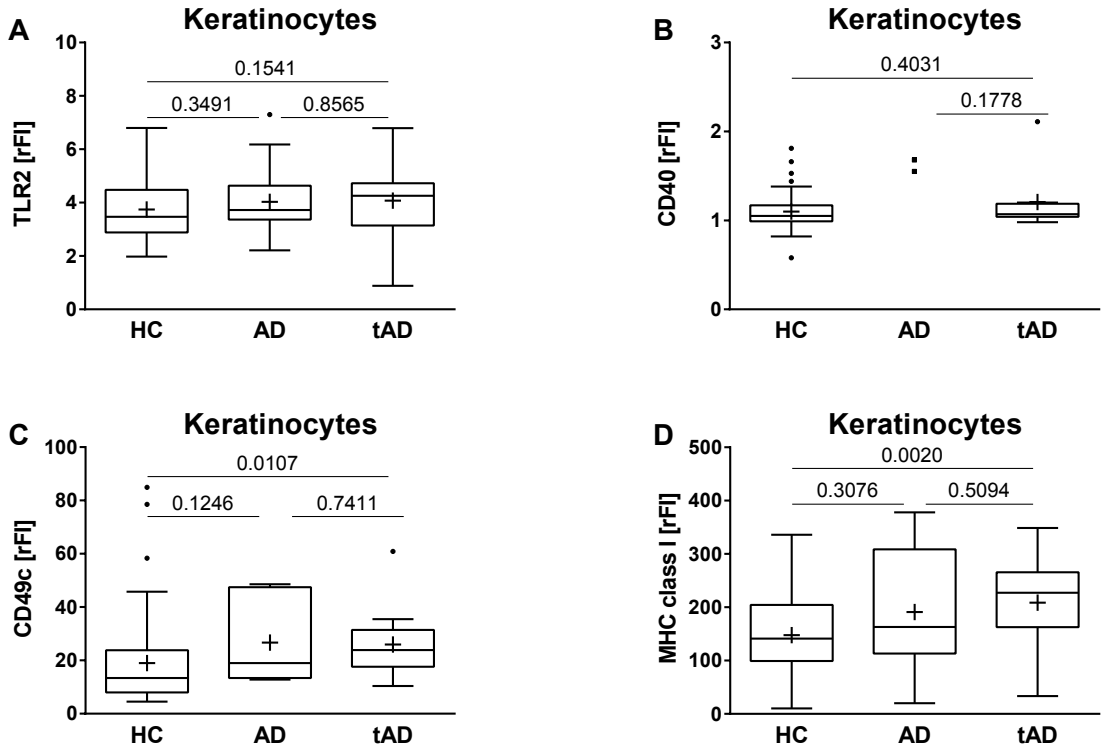

Figure 7.4. Receptor expression on freshly isolated KC from healthy and AD donors.
Keratinocytes (KC, CD1a^{neg}) were analysed by flow cytometry. TLR2 (n = HC:71, AD:20, tAD:24), CD40 (n = HC:43, AD:2, tAD:8), CD49c (n = HC:52, AD:6, tAD:14) and MHC class I (n = HC:64, AD:9, tAD:17) are presented as a box plot. The mean is marked by a + and the median by a vertical line inside the box. Outliers are marked as a black dot. Datasets with less than 4 samples are presented as individual values in black squares. The Wilcoxon matched-pairs signed rank test was used for connected samples.

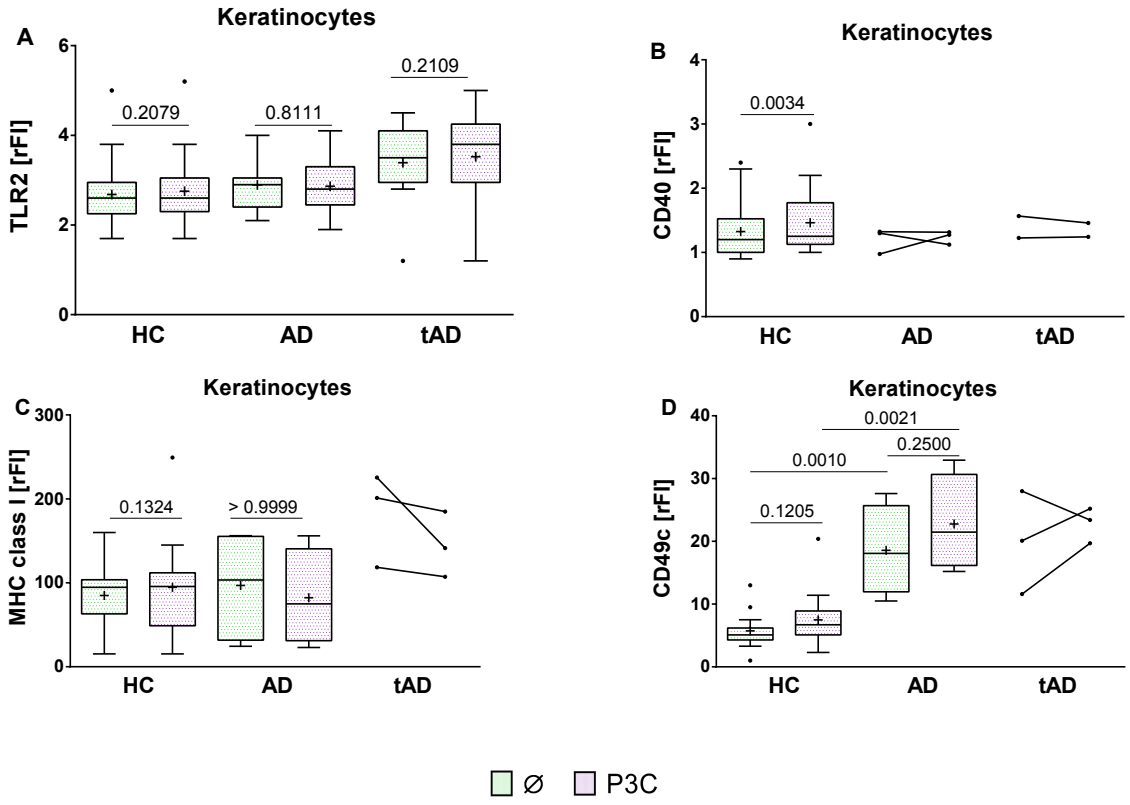

Figure 7.5. KC receptors are not influenced by P3C treatment.
Keratinocytes (KC, CD1a^{neg}) were analysed by flow cytometry. TLR2 (n = HC:37, AD:17, tAD:9), CD40 (n = HC:17, AD:3, tAD:2), MHC class I (n = HC:18, AD:4, tAD:3) and CD49c (n = HC:15, AD:4, tAD:3) are presented as a box plot. The mean is marked by a + and the median by a vertical line inside the box. Outliers are marked as a black dot. Control experiments (∅) are indicated in green and P3C in purple. Datasets with less than 4 samples are presented as connected lines from ∅ to P3C. The Wilcoxon matched-pairs signed rank test was used for connected samples.

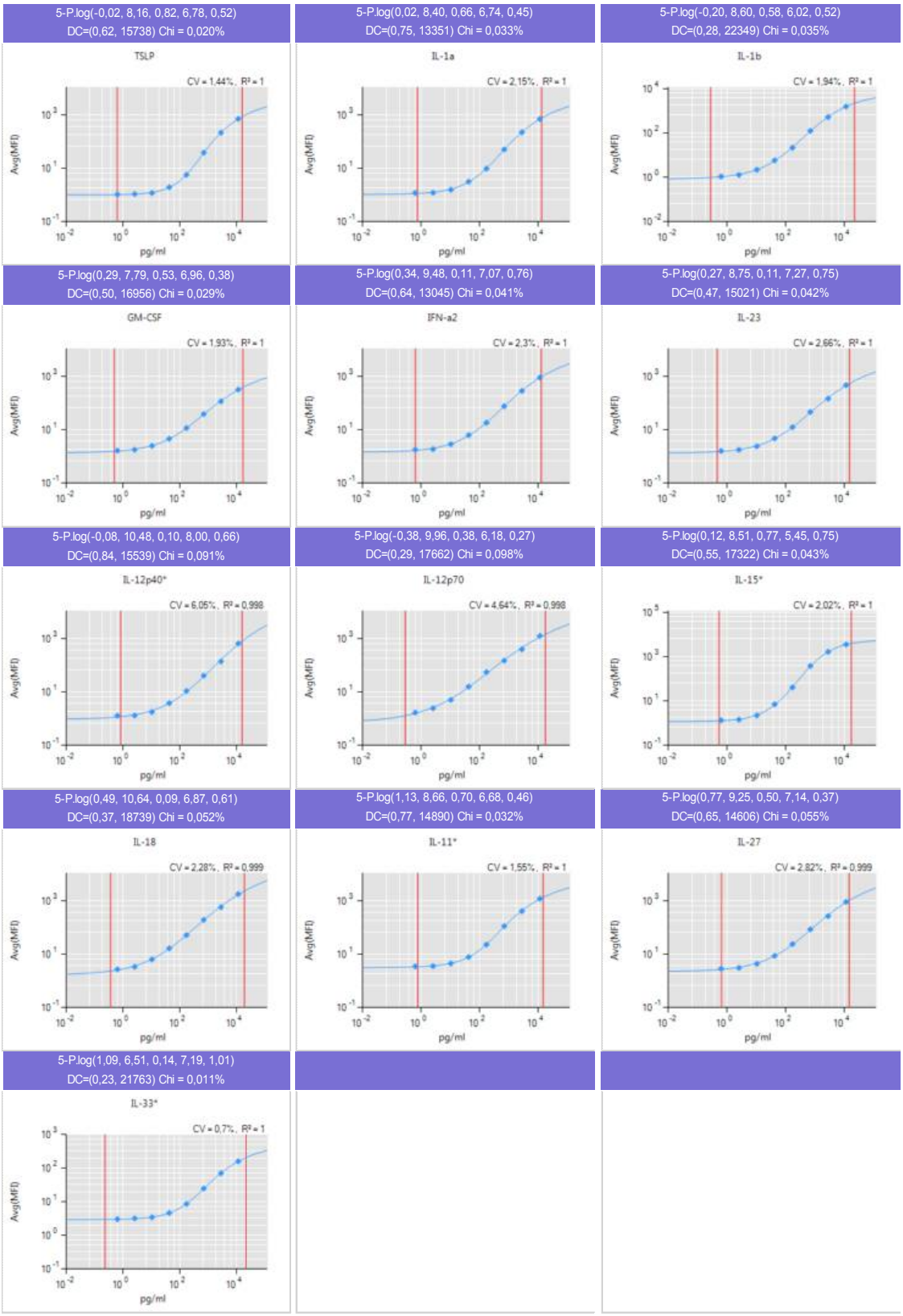

Figure 7.6. Cytokine standard curves - produced by *ex vivo* split thickness skin.
The supernatant was collected after 24 h culturing and analysed by flow cytometry using the LEGENDplex™ assay.

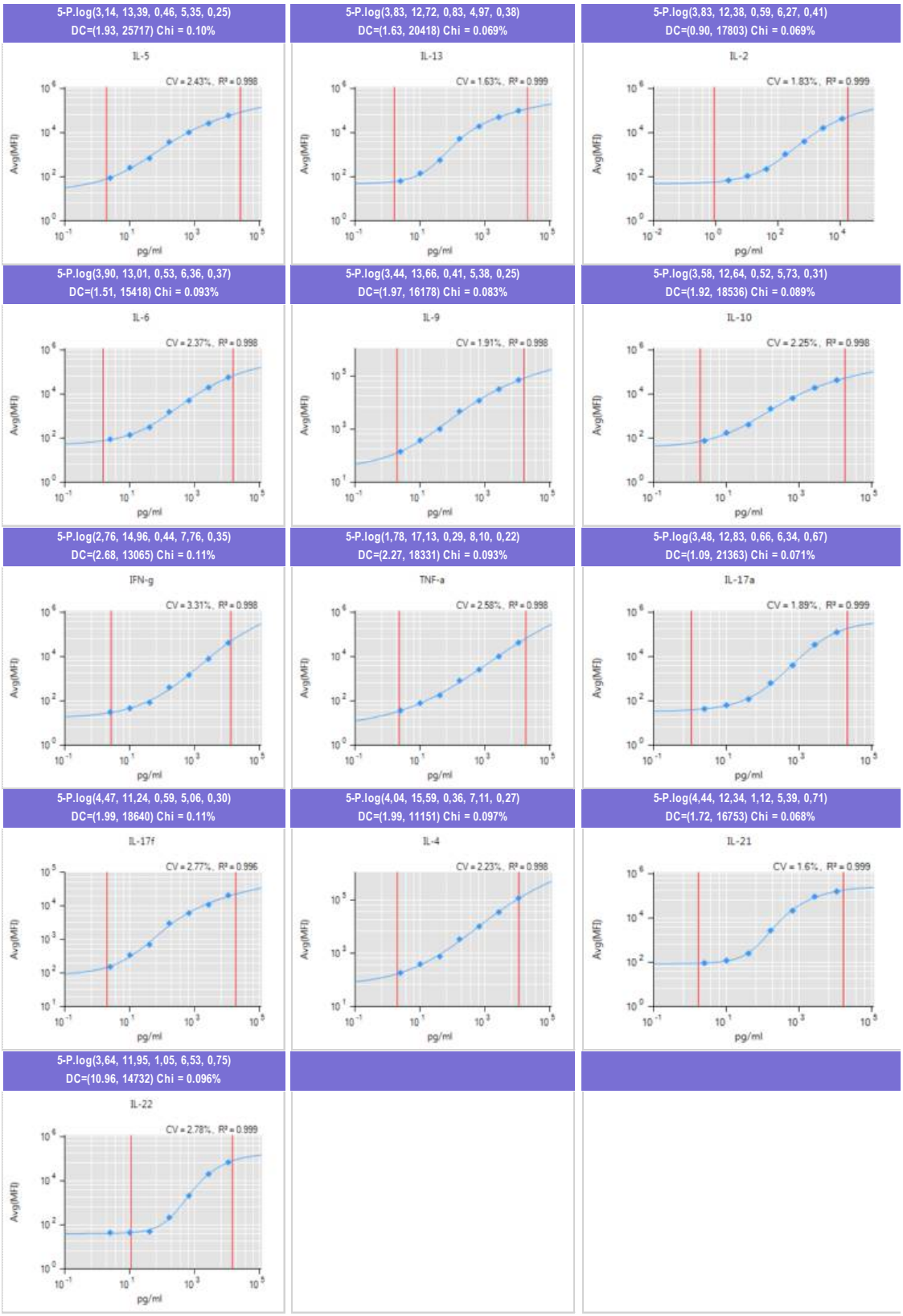

Figure 7.7. Standard curves of analysed Th cytokines produced by MLR.
The supernatant was collected after 7 days of co-culture and analysed by flow cytometry using the LEGENDplex™ assay.

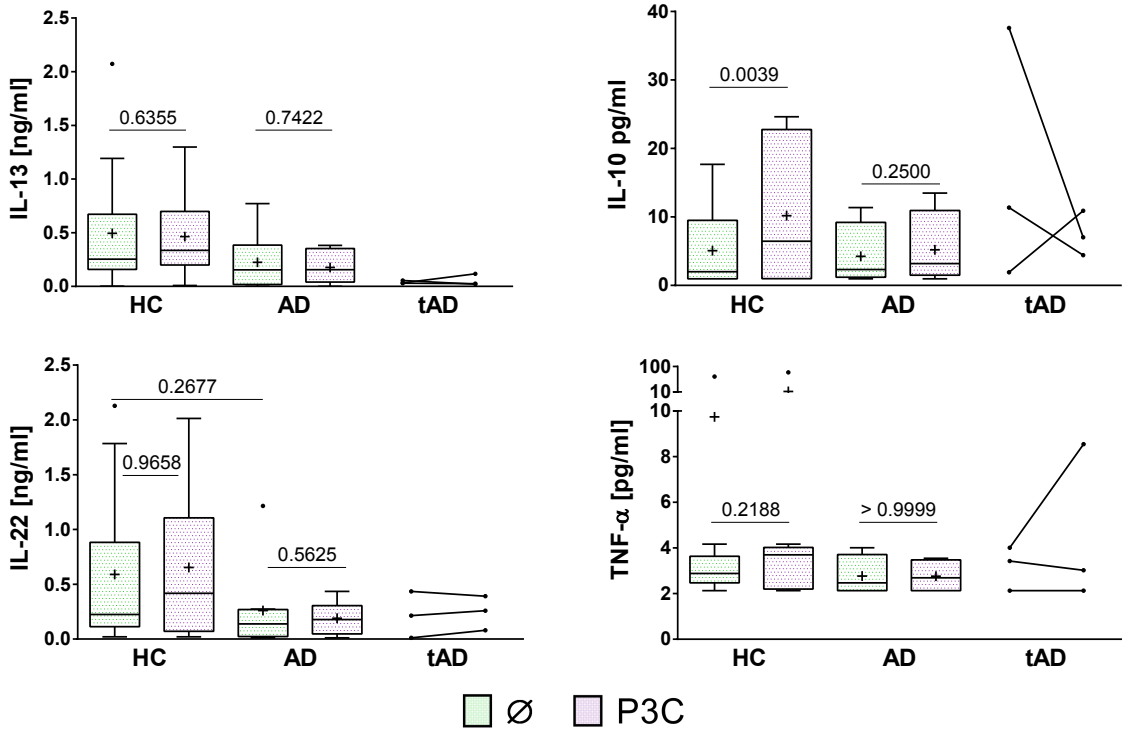

Figure 7.8. Cytokines in the MLR supernatant.
Ex vivo human skin was cultured with or without P3C for 24 h. Afterwards, an epidermal cell suspension was generated, which was mixed 1:1 with allogenic naïve $CD4^{pos}$ cells co-cultured for seven days. The supernatant was harvested and the cytokine expression of IL-13 (n = HC:13, AD:9, tAD:4), IL-10 (n = HC:9, AD:4, tAD:3), IL-22 (n = HC:13, AD:8, tAD:4) and TNF-α (n = HC:9, AD:4, tAD:3) was analysed by ELISA or FCM, respectively. The mean is marked by a + and the median by a vertical line inside the box. Outliers are marked as a black dot. Control experiments are indicated in green and P3C in purple. Datasets with less than 4 samples are presented as connected lines from ∅ to P3C. The Wilcoxon matched-pairs signed rank test was used for connected and the Mann-Whitney U for independent samples.

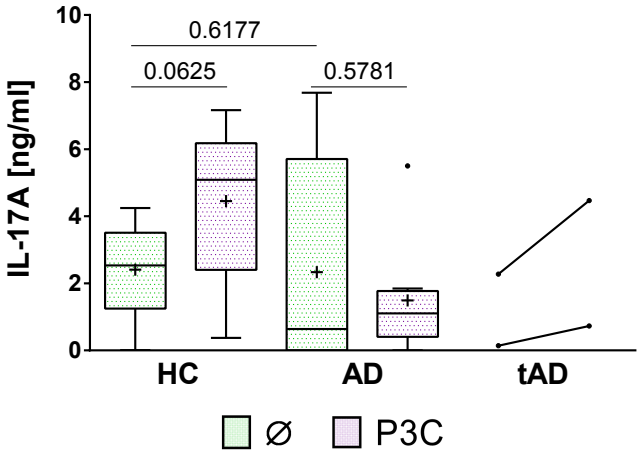

Figure 7.9. IL-17A expression analysed by ELISA.
Ex vivo human skin was cultured with or without P3C for 24 h. Afterwards, an epidermal cell suspension was generated, which was mixed 1:1 with allogenic naïve $CD4^{pos}$ cells co-cultured for seven days. The supernatant was harvested and the cytokine expression of IL-17A (n = HC:5, AD:8, tAD:2) was analysed by ELISA. The mean is marked by a + and the median by a vertical line inside the box. Outliers are marked as a black dot. Control experiments are indicated in green and P3C in purple. Datasets with less than 4 samples are presented as connected lines from ∅ to P3C. The Wilcoxon matched-pairs signed-rank test was used for connected and the Mann-Whitney U for independent samples. This figure was generated with the help of Kazumasa Iwamoto (`https://orcid.org/0000-0001-6183-6467`), who performed some ELISA.

7.2 Publications

- **Mladenov R**, Hristodorov D, Cremer C, Hein L, Kreutzer F, Stroisch T, Niesen J, Brehm H, Blume T, Brümmendorf TH, Jost E, Thepen T, Fischer R, Stockmeyer B, Barth S, Stein C. The Fc-alpha receptor is a new target antigen for immunotherapy of myeloid leukemia. Targeting and Elimination of Malignant Myeloid Cells. International Journal of Cancer 2015;137:2729–38.

- **Koch S**, Stroisch TJ, Vorac J, Herrmann N, Leib N, Schnautz S, Kirins H, Förster I, Weighardt H, Bieber T. AhR mediates an anti-inflammatory feedback mechanism in human Langerhans cells involving FcϵRI and IDO. Allergy 2017;72:1686–93.

- **Leib N**, Herrmann N, Koch S, Stroisch TJ, Schnautz S, Wilms H, Bieber T. MicroRNA-155 mediates downregulation of the high-affinity receptor for IgE through Toll-like receptor signaling. Journal of Allergy and Clinical Immunology 2018;141:425–429.e6.

- **Iwamoto K, Nümm TJ**, Koch S, Herrmann N, Leib N, Bieber T. Langerhans and inflammatory dendritic epidermal cells in atopic dermatitis are tolerized toward TLR2 activation. Allergy 2018;73:2205–13.

- **Kläschen S**, Nümm TJ, Herrmann N, Leib N, Maintz L, Bieber T. JAK1/2 inhibition impairs the development and function of inflammatory dendritic epidermal cells in atopic dermatitis, Journal of Allergy and Clinical Immunology, in revision

- **Herrmann N**, Nümm TJ, Iwamoto K, Leib N, Koch S, Majlesain Y, Kirins H, Schnautz S, Bieber T. Vitamin D_3-induced promotor-dissociation of PU.1 and YY1 results in FcϵRI reduction on dendritic cells in atopic dermatitis, Journal of Immunology, in revision

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