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Influence of keratinocyte derived mediators on CD4$^+$ T cell plasticity

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Abstract

Background: The human skin is one of the first contact sites for environmental factors and therefore, also crucial for setting the course of adequate immune responses. Keratinocytes are of particular importance in this system since they represent the most abundant cell type in the epidermis and have the ability to influence other cell types like dendritic cells or T cells. A functional crosstalk between keratinocytes and immune cells is often mediated by soluble mediators and is not only essential for the maintenance of skin homeostasis but also for reacting specifically to danger signals. One source of mediators which can impact this crosstalk and which is also sensitive to environmental factors are inflammasome complexes. These multiprotein complexes with the protease caspase-1 as key element can be activated by a multitude of danger signals and react with the activation of IL-1β and IL-18 as hallmark products. Among the known inflammasome triggers for keratinocytes are for instance UV-B irradiation or nigericin toxin. For monocytes an activation in response to pollen derived factors has also been demonstrated. However, neither the response of keratinocytes to pollen substances has been extensively investigated yet nor the influences on the crosstalk to other cells. Therefore, studies regarding these mechanisms represent a promising research subject.

Aim: The aim of the thesis project "Influence of keratinocyte derived mediators on $CD4^+$ T cell plasticity" is to elucidate the mechanisms of the T cell - keratinocyte crosstalk in the human system and to investigate whether this crosstalk can be influenced by environmental factors such as plant pollen or UV-B irradiation.

Methods: Soluble mediators from human primary keratinocytes were collected and used for co-culture experiments with differentiated T helper cell lines (Th1, Th2, Th17, Treg and Th0 control). Influences on T cell effector functions were measured by analysis of cell proliferation, cytokine release and mRNA expression. Besides steady state condition, keratinocyte derived mediators were also generated under IFN-γ provoked inflammatory conditions and under the impact of aqueous extracts from birch, ragweed and timothy grass pollen. To investigate the potential of aqueous pollen extracts (APEs) to impact on keratinocyte effector functions in general but also in particular on inflammasome mechanisms, keratinocytes of atopic and non-atopic donors were stimulated with APE in different concentrations and in combination with UV-B irradiation. For analysis of general

influences, changes in cell morphology, cell survival as well as cytokine release and mRNA expression were tested. Regarding inflammasome activation cell supernatants were analyzed for the hallmark cytokines IL-18 and IL-1β. The protein level of active caspase-1 and IL-1β/ IL-18 was determined by Western Blot, whereas influences on the mRNA level were tested in 2D culture as well as in a 3D skin model and analyzed by quantitative PCR.

Results: Co-culture experiments of different T cell lines with keratinocyte derived mediators revealed a dominating inhibitory effect of keratinocyte mediators on the secretion of nearly all investigated T cell cytokines (IFN-γ, TNF-α, TGF-β, IL-4, IL-5, IL-10, IL-13 and IL-22). Only the release of IL-17 by Th17 cells was induced in the presence of keratinocyte supernatants and seemed to be mediated by a non-protein factor. Experiments using mediators derived from ragweed APE stimulated keratinocytes showed similar results to mediators from unstimulated keratinocytes, whereas pollen extract from birch generally showed weaker effects. Data from analysis on mRNA level supported the protein data and demonstrated inhibiting effects on most of the tested cytokine mRNA and the transcriptions factors T-bet and GATA-3 as well as inducing effects on the transcription factors Foxp3 and RORC2. In addition, results revealed that pollen extracts of different species (ragweed, birch, timothy grass) can activate the inflammasome system in human primary keratinocytes. Elevated levels of IL-18 and IL-1β were measured and an increased protein level of the inflammasome key element, caspase-1, was shown in Western Blot analysis. Furthermore, it was observed that the combination with a second environmental factor, UV-B irradiation, led to even stronger inflammasome activation and that cells from atopic donors had a tendency to stronger reactions regarding IL-18 and IL-1β release than cells from non-atopic donors.

Conclusion: In summary, keratinocyte derived mediators seem to play a crucial role in orchestrating skin immune responses by influencing cytokine production of $CD4^+$ T cell subtypes in distinct ways. Since the influences are mostly of an inhibiting kind a suppression of inappropriate T cell reactions suggests itself as underlying pursuit. In contrast, the specific support of IL-17 release might have the aim to maintain important factors of skin homeostasis. Further results support the hypothesis that pollen can influence the immunological barrier of the skin by triggering the inflammasome in human keratinocytes per se and aggravating the effects of UV-B irradiation. Thus, pollen themselves can provide

a danger signal alone but also excite additive effects which may be important for the initiation and persistence of inflammatory skin reactions.

List of publications and grants

Oral presentations

Pollen and UV-B: A couple causing enhanced inflammasome activation in human primary keratinocytes

Daniela Dittlein, Stefanie Gilles, Julia Hiller, Carsten Schmidt-Weber, Jörg Durner, Heidrun Behrendt, Johannes Ring, Claudia Traidl-Hoffmann

Annual Meeting of ADF (Arbeitsgemeinschaft dermatologische Forschung), March 5th - 7th 2015, Talk in „Arbeitsgruppe experimentelle Allergologie" (AGEA), Ulm, Germany.

Pollen substances provoke inflammasome activation in human keratinocytes

Daniela Dittlein, Stefanie Gilles, Julia Hiller, Carsten Schmidt-Weber, Jörg Durner, Heidrun Behrendt, Johannes Ring, Claudia Traidl-Hoffmann

World Immune Regulation Meeting –VII (WIRM), March 19th -22nd, 2014, Davos, Switzerland.

Exposure to pollen substances activates the inflammasome machinery in human keratinocytes

Daniela Dittlein, Julia Hiller, Stefanie Gilles, Carsten Schmidt-Weber, Jörg Durner, Heidrun Behrendt, Johannes Ring, Claudia Traidl-Hoffmann

EAACI/WAO (European Academy of Allergy and Clinical Immunology/World Allergy Organization) Congress, June 22nd -26th 2013, Milan, Italy.

Pollen activate the NLRP3 inflammasome in human keratinocytes

Daniela Dittlein, Julia Hiller, Stefanie Gilles, Carsten Schmidt-Weber, Heidrun Behrendt, Johannes Ring, Claudia Traidl-Hoffmann

Annual Meeting of ADF (Arbeitsgemeinschaft dermatologische Forschung), March 13th - 16th 2013, Talk in „Arbeitsgruppe experimentelle Allergologie" (AGEA), Dessau, Germany.

Pollen induce inflammasome activity in human keratinocytes

Daniela Dittlein, Julia Hiller, Stefanie Gilles, Carsten Schmidt-Weber, Heidrun Behrendt, Johannes Ring, Claudia Traidl-Hoffmann

Allerg J 2013; 22 (1): 47

Annual Meeting of DGAKI (German Society of Allergy and Clinical Immunology), March 7th -8th 2013, Mainz, Germany.

Pollen as activator of the inflammasome in human keratinocytes

Daniela Dittlein, Julia Hiller, Stefanie Gilles, Carsten Schmidt-Weber, Heidrun Behrendt, Johannes Ring, Claudia Traidl-Hoffmann

EAACI (European Academy of Allergy and Clinical Immunology) Winter School, January 27th –30th 2013, Pichl, Austria.

Influence of keratinocyte derived mediators on CD4⁺ T cell cytokine secretion

Daniela Dittlein, Stefanie Eyerich, Kilian Eyerich, Carsten Schmidt-Weber, Johannes Ring, Heidrun Behrendt, Claudia Traidl-Hoffmann

DGAKI (German Society of Allergy and Clinical Immunology), 7th "Deutscher Allergiekongress", October 11th -13th 2012, Garching, Germany.

Impact of keratinocyte derived mediators on CD4⁺ T cell cytokine secretion

Daniela Dittlein, Stefanie Eyerich, Kilian Eyerich, Carsten Schmidt-Weber, Johannes Ring, Heidrun Behrendt, Claudia Traidl-Hoffmann

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Annual Meeting of DGAKI (German Society of Allergy and Clinical Immunology), March 22nd -23rd 2012, Mainz, Germany.

Poster presentations

Pollen and UV-B: A couple causing enhanced inflammasome activation in human primary keratinocytes

Daniela Dittlein, Stefanie Gilles, Julia Hiller, Carsten Schmidt-Weber, Jörg Durner, Heidrun Behrendt, Johannes Ring, Claudia Traidl-Hoffmann

Experimental Dermatology, 2015, 24, E19

Annual Meeting of ADF (Arbeitsgemeinschaft dermatologische Forschung), March 5th - 7th 2015, Ulm, Germany.

Pollen and UV-B: A couple causing enhanced inflammasome activation in human primary keratinocytes

Daniela Dittlein, Stefanie Gilles, Julia Hiller, Carsten Schmidt-Weber, Jörg Durner, Heidrun Behrendt, Johannes Ring, Claudia Traidl-Hoffmann

Winter School on Basic Immunology Research in Allergy and Clinical Immunology, EAACI (European Academy of Allergy and Clinical Immunology), February 5th - 8th, Les Arcs 1800, France.

Pollen substances provoke inflammasome activation in human keratinocytes

Daniela Dittlein, Stefanie Gilles, Julia Hiller, Carsten Schmidt-Weber, Jörg Durner, Heidrun Behrendt, Johannes Ring, Claudia Traidl-Hoffmann

World Immune Regulation Meeting –VII (WIRM), March 19th -22nd, 2014, Davos, Switzerland.

Exposure to pollen substances activates the inflammasome machinery in human keratinocytes

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EAACI/WAO (European Academy of Allergy and Clinical Immunology/World Allergy Organization) Congress, June 22nd -26th 2013, Milan, Italy.

Pollen activate the NLRP3 inflammasome in human keratinocytes

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Experimental Dermatology, 2013, 22, E4

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Impact of keratinocyte derived mediators on $CD4^+$ T cell effector functions

Daniela Dittlein, Stefanie Eyerich, Kilian Eyerich, Carsten Schmidt-Weber, Johannes Ring, Heidrun Behrendt, Claudia Traidl-Hoffmann

EAACI (European Academy of Allergy and Clinical Immunology) Congress, June 16th -20th 2012, Geneva, Switzerland.

Keratinocyte derived mediators and their influence on T cell effector functions

Daniela Dittlein, Stefanie Eyerich, Kilian Eyerich, Carsten Schmidt-Weber, Johannes Ring, Heidrun Behrendt, Claudia Traidl-Hoffmann

Experimental Dermatology, 2012, 21, E30

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Originalia

Substrate promiscuity of a rosmarinic acid synthase from lavender (Lavandula angustifolia L.)

Landmann C, Hücherig S, Fink B, Hoffmann T, Dittlein D, Coiner HA, Schwab W.

Planta. 2011 Aug; 234(2): 305-20.

Pollen and UV-B radiation strongly affect the inflammasome response in human primary keratinocytes

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Abbreviations

Abbreviation	
3D	Three dimensional
ABTS	2,2'-azino-bis 3-ethylbenzothiazoline-6-sulphonic acid
ACD	Allergic contact dermatitis
AE	Atopic eczema
AHR	Arylhydrocarbon receptor
AIM2	Absent in melanoma 1
Amb	Ambrosia artemisiifolia
AMP	Antimicrobial peptides
APC	Antigen presenting cell
APE	Aqueous pollen extract
ASC	Apoptosis-associated speck-like protein containing a CARD
ATP	Adenosine 5’-triphosphat
Bet	Betula pendula
BSA	Bovine serum albumine
CARD	Caspase activation and recruitment domains
CCL	C-Chemokine ligand
CCR	Chemokine receptor
CD	Cluster of differentiation
cDNA	Complementary DNA
CLA	Cutaneous lymphocyte antigen
cpm	Counts per minute
CXCL	CX-chemokine-ligand
DAMP	Danger-associated-molecular-pattern
DAPI	4'4',6-diamidino-2-phenylindole,6-diamidino-2-phenylindole
DC	Dendritic cell
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxid
DNA	Desoxyribonucleic acid
D-PBS	Dulbeccos modified eagle medium
EDTA	Ethylendiamintetraacetate
ELISA	Enzyme linked immuno absorbent assay

FACS	Fluorescence activated cell sorter
FCS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
Foxp3	Forkhead box P3
GM-CSF	Granulocyte macrophage stimulating factor
H&E	Hematoxylin and eosin
h.i.	Heat inactivated
HIF-1α	Hypoxia-inducible factor 1-alpha
HLA	Human leukocyte antigen
HU-DC	Human dendritic cell medium
IDEC	Inflammatory dendritic epidermal cell
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
Inv	Involucrin
K14	Keratin 14
KS	Keratinocyte supernatant
LC	Langerhans cell
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
MACS	Magnetic cell sorting
MAPK	Mitogen-activated protein kinase
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MoDC	Monocyte-derived dendritic cells
mRNA	Messenger RNA
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK cell	Natural killer cell
NLR	NOD-like receptor
NLRP	NOD-like receptor-pyrin-containing
NOD	Nuclear oligomerization domain
PALM	Pollen associated lipid mediators
PAMP	Pathogen-associated molecular pattern

PAR-2	Protease-activated receptor 2
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cell
PE	Phycoerythrin
PFA	Para-formaldehyd
PGE2	Prostaglandin 2
Phl	Phleum pratense
PI	Propidium iodide
Pin	Pinus sylvestris
PRR	Pattern recognition receptor
qPCR	Quantitative polymerase chain reaction
R	Receptor
rh	Recombinant human
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SEM	Standard error of the mean
siRNA	Small interference RNA
STAT	Signal transducers and activators of transcription
TBS-T	Tris-buffered saline -Tween
TCR	T cell receptor
TF	Transcription factor
Tfh	Follicular helper T cell
TGF-β	Transforming growth factor β
Th	T helper cell
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Treg	Regulatory T cell
TSLP	Thymic stromal lymphopoietin
U	Unit
UV	Ultra violet

1 Introduction

Mechanical insults, pollutants, UV light and pathogenic microorganisms, a conglomeration of environmental factors the human body has to withstand every day of its life. Luckily evolution has, in the course of time, developed a protective barrier to shield our organism from such threats: the skin. Its story began 500 million years ago with a primitive integument in Cnidarians which formed the body shape and continues through present time with being a complex and multifunctional organ (Chuong *et al.*, 2002). Today, the mammalian skin forms the boundary between organism and environment, shielding the body not only from harm, but also preventing heat and water loss and serving as a sensitive sensory organ. This vital system of protection and its role as interface to the outside world is achieved by a complex, multi-layered structure and the cooperation of many different cell types (De Benedetto *et al.*, 2012; Feingold *et al.*, 2007).

1.1 Overview of skin components

1.1.1 Skin structure and keratinocytes as central component

The ability of the human skin to carry out multiple roles of maintenance and protection is very closely related to its structure. As shown in figure 1 the skin consists of two major compartments 1. the epidermis, subdivided in four strata, and 2. the underlying dermis (Di Meglio *et al.*, 2011). The most numerous cell type present in the four epidermal layers is the keratinocyte (Pasparakis *et al.*, 2014). Starting from the basement membrane, which connects the epidermal with the dermal part, the *stratum basale* is the bottom layer of keratinocytes. It contains undifferentiated basal cells, which divide constantly and characteristically produce basal structural proteins like keratin 5 and 14. Starting to differentiate, the keratinocytes move on in the *stratum spinosum* and change their keratin production to keratin 1 and 10. Further maturation lets the keratinocytes wander to the *stratum granulosum* where the production of keratins, proteins and lipids is further enhanced (Nestle *et al.*, 2009). Desmosomes, complexes of adhesion proteins, connect the keratinocytes of the granular layer with the cells from the outer layer the *stratum corneum* (Proksch et *al.*, 2008). Here, the keratinocytes, now called corneocytes, form an enucleated

shield which effectively prevents transepidermal water loss (TEWL) but also acts as a scaffold for microflora (Heath and Carbone, 2013).

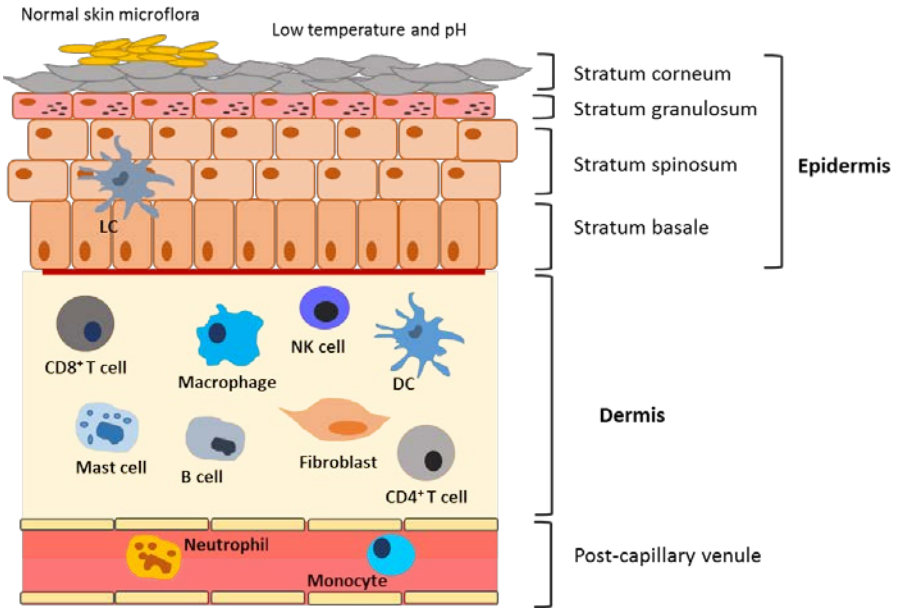


Figure 1: Anatomy of the human skin. The protective epidermal barrier of the skin contains four keratinocyte layers: the stratum basale, the stratum spinosum, the statum granulosum and the outermost layer, the stratum corneum. The layers form a physical barrier and provide home for Langerhans cells (LCs), T cells and on the surface for normal skin microflora. The dermis is composed of collagen, elastic tissue and resticular fibres and harbors many specialized cells. In case of inflammation immune cells can rapidly migrate from post cappillary venules to the skin and contribute to immune responses. *Modified from Miller et al., 2011 and Nestle et al., 2009.*

Although the physical barrier consists mainly of the stratum corneum, the nucleated epidermis parts also provide components contributing to the skin´s barrier function. Important components are for example the cell–cell junctions (tight junctions, desmosomes, gap junctions) and the associated cytoskeletal proteins (keratins, filaggrin) (Kubo *et al.*, 2012; Proksch *et al.*, 2008). In addition, calcium plays an important role for cell-cell adhesion and epidermal differentiation. The calcium concentration is highest in granular layers, where it is an important regulator of protein synthesis, and lowest in the stratum corneum (Proksch *et al.*, 2008). Aside from keratinocytes the epidermal zones also contain non-epithelial cells. Among them, melanocytes, Langerhans cells and also T cells (mainly $CD8^+$) which can be found in the stratum basale and spinosum (Nestle *et al.*, 2009). However, in comparison, the dermis has a far greater cell diversity than the epidermis. The dermal zone is not only rich in extracellular matrix but also contains stromal cells (fibroblasts and fibrocytes), structural cells of blood and lymph vessels and nerve cells.

Furthermore, specialized immune cells, including dendritic cells (DCs), $CD4^+$ T cells, γδ-T cells or natural Killer (NK) cells, macrophages and mast cells, reside in the dermis or migrate through it and contribute vitally to the skins immune barrier function (Nestle *et al.*, 2009; Pasparakis *et al.*, 2014).

1.1.2 Dendritic cells

Dendritic cells are a heterogeneous population of cells with the main tasks of antigen uptake, processing and presentation to T cells (Novak, 2012). Therefore, DCs play as professional antigen presenting cells (APCs) a crucial role in initiating and orchestrating adaptive immune responses. Progenitors of DCs derive from the bone marrow and give rise to several subtypes, differing in their location, migration patterns and specialized immunological roles (Haniffa *et al.*, 2015). Resident DCs are located in the lymphoid tissues and take up antigens delivered from the lymph and bloodstreams to present them via major histocompatibility complex (MHC) molecules to local T cells. In contrast migratory tissue DCs cycle between peripheral tissues and lymph nodes where they present tissue sampled antigens to naïve T cells (Boltjes and van Wijk, 2014). In peripheral blood, three main DC subsets are described: plasmacytoid DC (pDC) and two types of conventional DC (cDCs): $CD1a^+CD1c^+$ and $CD141^{hi}CD14^-$ (MacDonald *et al.*, 2002). While pDCs are mainly characterized by their potential to produce large amounts of type I interferons in anti-viral responses, activated cDCs produce cytokines such as interleukin- 6 (IL-6), IL-12, IL-15, IL-18, and IL-23 (Altfeld *et al.*, 2011; Gregorio *et al.*, 2010).

As immune sentinels DCs also play a central role in skin immunity and are present in the epidermis and the dermis (Haniffa *et al.*, 2015). In steady state the epidermis harbours only Langerhans cells (LCs), which can be identified on the expression of CD11c, CD45, $CD1a^{hi}$, CD207 (Langerin), CD324 (E-cadherin), CD326 (EpCAM), CCR6, CD83, FcεRI and HLA-DR as well as the presence of Birbeck granules (Merad *et al.*, 2008). LCs are situated in the basal and suprabasal layers of the epidermis and interact closely with neighbouring keratinocytes through E-cadherin (Tang *et al.*, 1993). Besides their ability to stimulate inflammatory T cells responses, mice studies raised the possibility that LCs may also have an immune inhibitory capacity by inducing regulatory T cells (Tregs) (Loser and Beissert, 2012; Yoshiki *et al.*, 2009). During inflammation, an additional subset of DCs has been described in the epidermis of atopic eczema patients, the so called inflammatory dendritic epidermal cells

(IDECs) (Wollenberg *et al.*, 1996). IDECs are characterized by following expression pattern: $CD11c^+$, HLA-DR^+, Lin^-, $CD1a^+$, $CD206^+$, $CD36^+$, $Fc\varepsilon R^{hi}$, IgE^+, $CD1b/c^+$,$CD11b^+$ and DC-$SIGN^+$ (Wollenberg *et al.*, 2002). Besides the high expression of IgE receptor (FcεRI), both activated LCs and IDECs express the receptor for the Th2 response promoting cytokine thymic stromal lymphopoietin (TSLP), making them sensitive for TSLP produced by keratinocytes (Wang and Xing, 2008).

The dermal part of the skin harbours at least three different types of cDCs in the steady state. On the one hand, two types of $CD1c^+$ DCs and on the other hand the $CD141^+$ DCs (Gros and Novak, 2012). The $CD1c^+$ population can be further subdivided into $CD1a^+$ $CD11c^+$ DCs which have a mature phenotype and are potent inducers of T cells (Angel *et al.*, 2006), and $CD14^+$ DCs, which are assumed to polarize naïve $CD4^+$ T cells into follicular helper T cells (Klechevsky *et al.*, 2008). $CD141^+$ DCs in contrast, are proposed as equivalents of mouse $CD8\alpha^+$ DCs and to cross present antigens to $CD8^+$ T cells (Bachem *et al.*, 2010). Normally absent in the skin, pDCs can start to migrate into the dermis under inflammatory conditions. For example pDCs are assumed to be early key players in psoriatic lesions and to support the migration of so called inflammatory dermal DCs, a $CD11c^+CD1c^-$ cell population, which contributes to chronic psoriasis pathology (Zaba *et al.*, 2009). Taken together skin DCs form in health and disease a complex network, contributing to tissue homeostasis as well as to skin immunity (Coquerelle and Moser, 2010).

Table 1: Dendritic cells in the skin. Adapted from Merad et al., 2008; Wollenberg et al. 1996, Gros and Novak 2012; Angel et al., 2006; Bachem et al., 2010 and Zaba et al., 2009.

	state	**main group**	**supgroup I**	**subgroup II**
Epidermis	steady state	LCs		
	inflamed state	LCs and IDECs		
Dermis	steady state	cDCs	$CD1c^+$	$CD1a^+CD11^+$
				$CD14^+$
			$CD141^+$	
	inflamed state	cDCs		
		pDCs		
		inflammtory DCs	$CD11c^+CD1c^-$	

LC = Langerhans cell; IDEC = inflammatory dendritic epidermal cells; cDC = conventional dendritic cell; pDC = plasmacytoid dendritic cells

1.1.3 T cells

Although dendritic cells can be seen as initiators of adaptive immune responses, they would be useless without cells receiving and executing their signals. Therefore, T cells with their ability to interact with DCs and to transform information into action, are likewise essential for an effective immune reaction.

1.1.3.1 T cell subpopulations

Originating from hematopoietic stem cells in the bone marrow, T cells develop further in the thymus from where they are only released after a strict selection process (Takahama, 2006). Successfully audited naïve T cells circulate between blood stream and peripheral lymphoid tissues until they meet their specific antigen presented as *MHC : peptide complex* on the surface of an antigen presenting cell (Goldrath and Bevan, 1999). This encounter induces the differentiation of naïve T cells into effector cells, which can subsequently contribute to immune reactions (Kapsenberg, 2003). In general, two main subtypes of T cells are described. The first one is characterized by the surface molecule CD4, which is necessary for the recognition of antigens presented by MHC class II molecules. The second subtype is characterized by the expression of the surface molecule CD8 which in turn is necessary for the recognition of antigens presented by MHC class I molecules (Zenewicz *et al.*, 2009). Naïve $CD8^+$ T cells differentiate in response to antigen recognition to cytotoxic effector T cells that recognize and kill infected cells (Goldrath and Bevan, 1999). In contrast to $CD8^+$ cells, naïve $CD4^+$ T cells can differentiate further to several different T helper (Th) cell subsets: Th1, Th2, Th9, Th17, and Th22 (Dardalhon *et al.*, 2008; Jutel and Akdis, 2011). Each of these subsets is identified by the expression of characteristic transcription factors and the production of certain cytokines, which enable the Th cells to exert specific effects on other cell types, e.g. on B cells for antibody class switching (Cosmi *et al.*, 2014). Another specialized type of $CD4^+$ cell, which is named after its primary localization in the lymphoid tissues, is the follicular helper T (Tfh) cell (Breitfeld *et al.*, 2000). This subtype is characterized by IL-21 secretion and specialized to assist B cells during their differentiation process (Ma and Deenick, 2014). Furthermore, $CD4^+$ cells can also become regulatory T cells which provide self-tolerance and prevent self-destruction by suppressing immune responses and inflammation (Ansel *et al.*, 2003). As shown in figure 2 the polarization of naïve $CD4^+$ T cell to the named subsets is mainly influenced by three signals (Kapsenberg, 2003). *Signal 1* is the T cell receptor (TCR) specific recognition of the antigen presented by

MHC II molecules on the DCs cell surface. *Signal 2* is represented by an additional binding of co-stimulatory molecules and *signal 3* derives from the microenvironment (e.g. cytokines released by DCs or tissue derived factors).

For example, the key cytokine triggering **Th1** polarization is the DC derived IL-12, which can be supported by the presence of IL-18, IL-23, IL-27 and type I interferons (Biedermann *et al.*, 2004). In addition, the transcription factor T-bet was demonstrated to be essential for regulating the Th1 lineage commitment and for the optimal production of the Th1 signature cytokine IFN-γ (Schmitt and Ueno, 2015). The **Th2** subset typically produces the cytokine IL-4, which is also needed for the initial polarization of Th2 cells (Parronchi *et al.*, 1992). The IL-4 signal is transduced by STAT6 which is together with the transcription factor GATA-3 characteristic for the Th2 type. With the release of the cytokines IL-4, IL-5 and IL-13, Th2 cells are an effective instrument to combat extracellular parasites (Urban *et al.*, 1998). However, in industrial countries with a low risk of parasite infections, the Th2 response has turned into a burden for many people, since its IgE supporting nature is the fundament of allergic reactions (Ansel *et al.*, 2003; Pulendran and Artis, 2012). Initially the Th2 subtype was also described as the main source of the cytokine IL-9. However, this was proven false when another distinct Th cell subset primarily producing IL-9 was identified and accordingly named **Th9** (Dardalhon *et al.*, 2015; Kaplan *et al.*, 2015; Veldhoen *et al.*, 2008). Especially the presence of IL-4, TGF-β and IL-21, as well as the expression of the transcription factors PU.1, GATA-3 and IRF-4 play an important role in the polarization of this newly described subtype (Dardalhon *et al.*, 2008; Staudt *et al.*, 2010; Veldhoen *et al.*, 2008). It is assumed that Th9 cells are important for the defence against extracellular pathogens (Schlapbach and Simon, 2014). Also crucial for the host defence against extracellular bacteria and fungi (Bettelli *et al.*, 2007) is the **Th17** subset. Its lineage commitment is induced by a complex cytokine cocktail of IL-6, IL-23, TGF-β and IL-1β and is characterized by the expression of STAT3, the transcription factor RORC and the chemokine receptor CCR6 (Manel *et al.*, 2008; Schmitt and Ueno, 2015). The release of the cytokines IL-17, IL-22 and IL-26 allows Th17 cells to contribute actively in immune responses which are often connected to epithelial tissue. Although, IL-22 secretion was for many years mainly attributed to Th17 and Th1 cells, it turned out that a Th subset producing IL-22 exists, which is relatively independently of IL-17 and IFN-γ release (Duhen *et al.*, 2009; Trifari *et al.*, 2009). This new **Th22** subtype was demonstrated to be relevant for tissue homeostasis and for wound repair (Eyerich *et*

al., 2009). For the polarization into Th22 cells, signalling via the aryl hydrocarbon receptor (AHR) is assumed to be necessary, but a distinctive characteristic transcription factor is still missing for this subtype (Trifari *et al.*, 2009).

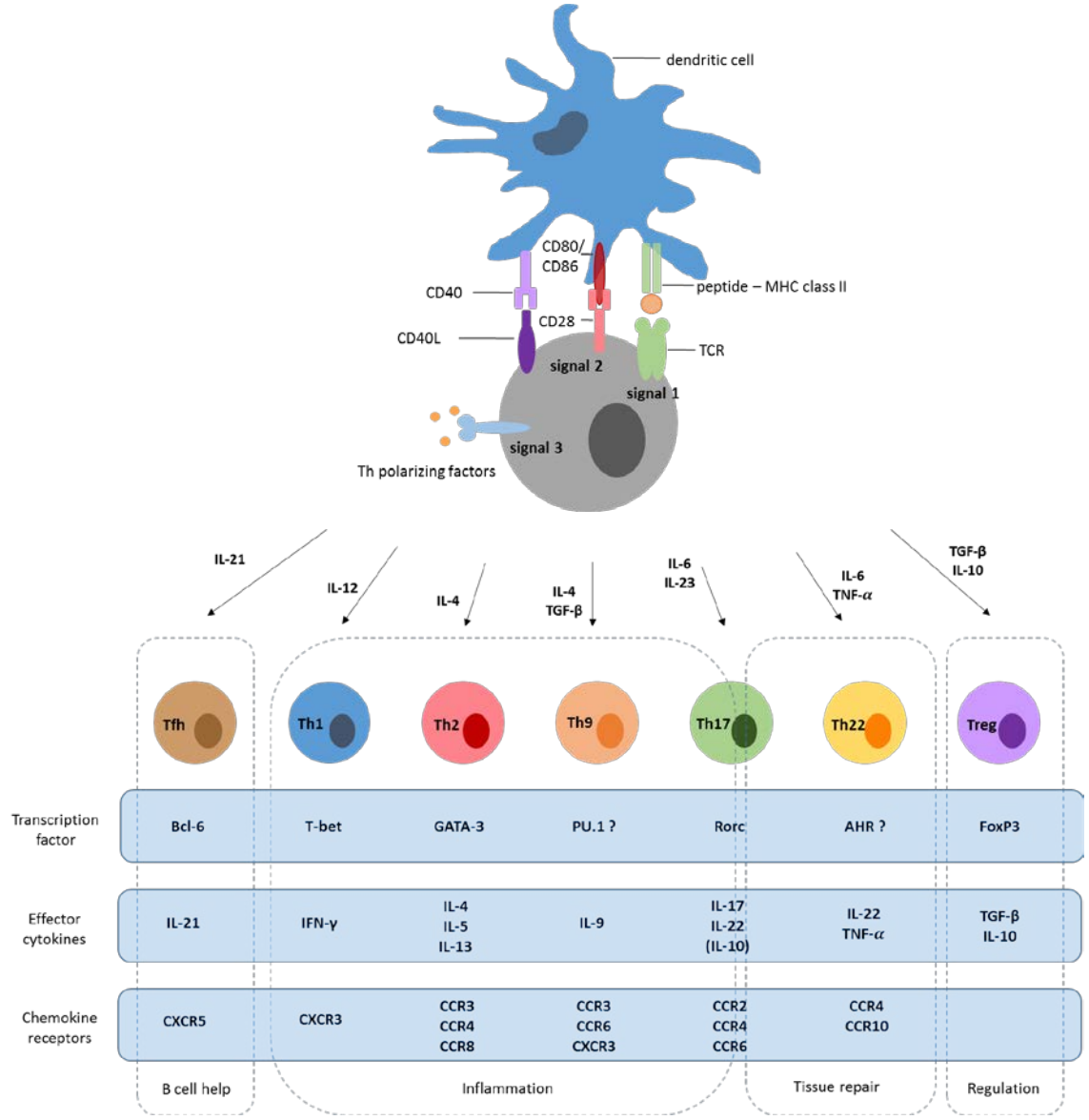


Figure 2: T cell stimulation and CD4⁺ T cell polarization by dendritic cells. Activation and polarization of naïve CD4⁺ T cells requires three signals. Signal 1 is the antigen- specific signal mediated through TCR binding the MCH class II : antigen peptide complex. Signal 2 is the co-stimulatory signal, mainly triggered by the binding of CD28 with CD80/86. Signal 3 is the polarizing signal mediated by soluble or membrane-bound factors. To date six distinct T helper cell subgroups are known to emerge from naïve CD4⁺ T cells (Tfh, Th1, Th2, Th9, Th17 and Th22), which are complemented by the group of regulatory T cells (Tregs). Classically, Th cells are grouped according to their cytokine secretion pattern (effector cytokine panel, middle). Furthermore, expression of lineage-specific transcriptions factors (upper panel) or chemokine receptors (lower panel) is used to assign CD4⁺ T cells to a certain subset. In addition, Th cells can be categorized functionally into B –cell help, inflammation, tissue repair and regulation subsets (dashed lined boxes). *Modified from Kapsenberg, 2003 and Eyerich & Zielinski, 2014.*

Regarding **regulatory T cells** two different main groups exist: natural Tregs (nTregs, which are thymus derived, express CD4, CD25 and Foxp3 and secrete IL-10 and TGF-β) and inducible Tregs (iTregs, which originate only after antigen encounter from naïve $CD4^+$ T cells in the periphery) (Peterson, 2012). The group of iTregs can be further subdivided in a) T regulatory type 1 (Tr1) cells expressing CD4 and CD25 but no Foxp3 and exerting their regulatory function primarily via the production of IL-10; b) Th3 cells expressing CD4, CD25 and Foxp3 and producing large amounts of TGF-β and c) $CD4^+CD25^+Foxp3^+$ cells producing both, IL-10 and TGF-β (Beissert *et al.*, 2006; Roncarolo *et al.*, 2006). For the induction of Tregs mainly the cytokines IL-10 (Tr1), TGF-β (Th3) and a combination of TGF-β, IL-2 and retinoic acid ($CD4^+CD25^+Foxp3^+$) are of importance. Besides the already named markers, also the expression of CTLA-4, GITR, CD127, GARP, CD121a, CD121b can be used to identify Tregs (Hoeppli *et al.*, 2015). To accomplish in their tasks of deleting autoreactive T cells, inducing tolerance and suppressing inflammation, Tregs generally have four strategies: 1. Secretion of inhibitory cytokines like IL-10 and TGF-β; 2. Induction of apoptosis in effector lymphocytes by a granzyme-perforin mediated mechanism; 3. Depriving effector T cells of cytokines leading to apoptosis or 4. Inhibition of the DC function (Vignali *et al.*, 2008). These skills make Tregs very effective immune regulators which are indispensable for a balanced immune response.

In addition to the already described T cells subset, there is a special group of T cells, not expressing the classical TCR consisting of an α and β chain but belonging to the γδ TCR expressing lineage (0.5 - 10 % of all T cells) (Adams *et al.*, 2015; Wu *et al.*, 2009). Since these γδ T cells are the only major set of tissue resident T cells and have a restricted TCR repertoire matched to the respective tissue they are especially interesting regarding local tissue immune reactions (Wu *et al.*, 2009). Moreover, γδ T cells recognize antigens without the need for processing and presentation by classical MHC molecules which makes them sensitive for non-protein antigens like lipids (Hayday, 2000).

1.1.3.2 T cell plasticity

Although the different Th subsets are often described as distinctive, there is still a certain level of plasticity, which allows the cells to adopt other phenotypes under certain circumstances (Figure 3).

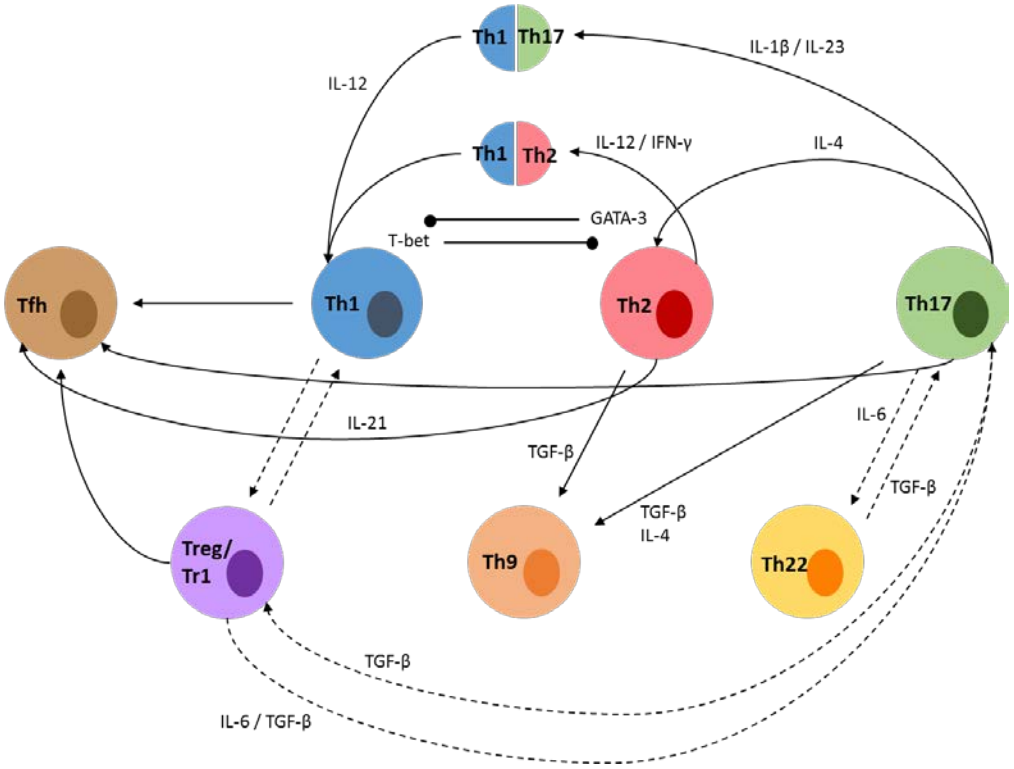


Figure 3: Plasticity of human T helper cells. Naïve $CD4^+$ T cells can differentiate to various types of effector cells (Tfh, Th1, Th2, Th9, Th17, Th22 and Tregs) and fulfil lineage specific tasks. In response to certain factors, however, the lineage commitment can become loose and the subsets can switch lineage. Depending on the microenvironment and the subset either total switches or developments to hybrid cells (displayed as two-coloured cells) are possible. Reciprocal inhibition of the transcription factors T-bet and GATA-3 mostly prevents plasticity between Th1 and Th2 and makes it a rare phenomenon. In contrast, Th17 and Treg cells are presumed to be highly plastic and to be able to switch with the help of specific lineage factors into several different Th subsets. One direction developments are depicted by continuous arrows, reciprocal relationships by dashed arrows. *Modified from Geginat et al., 2014 and Ivanova et al., 2015.*

Especially Treg and Th17 cells have shown a reciprocal relationship in many studies (Nistala and Wedderburn, 2009). So it was demonstrated in mice that dependent of TGF-β1, Th17 cells can transdifferentiate into Tr1 cells and contribute to the resolution of inflammation (Gagliani *et al.*, 2015). In return iTregs, which appear to be less stable than nTregs, seem to be susceptible to transit towards Th17 like cells in an inflammatory environment and the presence of IL-6 and TGF-β (Murphy and Stockinger, 2010) or IL-2 and IL-1β (Deknuydt *et al.*, 2009). Although there also exists a Th1 like phenotype co-expressing Foxp3 and T-bet

in mice (Stock *et al.*, 2004), Th17 cells seem in comparison to Treg and also in general the most plastic subtype. For example Th17 cells can not only acquire Treg features but also switch under the influence of IL-12 from IL-17 producing cells to hybrid cells producing both IL-17 and IFN-γ and further on even to IFN-γ only producing Th1 cells (Annunziato *et al.*, 2015; Lexberg *et al.*, 2010). Moreover, Th17 cells can adopt Th2 phenotype characteristics under the influence of IL-4 (Annunziato *et al.*, 2012; Lexberg *et al.*, 2008). In contrast to this, the Th1 /Th2 balance seems to be more stable, as both of them are regulated by mutually supressing transcription and signalling factors (Biedermann *et al.*, 2004). Nevertheless, it could be demonstrated that even stably committed Th2 cells can express the IL-12Rβ2 and co-produce IL-4 and IFN-γ in response to viral infection (Hegazy *et al.*, 2010). Furthermore, Th1 cells can switch to IL-10 producing Tr1 cells (Cope *et al.*, 2011) and Th2 cells were shown to switch to IL-9 producing Th9 cells upon stimulation with TGF-β (Veldhoen *et al.*, 2008), whereas IL-21 let adopt them the Tfh phenotype (Glatman Zaretsky *et al.*, 2009). In summary, T cell plasticity is a frequently occuring phenomenon and can be a possiblity to ensure the most effective immune response.

1.1.3.3 T cells in the skin

With approximately 2 x 10^{10} cells residing in the entire skin surface, T cells are more than twice abundant in the skin than in the blood and contribute essentially to epithelial immunity (Clark *et al.*, 2006). Under non-inflammatory condition the epidermis harbours primarily $CD8^+$ T cells, whereas the dermis shows an equal number of $CD4^+$ and $CD8^+$ cells (Bos and Kapsenberg, 1993). However, under inflammatory conditions, e.g. psoriatic or atopic skin lesions, augmented numbers of $CD4^+$ T cells can also enter the epidermal compartment (Biedermann *et al.*, 2004). Independent of their localization most of the skin residing T cells belong to the memory type (Bos and Kapsenberg, 1993). These cells have already seen an antigen in skin-draining lymphnodes once and are imprinted by this encounter with the expression of the skin homing adressins cutaneous lymphocyte antigen (CLA) and CCR4 (Campbell and Butcher, 2002; Clark *et al.*, 2006). For the recruitment into the skin, T cells roll along the endothelial lumen mediated by e.g. E-selectin and its ligand CLA, and then follow a chemokine gradient (Islam and Luster, 2012). Contrary to the former opinion that T cells migrate primarily under inflammatory conditions to the skin, recent studies showed that 98 % of all CLA^+ T cells, including naïve T cells, already reside under physiological conditions in the skin (Clark *et al.*, 2006).

Regarding Th subsets there are differences not only in their frequency in the skin but also in their contribution to the maintenance of skin homeostasis and integrity. For example Th1 and Th2 cells are not very frequent in the human skin, although during certain immune disorders such as autoimmune (psoriasis) or allergic diseases (atopic eczema (AE)) they can play an important role (Biedermann *et al.*, 2004). In contrast the subtypes Th17 and Th22 are quite frequent in the skin and have more extensive duties than Th1 and Th2 cells. To provide barrier integrity and to ensure protection against extracellular pathogens, Th17 and Th22 cells have the tasks of a) inducing an epithelial innate immune response b) recruiting immune cells via chemokine induction and c) inducing regeneration of epithelial tissue after an inflammation (Eyerich and Zielinski, 2014). For this purpose especially the release of the subset signature cytokines IL-17 and IL-22 is important since they can trigger keratinocytes to produce a variety of cytokines, chemokines and antimicrobial peptides (Weaver *et al.*, 2013). Although, Th17 and Th22 cells both produce IL-22 and can work together, there are distinctive differences between the subtypes. IL-17 release by Th17 cells is in general a strong pro-inflammatory inductor, which can also reinforce Th1 immune responses. In contrast to this, IL-22 on its own, has a more protective and regenerative effect on keratinocytes and plays an important role in wound repair (Eyerich and Zielinski, 2014). Besides Th1, Th2, Th17 and Th22 cells the relatively newly described Th9 subset is also found in human skin. Th9 cells were shown to increase the production of pro-inflammatory cytokines by other Th cells and since a large proportion of Th9 cells is specific for *Candida albicans,* the subset also seems to be involved in fungus immune responses. In addition, Th9 cells can be found within both, lesions of psoriasis and atopic eczema patients, although only in psoriasis the frequency is higher compared to healthy skin (Schlapbach and Simon, 2014). Around 10 % of the T cells in skin are $CD4^+$ $Foxp3^+$ Tregs and seem to be relevant in many diseases e.g. in psoriasis where the balance of Th17 and Treg cells is an important regulator of disease severity (Marwaha *et al.*, 2012). In addition, 80 % of blood $Foxp3^+$ Tregs express CLA, meaning that the skin is likely to be a major target for peripheral Treg cell homing (Sather *et al.*, 2007).

Besides the mentioned subsets also skin γδ-T cells have an important role in tissue homeostasis and repair. In humans they make up 2-9 % of total T cells in the dermis and 1-10 % in the epidermis (Hayday and Tigelaar, 2003). γδ-T cells can produce growth factors (e.g. fibroblast growth factor 7 (FGF7), FGF9, keratinocyte growth factor and insulin-like

growth factor 1) which promote the survival of keratinocytes (Witherden and Havran, 2013), but can also exert pro-inflammatory action through the production of mediators such as IL-17 (Laggner *et al.*, 2011).

1.1.4 Innate lymphoid cells in the skin

Besides keratinocytes, DCs and T cells, skin immunity and homeostasis also depend on several other cell types. Among them, the group of innate lymphoid cells (ILCs) which was initially only represented by nature killers (NK) cells. However, in the last few years three additional types of ILCs were identified, namely ILC1, ILC2 and ILC3. Their transcriptional programs and effector mechanisms resemble those of T helper subsets which is also reflected in their naming (Diefenbach *et al.*, 2014). Thus, ILC1s produce mainly the Th1 subset associated cytokine IFN-γ, ILC2s express with IL-5, IL-9, and IL-13 a Th2 profile, and ILC3s find their counterpart in Th17 cells expressing IL-22 and/or IL-17 (Halim and McKenzie, 2013; Licona-Limon *et al.*, 2013; Spits, 2013; Walker and McKenzie, 2013). Although these ILCs seem to mirror the phenotypes and functions of T cells, they are part of the innate immune system and differ distinctly in several points from T cells. For instance, ILCs derive from a common lymphoid progenitor and do not express any cell lineage markers linked to other immune cells. Moreover, they do not express antigen receptors or undergo clonal selection and expansion when stimulated (Eberl *et al.*, 2015). ILCs are located throughout tissues, but often in close proximity to epithelial tissue where they are proposed to play the role of translators of epithelial-derived signals to other components of the immune system (Mjosberg and Eidsmo, 2014). Recent studies have already shown that ILC2s can interact via the expression of MHC-II molecules with $CD4^+$ T cells and support the induction of Th2 cells (Oliphant *et al.*, 2014). In the skin, mainly type 2 and 3 ILCs were described and also found to be associated with the pathology of AE and psoriasis, respectively (Kim, 2015). Furthermore, ILC2s express the IL-33 receptor ST2 and respond after ST2 stimulation with the production of IL-5 and IL-13. This effect can be enhanced by the presence of IL-25 and TSLP, which are, as IL-33, factors typically derived from keratinocytes (Salimi *et al.*, 2013; Teunissen *et al.*, 2014). In contrast to type 2 and 3, ILC1s seem to be a rare population in skin (Teunissen *et al.*, 2014). Nonetheless, a role of ILC1 in allergic contact dermatitis is currently being discussed and under investigation (Kim, 2015).

1.1.5 Macrophages, mast cells and basophils in the skin

In addition to DCs, T cells and ILCs, macrophages are another important immune cell type within the skin. Depending on the type, macrophages can have pro- or anti-inflammatory functions. While M1 type macrophages are assumed to contribute to skin inflammation, the regulatory M2 macrophages might exert a dampening effect on inflammation via the production of IL-10 and TGF-β (Fuentes-Duculan *et al.*, 2010; Mantovani *et al.*, 2013).
Similarly to macrophages also mast cells, a type of tissue resident granulocytes, can act either pro- or anti-inflammatory in the skin. For example, mast cells can store pre-formed pro-inflammatory mediators in granules which can be rapidly released after activation, e.g. by antigen binding via IgE receptor. On the other hand, mast cells can also produce IL-2 leading to an accumulation of regulatory T cells in the skin (Galli *et al.*, 2008). Basophils, another type of granulocytes, can also release pro-inflammatory mediators like histamine, leukotrienes or proteolytic enzymes, but are actually recruited to the skin in abundance only under inflammatory conditions. Since basophils are also able to produce IL-4 and IL-13 in response to IgE binding, they can actively contribute to the development of a Th2 response in the skin. In the presence of the keratinocyte-derived cytokines TSLP, IL-33 and IL-18, basophils can reach even further enhanced production of Th2 response related cytokines (Borriello 2014).

All in all, the skin is a highly diverse network of cells communicating and interacting in a myriad of ways which is often influenced and orchestrated by the first signals derived from keratinocytes.

1.2 Keratinocytes as immune sentinels

To fulfil its role as protective barrier, the skin must be able to detect danger signals and to initiate a rapid, efficient and effective response. For this purpose keratinocytes possess several first line mechanisms which, however, are not enough to shield the skin against all possible insults. Therefore, keratinocytes also need to function as a link to specific immunity to ensure an effective second line of defence involving immune cells like DCs or T cells. Essential for this link between keratinocytes and skin infiltrating immune cells is a sophisticated system of receptor expression and mediator production as it is described below (Suter *et al.*, 2009) (Figure 4).

1.2.1 Recognition of external and internal signals: Sensing via receptors

One major group of receptors for the recognition of external danger signals is the family of **Toll like receptor (TLRs)**. As type I transmembrane receptors TLRs can recognize several pathogen-associated molecular patterns (PAMPs) from bacterial, viral and fungal sources as well as danger-associated molecular patterns (DAMPs) like endogenous molecules released from dying cells (Newton and Dixit, 2012). TLRs mainly trigger signalling pathways leading to the activation of the transcription factor NF-κB as well as mitogen-activated protein kinase (MAPK) cascades which drive the transcriptional induction of several cytokine, chemokine, and adhesion molecule genes (Baker *et al.*, 2003; Miller, 2008). In human ten different TLR types are described which are located either on the cell surface to detect extracellular danger signals (TLR1, TLR2, TLR4, TLR5 and TLR6) or intracellularly in endolysosomes for the detection of nucleic acids (TLR3, TLR7, TLR8, TLR9 and TLR10) (Lebre *et al.*, 2007; Li *et al.*, 2013; Morizane *et al.*, 2012). Human keratinocytes can express TLR1-9, although not all TLR types are constitutively expressed and at least for TLR4 contradictory data exist (Lebre *et al.*, 2007; Mempel *et al.*, 2003; Song *et al.*, 2002). Moreover, the TLR expression in keratinocytes can also vary within the different layers of the epidermis and under inflammatory disease conditions (Baker *et al.*, 2003; Lai and Gallo, 2008).

Besides TLRs, keratinocytes also express **nucleotide-binding-and-oligomerization domain (NOD) like receptors (NLRs)**, which are intracellular sensors of PAMPs and can be divided into three subfamilies: 1. NODs, such as NOD1 and NOD2, which respond to bacterial peptidoglycan (Franchi *et al.*, 2009). 2. NLRPs (NOD-like receptor-pyrin-containing; also

known as NALPs) and 3. IPAF (ICE-protease-activating factor) (Conforti-Andreoni *et al.*, 2011). The latter two, NLRPs and IPAF, are both involved in the formation of multiprotein complexes called inflammasomes, which are responsible for the activation and the release of the pro-inflammatory cytokines IL-1β and IL-18 (Watanabe *et al.*, 2007). To date several different inflammasomes (NLRP1, -2, -3, -6, -7, -12, NLRC4, AIM2, RIG-I and IFI16) have been identified of which at least NLRP3, AIM2 and NLRP1 are active in human keratinocytes (Dombrowski *et al.*, 2011; Lamkanfi and Dixit, 2014; Watanabe *et al.*, 2007). The different inflammasome types can be distinguished by their structure and the sensed danger signals but share a common composition. This comprises at least two modules: a cytoplasmic sensor protein either from the NLR family or the PYHIN (pyrin domain (PYD) and HIN domain) family and the effector protein pro-caspase-1. For the sensor proteins missing a CARD (caspase activation and recruitment domain) motif the recruitment of pro-caspase-1 additionally requires the adaptor protein ASC (apoptosis-associated speck-like domain containing a caspase recruitment domain). After recruitment, pro-caspase-1 molecules are cleaved and become proteolytically active allowing the processing of IL-1β and IL-18 pro-forms (Rathinam *et al.*, 2012). To start and finish this cascade two activation signals are necessary. One priming signal is the activation via TLR or Tumor necrosis factor (TNF) receptor, which triggers the NF-κB pathway and subsequently the expression of IL-1β and IL-18 pro-forms (Latz *et al.*, 2013). Signal-2-trigger factors for the activation of caspase-1 are manifold (e.g. anthrax toxin, asbestos, nucleic acids) but the exact mechanisms are poorly understood (Strowig *et al.*, 2012). So far the NLRP3 inflammasome is the most studied type and in comparison to other inflammasomes also the one with the highest diversity in its trigger factors (Newton and Dixit, 2012). Among these factors are bacterial, viral and fungal pathogens, pore-forming toxins, uric acid crystals and also ATP (Lamkanfi and Dixit, 2014). However, the exact molecular mechanisms leading to an activation are also not known for NLRP3 and a single event unifying the diverse agents has not emerged yet. Nevertheless, it is assumed that events like K^+ efflux, Ca^{2+} influx (Munoz-Planillo *et al.*, 2013) or production of ROS do play a role in the assembly of the NLRP3 complex (Zhou *et al.*, 2011). In addition, it was shown that in THP1 cells as well as in human keratinocytes the protease caspase-4 is required for NLRP3 inflammasome activation (Sollberger *et al.*, 2012). In contrast to NLRP3, the AIM2 (absent in melanoma 2) inflammasome is far more specific concerning its trigger factors which comprise solely double stranded DNA of self and non-

self-origin (Hornung *et al.*, 2009; Schroder and Tschopp, 2010). The activation of AIM2 can be enhanced by the presence of type I and II IFNs, as it occurs in response to viral or bacterial pathogens (Fernandes-Alnemri *et al.*, 2010). Besides AIM2 and NLRP3, the NLRP1 complex is the third inflammasome type which is described to be active in human keratinocytes (Watanabe *et al.*, 2007). In macrophages the bacterial anthrax toxin and muramyl dipeptide are direct ligands leading to the assembly of NLRP1, whereas in human keratinocytes UV-B irradiation can also serve as trigger for an activation (Hsu *et al.*, 2008; Watanabe *et al.*, 2007).

Although keratinocytes possess with the expression of TLRs and NLRs efficient tools for recognizing danger signals, they express further receptors complementing the general sensing range. Among them are for example C-type-lectin receptors allowing the cells to recognize carbohydrate structures e.g. from fungal pathogens. Furthermore, keratinocytes express receptors of the complement system as well as Fc receptors such as FcγRI, FcγRII and FcγRIII (Cauza *et al.*, 2002; Szolnoky *et al.*, 2001; Tigalonowa *et al.*, 1990). Together with the TLRs and NLRs these receptors make keratinocytes to an even more efficient barrier for frontline detection of pathogen invasion.

Of equal importance to the sensing of external danger signals is the receiving of internal signals, e.g. from other cells or also via an autocrine feedback pathway. For this purpose keratinocytes express a wide range of cytokine and chemokine receptors as well as receptors for growth factors (see Table 2) (Suter *et al.*, 2009). This makes them sensitive for example for pro-inflammatory cytokines like IL-1, IL-18, TNF-α or IFN-γ or the cytokines IL-17 and IL-22, which are important for the induction of antimicrobial peptides production (Weaver *et al.*, 2013). In addition, keratinocyte activation via its receptors can also alter the proliferation and differentiation behaviour of cells and by this influence on epidermal stability (Suter *et al.*, 2009).

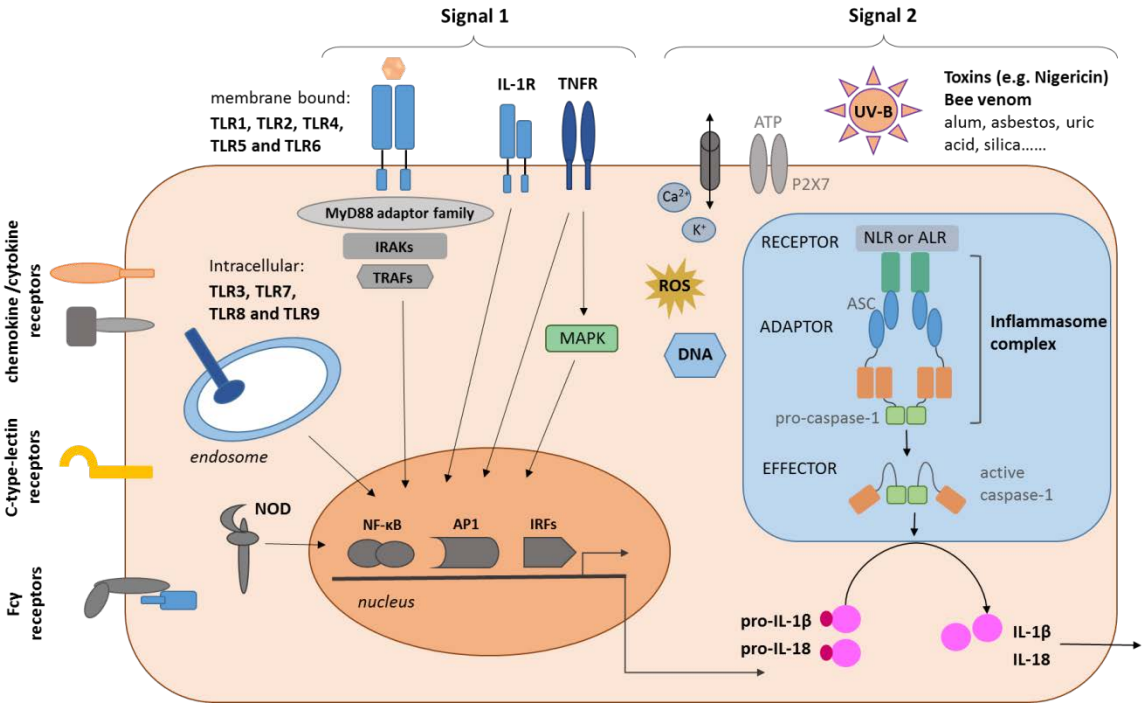


Figure 4: Recognition of external and internal signals in human keratinocytes. Keratinocytes display several different receptor families (TLRs, NLRs, Fcγ-R, C-type lectin receptors and cytokine/chemokine receptors), which can sense PAMPS / DAMPs or soluble messenger factors from the environment and from internal sources. Distinct signal pathways e.g. via MyD88 or MAPKs ensure an effective signal transmission and lead to the transcription of pro-inflammatory mediators. In addition, keratinocytes possess inflammasome complexes contributing to immune reactions by sensing danger signals (e.g. ROS, toxins, UV-B,) and the subsequent activation of the pro-inflammatory cytokines IL-1β and IL-18. Upon transcriptional induction by PRRs (signal 1), IL-1β and IL-18 are synthesized as biologically inactive proteins, which are subsequently processed by the cysteine protease caspase-1. Conversion of procaspase-1 itself into an enzymatically active form is mediated by the inflammasome (signal 2). There are distinct types of inflammasomes, differentiated by their protein constituents, activators, and effectors. A basic inflammasome complex consists of a cytosolic sensor (NLR or a member of the ALR family), the adaptor ASC, and the effector molecule procaspase-1. Functionally expressed in keratinocytes are the inflammasome types NLRP1, NLRP3 and AIM2. Factors stimulating these inflammasome complexes are depicted in bold letters. ATP, activating the Pannexin receptor/ion channel P2X7, is a known inductor of NLRP3 in monocytes, however, not effective in keratinocytes and therefore marked in grey. *Modified from Miller 2007; Newton 2012, Conforti- Andreoni 2011 and Atianand 2013.*

TLR (Toll-like receptor), NLR (NOD-like receptor), PAMP (Pattern- associated- molecular- pattern), DAMP (danger-associated-molecular-pattern), MAPK (mitogen-activated protein kinase), ROS (reactive oxygen species), PRR (Pattern recognition receptor), ALR (AIM2-like receptor), ASC (apoptosis-associated speck-like protein containing a CARD), CARD (Caspase activation and recruitment domains*),* ATP (adenosine triphosphate).

1.2.2 Tools of defence

To defend themselves and protect the skin´s integrity keratinocytes possess several direct mechanisms but can also recruit and activate other immune cells. In addition, the interaction with commensal bacteria residing on the skin surface can provide a protective barrier against cutaneous pathogens (Figure 5).

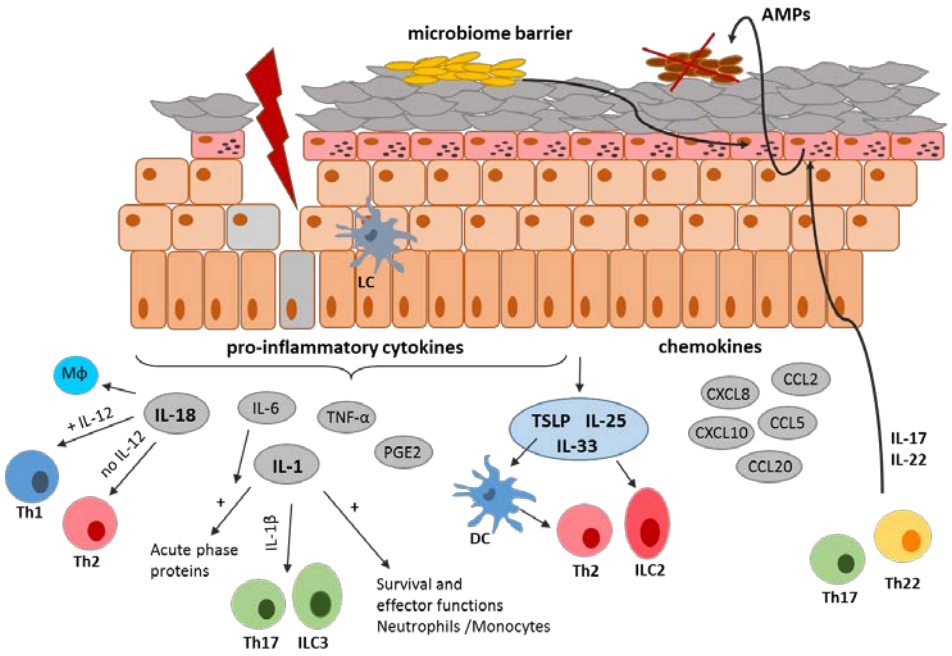


Figure 5: Tools of defence in human keratinocytes. Keratinocytes possess several tools of defence preventing pathogen invasion or supporting immune responses under inflammatory conditions. Depicted are the microbiome barrier, the production of antimicrobial peptides (AMPs), the production of pro-inflammatory cytokines and their effects on immune cells as well as the production of chemokines for the recruitment of immune cells into the skin. *Adapted from Suter 2009, Garlanda 2013, Salimini 2014 and Leung 2014.*

1.2.2.1 Antimicrobial peptides (AMPs)

Keratinocytes are an important source of cationic AMPs, namely β-defensins and cathelicidins as well as Ca^{2+} sensing S100 family members and RNases (Heath and Carbone, 2013; Nestle *et al.*, 2009). β-defensins and cathelicidins are mainly expressed in the upper layers of the epidermis and can kill a wide range of microbes by forming pores in their membranes (Suter *et al.*, 2009). In addition, they can act as chemoattractants and mediate the migration of immune cells (Yang and Oppenheim, 2009). During skin infection the local production of AMPs by keratinocytes can be increased by T cell-derived cytokines, in particular IL17-A and IL-22 (Weaver *et al.*, 2013).

1.2.2.2 Keratinocyte derived cytokines and chemokines

Keratinocytes can produce a wide range of cytokines and chemokines to exert specific effects on other cell types (see Table 2). This includes for example GM-CSF, which induces differentiation and activation of DCs, IL-10 as regulatory cytokine and also members of the IL-1 family (e.g. IL-1, IL-18, and IL-33) as well as TNF-α which can trigger an independent inflammatory reaction (Suter *et al.*, 2009). For example IL-1β and IL-18, which are unified in their dependence on inflammasome mediated activation, are central mediators in the control of inflammation and immune response. Since both isoforms of IL-1, α and β, bind to the same receptor (IL-1R1) they have similar biological properties. This includes the induction of fever, vasodilation and hypotension as well as the enhancement of pain sensitivity and influence on differentiation and function of innate and adaptive lymphoid cells (Dinarello, 2009). Despite these similarities the α and β forms also differ in several ways. This comprises their activation, expression pattern and partly their impact on immunity (Sims and Smith, 2010). For example IL-1α is already fully active in its pro-form and needs no further proteolytic activation by caspase-1 to function as a mediator of the early phase in sterile inflammation (Chen *et al.*, 2007; Rider *et al.*, 2011). For IL-1β, in contrast, it is known that it depends on caspase-1 to become activated and that it efficiently promotes the development of Th17 and ILC3 cells (Annunziato *et al.*, 2007; Garlanda *et al.*, 2013). Like IL-1, the cytokine IL-18 has several different effects and is an important component of T cell polarization, natural killer (NK) cell responses and the interplay between macrophages and NK cells (Mailliard *et al.*, 2005; Smith, 2011). For example, IL-18 can work synergistically together with IL-12 to induce Th1 cell differentiation, but in absence of IL-12 it can also promote Th2 responses (Konishi *et al.*, 2002; Nakanishi *et al.*, 2001). In addition, IL-18 can enhance the generation and function of Th17 cells, however, less effective than IL-1β (Chung and Dong, 2009). In keratinocytes, unlike to macrophages, not only the IL-18 but also the IL-1 pro-form is constitutively expressed, making it easier to achieve a fast activation of the cytokines (Feldmeyer *et al.*, 2007; Olaru and Jensen, 2010). Notably, all three cytokines IL-1α, IL-1β and IL-18 are characterised by lacking a signal peptide for protein secretion via the classical endoplasmatic reticulum/Golgi pathway (Lamkanfi, 2011). Therefore, they are released from the cell by a poorly understood pathway termed *unconventional protein secretion* (Nickel and Rabouille, 2009). Nevertheless, recent studies have indicated that caspase-1 itself may play a role in the

secretion of several leaderless proteins including also IL-1α (Gross *et al.*, 2012; Keller *et al.*, 2008). Besides IL-1 and IL-18, keratinocytes express with IL-33 another cytokine belonging to the IL-1 family. Together with TSLP and IL-25, which are produced by keratinocytes as well, IL-33 is a known inducer of Th2 response. In response to pathogenic stimuli, keratinocytes can also secrete several chemokines including CXCL8 (IL-8), CXCL10 (IP10), CCL20 (MIP-3 α) and CCL5 (RANTES), which subsequently recruit T cells and innate effector cells to the skin (Heath and Carbone, 2013).

Table 2: Receptors and molecules expressed by human keratinocytes. Adapted from Suter et al., 2009 and Bernard et al., 2012.

Adhesion molecules	ICAM; E-Cadherin; p120 catenin
Interleukins	IL-1; IL-6; IL-8; IL-10; IL-12; IL-15; IL-17; IL-18; IL-23; IL-25; IL-33
Interferons	IFN-α, -β and - γ
Cytokines	TNF-α; TGF-α and -β; βFGF; PDGF; TSLP; PGE2
Growth factors	G-CSF; M-CSF; GM-CSF
Chemotactic factors	CXCL8 (IL-8); CCL5 (RANTES); CCL2 (MCP-1); CXCL10 (IP10); CCL17 (TARC); CCL20 (MIP-3α); CCL22 (MDC); CCL27 (CTACK)
Receptors	TLR1-9; NLRs; CD14 (co-receptor); IL-1R; IL-4R; IL-8R; IL-10R; IL-13R; IL-17R; IL-21R; IL-22R IL-18R; IL-31R; IFN-γR; TNF-αR; EGFR; TGF-βR
Immun. molecules	MHC- I; MHC-II; CD80/CD86; CD40

1.2.2.3 Interactions between skin and its microflora

Although the skin possesses several mechanisms to defend itself from pathogenic bacteria, it is at the same time host of a myriad of commensal microbes. The four major groups of bacteria on the skin are: *Firmicutes*, *Actinobacteria, Bacteroidetes* and *Proteobacteria* (Hannigan and Grice, 2013). Among them are for instance *Staphylococcus epidermidis*, *propionibacterium*, *coryenebacteria* and *lactobacilli,* which reside in different compositions on the tissue surface as well as in its appendages such as hair follicles and sebaceous glands (Belkaid and Segre, 2014; SanMiguel and Grice, 2015). Recent studies showed that this microbial colonization is most of the time extremely beneficial for the human body since it can form a shielding microbiome barrier that protects the skin from infection by competing with pathogens and influencing local immune responses (Pasparakis *et al.*, 2014). However, the microbiome´s composition has to be well balanced, otherwise negative effects and skin inflammation can be a consequence (Pasparakis *et al.*, 2014). To ensure a stable skin homeostasis the skin immune system and the microbiota are in constant communication which primarily takes place in the stratum corneum since this layer is inhabited by the vast

majority of the microorganisms (SanMiguel and Grice, 2015). For example *S. epidermidis* can enhance via TLR signalling the production of AMPs by keratinocytes and therefore support the immune response to group A Streptococcus and HPV infection (Lai *et al.*, 2010; Wanke *et al.*, 2011). In addition, the presence of *S. epidermidis* enhances the expression of tight junctions strengthening the physical barrier of the skin (Yuki *et al.*, 2011) and was also shown to induce a protective IL-17A^{+}CD8^{+} T cell population via DC interaction (Naik *et al.*, 2015). As skin commensals can modulate cutaneous immunity, they can also be influenced in reverse by the host immunity. Studies in this context revealed that in mice skin microbial diversity and richness decreased when the complement system, a central component of innate immunity, was blocked (Chehoud *et al.*, 2013). Furthermore, also patients suffering from immunodeficiency syndromes, e.g. the STAT-3 deficient hyper IgE syndrome or chronic mucocutaneous candidiasis, have altered microbial colonization which can lead to suppressed immune responses to pathogens (Oh *et al.*, 2013; Smeekens *et al.*, 2014).

1.3 Dysregulated skin immune responses and inflammatory mechanisms

Since most skin diseases have a complex pathology, the involvement of keratinocytes is a central point and uncovering underlying mechanisms is crucial for developing new therapies. Especially the cross talk between keratinocytes and other immune cells which is often mediated by soluble factors is an important target. Therefore, the activation of inflammasome complexes as source of the pro-inflammatory cytokines IL-1β and IL-18 has also been proposed as a potential contributor to several dysregulated skin responses. Among them are also the following inflammatory diseases: atopic dermatitis, allergic contact hypersensitivity and psoriasis (Figure 6) .

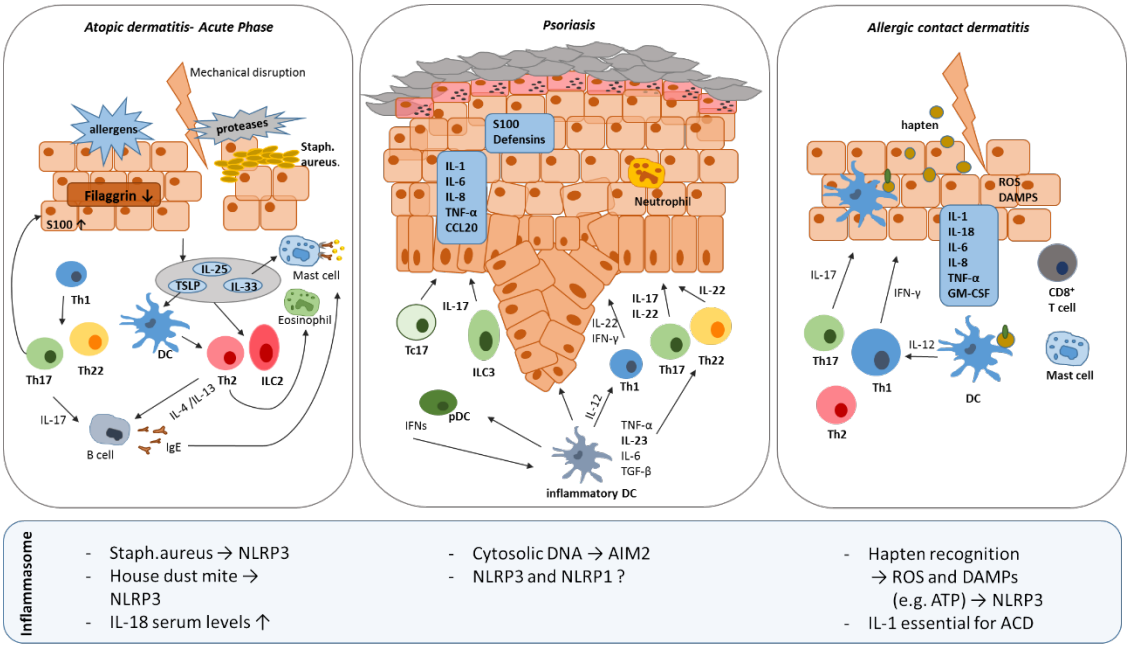


Figure 6: Dysregulated skin immune responses and the involvement of inflammasome associated mechanisms. From left to right are depicted the disease patterns of a) acute atopic dermatitis, b) psoriasis and c) allergic contact dermatitis (ACD). Underneath (blue box) the respective contribution of inflammasome complexes is listed. *Modified from Cevikbas 2012, Di Meglio 2011, Nestle 2009, Diani 2015, Crow 2012, Salimi 2014, Peiser 2013, Gittler 2013 and Kaplan 2012.*

1.3.1 Atopic dermatitis

Atopic dermatitis (AD), also known as atopic eczema (AE), is a multifactorial disorder which clinically manifests in skin lesions characterized by intensive itching and erythematous papules (Spergel, 2008). It is among the three most atopic diseases and often the initial step in the so-called “atopic march” preceding the development of allergic rhinitis and asthma (Leung and Guttman-Yassky, 2014). AD pathogenesis is closely associated with an

unbalanced immune response and epidermal barrier dysfunctions making the skin very dry and sensitive to environmental insults like allergens or microbial colonization (Kuo *et al.*, 2013). According to genetic studies, mutations in the gene encoding the filament-aggregating protein filaggrin are often the underlying reason for this impaired skin barrier function (Irvine *et al.*, 2011). However, only 20 – 40 % of AD patients indeed carry a filaggrin mutation indicating that there are further causes for an impaired barrier function (Mohiuddin *et al.*, 2013). Consistently recent studies revealed that Th2 and Th22 cytokines (IL-4, IL-13, IL-31 and IL-22) can also mediate barrier dysfunctions by weakening the tight junction system or supressing the terminal differentiation of keratinocytes (Leung and Guttman-Yassky, 2014). Moreover, the Th2 cytokines IL-4 and IL-13 can also disturb the skin defence by suppressing the production of AMPs in keratinocytes (Gittler *et al.*, 2012).

Besides the skin's barrier function also the general disease pattern of AD is strongly influenced and defined by cytokines released by immune cells and keratinocytes. Thus, the acute phase of AD with a cutaneous allergic reaction is predominated by Th2 cytokines, whereas the chronic phase is characterized by a shift in the Th1 direction with increased IFN-γ levels and general inflammation (Hamid and Minshall, 1996). However, the last years of research showed that a simple separation in Th2 and Th1 phase is not sufficient. For instance, the acute phase is not only associated with Th2 cytokines but also with Th22 and Th17 derived cytokines which can up-regulate the pro-inflammatory epidermal differentiation complex cluster-encoded S100A genes (Gittler *et al.*, 2013; Nukui *et al.*, 2008). In addition, also the switch to Th1 is not complete and still accompanied by Th2, Th17 and Th22 activated cells, making the AD pathophysiology even more complex (Gittler *et al.*, 2013). Influences on this system can also come directly from keratinocytes, e.g. by the release of the cytokines TSLP, IL-25 (IL-17E) and IL-33 which promote Th2 responses leading to elevated infiltration by mast cells, eosinophils , and allergen specific IgE (Liu *et al.*, 2011; SanMiguel and Grice, 2015). Furthermore, the cytokines are also important for the induction of type 2 ILCs which additionally contribute to the predominated Th2 milieu in acute AD flares (Salimi *et al.*, 2013). All three cytokines as well as the IL-33 receptor ST2 are overexpressed in acute and chronic AD skin and can interplay to promote inflammatory skin reactions (Kuo *et al.*, 2013). In addition, it was shown that also changes in the microbiome characterize acute flares, namely decreased diversity and richness which restores after flare remission (Kong *et al.*, 2012).

Furthermore, several studies suggested an implication of inflammasome associated processes in the pathology of AD. For instance AD patients often suffer from an elevated *Staphylococcus aureus* skin colonization, which is known to activate the NLRP3 inflammasome in keratinocytes and trigger IL-1β release (Miller and Cho, 2011; Olaru and Jensen, 2010). In addition, the classical type I allergen Der p 1 from house dust mite was shown to induce inflammasome driven reactions in human primary keratinocytes and is proposed as contributing factor to AD flares (Dai *et al.*, 2011). IL-1β itself is also known to mediate several other diseases, e.g. CAPS or Schnitzler syndrome which share the hallmark of urticarial rash with a typical allergic skin reaction (Contassot *et al.*, 2012). Additionally, an association with enhanced expression of IL-1 cytokine in the stratum corneum could be demonstrated for AD patients with filaggrin loss-of-mutations (Kezic *et al.*, 2012). Another implication of inflammasome reaction in allergic disease was shown for bronchial asthma, suggesting also roles in another atopic diseases (Kim *et al.*, 2014). For the course of AD also IL-18 seems to be particularly important since it can drive Th1 and Th2 reactions depending on the microenvironment and can accordingly contribute to changes in the T cell response (Konishi *et al.*, 2002; Nakanishi *et al.*, 2001)). Moreover, murine studies demonstrated that an over-secretion of IL-18 from epidermal cells resulted in AD-like skin eruptions (Konishi *et al.*, 2002).

1.3.2 Allergic contact dermatitis

Likewise to atopic dermatitis the allergic contact dermatitis (ACD) reaction belongs to the group of allergic related skin disorders and is also assumed to be influenced by inflammasome associated mechanisms (Gittler *et al.*, 2013; Weber *et al.*, 2010). The disease falls in the category of delayed-type hypersensitivity which is caused by haptens, e.g. nickel, activating antigen-specific T cells. ACD often occurs as an occupational disease, for instance in form of organic chemicals allergy, and with a prevalence of 7% it is quite spread in general population (Diepgen, 2003; Heine *et al.*, 2004; Thyssen *et al.*, 2007). Regarding inflammasome involvement, studies in human and mice demonstrated that the sensitization phase of ACD is indeed dependent on activated caspase-1, IL-1β and IL-18 and that signalling via the IL-1 receptor is necessary both in the sensitization period and elicitation phase (Antonopoulos *et al.*, 2001; Kish *et al.*, 2012; Zepter *et al.*, 1997). Although, Langerhans cells are often contributing in this reaction, the potent hapten

trinitrochlorobenzene (TNCB) can induce in human keratinocytes also directly a caspase-1 dependent IL-1β activation (Watanabe *et al.*, 2007). Clinical relevance of the inflammasome involvement manifests in the fact that the use of anti-IL-1 antagonists suppresses the symptoms of contact hypersensitivity (Kondo *et al.*, 1995).

1.3.3 Psoriasis

With a prevalence of about 2% in Europe and North America psoriasis is a wide spread inflammatory skin disease which, however, is not allergy related but classified as a chronic autoinflammatory disorder (Christophers, 2001; Diani *et al.*, 2015). It is characterized by keratinocytes proliferating at ten times the rate of non-diseased skin cells and fail to mature properly, resulting in inflamed skin lesions known as plaques (Crow, 2012). Psoriatic episodes are mainly mediated by DCs and T cells accompanied by a complex feedback loop to other cells including keratinocytes (Boehncke and Schön, 2015). Likewise other autoimmune-type diseases, psoriasis initially has been defined as a Th1 type dominated disorder. However, in the last years it became clear that also Th17 and Th22 cells essentially contribute to its pathophysiology (Eyerich *et al.*, 2010). The cross-talk between the skin epithelial cells and the migrated immune cells is mainly mediated by cytokines such as TNF-α, IFN-γ, IL-22 and IL-17 but also the inflammasome related cytokine IL-1 (Boehncke and Schön, 2015; Diani *et al.*, 2015). Therapeutically antibodies directed against IL-17 and TNF-α or inhibitors of the cytokines showed good results (Gottlieb *et al.*, 2005; Papp *et al.*, 2012). In addition, it could be demonstrated that cytosolic DNA can trigger AIM2 inflammasome and IL-1β activation in psoriasis (Reinholz *et al.*, 2013). These findings and genetic studies suggesting an involvement of NLRP1 and NLRP3 (Ekman *et al.*, 2014; Salskov-Iversen *et al.*, 2011), make also the inflammasome a potential target in psoriasis therapy.

1.4 Impact of environmental factors on skin immunity

Although the skin is composed of several barriers shielding the human body from harm, its integrity can be impaired by exogenous factors. Among these factors are germs and chemicals, but also plant pollen and ultraviolet (UV) irradiation. The latter two are particular important since the human body is automatically exposed to them during outside stays and complete avoidance is impossible. Therefore, the impact of plant pollen and UV

light on skin immunity is of certain importance and described in detail in the following sections.

1.4.1 Plant pollen derived substances as immune modulating factors

From a medical point of view plant pollen are primarily allergen carriers causing allergic reactions like hay fever, asthma or atopic skin eczema (Dharajiya *et al.*, 2008; Werfel *et al.*, 2015). However, for plants, pollen grains are containers of the male genetic material and therefore essential for reproduction and survival. In angiosperms pollen grains are found in the anthers of stamens, from where they have to be transported to the pistil, the female reproductive organ (Bleckmann *et al.*, 2014). This process, called pollination, can be mediated by insects, water or wind, where transportation by wind can overcome long distances (Aličić, 2014; Sofiev, 2013). Once a pollen grain reaches a suitable pistil it starts germinating and produces the pollen tube transporting the sperm cells to the ovule (Bleckmann *et al.*, 2014). However, in anticipation of germination the landing on any moist ground, which includes human epithelia, is for pollen in general a signal to release many bioactive substances (Gunawan *et al.*, 2008). Among these substances are often molecules with allergenic potential, for example the major allergen from ragweed, Amb a I, which is a pectate lyase and originally meant for modifying pectin in the intine and the pollen tube (McCormick, 2013). Apart from allergens also proteases, lipids or NAD(P)H oxidases with ROS-generating activity are released upon hydration by the pollen grain (Gunawan *et al.*, 2008). Although these factors are necessary for the plant they can have negative effects on the human system. For example NAD(P)H oxidases play an important role in pollen tube growth, but in humans the production of ROS can amplify allergic immune responses (Dharajiya *et al.*, 2008). The same is true for proteases, which are needed for example in the pollen-pistil interaction (Radlowski, 2005), but which can also destroy the protective tissue barrier of human skin epithelia and thereby facilitate the passage of allergens (Takai and Ikeda, 2011). In addition, it could be demonstrated that lipids, a main component of the pollen grain´s cell wall, show homology to a molecule group important for inflammatory reactions in humans, the eicosanoids (Gilles *et al.*, 2009a). These so called Pollen-associated-lipid-mediators (PALMs) can be divided structurally into two groups. The LTB_4 like PALMS, which are monohydroxylated derivates of the linoleic and α-linolenic acid, and the group of phytoprostanes which are formed in a non-enzymatic oxygen radical catalysed

reaction from α-linolenic acid (Gilles *et al.*, 2012). PALMs of the LTB_4 like class were shown to induce chemotaxis and activation of human neutrophils and eosinophils, whereas birch pollen–derived E1 phytoprostanes inhibit the production of IL-12p70 in human dendritic cells via blocking NF-κB translocation (Gilles *et al.*, 2009b; Plotz *et al.*, 2004; Traidl-Hoffmann *et al.*, 2002; Traidl-Hoffmann *et al.*, 2005). Besides nasal and lung epithelia the skin is one of the biggest contact sides for pollen. Thus, keratinocytes are also direct targets of all released pollen substances. Studies showed that pollen allergen is not only processed by Langerhans cells in the skin but can also be internalized by keratinocytes. An accumulation of the allergens in the keratinocyte lysosomes hints to an internal processing necessary for MHC-class II presentation (Blume *et al.*, 2009). Furthermore, it could be shown that sensitized patients react with skin eczema to atopy patch tests with native pollen grains (Eyerich *et al.*, 2008). The fact that pollen substances can act as trigger factor for AD is supported by another study, demonstrating that total exposure of AD patients with grass pollen in an environmental challenge chamber results in a pronounced eczema flare up at air exposed skin areas (Werfel *et al.*, 2015).

Since the contact with wind transported pollen is most likely for humans, mainly pollen from anemophilous plants are of interest in allergy aspects. Among them *Ambrosia artemisiifolia* (common/short ragweed) is described as exceptionally aggressive not only in its spread rate but also regarding its allergenicity. The plant which originates from North America is since the beginnings of the 1990s also on the rise in Europe. *A. artemisiifolia* is an annual weed which has its flowering season from August until end of September and produces high amounts of pollen grains with up to 60 000 seeds in large specimens (Crompton, 1975; Taramarcaz *et al.*, 2005). Therefore, the majority of late summer hay fever can be attributed to *A. artemisiifolia* pollen of which 5 -10 grains per cubic meter of air are already sufficient to provoke allergic reaction in sensitized patients (Crompton, 1975; Mandrioli, 1998). High NAD(P)H oxidase contents (Dharajiya *et al.*, 2008) and strong protease activity (Gunawan *et al.*, 2008) are assumed to contribute to the high allergenic potential of *A. artemisiifolia*. Air pollution and climate change are additional factors which can have impact on pollen. For example diesel exhausting particles but also high environmental ozone levels were shown to enhance the allergenicity of pollen (Beck *et al.*, 2013; Kanter *et al.*, 2013). These influences make pollen to an even more resilient danger signal for human cells and therefore also more likely to provoke skin reactions.

1.4.2 UV-B irradiation as immune modulating factor

Even though solar irradiation is necessary for vitamin D3 production and gives most people a feeling of well-being, the associated exposure to ultraviolet (UV) light is not always beneficial to the human skin. This is based on the nature of UV radiation, which allows an interaction with organic molecules and can provoke chemical reactions. Depending on the wavelength UV-light is divided into three categories: UV-C (200 – 290 nm), UV-B (290 – 320 nm) and UV-A (320 – 400 nm) (Totonchy and Chiu, 2014). Although the stratospheric ozone effectively blocks wavelengths shorter than 290 nm and 70 – 90 % of UV-B, a significant portion of UV radiation still reaches the human skin (Duthie *et al.*, 1999). The stratum corneum of the epidermis can absorb most of this radiation, however, the longer wavelengths, like UV-B, do still partly penetrate the epidermal layers and can cause severe photodamage (Nasti and Timares, 2012). The mildest forms of UV-B provoked reactions are "sunburns" with temporary erythema and inflammation. But excessive and prolonged exposure to UV-light can also cause permanent skin damage, photo-ageing and DNA damage and increases the likelihood of developing skin cancer (Leighton *et al.*, 2013). Although the mechanisms responsible for these effects are often more than complex, some of them have already been investigated successfully. For example, the UV-B caused isomerization of urocanic acid in the epidermis has been identified as a trigger for reactive oxygen species (ROS) production, resulting in oxidative DNA and protein damage and in downstream immune suppression (Hart *et al.*, 1993). Regarding DNA mutations it was shown that UVB leads to cyclobutane pyrimidine dimers and pyrimidine-pyrimidone photoproducts which contribute to the high proportion of p53 mutations in squamous cell carcinomas (Lee *et al.*, 2013). In addition, UV-B modifies cell signalling mechanisms leading to a local microenvironment that favours Th2 immune responses and suppresses Th1 driven reactions (Duthie *et al.*, 1999). The Th2 milieu is enhanced further by the UV-B induced production of high IL-33 levels in keratinocytes (Byrne *et al.*, 2011). Another mechanism known to be involved in the response to UV-B irradiation is the activation of NLRP3 and NLRP1 inflammasomes in keratinocytes. As described in section 1.2.1 inflammasome activation is the source of the cytokines IL-1β and IL-18 which drive inflammation and can direct immune reactions. For example IL-1 release can stimulate skin cells to synthesize prostaglandins as well as to produce the pro-inflammatory cytokines TNF-α and IL-6 (Duthie *et al.*, 1999). Although the exact mechanisms leading to the

inflammasome activation after UV-B irradiation are not completely understood, the two factors ROS and Ca^{2+} influx are considered to play an important role (Feldmeyer *et al.*, 2007; Heck *et al.*, 2003). Despite the negative effects UV-light can have in higher dosages it is used in lower dosages also as therapy for skin diseases e.g. psoriasis, vitiligo and atopic dermatitis (Totonchy and Chiu, 2014). In AD the immunesuppressive effects of narrowband UV-B, including suppression of the antigen-presenting function of Langerhans cells, the induction of apoptosis in infiltrating T cells and a reduction of S. *aureus* colonization (Garritsen *et al.*, 2014), are assumed to improve disease symptoms. However, many studies showed contradictory results and it seems that only chronic and not acute flares of AE respond to UV-B therapy at all (Darsow *et al.*, 2013; Garritsen *et al.*, 2014).

In summary UV-B light is an environmental factor which exerts many effects through different mechanisms in the human skin and can therefore not to be underestimated in its role as immunomodulator.

2 Aim of the Study

Being able to produce and secrete a broad spectrum of different immunomodulatory mediators, keratinocytes seem to be predestined for the interaction with other cell types and for transmitting information. An important partner in this crosstalk are T helper cells which are known to play essential roles in maintaining skin homeostasis but also in inflammation and skin defence reactions. To understand the underlying mechanisms of skin diseases with involvement of T helper cells (e.g. psoriasis or atopic dermatitis), it is crucial to figure out how interactions with epithelial cells contribute to the orchestrating of immune responses. Hence, research in this area is much-needed, especially since former studies in this field mainly concentrated on effects T cells can have on keratinocytes but not the other way round. In addition, nearly no data is available regarding the influences keratinocytes can exert on distinct $CD4^+$ T cells subsets by the production of soluble mediators. Likewise nearly totally neglected was the influence of environmental factors on the cell cross talk in the human skin. For instance, plant pollen are a known risk factor not only for allergic rhinitis and asthma, but also for eliciting eczematous reactions on the skin of patients with atopic dermatitis. A modulation in the secretion and production of keratinocyte derived mediators in this process seems to be unavoidable, but to what extent and by which mechanisms is still unknown. The same is partly true for UV-B radiation, which is another environmental factor difficult to avoid during outdoor days and which can cause in higher dosages skin damage. Aside from that it has also been mostly disregarded that a combination of environmental factors may be more effective than single factors in provoking reactions and modulating immune responses e.g. by inducing inflammasome activation.

Thus, the aim of this study is to test on the one hand the modulating effect of keratinocyte derived mediators on the function and plasticity of different T helper cell lines and on the other hand to investigate the potential of the two environmental factors, plant pollen and UV-B, to influence keratinocytes in their effector function in general but also in particular by activating inflammasome mechanisms (Figure 7).

Results from these studies will provide a deeper insight in disease mechanisms as well as the chance to find new points of contact where environmental influences, e.g. allergy

provoking substances, may cause immune reactions in skin by modulating the production and secretion of messenger molecules from keratinocytes.

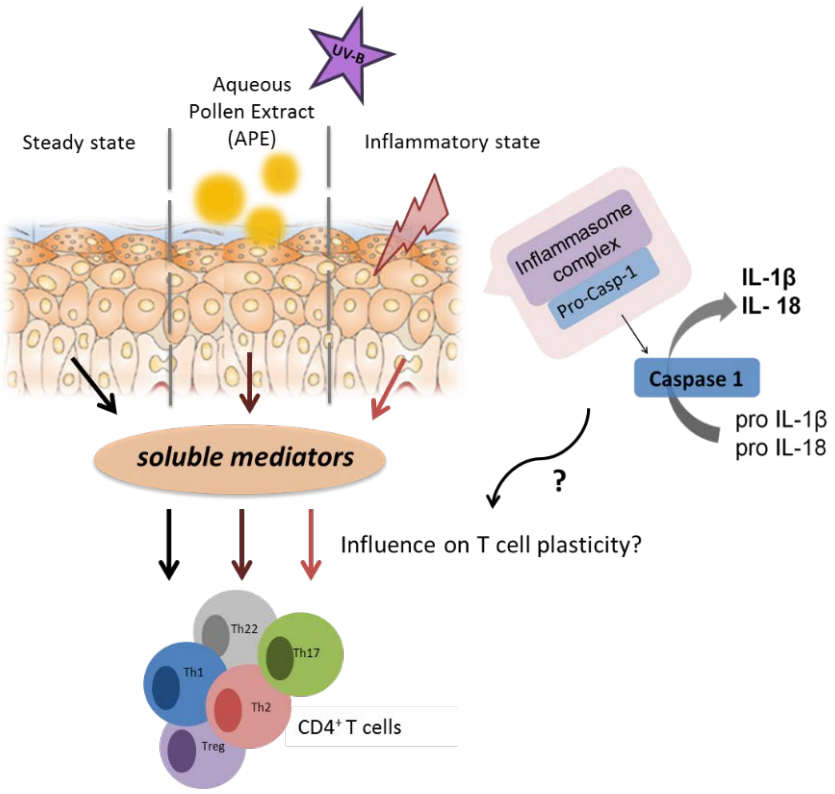


Figure 7: Aim of the study. For a better understanding of disease mechanisms and to define the potential of environmental factors to influence skin immune responses the crosstalk between keratinocytes and T helper subsets via soluble mediators is investigated.

3 Material and Methods

3.1 Material

3.1.1 Reagents

Table 3: Reagents

Reagent	Supplier
10xMEM	Life technologies, Carlsbad, CA; U.S.A.
2-Mercapto-Ethanol	Sigma-Aldrich, München; Germany
^{3}H-Thymidine	Hartmann Analytik, Göttingen; Germany
4',6-diamidino-2-phenylindole (DAPI)	Sigma-Aldrich, München; Germany
ABTS	Roche Diagnostics, Mannheim; Germany
Acetone	Merck, Darmstadt; Germany
Adenine	Sigma-Aldrich, München; Germany
Adenosintriphosphat (ATP)	Merck Millipore, Darmstadt, Germany
AIM-V serum free medium	Life technologies, Carlsbad, CA; U.S.A.
Albumin from bovine serum (BSA)	Sigma-Aldrich, München; Germany
Aqua ad injectabilia	Laboratori Diaco Biomedicali, Trieste; Italy
autoMACS rinsing solution	Miltenyi Biotech, Bergisch Gladbach; Germany
autoMACS running buffer	Miltenyi Biotech, Bergisch Gladbach; Germany
Calciumchloride	Merck, Darmstadt; Germany
Caspase inhibitor Ac-YVAD-cmk	Sigma-Aldrich, München; Germany
CD14^{+} micro-beads (human)	Miltenyi Biotech, Bergisch Gladbach; Germany
CD4^{+} T cell isolation kit II (human)	Miltenyi Biotech, Bergisch Gladbach; Germany
Citrat-monohydrate	Merck, Darmstadt; Germany
Citric acid (0,1 M)	Merck, Darmstadt; Germany
Collagen I, rat tail	Life technologies, Carlsbad, CA; U.S.A.
Cryo-embedding compound	Microm International, Waldorf; Germany
Cytotoxicity Detection Kit (LDH)	Roche Diagnostics, Mannheim; Germany
DEPC treated water (pyrogen free)	Life technologies, Carlsbad, CA; U.S.A.
DermaLife Calcium-free basal medium	Lifeline Cell Technology, U.S.A.
DermaLife K Cell Culture Medium	Lifeline Cell Technology, U.S.A.
DermaLife K Cell Culture Medium Components	Lifeline Cell Technology, U.S.A.
Dimethylsulfoxid (DMSO)	Merck, Darmstadt; Germany
DMEM high Glucose	Life technologies, Carlsbad, CA; U.S.A.

DMEM high Glucose, no Ca^{2+}, no Glutamine	Life technologies, Carlsbad, CA; U.S.A.
DMSO, cell culture grade	Applichem, Darmstadt; Germany
D-PBS w/o Ca/Mg	Life technologies, Carlsbad, CA; U.S.A.
ECL Prime Western Blot Reagent	GE Healthcare, Freiburg; Germany
EDTA (0,05 %, pH 8,0)	Life technologies, Carlsbad, CA; U.S.A.
Epidermal growth factor (EGF)	Sigma-Aldrich, München; Germany
Ethanol absolute	Merck, Darmstadt; Germany
F12 + Glutamax Nutrient Mixture	Life technologies, Carlsbad, CA; U.S.A.
Fast Start Universal SYBR Green Master (Rox)	Roche Diagnostics, Mannheim; Germany
Fetal calf serum (FCS) Hyclone II	Perbio Science, Bonn; Germany
First strand cDNA synthesis kit	Thermo Fisher Scientific, Schwerte, Germany
First strand cDNA Synthesis kit	Applied Biosystems (Life technologies) Carlsbad, CA; U.S.A.
Formaldehyde (min 37%)	Merck, Darmstadt; Germany
Gentamycine	Life technologies, Carlsbad, CA; U.S.A.
Heparin-Natrium 250.000 U	Ratiopharm, Ulm; Germany
High capacity cDNA reverse transcription Kit	Life technologies, Carlsbad, CA; U.S.A.
Hydrochloric acid (HCl)	Merck, Darmstadt; Germany
Hydrocortisone	Sigma-Aldrich, München; Germany
Hydrogen peroxide (H_2O_2)	Sigma-Aldrich, München; Germany
IMDM medium	Life technologies, Carlsbad, CA; U.S.A.
Insulin	Sigma-Aldrich, München; Germany
Keratinocyte Basal Medium	PromoCell, Heidelberg; Germany
Laemmli buffer	VWR International, Darmstadt, Germany
L-Glutamine	Life technologies, Carlsbad, CA; U.S.A.
Lipofectamine RNAiMAX Reagent	Life technologies, Carlsbad, CA; U.S.A.
Lipopolysaccharide	Invivogen, San Diego; U.S.A.
Lymphoprep	Axis Shield, Oslo, Norway
M199 medium	Life technologies, Carlsbad, CA; U.S.A.
Maxima SYBR Green/Rox qPCR Master Mix 2x	Thermo Fisher Scientific, Schwerte, Germany
MEM Non-Essential Amino Acids Solution	Life technologies, Carlsbad, CA; U.S.A.
Methanol	Sigma-Aldrich (Fluka), München; Germany
Mitomycine C	Sigma-Aldrich, München; Germany
Mounting Medium	Sigma-Aldrich, München; Germany
Naïve T cell isolation kit (human)	Miltenyi, Bergisch Gladbach; Germany
Nigericin	Sigma-Aldrich, München; Germany
Non-essential Amino Acids	Life technologies, Carlsbad, CA; U.S.A.

NuPAGE MOPS SDS Running Buffer (20x)	Life technologies, Carlsbad, CA; U.S.A.
NuPAGE Transfer buffer (20x)	Life technologies, Carlsbad, CA; U.S.A.
PapPen Liquid Blocker	Kisker, Steinfurt; Germany
Paraformaldehyde (PFA)	Sigma-Aldrich, München; Germany
Penicillin-Streptomycin	Life technologies, Carlsbad, CA; U.S.A.
Prime Blocking Reagent	GE Healthcare, Freiburg; Germany
Progesterone	Sigma-Aldrich, München; Germany
Propidiumiodide (PI)	Sigma-Aldrich, München; Germany
Protease inhibitor tablets	Roche Diagnostics, Mannheim; Germany
Restore Western Blot Stripping buffer	Thermo Fisher Scientific, Schwerte, Germany
rh GM-CSF	PromoKine, Heidelberg; Germany
rh IFN-γ	R&D Systems, Wiesbaden; Germany
rh IL-12	PromoKine, Heidelberg; Germany
rh IL-1β	Miltenyi, Bergisch Gladbach; Germany
rh IL-2 Proleukin	Novartis, Basel, Switzerland
rh IL-23	PromoKine, Heidelberg; Germany
rh IL-4	PromoKine, Heidelberg; Germany
rh IL-6	PromoKine, Heidelberg; Germany
rh TGF-β	PromoKine, Heidelberg; Germany
rh TGF-β1 (ELISA Standard)	R&D Systems, Wiesbaden; Germany
rh TNF-α	R&D Systems, Wiesbaden; Germany
RNA Isolation Kit	Qiagen, Venlo; the Netherlands
RPMI 1640 + L-Glutamine	Life technologies, Carlsbad, CA; U.S.A.
Saponin from Quillaja bark	Sigma-Aldrich, München; Germany
Seeblue prestained protein standard	Life technologies, Carlsbad, CA; U.S.A.
Sodium Azide (NaN_3)	Merck, Darmstadt; Germany
Sodium chloride (NaCl)	Carl Roth, Karlsruhe; Germany
Sodium hydrogen carbonate ($NaHCO_3$)	Merck, Darmstadt; Germany
Sodium hydroxide (NaOH)	Merck, Darmstadt; Germany
Sodium-Pyruvate ($C_3H_3NaO_3$)	Life technologies, Carlsbad, CA; U.S.A.
Streptavidin-horseradish peroxidase	R&D GE Healthcare UK limited, Wiesbaden
Sucrose	Sigma-Aldrich, München; Germany
Sulphuric acid (H_2SO_4)	Merck, Darmstadt; Germany
Tetramethylbenzidine (TMB)	Sigma-Aldrich (Fluka), München; Germany
Transferrin	Sigma-Aldrich, München; Germany
Trichloroacetic acid	Merck, Darmstadt; Germany
Triiodothreonin (T3)	Sigma-Aldrich, München; Germany
Tris(hydroxymethyl)-aminomethan	Sigma-Aldrich (Fluka), München; Germany
Triton X	Sigma-Aldrich (Fluka), München; Germany

Trypanblue 0.4% solution	Life technologies, Carlsbad, CA; U.S.A.
Trypsin 0.05% EDTA	Sigma-Aldrich, München; Germany
Tween 20 detergent	Merck Millipore (Calbiochem), Darmstadt, Germany

3.1.2 Antibodies and ELISA/Multiplex Kits

All used antibodies and assay kits are directed against human antigens.

Table 4: ELISA Kits

ELISA Kit	Targets	Supplier
OptEIA ELISA Kit	IL-1β; IL-10; IL-4; GM-CSF; IL-8; IP-10, TNF-α; CCL5	BD Biosciences, Heidelberg; Germany
Quantikine ELISA Kit	IL-17; IL-22; IFN-γ; IL-1α; CCL-20	R&D Systems, Wiesbaden; Germany
Paired Antibodies	IL-6; IL-12p70; IL-23; IL-18	eBioscience, Alasdar Stewart; UK

Table 5: TGF-β ELISA antibodies

Target	Specification	Supplier
TGF-β1	soluble receptor II Fc/Chimera	R&D Systems, Wiesbaden; Germany
TGF-β1	Biotin conjugated	R&D Systems, Wiesbaden; Germany

Table 6: Multiplex Kits

Kit	Target	Supplier
Th17 magnetic bead panel	IL-10; IL-17; IL-22; IFN-γ; IL-4; IL-5; IL-9; IL-21; TNF-α; IL-1β	Merck Millipore, Darmstadt, Germany
Th17 magnetic bead panel	IL-1α; IL-8; CCL2; CCL3; CCL4; G-CSF	Merck Millipore, Darmstadt, Germany

Table 7: Primary antibodies for immunofluorescence stainings

Target	Source	c(stock)	Dilution	Supplier
IL-18	rabbit	0.2 mg/ml	1 : 50	Santa Cruz Biotechnology, Heidelberg; Germany
IL-1β	mouse	0.5 mg/ml	1 : 50	Abcam, Cambridge; UK
Keratin 14	mouse	n.s.	1 : 50	Sigma-Aldrich, München; Germany
Involucrin	mouse	n.s.	1 : 50	Sigma-Aldrich, München; Germany
Iso IgG	mouse	1 mg/ml	1 : 50	eBioscience, Alasdar Stewart; UK
IsoIgM	mouse	0.5 mg/ml	1 : 50	eBioscience, Alasdar Stewart; UK

n.s. = not specified; Iso = isotype control

Table 8: Primary antibodies for Western Blot

Target	Source	c(stock)	Dilution	Supplier
β-Actin	mouse	n.s.	1 : 10 000	Santa Cruz Biotechnology, Heidelberg; Germany
GAPDH	mouse	n.s.	1 : 5 000	Thermo Fisher Scientific, Schwerte, Germany
Caspase-1	rabbit	n.s.	1 : 1000	Cell signaling Technology, Leiden; the Netherlands
IL-18	rabbit	0.2 mg/ml	1 : 1000	Santa Cruz Biotechnology, Heidelberg; Germany
IL-1β	mouse	0.5 mg/ml	1 : 1000	Abcam, Cambridge; UK

n.s. = not specified

Table 9: Primary Antibodies for FACS

Target	Source	Dilution	Supplier
Keratin 14	mouse	1 : 50	Sigma-Aldrich, München; Germany
Involucrin	mouse	1 : 50	Sigma-Aldrich, München; Germany
CD4 FITC	mouse	1 : 100	BD Biosciences, Heidelberg; Germany
CD45RA PE	mouse	1 : 100	BD Biosciences, Heidelberg; Germany
CD1a PE	mouse	1 : 100	BD Biosciences, Heidelberg; Germany
CD45RO PE	mouse	1 : 100	BD Biosciences, Heidelberg; Germany
CD14 FITC	mouse	1 : 100	eBioscience, Alasdar Stewart; UK
CD25 PE	mouse	1 : 100	BD Biosciences, Heidelberg; Germany
CD40 FITC	mouse	1 : 100	BD Biosciences, Heidelberg; Germany
CD80 FITC	mouse	1 : 100	BD Biosciences, Heidelberg; Germany
CD83 PE	mouse	1 : 100	eBioscience, Alasdar Stewart; UK
CD86 APC	mouse	1 : 100	BD Biosciences, Heidelberg; Germany
HLA-DR APC	mouse	1 : 200	eBioscience, Alasdar Stewart; UK
HLA-ABC FITC	mouse	1 : 100	BD Biosciences, Heidelberg; Germany
Iso FITC	mouse	1 : 100	BD Biosciences, Heidelberg; Germany
Iso PE	mouse	1 : 100	BD Biosciences, Heidelberg; Germany
Iso APC	mouse	1 : 100	BD Biosciences, Heidelberg; Germany

FITC = Fluorescein isothiocyanate (Alexa Fluor 488); PE = Phycoerythrin; APC = Allophycocyanin; CD = Cluster of differentiation; Iso = isotype.

Table 10: Antibodies for cell culture use

Target	Source	c(stock)	Dilution	Supplier
CD3	mouse	1 mg/ml	1 : 1000	BD BD Biosciences, Heidelberg; Germany
CD2	mouse	2 mg/ml	1 : 400	SIAF, Davos; Switzerland
CD28	mouse	1 mg/ml	1 : 1000	BD Biosciences, Heidelberg; Germany
IL-4	rat	1 mg/ml	1 : 200	eBioscience, Alasdar Stewart; UK
IFN-γ	mouse	1 mg/ml	1 : 1000	eBioscience, Alasdar Stewart; UK
IL-12	mouse	1 mg/ml	1 : 40 000	eBioscience, Alasdar Stewart; UK

Table 11: Secondary Antibodies for FACS, immunofluorescence and Western Blot

Antibody	Source	c(stock)	Dilution	Supplier
α-mouse IgG -HRP	goat	0.4 mg/ml	1 : 5000	Santa Cruz Biotechnology, Heidelberg; Germany
α-rabbit IgG-HRP	goat	0.4 mg/ml	1 : 5000	Santa Cruz Biotechnology, Heidelberg; Germany
α-mouse IgM PE	goat	0.4 mg/ml	1 : 100	Santa Cruz Biotechnology, Heidelberg; Germany
α-mouse IgG PE	donkey	0.4 mg/ml	1 : 100	Santa Cruz Biotechnology, Heidelberg; Germany
α-rabbit IgG FITC	goat	0.4 mg/ml	1 : 100	Santa Cruz Biotechnology, Heidelberg; Germany

3.1.3 Primer

All DNA Primer were ordered from metabion international AG or Microsynth AG, respectively. After delivery primers were reconstituted in RNase/DNase free DEPC water to a concentration of c = 100 pmol/µl and stored at -20°C. The final concentration per reaction well was 1,6 pmol/µl.

Table 12: Primer sequences for qPCR

Primer		Sequence from 5`-3`
Elongationfactor 1α	fwd	CTG AAC CAT CCA GGC CAA AT
	rev	GCC CTG TGG CAA TCC AAT
GAPDH	fwd	GAA GGT GAA GGT CGG AGT
	rev	GAA GAT GGT GAT GGG ATT
Interleukin-1β	fwd	AAA AGC TTG GTG ATG TCT CG
	rev	TTT CAA CAC GCA GGA CAG G
Interleukin 18	fwd	GCT GAA CCA GTA GAA GAC AAT TG

	rev	ATC TGA TTC CAG GTT TTC ATC ATC T
Caspase 1	fwd	GAA TGT CAA GCT TTG CTC CCT AGA
	rev	AAG ACG TGT GCG GCT TGA TGA CT
AIM2	fwd	AGC AAG ATA TTA TCG GCA CAG
	rev	GGA CTA CAA ACA AAC CAT TCA C
NLRP3	fwd	CTT CTC TGA TGS GGC CCA AG
	rev	GCA GCA AAC TGG AAA GGA AG
TSLP	fwd	GGC CAC ATT GCC TTA CTG AA
	rev	TCC TCT TCT TCA TTG CCT GAG
PAR2	fwd	CCA TCC AAG GAA CCA ATA GA
	rev	CTG AGG CAG GTC ATG AAG A
HIF-1α	fwd	GAT TTT GGC AGC AAC GAC AC
	rev	TGA ATC TGG GGC ATG GTA AA
Filaggrin	fwd	AAG GTT CAC ATT TAT TGC CAA A
	rev	GGA TTT GCC GAA ATT CTT TT
Interleukin 17A	fwd	TCA TCC ATC CCC AGT TGA TT
	rev	TTC GTC GGA TTG TGA TTC CT
Interleukin 22	fwd	GCA GGC TTG ACA AGT CCA ACT ACT
	rev	GCC TCC TTA GCC AGC ATG AA
IFN-γ	fwd	TCT CGG AAA CGA TGA AAT ATA CAA GTT
	rev	GTA ACA GCC AAG AGA ACC CAA AA
Interleukin 4	fwd	ACA GCC TCA CAG AGC AGA AGA CT
	rev	GTG TTC TTG GAG GCA GCA AAG
T-Bet	fwd	GAT GCG CCA GGA AGT TTC AT
	rev	GCA CAA TCA TCT GGG TCA CAT T
GATA-3	fwd	GCG GGCTCT ATC ACA AAA TGA
	rev	GCT CTC CTG GCT GCA GAC AGC
RORC2	fwd	TCA TCC ATC CCC AGT TGA TT
	rev	TTC GTC GGA TTG TGA TTC CT
Foxp3	fwd	GAA ACA GCA CAT TCC CAG AGT TC
	rev	ATG GCC CAG CGG ATG AG
TGF-β	fwd	AAA TTG AGG GCT TTC GCC TTA
	rev	GAA CCC GTT GAT GCT CAC TTG
Interleukin 10	fwd	GTG ATG CCC CAA GCT GAG A
	rev	CAC GGC CTT GCT CTT GTT TT
Interleukin 5	fwd	AGC TGC CTA CGT GTA TGC CA
	rev	GCA GTG CCA AGG TCT CTT TCA

fwd = forward primer; rev = reverse primer

3.1.4 siRNA

For siRNA transfection experiments siRNA-SMART pool mixes from Thermo Scientific were used and dissolved according to manufacturer´s instruction in DEPC water with a final concentration of 20 µM.

Table 13: siRNA sequences

Target	Company index label	Sequence
Caspase-1	Target 1 J-004401-06	GGAAGACUCAUUGAACAUA
	Target 2 J-004401-07	GAUGGUAGAGCGCAGAUGC
	Target 3 J-004401-08	CCGCAAGGUUCGAUUUUCA
	Target 4 J-004401-09	GAGUGACUUUGACAAGAUG
AIM-2	Target 1 J-011951-05	GCACAGUGGUUUCUUAGAG
	Target 2 J-011951-06	UCAGACGAGUUUAAUAUUG
	Target 3 J-011951-07	GAAAGUUGAUAAGCAAUAC
	Target 4 J-011951-08	GUUCAUAGCACCAUAAAGG
Non-target control (scramble)	Target 1	UGGUUUACAUGUCGACUAA
	Target 2	UGGUUUACAUGUUGUGUGA
	Target 3	UGGUUUACAUGUUUUCUGA
	Target 4	UGGUUUACAUGUUUUCCUA

3.1.5 Media and buffers

All media were sterile filtered and stored at 4°C.

Table 14: Keratinocyte/Feeder medium

Ingredient	Stock	ml of stock	Final
DMEM high Glucose		300 ml	
F12/Glutamax Mixture		150 ml	
Glutamine	200 mM	10 ml	4 mM
Adenine	87.4 mM	1 ml	174.8 µM
Hydrocortisone	7,7 mg/ml	1 ml	42 µM
Triiodothyronin (T3)	2 µM	0.5 ml	20 nM
Transferrin	5 mg/ml	0.5 ml	5 µg/ml
Choleratoxin	0.84 µg/ml	0.5 ml	0.84 ng/ml
Insulin (recombinant)	4 mg/ml	0.5 ml	4 µg/ml
Epidermal growth factor	1 mg/ml	0.5 ml	1 µg/ml
Penicillin-Streptomycin	10 000 U/ml	5 ml	100 U/ml
FCS		50 ml	

Table 15: Fibroblast and 3T3 medium

Ingredient	Stock	ml of stock	Final
DMEM high Glucose		395 ml	
FCS		100 ml	20 %
Penicillin-Streptomycin	10 000 U/ml	5 ml	100 U/ml

Table 16: Complete RPMI (cRPMI) medium

Ingredient	Stock	ml of stock	Final
RPMI 1640		420 ml	
FCS		50 ml	10 %
Pen/Strep/Kana	10 mg/ml	10 ml	2 mg/ml
2 mM L-Glutamine	200 mM	10 ml	4 mM
Sodium pyruvate	100 mM	10 ml	20 mM
Non-Essential Amino Acids	100x	10 ml	2x
β-Mercaptoethanol	50 mM	0.5 ml	50 µM

Table 17: Media for 3D skin model cultures

Ingredient	c(stock)	ml of stock			c(final)
		Epiderm I	***Epiderm II***	***Cornification***	
DMEM (w/o $CaCl_2$)		360 ml	-	-	
DMEM high Glucose		-	360.5 ml	236 ml	
F12		120 ml	120 ml	236 ml	
Glutamine	200 mM	10 ml	10 ml	10 ml	4 mM
Adenine	87.4 mM	0.23 ml	0.23 ml	0.23 ml	40 µM
Hydrocortisone	21.2 mM	0.024 ml	0.024 ml	0.024 ml	1 µM
Triiodothyronin (T3)	2 µM	5 ml	5 ml	5 ml	20 nM
Transferrin	5 mg/ml	1 ml	1 ml	1 ml	10 µg/ml
Insulin	4 mg/ml	1.25 ml	1.25 ml	1.25 ml	10 µg/ml
Progesterone	2 µM	0.5 ml	0.5 ml	-	2 nM
$CaCl_2$	2 M	0.5 ml	-	-	0.2 M
FCS		2.5 ml	2.5 ml	-	0.5 %
FCS				10 ml	2.0 %

Table 18: HuDC medium

Ingredient	Stock	ml of stock	Final
RPMI 1640		450 ml	
FCS		50 ml	10 %
Gentamycine	10 mg/ml	1 ml	20 µg/ml
L-Glutamine	200 mM	5 ml	2 mM

β-Mercaptoethanol	50 mM	0.5 ml	50 µM

Table 19: Keratinocytes growth medium (DermaLife K medium, Lifeline cell technology)

Ingredient	Stock	ml of stock	Final
DermaLife basal		475.5 ml	
Penicillin-Streptomycin	10 000 U/ml	5 ml	100 U/ml
L-Glutamine LifeFactor	200 mM	15 ml	6 mM
Hydrocortisone	0.1 mg/ml	0.5 ml	100 ng/ml
rh Insulin LifeFactor	0.5 mg/ml	0.5 ml	0.5 µg/ml
Epinephrine LifeFactor	100 µM	0.5 ml	1 µM
Extract P^{TM} LifeFactor		2 ml	4%
Apo-Transferrin	0.5 mg/ml	0.5 ml	5 µg/ml
rh TGF-α LifeFactor	100 ng/ml	0.5 ml	100 ng/ml

Table 20: Freezing medium

Ingredient	ml of stock	Final
DMEM/F12	250 ml	50 %
FCS	200 ml	40 %
DMSO	50 ml	10 %

Table 21: 10x Tris-buffered saline (TBS), pH 7.6 (HCl)

Ingredient	Quantity
Tris	60.55 g
Sodium Chloride	87.66 NaCl
ddH_2O	1000 ml

Table 22: 1x Tris-buffered saline Tween (TBS-T)

Ingredient	Volume
10x TBS	100 ml
ddH_2O	1000 ml
Tween 20	0.5 ml

Table 23: FACS buffer

Ingredient	Volume
PBS w/o Ca/Mg	450 ml
FCS	50 ml
Sodium azide	0.1 ml

3.1.6 Consumable material

Table 24: Consumable material

Material	Supplier
Adhesion slides SuperFrost® Plus	Thermo Fisher Scientific, Schwerte; Germany
Amicon centrifugation filter units	Merck Millipore, Darmstadt; Germany
Butterfly needles	Dahlhausen, Köln; Germany
Cover slips (24 x 60 mm)	Hirschmann Laborgeräte, Eberstadt; Germany
Cryotubes 1.8 ml	Nunc, Roskilde; Denmark
EDTA-Monovettes	Sarstedt, Nümbrecht; Germany
FACS tubes	Thermo Fisher Scientific, Schwerte; Germany
Filter tips (TipOne)	Starlab, Hamburg; Germany
Glass fibre filter	Perkin Elmer, Rodgau; Germany
Heatsealing paper	Perkin Elmer, Rodgau; Germany
Maxisorp plates (96 well)	Nunc, Roskilde; Denmark
MeltiLex scintillation sheets	Perkin Elmer, Rodgau; Germany
Microtubes 2 ml PP, sterile	Sarstedt, Nürnbrecht; Germany
Multiplex plates	Merck Millipore, Darmstadt; Germany
NuPAGE Bis-Tris Gels	Life technologies, Carlsbad, CA; U.S.A.
PCR foil MicroAmp	Applied Biosystems (Life technologies) Carlsbad, CA; U.S.A.
PCR tubes	Eppendorf, Hamburg; Germany
Perfusor syringes	Braun, Melsungen; Germany
Pipette tips	Eppendorf, Hamburg; Germany
Pipettes (1, 5, 10, and 25 ml)	Greiner Bio-One, Frickenhausen; Germany
Printed Filtermat (β-counter)	Perkin Elmer, Rodgau-Rüdesheim; Germany
PVDF Immobilon® Membrane	Merck Millipore, Darmstadt; Germany
QiaShredder Columns	Qiagen, Hilden; Germany
qPCR plates 384 well I	Bio-Rad, München; Germany
qPCR plates 384 well II	Thermo Fisher Scientific, Schwerte; Germany
Reaction tubes (0.5; 1.5; 2 ml)	Eppendorf, Hamburg; Germany
Reaction tubes (15 ml; 50 ml)	Sarstedt, Nürnbrecht; Germany
RNeasy Mini Kit for RNA Isolation	Qiagen, Hilden; Germany
Sample discs for osmometer SS-033	WESCOR, Utah, U.S.A.
Sterile filter device (250 ml; 500 ml)	Sarstedt, Nürnbrecht; Germany
Syringe filter units (0.22; 0.45 µm)	Merck Millipore, Darmstadt; Germany
Thincert plate, 12 well, PS, LID, sterile	Greiner Bio-One, Frickenhausen; Germany
Thincerts cell culture inserts (3 µm pores)	Greiner Bio-One, Frickenhausen; Germany
Tissue culture flask (25; 75 and 175 cm^2)	Greiner bio-one, Frickenhausen; Germany

Tissue culture plates (96; 48; 24; 12 and 6 well)	Corning Incorporated (Falcon); Tewksbury, MA; U.S.A.
Tungsten Carbide Beads 5 mm	Qiagen, Hilden; Germany
Whatman Paper	Nunc, Roskilde; Denmark

3.1.7 Instruments

Table 25: Instruments

Device	Supplier
7900 HT Fast Real-Time PCR System	Applied Biosystems (Life technologies) Carlsbad, CA; U.S.A.
autoMACS Pro Separator	Miltenyi Biotech, Bergisch Gladbach; Germany
Bio-Plex 200 system	Bio-Rad, München; Germany
Bio-Plex Pro Wash Station	Bio-Rad, München; Germany
Cell counter Sysmex KX21N	Sysmex, Norderstedt; Germany
Centrifuge 5417R	Eppendorf, Hamburg; Germany
Centrifuge Megafuge 1R	Heraeus, Hanau; Germany
ChemoCam Imager for ECL Western Blot detection	Intas, Göttingen; Germany
Cryostat	Leica, Nussloch; Germany
FACS Calibur	Becton Dickinson, Heidelberg; Germany
FACS Fortessa	Becton Dickinson, Heidelberg; Germany
Incubator	Heraeus, Hanau; Germany
Liquid scintillation β Counter	Perkin Elmer, Rodgau; Germany
Magnetic stirrer RCT basic	IKA Werke, Staufen; Germany
Micro scale	MC1 Research, Sartorius, Göttingen; Germany
Microbeta Filtermate Harvester	Perkin Elmer, Rodgau; Germany
Microplate Reader Mithras LB940	Berthold Technologies, Zug; Switzerland
Microplate spectrophotometer Epoch	Biotek, Bad Friedrichshall; Germany
Microplate washer Hydrospeed	Tecan, Männedorf; Switzerland
Microscope Axiovert 200M	Zeiss, Jena; Germany
Microscope Axiovert 25	Zeiss, Jena; Germany
Microscope DFL420	Leica, Solms; Germany
Microsealer	Perkin Elmer LAS, Rodgau; Germany
Multichannel Pipettes	Brand, Wertheim; Germany
Multifuge 1 L-R	Heraeus, Hanau; Germany
NanoDrop 2000	Thermo Fisher Scientific, Schwerte; Germany
Osmometer VAPRO Pressure 5520	WESCOR, Utah, U.S.A
PCR machine TC-412	Techne Inc, Burlingtion, NJ; U.S.A.
pH meter SenTix 81	WTW GmbH, Weilheim; Germany

Pipettes with diposable tips	Eppendorf, Hamburg; Germany
Power supply LNG350-06	Heinzinger Electronic; Rosenheim; Germany
Shaker UnitWist RT	Uni-Equip, Martinsried; Germany
Special accuracy weighing machine	Kern 770, Witten; Germany
Special accuracy weighing machine Mettler Tolego XS105 Dual Range	WTM Wägetechnik, München; Germany
Suction blister device PTC 3300 vac	Innokas Medical Ltd; Kempele; Finland
Thermo shaker TS100	Kisker Biotech; Steinfurt, Germany
TissueLyser	Qiagen, Hilden; Germany
UV system UV 800K	Waldman, Villingen-Schwenningen; Germany
ViiATM 7 Real-Time PCR System	Applied Biosystems (Life technologies) Carlsbad, CA; U.S.A.
Washer for proliferation plates	Perkin Elmer, Rodgau; Germany
Waterbath	Julabo, Seelbach; Germany
Weighing machine Kern 770	Kern & Sohn GmbH, Balingen; Germany
Xcell II blot module for protein gel electrophoresis and Western blotting	Life technologies, Carlsbad, CA; U.S.A.

3.2 Methods

3.2.1 Donors

Blood cells, keratinocytes and fibroblasts were obtained from non-atopic individuals as well as from patients allergic to grass and birch pollen. Non-atopic subjects were defined by a normal total IgE titer below 50 kU/l in the serum, a negative radio-allergen-sorbent-test (RAST) against nine common environmental allergens (D. *pteronyssinus*, cat danders, wheat flour, celeriac, timothy grass, secale cereale, birch, hazel, and mugwort) and the absence of atopic diseases in history.

3.2.2 Isolation and cultivation of human primary keratinocytes

Primary human keratinocytes were isolated from the epidermis by suction blister method as described earlier (Traidl *et al.*, 2000). Briefly, single-cell suspensions of epidermal cells from suction blisters' roofs were prepared by trypsinization of the blister roof and seeded on a feeder layer of 3T3/J2 fibroblasts treated with 10 µg/ml mitomycine C for 2 h. First-passage keratinocytes were cryopreserved in liquid nitrogen. Experiments were performed in sterile 6 or 12 well tissue culture plates with second-passage keratinocytes grown in DermaLife keratinocyte growth medium to 80-90 % confluence.

3.2.3 3D skin model

For 3D skin cultures the protocol by Carlson et al., 2008, was adapted with following changes to a 12 well format. Human primary fibroblasts derived from bunch biopsies were used to generate a two layered type I collagen matrix on polyethylene terephthalate membranes in hanging cell culture inserts. Membranes were coated with 350 µl acellular collagen before a second collagen layer containing $2x10^4$ fibroblasts in a volume of 750 µl fibroblast medium was added (step 1 and 2). After incubation of the polymerized matrix for 5–7 days (step 3), 1.25×10^5 second-passage human primary keratinocytes were seeded in 15 µl epidermal I medium on the matrix and incubated for six days in submerge culture (step 4). For the first culture period epidermal medium I was used and after 48 h medium change was performed. After four - five days epidermal medium was exchanged with epidermal medium II (step 5). The composite culture was raised after two or three further incubation days to air-liquid interface by removing all media from the upper chamber and

adding cornification medium only in the lower chamber of the well (step 6). The tissue was ready for stimulation seven days later.

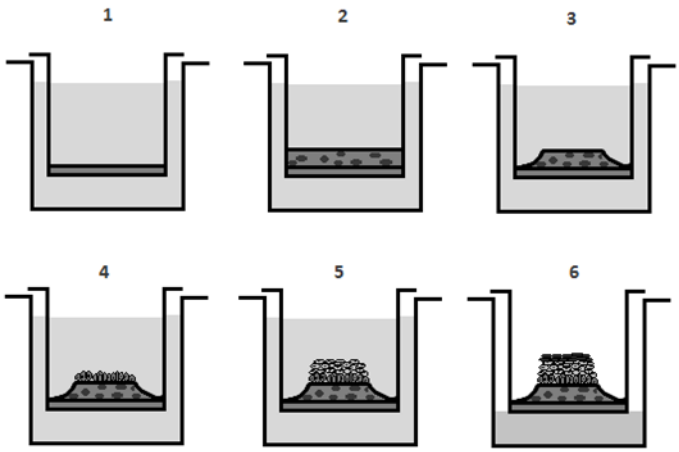


Figure 8: **Preparation steps for 3D skin models.** Adapted from Carlson et al., 2008.

3.2.4 Preparation of APE and APE fractions

Pollen of *Betula pendula*, *Phleum pratense* and *Pinus sylvestris* were collected in the spring of 2012 and 2013 during flowering season in southern Bavaria. Pollen was extracted by sieving catkins and heads with a 100 µm sieve followed by a 70 µm sieve and stored at −80 °C until further processing. Pollen from *Ambrosia artemisiifolia* was provided by Helmholtzzentrum München BIOP where ragweed plants were grown in fully air-conditioned greenhouse cabins under controlled conditions.

Aqueous pollen extracts (APE) were generated by incubation of pollen grains in DermaLife Basal medium for 30 min at 37 °C. After centrifugation (10 min, 4000 rpm, 4 °C) supernatants were filtered by using syringe driven filter units (0.22 µm). Endotoxin contamination was ruled by conducting limulous amoebocyte lysate (LAL) assays.

To subdivide APE into protein and non-protein containing fractions the pollen extract was centrifuged with 3 kDa cut off centrifugation filter units according to manufacturer's instructions. For heat inactivation APE was incubated 10 min at 95 °C and centrifuged to remove heat-debris (10 min, 1200 rpm).

3.2.5 Medium testings

Tests for identifying the optimal medium for co-culture experiments were conducted with human primary keratinocytes after reaching 80 % of confluence in 6 well tissue culture plates. Cell were treated ± IFN-γ (300 U/ml) for 6 h and afterwards incubated at 37 °C for 18 h with 1 ml of IMDM, RPMI, M199, DMEM, KGM 2, DermaLife Basal, AIM-V or F12 medium, respectively. Subsequently cell morphology was checked by microscope and supernatants were collected for TNF-α and IP-10 release analysis. Cells were harvested by trypsinization and stained with propidium iodide, Keratin 14 and Involucrin for FACS analysis.

For tests regarding a 72 h incubation period with T cells, following media were tested in a 1 : 3 dilution with AIM-V medium: IMDM, M199, DMEM, DermaLife Basal and F12. Effects on cell death were analyzed by propidium iodide FACS staining and effects on cytokine release by ELISA.

3.2.6 Generation of keratinocyte supernatants for co-culture experiments

Keratinocyte cell supernatants for T cell co-cultures experiments were generated in 6 well cell culture plates as follows. Human primary keratinocytes were pre-stimulated at 80 % confluence with IFN-γ (300 U/ml) for 6 h in DermaLife keratinocyte growth medium + supplements but without hydrocortisone. Subsequently cells were washed three times with D-PBS and stimulated with APE in a total volume of 1 ml DermaLife Basal medium/well. After 24 h at 37 °C, 5 % CO_2, cells were washed five times with D-PBS and then incubated overnight in DMEM high Glucose medium at 37 °C, 6.5 % CO_2. For experiments with no APE stimulation, cells were washed three times with PBS directly after pre-stimulation and incubated overnight in DMEM high Glucose. Cell-free supernatants were harvested and stored at -80 °C until use.

For acetone protein precipitation keratinocyte supernatants and medium control, respectively, were diluted 1 : 4 with ice cold acetone and subsequently incubated for 2 h at -20 °C. Afterwards the acetone mix was centrifuged (10 min, 14 000 g) and the resulting protein-free supernatant was transferred to a new tube. Acetone parts were allowed to evaporate from the uncapped tube under the sterile bench.

3.2.7 Isolation of PBMCs and generation of monocyte derived dendritic cells

Peripheral blood mononuclear cells (PBMCs) were isolated from human heparin treated blood by density gradient centrifugation. For this purpose 10 ml Ficoll-Lymphoprep separation medium was overlayed with 25 ml of 1 : 1 D-PBS diluted blood. After centrifugation (2200 rpm w/o brake, 15 min, RT), desired cells were removed from the interphase and washed three times with D-PBS supplemented with 5 mM EDTA (centrifugation step 1: 1600 rpm, 10 min; centrifugation steps 2 and 3: 1200 rpm; 10 min). Monocytes were isolated from PBMCs by magnetic cell sorting (MACS) technology using human $CD14^+$ MicroBeads according to manufacturers' instructions. In vitro immature dendritic cells, so called monocyte derived dendritic cells (MoDCs), were obtained by culturing monocytes in the presence of Interleukin 4 (IL-4) c = 50 U/ml and the granulocyte-macrophage-colony-stimulatory factor (GM-CSF) c = 50 U/ml in HuDC medium for five days at 37 °C, 5 % CO_2. Maturation of MoDCs was induced on day five by LPS (c = 100 ng/ml) treatment for 24 h.

3.2.8 Isolation of naïve $CD4^+$ T Cells and subset differentiation

$CD4^+CD45RA^+$ naïve T cells were isolated from PBMCs by magnetic cell sorting (MACS) technology. For a negative selection process the human naïve $CD4^+$ T Cell Isolation Kit II was used according to manufacturers' instructions. FACS staining for CD4, CD45RO and CD45RA was used as purity check.

Differentiation of naïve $CD4^+$ $CD45RA^+$ T cells into different subsets (Th1, Th2, Th17, Treg and control Th0) was achieved during a culture period of seven days in 12 or 48 well not tissue treated cell culture plates by adding subset specific cytokines and antibodies. Two different protocols were tested and are described in following passages:

I. For T cell subset differentiation in 12 well plates the wells were coated with αCD3 (c = 1 µg/ml) for 2 h at 37 °C and washed with D-PBS before use. Subsequently 1.5 x 10^6 naïve T cells were seeded in a volume of 1.5 ml AIM-V medium/well. To induce differentiation following cytokine/antibody cocktails were used:

Table 26: Cytokines and antibodies for T cell differentiation in 12 well plates

	Cytokine / antibody	**c_{end}**
Th1	αIL-4	5 µg/ml
	IL-12	250 ng/ml
	αCD28	1 µg/ml
Th2	αIL-12	25 ng/ml
	IL-4	25 ng/ml
	αIFNγ	1 µg/ml
	αCD28	1 µg/ml
Th17	IL-6	10 ng/ml
	IL-23	30 ng/ml
	IL-1β	20 ng/ml
	αIL-4	5 µg/ml
	αIFNγ	1 µg/ml
	TGFβ	3 ng/ml
	αCD28	1 µg/ml
Th0	αCD28	1 µg/ml

On day three and five half of the medium was replaced by fresh AIM-V medium supplemented with 40 U/ml IL-2 in case of Th0, Th1 and Th2. The Th17 line did not receive any IL-2. On day seven cells were harvested and restimulated in 96 well plates with αCD3/αCD28.

II. For subset differentiation in 48 well plates naïve $CD4^+$ T cells were seeded with a concentration of 0.5 x 10^6 cells/500 µl AIM-V medium for each well. For general activation a mixture of αCD28 + αCD3 + αCD2 =CD mix was used. For the induction of the different T cell lines following cytokine/antibody cocktails were added :

Table 27: Cytokines and antibodies for T cell differentiation in 48 wells

	Cytokine / antibody	**c_{end}**
Th1	αIL-4	5 µg/ml
	IL-12	25 ng/ml
	IL-2	100 U/ml
	CD-mix (1 : 400)	
Th2	αIL-12	5 µg/ml
	IL-4	40 ng/ml
	IL-2	100 U/ml
	CD-mix (1 : 400)	
Th17	IL-6	30 ng/ml
	IL-23	30 ng/ml
	IL-1β	20 ng/ml
	αIL-4	5 µg/ml
	αIFNγ	5 µg/ml
	TGFβ	5 ng/ml
	CD-mix (1 : 400)	

Treg	αIL-4	5 µg/ml
	αIL-12	5 µg/ml
	αIFNγ	5 µg/ml
	TGFβ	5 ng/ml
	IL-2	100 U/ml
	CD-mix (1 : 400)	
Th0	IL-2	100 U/ml
	CD-mix (1 : 400)	

After 48 h cells were splitted and fed with fresh cRPMI medium supplemented with IL-2 (100 U/ml). For the Th2 subset IL-4 (40 ng/ml) was added additionally. The Th17 subset received fresh cRPMI medium without any supplements

3.2.9 Co-culture of T cells with keratinocyte derived mediators

For co-culture experiments comprising keratinocyte derived mediators and different T cell subsets, αCD3 coated 96 well flat bottom plates were used (c = 1 µg/ml for 2 h at 37 °C). 0.8 -1.0 x 10^5 T cells were seeded in a volume of 150 µl AIM-V medium per well containing αCD28 in a concentration of 1 µg/ml. 50 µl keratinocyte supernatant and control medium, respectively, was added to reach a total volume of 200 µl/well and a dilution of 3 : 1 for the keratinocyte mediators. For each sample triplicates were performed. After a 72 h incubation period at 37 °C, 5 % CO_2 in the presence of keratinocyte supernatants, T cell supernatants were harvested and cells were used for thymidine proliferation assay, propidiumiodide FACS staining or RNA isolation.

3.2.10 Proliferation Assay

T cell proliferation was examined by measuring T cells mitotic activity via incorporation of radioactive labeled thymidine. After 72 h of restimulation cells were pulsed in 96 well flat bottom plates with 1 nCi/ml ^{3}H-thyimidine and afterwards incubated for six hours at 37 °C, 5 % CO_2. Subsequently cells were separated from the supernatant by a suction process holding back the cells on a filter paper. The filter was dried and sealed with a scintillation film making it possible to measure the radioactivity in a β-counter device. The dose of radioactivity, measured in counts per minute (cpm), is directly proportional to the amount of synthesized DNA and consequently to the proliferation activity of the cells.

3.2.11 ELISA and Multiplex

For cytokine quantification ELISA kits and Multiplex assays were used according to manufacturer´s instructions (see Table 4).

3.2.12 Inflammasome activation assays with APE and UV-B

Human primary keratinocytes were stimulated at 80-90 % confluence with aqueous pollen extracts (APE) in concentration ranges between 1.25 mg/ml to 10 mg/ml in DermaLife Basal medium. For inflammasome activation assays in 6 well tissue culture plates, cells were washed three times with D-PBS and subsequently stimulated either alone with APE and APE fractions in a total volume of 1 ml/well or in combination with a preceding overnight (18 h) IFN-γ (300 U/ml) /TNF-α (100 ng /ml) stimulation. After incubation at 37 °C, 5 % CO_2 for certain time periods, supernatants and cells were harvested for cytokine quantification, Western Blot analysis or RNA isolation.

3D skin cultures were stimulated with APE by adding 15 µl of APE in desired concentration on top of the tissue.

For UV-B treatment cells were irradiated with 90 mJ/cm^2 UV-B using an UV therapy device. Supernatants and cells were harvested after 1 h or 4 h of stimulation.

3.2.13 Inhibition of caspase-1 by caspase inhibitor

To inhibit caspase-1 activity in keratinocytes, cells were cultured in 6 well plates until they reached 80 – 90 % confluence and subsequently incubated with the caspase inhibitor Ac-YVAD-cmk in a concentration of 50 – 100 µM in Dermalife Basal medium for 45 min at 37 °C, 5% CO_2. DMSO served as solvent control. Afterwards cells were not washed but stimulants simply added to the volume. Successful inhibition of caspase-1 activity was controlled by measuring the IL-1β and IL-18 release by ELISA.

3.2.14 siRNA transfection

For siRNA transfection experiments siRNA-SMART pool mixes from Thermo scientific were used (see Table 13):

- L-004404 00-0005, ON-TARGETplus Human CASP1 (834) siRNA-SMARTpool 5 nmol
- L-011951-00-0005, ON-TARGETplus Human AIM2 (9447) siRNA-SMARTpool 5 nmol
- D-001810-1005, ON-TARGETplus Non-targeting Pool, 5 nmol (scramble control)

Cells were cultured in 12 well tissue culture plates and transfected according to manufacturer´s instruction using Lipofectamine RNAiMAXReagent as transfection reagent. However, divergent from the normal protocol, DermaLife basal medium was used as culture medium and as final volume/well 100 µl with a concentration of 20 pmol of siRNA was chosen. After 6 h of incubation further 400 µl medium were added to avoid dehydration of the cells. Transfection success was controlled after 48 h by harvesting the cells and determination of caspase-1 and AIM2 mRNA levels by qPCR.

3.2.15 RNA isolation and quantitative real-time PCR

3.2.15.1 RNA isolation and quantitative real-time PCR: Keratinocytes

Total RNA extraction from keratinocytes and the epidermal part of 3D skin cultures was performed using TissueLyser technique for cell disruption and RNeasy kit with on-column DNase digestion according to manufacturer's instructions. RNA concentration was measured using the NanoDrop 2000 spectrophotometer. cDNA synthesis was performed using the high capacity cDNA reverse transcription Kit (Applied Biosystems). mRNA expression was quantified on the ViiATM 7 Real-Time PCR System using FastStart Universal SYBR Green Master (Rox) Mix and primers listed in Table 12. Expression levels were normalized to the house-keeping genes EF1α and GAPDH. Relative changes in genes expression were analyzed using the comparative C_T ($2^{-\Delta\Delta CT}$) method.

3.2.15.2 RNA isolation and quantitative real- time PCR: T cells

Total RNA extraction from T cells was performed using QIAshredder homogenizer and RNeasy Mini kit with on-column DNase digestion according to manufacturer's instructions. RNA concentration was measured using the NanoDrop 2000 spectrophotometer. cDNA synthesis was performed using the first strand cDNA synthesis kit (Life technologies). mRNA expression was quantified on the 7900 HT Fast Real-Time PCR System using Maxima SYBR Green/Rox qPCR Master Mix 2x. Primers used for this experimental part are listed in Table 12. Expression levels were normalized to the house-keeping gene EF1-α. Relative changes in genes expression were analyzed using the comparative C_T ($2^{-\Delta\Delta CT}$) method.

3.2.16 Protein quantification

Protein quantification was either performed using the Coomassie (Bradford) Protein Assay Kit or the Pierce™ BCA Protein Assay Kit following the manual instructions for 96 well plate sample analysis.

3.2.17 LDH Assay

Cell death was quantified using the cytotoxicity detection Kit (LDH) from Roche according to manufacturer's instructions. 100 % cell death was determined by lysing the cells in 0.5 % Triton X-100.

3.2.18 Western Blot

For immunoblotting cells were lysed in D-PBS/protease inhibitor cocktail by repeated freeze-thaw cycles and by direct lysis in Laemmli buffer, respectively. Cell supernatants were concentrated with 3 kDa cut off centrifugal filters units and protein amount was determined by Bradford assay. Proteins were separated on sodium dodecyl sulphate-polyacrylamide gels (Bis-Tris) and then transferred to polyvinylidene difluoride membranes using wet blot system. The membranes were blocked with 5 % milk powder and 5 % BSA, respectively, in 1×TBS 0.5 % Tween-20 and then incubated overnight at 4 °C with primary antibodies as listed in table 8. Appropriate HRP-conjugated secondary antibodies (table 11) were applied in 1×TBS 0.5 % Tween-20 for 1 h at RT. Protein levels were detected using ECL reagent on a Chemiluminescence system. Equal amount of protein sample loading was verified by detecting β-Actin or GAPDH protein expression. Data analysis and normalization calculations were performed using the 1D software (Intas).

3.2.19 Histology and staining procedures

Samples obtained from 3D skin models for cryo-sectioning were harvested and directly incubated overnight in D-PBS 2 M Sucrose. Afterwards the tissue was embedded on dry ice in embedding compound. 7-10 µm sections were produced using a Cryostat cutting device. For immunofluorescence staining sections were fixed with methanol (- 20 °C; 5 min) and subsequently treated with D-PBS 5 % saponin for permeabilisation (10 min). Afterwards sections were surrounded with a circle of blocking liquid to allow a staining using low volume in form of drops applied by pipette. After blocking with D-PBS 1 % BSA sections

were incubated with primary antibodies diluted in D-PBS 1 % BSA for 1 h at RT and in humid environment (see Table 7). Secondary antibodies (see Table 11) were applied diluted in D-PBS 1 % BSA and incubated for 1 h in the dark. As last step the sections were treated 1 minute with DAPI 0.5 µg/ml diluted in D-PBS. Between all steps washing steps with D-PBS were conducted. For preservation mounting medium was added on each section and cover slips were applied. Sections were stored in the dark at 4 °C. Immunofluorescence stainings of single cells grown on glass slides were conducted in the same manner as described above.

Samples of 3D skin models for paraffin embedding were transferred to 4 % Formalin solution directly after harvest. Afterwards samples were handed over to the department of dermatology of the Technische Universität München for further processing. There 8 µm sections were produced and Hematoxylin/Eosin (H&E) staining was performed to check morphology. Immunohistochemical staining was done using the automatic IHC stainer Bond Max (Leica Biosystems).

3.2.20 Osmolarity measurement

Osmolarity of keratinocyte medium with and without APE addition was measured using the Osmometer VAPRO pressure 5520 (WESCOR, Utah) according to manufacturer's instructions.

3.2.21 FACS staining

All FACS stainings were performed in 96 well round bottom plates and analyzed by FACS Calibur. Data analysis was performed using the CellQuest Pro software.

For propidium iodide (PI) staining approximately $1x10^5$ cells were resuspended in 200 µl FACS buffer and PI was added in a concentration of 0.05 mg/ml.

FACS stainings of human primary keratinocytes with antibodies against Keratin 14, Involucrin and corresponding control isotypes were performed as follows. Approximately $1x10^5$ cells were resuspended in FACS buffer and fixated by treating with 2 % PFA for 10 min. Cells were washed with FACS buffer and subsequently resuspended in 100 µl FACS buffer 1% saponin containing primary antibodies in appropriate dilution (see table 9). After 30 min at room temperature cells were washed with FACS buffer 1% saponin. Secondary antibodies were added in a volume of 100 µl FACS buffer 1% saponin (Keratin 14: goat anti-

mouse IgM FITC; Involucrin: goat anti mouse IgG FITC). After further 30 min of incubation in the dark, cells were washed with FACS buffer 1% saponin and then analyzed. Expression levels were calculated by difference in mean fluorescence intensity (MFI) to isotype controls.

FACS stainings of naïve T cells, monocytes and dendritic cells were performed using directly fluorescence dye labeled antibodies (Table 9) which were diluted in 50 µl FACS buffer and then used to resuspend the harvested cells. After 1 h of incubation at 4°C and in the dark cells were washed with FACS Buffer and analyzed.

3.2.22 Statistics

For all statistical analyses GraphPad Prism version 5.0 was used. In case of data normalization measured values were normalized to the control mean. Data are shown in general ± standard error of the mean (SEM). Statistical significance was tested in cases of comparisons between two groups using Mann Whitney test or Wilcoxon matched-pairs signed rank test. For comparing three or more paired groups, parametric data were analyzed using a one-way ANOVA followed by Tukey post hoc analysis. Data were considered significant at $p < 0.05$.

4 Results

4.1 Optimization of medium conditions for co-culture experiments

Cell culture media are optimized in their composition for distinct cell types supporting growth and function in the best possible way. Therefore, co-culture experiments comprising different cell types with different needs are challenging. To identify a cell culture medium which is suitable for both, generation of keratinocyte mediators and co-culture experiments with T cells, several media were tested regarding their effects on cell death, cell morphology, expression of differentiation markers and cytokine release.

4.1.1 Influence of keratinocyte media on CD4⁺ T cells

The most convenient way for co-culture experiments with keratinocyte supernatants and T cells would be to generate keratinocyte supernatants in keratinocyte medium and use the supernatants later on in a mix of T cell medium. To check the feasibility of this setting, T cell cytokine release in pure AIM-V medium was compared with cytokine release in a 3 : 1 mix of AIM-V medium and keratinocyte medium.

Two different keratinocyte media, DermaLife and KGM 2, were tested for their effects on different CD4⁺ T helper cell subsets (Th1, Th2, Th17 and Th0 control).

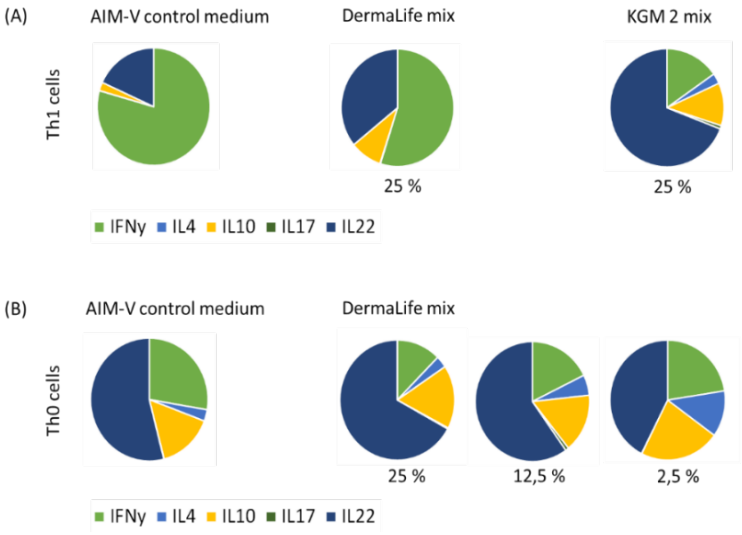


Figure 9: Effects of keratinocyte growth media on T cell cytokine release. CD4⁺ T cells were incubated in a mix of AIM-V medium with either DermaLife or KGM 2 medium for 72 h. T cell cytokine release (IFN-γ, IL-4, IL-10, IL-17 and IL-22) was measured by ELISA. **(A)** Influence of 25 % keratinocyte growth media on the cytokine profile of Th1 cells. **(B)** Concentration dependent influence of DermaLife keratinocyte medium on Th0 cytokine profile (25 %, 12.5 % and 2.5 % in AIM-V medium).

As a representative example for all T cell subsets figure 9 shows the influence of keratinocyte media on the Th1 cytokine profile. It demonstrates that both tested keratinocyte media, DermaLife and KGM 2, caused a shift to high IL-22 release on the cost of other cytokines. In the case of Th1 and Th0 cells, IFN-γ release was strongly inhibited by the presence of 25 % keratinocyte medium, whereas IL-22 release was induced (Figure 9A and B). Besides the favoring of IL-22 secretion it could be observed that in lower concentrations (12.5 % and 2.5 %) DermaLife medium induced IL-4 secretion in Th0 (Figure 9B) and Th2 cells (data not shown). Due to these media effects, both keratinocyte media were not considered as suitable for T cell culturing. In consequence it became necessary to test other T cell and keratinocyte media and to optimize the experimental set up to exclude falsifying medium effects.

4.1.2 Medium effects on keratinocyte cultures

In order to find an alternative to DermaLife medium for culturing keratinocytes, the following basal media were tested over a culture period of 18 h (± 6 h IFN-γ pre-stimulation): KGM 2, RPMI, M199, F12, AIM-V, DMEM, IMDM and PBS as buffer negative control. Three independent experiments with cells from healthy donors were conducted, although not all media were tested three times since some of them were excluded early after obvious negative effects on the cells.

Medium effects on keratinocyte morphology and survival

Human primary keratinocytes cultured in different media revealed after only 18 h differences in their morphology (Figure 10). In comparison to cells cultured in the keratinocyte media DermaLife and KGM 2, which showed intact and glowing edges, especially AIM-V medium treated cells showed clear signs of cell death with vesicle formation. Similar to this, PBS incubated wells showed puffed up cells detaching from the plate. Cells cultured in M199 and F12 medium also seemed to be more flattened and less shiny in their appearance than in keratinocyte medium. However, for DMEM, IMDM and RPMI cell morphology appeared to be good and similar to control keratinocyte media.

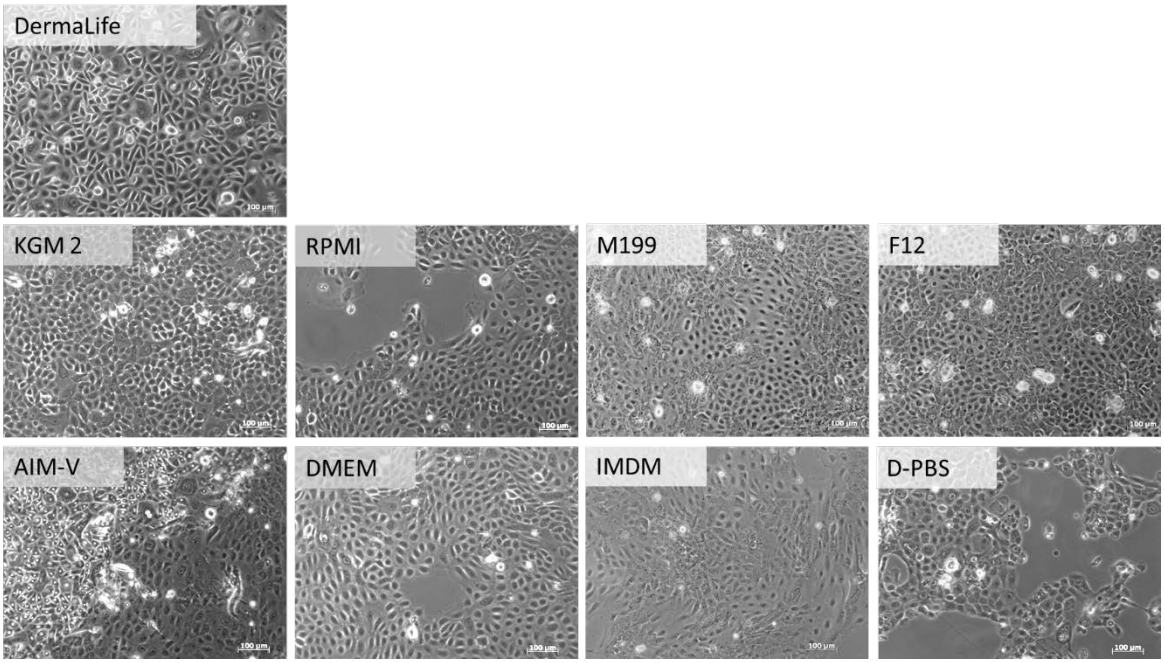


Figure 10: Effects of different cell culture media on the morphology of human primary keratinocytes. Keratinocytes of healthy donors were incubated at 80-90 % confluence with different cell culture media for 18 h and changes in morphology were checked by microscope (magnification = 100x).

Besides morphology, cell survival was checked by propidium iodide (PI) FACS staining. AIM-V medium treated cells were excluded from this test due to negative morphological results. Results of the remaining media showed that under steady state conditions no clear effect of the media on cell death was observable. However, IFN-γ pre-stimulated cells had a tendency to a higher percentage of PI positive cells in M199 and RPMI medium (Figure 11).

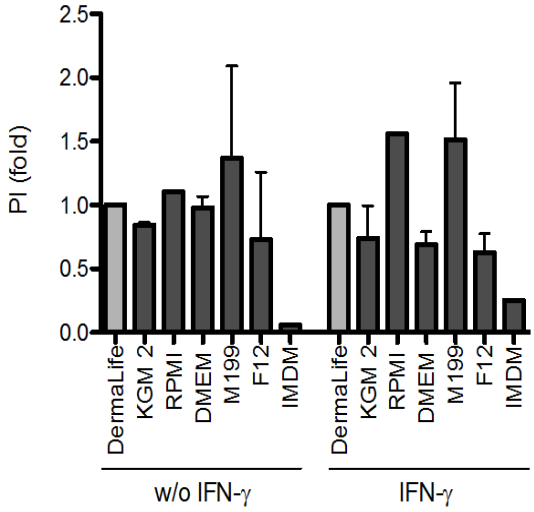


Figure 11: Effects of different cell culture media on the percentage of propidium iodide (PI) positive cells. Human primary keratinocytes (with and without IFN-γ pre-stimulation) were incubated for 18 h with different media and cell death rate was checked subsequently by PI FACS staining. Data is shown normalized on control values of DermaLife keratinocyte medium as fold induction ± SEM.

To check influences regarding the expression of differentiation markers, keratinocytes were stained for Involucrin and keratin 14 (K14) and analyzed by FACS. Involucrin, mainly expressed by differentiated keratinocytes, was influenced only to a minor degree by most of the media compared to keratinocyte media (Figure 12A) However, RPMI conditions showed a down regulation of Involucrin as well as DMEM did in the IFN-γ induced inflammatory state. Keratin 14, a marker for basal keratinocytes, was clearly affected by M199 and RPMI medium, both inducing higher expression in comparison to DermaLife control (Figure 12).

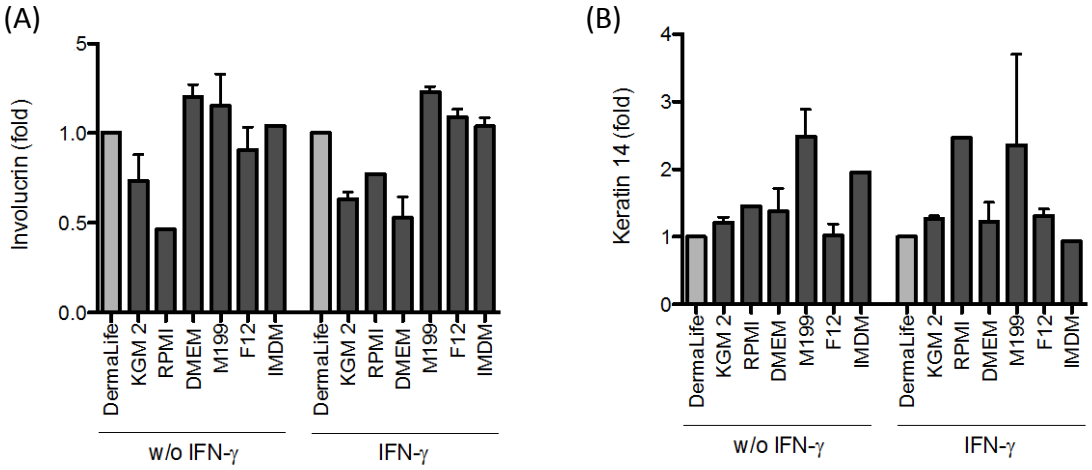


Figure 12: **Effects of cell culture media on the expression of the differentiation markers Involucrin and K14**. Human primary keratinocytes (with and without IFN-γ pre-stimulation) were incubated for 18 h with different media and subsequently analysed by FACS for **(A)** Involucrin and **(B)** K14 expression. Data is shown normalized on control values of DermaLife keratinocyte medium as fold induction ± SEM.

Medium effects on keratinocyte cytokine release

Effects of the different media on the release of the inflammatory cytokines IP10 and TNF-α were measured by ELISA in keratinocyte cell supernatants. Results revealed that especially M199 induced IP10 and TNF-α secretion under steady state as well as under inflammatory conditions. Besides M199 also IMDM and RPMI led to increased levels of TNF-α in the keratinocytes' supernatant when cells were pre-stimulated with IFN-γ (RPMI not tested for steady state) (Figure 13).

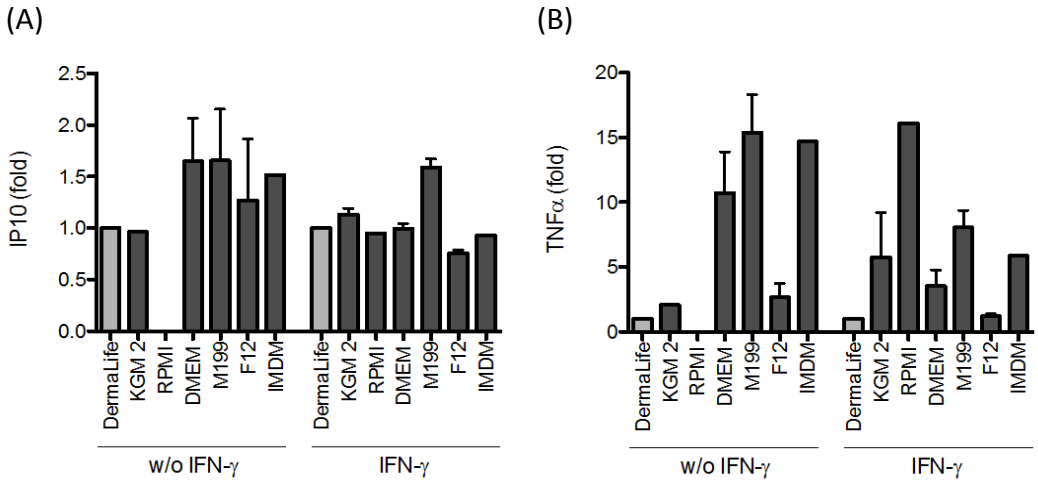


Figure 13: Effects of cell culture media on keratinocytes' IP10 and TNF-α cytokine release. Human primary keratinocytes (with and without IFN-γ pre-stimulation) were incubated for 18 h with different media and supernatants were analysed by ELISA for **(A)** IP10 and **(B)** TNF-α expression. Data is shown normalized on control values of DermaLife keratinocyte medium as fold induction ± SEM.

4.1.3 Medium effects on CD4⁺ T cells

As keratinocytes were tested in 4.1.2 also CD4⁺ T cells were tested regarding their tolerance to different culture media. For this purpose CD4⁺ T cells of different subsets (Th1, Th2, Th17 and Th0 control) were incubated for 72 h with different media in a mixture of 1 : 3 with serum free AIM-V medium.

Medium effects on T cell survival

Propidium iodide FACS staining presented in figure 14 showed that in comparison to AIM-V control the media DermaLife and F12 had no effect on cell survival. M199, however, led to higher cell death rate in nearly all T cells types which is reflected by an increase in PI

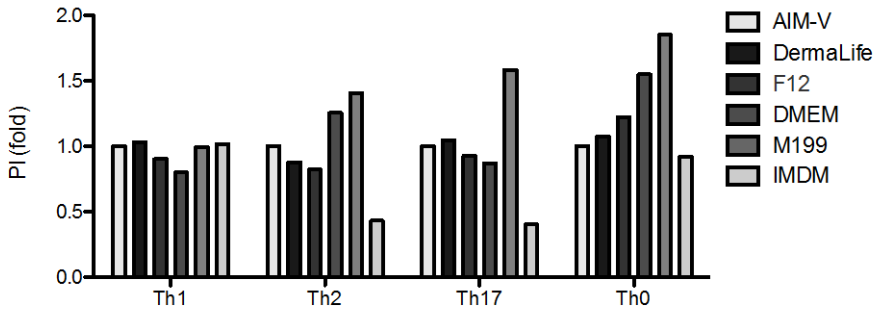


Figure 14: Effects of different cell culture media on the survival of CD4⁺ T cells. T cell subsets (Th1, Th2, Th17 and control Th0) were incubated for 72 h with different cell culture media in a mixture of 1:3 with AIM-V medium. Cell survival was checked by PI FACS staining. Data is shown normalized to AIM-V control as fold induction.

positive cells. In addition, DMEM showed a tendency to a higher PI rate in Th2 and Th17 cells, whereas the use of IMDM had a rather protective effect by preventing cell death.

Medium effects on T cell cytokine release

ELISA data of T cell supernatants shown in figure 15A revealed that M199 and IMDM medium inhibited IFN-γ release as strong as both of the tested keratinocyte media (DermaLife and KGM 2). Only DMEM and F12 did not decrease the IFN-γ secretion in comparison to AIM-V control medium (Figure 15A). Moreover, IL-4 secretion of Th2 cells was down-regulated by all tested media compared to control, although the effect was strongest for F12 and less prominent for DMEM medium (Figure 15B). For IL-10 and IL-17 (Figure 15C and D) M199 was the only medium with a clear tendency to decrease cytokine release. In contrast to the inhibition of most cytokines, an inducing effect for IL-22 secretion was observed for the two keratinocyte media but also for IMDM (Figure 15E).

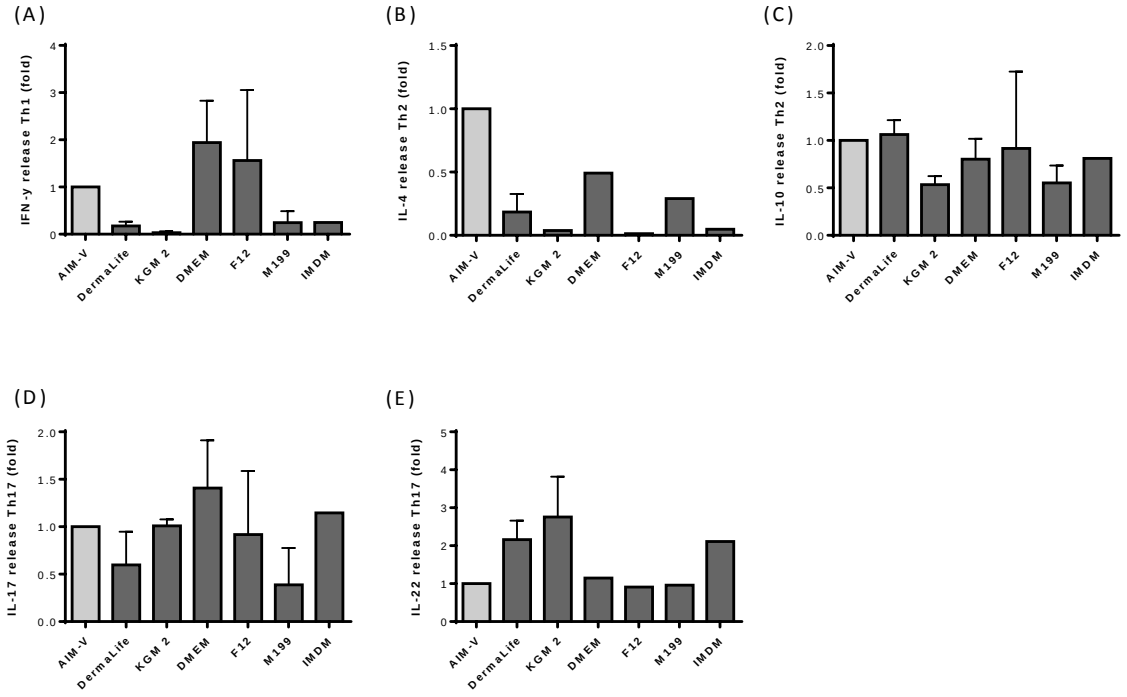


Figure 15: Effects of different cell culture media on the cytokine release of CD4⁺ T cells. T cell subsets Th1, Th2, and Th17 were incubated for 72 h with different cell culture media in a mixture of 1:3 with AIM-V medium. Cytokine release was checked by ELISA: **(A)** IFN-γ, **(B)** IL-4 **(C)** IL-10 **(D)** IL-17A and **(E)** IL-22. Data is shown normalized to AIM-V control as fold induction ± SEM.

Based on the results of 4.1.2 and 4.1.3 Table 28 represents summarized data of medium effects on keratinocytes and T cells. It demonstrates that only DMEM medium was able to

achieve no clear negative result in all tested categories. Therefore, DMEM was chosen for the generation of keratinocyte supernatants which were used in all following T cell co-culture experiments.

Table 28: Summarized data of medium tests for the effects on keratinocytes and CD4$^+$ T cells. Left column represents all tested media and head row shows all tested criteria. + = no negative effect; ++ = positive effect; - = negative effect and - - = extreme negative effect.

	Morphology	PI	Involucrin	K14	IP10	TNF-α	T cell cytokines
DermaLife	++	+	+	+	+	+	- -
KGM 2	++	+	+	+	+	+	- -
RPMI	+	-	-	-	+	- -	n.t.
DMEM	+	+	+	+	+	+	+
IMDM	-	++	+	+	+	- -	- -
M199	-	- -	-	-	- -	- -	-
F12	+	+	+	+	+	+	- -
AIM-V	- -	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.

n.t. = not tested

4.2 Influence of keratinocyte derived mediators on CD4$^+$ T cell effector function

Keratinocytes can produce large amounts of cytokines and chemokines, which exert influences not only on neighbouring keratinocytes but also on other cell types residing in the skin or migrating into the skin. To study the modulating effects of keratinocyte derived mediators on the function and plasticity of different T helper (Th) cell subsets, co-culture experiments were conducted. For this purpose keratinocyte supernatants were generated under steady state or inflammatory conditions provoked by IFN-γ pre-stimulation. In addition, keratinocytes were stimulated in a second round of experiments with aqueous pollen extract (APE) from birch and ragweed, reflecting the contact to plant pollen substances. For all three settings, cell-free keratinocyte supernatants were collected and used for co-cultures with differentiated T cell lines (Th1, Th2, Th17, Treg and Th0 control) derived from naïve CD4$^+$ T cells of healthy blood donors. To reveal the impact of the keratinocyte derived mediators on T cell effector functions following parameters were chosen: cell proliferation, cytokine release and mRNA expression of typical T cell cytokines and transcription factors.

4.2.1 Influence of keratinocyte derived mediators on T cell proliferation

To study the influence of keratinocyte derived mediators on the proliferation of CD4$^+$ T cells, co-culture experiments were conducted as follows. Different T cell lines (Th1, Th2,

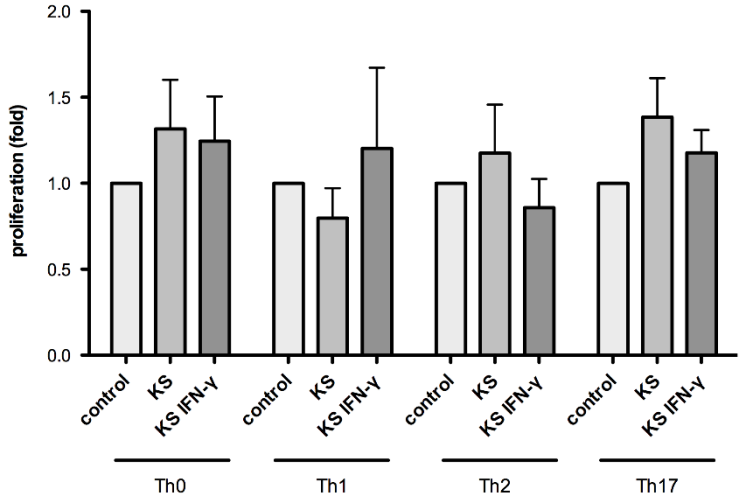


Figure 16: Effects of keratinocyte derived mediators on T cell proliferation. Different CD4$^+$ T cell subsets (Th1, Th2, Th17 and control Th0) were incubated for 72 h with supernatants of unstimulated or IFN-γ pre-stimulated keratinocytes. T cell proliferation was measured by ^{3}H thymidine incorporation assay. Data is normalized to medium control and shown ± SEM; n = 10. KS = keratinocyte supernatant.

Th17 and Th0 control) were incubated with keratinocyte supernatants for 72 h. Cell proliferation of the T cells was subsequently measured by ^{3}H thymidine incorporation assay. As shown in figure 16 keratinocyte supernatants had no significant effects on T cell proliferation in all tested T cell lines in comparison to medium control. However, a tendency to slightly higher proliferation in Th17 and Th0 cells could be observed.

4.2.2 Influence of keratinocyte derived mediators on T cell cytokine release

To determine the influence of keratinocyte derived mediators on T cell cytokine release, $CD4^{+}$ T cell subsets were restimulated with αCD3/CD28 and incubated 72 h in the presence of keratinocyte supernatants. Besides total supernatants from steady state and IFN-γ induced inflammatory state keratinocytes, protein free supernatants were also used in this setting to check for protein dependent effects. Changes in the T cell cytokine profile were measured by Multiplex or ELISA technique. As key cytokines for the respective T cell subsets were chosen: IFN-γ and TNF-α for Th1; IL-4, IL-5 and IL-10 for Th2; IL-17 for Th17; IL-22 for Th0, Th1 and Th17; and TGF-β for Tregs.

4.2.2.1 Effects of mediators from untreated and IFN-γ treated keratinocytes

4.2.2.1.1 Effects of total keratinocyte supernatants

Results of co-culture experiments showed that total keratinocyte supernatants exerted an an inhibitory effect on the secretion of nearly all investigated T cell cytokines. As shown in figure 17A secretion of IFN-γ, the key cytokine of Th1 cells, was clearly down regulated by the presence of keratinocyte supernatants in comparison to control. However, this was only true for unstimulated keratinocytes since IFN-γ pre-stimulated keratinocytes did not inhibit the T cell IFN-γ release. Similar to this finding the presence of keratinocyte supernatants regulated the IL-22 cytokine secretion in Th1 and Th0 cells (Figure 17B). Under steady state conditions IL-22 release was attenuated significantly compared to the release of control cells in AIM-V medium, whereas cells incubated with mediators derived from IFN-γ pre-stimulated keratinocytes did not provoke a down regulation of IL-22 secretion. In contrast IL-10 cytokine release of Th0 and Th2 cells was inhibited by both, keratinocyte mediators derived from unstimulated and IFN-γ pre-stimulated cells (Figure 17E). In addition, the presence of keratinocyte supernatants provoked a down regulation of IL-4 in Th2 cell co-cultures (Figure 17D).

Besides the inhibiting effects observed for IFN-γ, IL-22, IL-10 and IL-4, the only cytokine showing a clearly enhanced secretion in response to keratinocyte derived mediators was IL-17. Both, mediators derived from unstimulated and IFN-γ pre-stimulated keratinocytes, led in Th17 cells to increased IL-17 secretion (Figure 17E). Results for this phenomenon were highly significant and exceeded the four times fold mark.

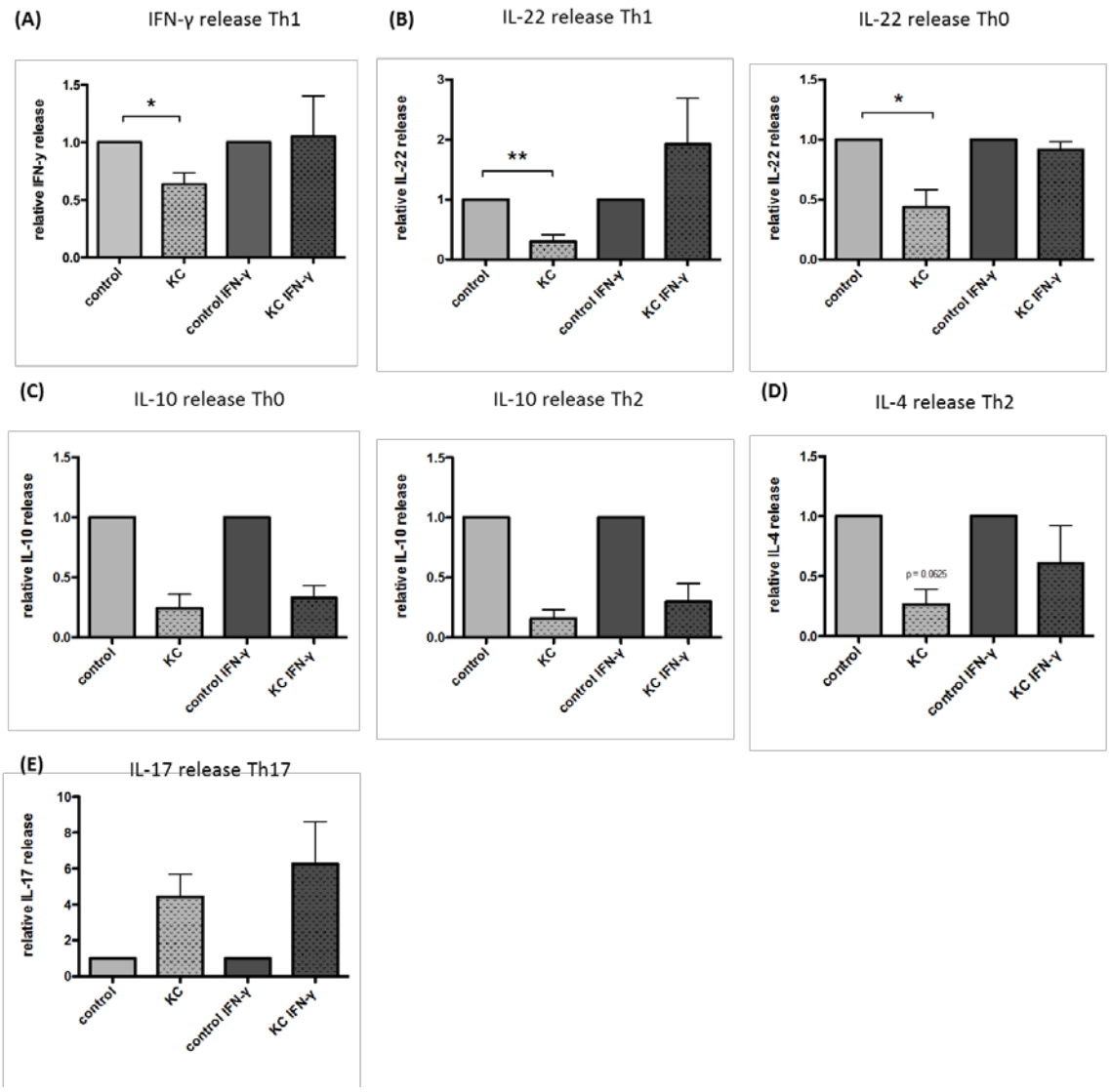


Figure 17: T cell cytokine relase modulation by keratinocyte derived mediators. T cell subsets (Th1, Th2, Th17 and control Th0) generated in 12 well plates were incubated for 72 h with supernatants of unstimulated (KC) or IFN-γ pre-stimulated keratinocytes (KC IFN-γ). T cell cytokine release (IFN-γ, IL-22, IL-10, IL-4 and IL-17) was measured by ELISA. **(A)** IFN-γ release (Th1 unstim. n = 10, prestim. n = 7); **(B)** IL-22 release (unstim. n = 8, prestim. n =4); **(C)** IL-10 release (Th0 unstim. n = 5, prestim. n = 4 and Th2 n = 5); **(D)** IL-4 release (unstim. n = 5, prestim. n=3) and **(E)** IL-17 release (unstim. n=5, prestim. n= 3). Data is normalized to corresponding medium control and shown ± SEM. * $p < 0.05$ and ** $p < 0.01$, Wilcoxon matched-pairs signed rank test.
control = medium control; KC = keratinocyte supernatant; control IFN-γ = medium control for T cells treated with supertatants from IFN-γ treated keratinocytes; KC IFN-γ = supernatant of IFN-γ treated keratinocytes.

4.2.2.1.2 Effects of protein-free keratinocyte supernatants

To identify the active components in keratinocyte supernatants causing the effects described in 4.2.2.1.1, keratinocyte supernatants were treated with acetone for protein precipitation and removal.

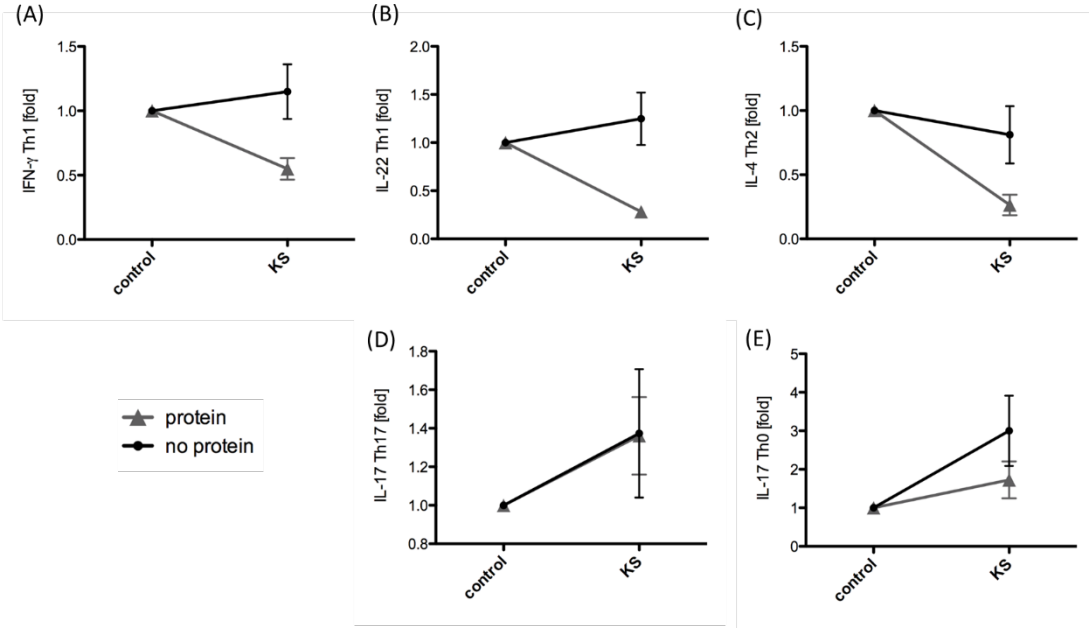


Figure 18: Effects of protein-free keratinocyte supernatants on T cell cytokine release. T cell subsets (Th1, Th2, Th17 and Th0 control) generated in 12 well plates were incubated for 72 h with total keratinocyte supernatants (protein) or protein-free keratinocyte supernatant (no protein). T cell cytokine release (IFN-γ, IL-22, IL-4 and IL-17) was measured by ELISA. **(A)** IFN-γ release Th1 (n = 3); (**B)** IL-22 release Th1 (n = 3); **(C)** IL-4 release Th2 (n = 2); **(D)** IL-17 release Th17 (n = 3); **(E)** IL-17 release Th0 (n = 3). Data is normalized to corresponding medium control and shown as fold induction ± SEM. KS = keratinocyte supernatant.

Experiments revealed that in comparison to protein containing supernatants, which dampened the cytokine release, the non-protein fractions did not exert an inhibiting effect on the T cells. Figure 18 illustrates this finding by showing that in Th1 cells both, IFN-γ and IL-22 release (Figure 18A and B) and in Th2 cells the IL-4 release (Figure 18C), were not inhibited when proteins were removed from keratinocyte supernatant. In contrast IL-17 release was induced in Th17 and Th0 cells regardless if protein-containing or protein-free keratinocyte supernatants were used in T cell cultures (Figure 18D and E).

4.2.2.2 Effects of mediators from APE treated keratinocytes

Besides the two conditions, steady state and inflammatory state, which were described in the preceding passages, a third setting with the aim to simulate the contact of skin with

plant pollen was tested. For this purpose keratinocytes of healthy donors were treated with APE of birch (Bet) or ragweed (Amb) and the emerging effects on the cytokine release of $CD4^+$ T cell subsets were analysed by TGF-β ELISA and Multiplex for seven other cytokines (IFN-γ, TNF-α, IL-22, IL-5, IL-4, IL-10 and IL-17). Results are presented according to the particular T cell line and its assigned standard cytokines. All experiments were also conducted with supernatants of keratinocytes pre-stimulated with IFN-γ. However, the resulting data were very similar to no pre-stimulation settings and showed no additional effects. Therefore, figures in this passage do not include IFN-γ pre-stimulation data, which are, however, attached in the appendix (Figure A1 – A4). All other results are shown normalized to control and in comparison to the results of T cells co-cultured with mediators derived from unstimulated keratinocytes.

ELISA analysis for **TGF-β cytokine release** revealed that keratinocyte derived mediators exert different effects on the T cell subsets (Figure 19). Compared to medium control, Treg and Th0 cells were not affected in their TGF-β release by the presence of keratinocytes supernatants obtained under steady conditions. However, Th1 and Th2 cells seemed to be inhibited in their TGF-β secretion when incubated with these supernatants. In contrast the mediators derived from APE stimulated keratinocytes hardly affected cytokine release of Th0, Th1 and Th2 cells, although a slight induction was observable in Tregs. On Th17 cells

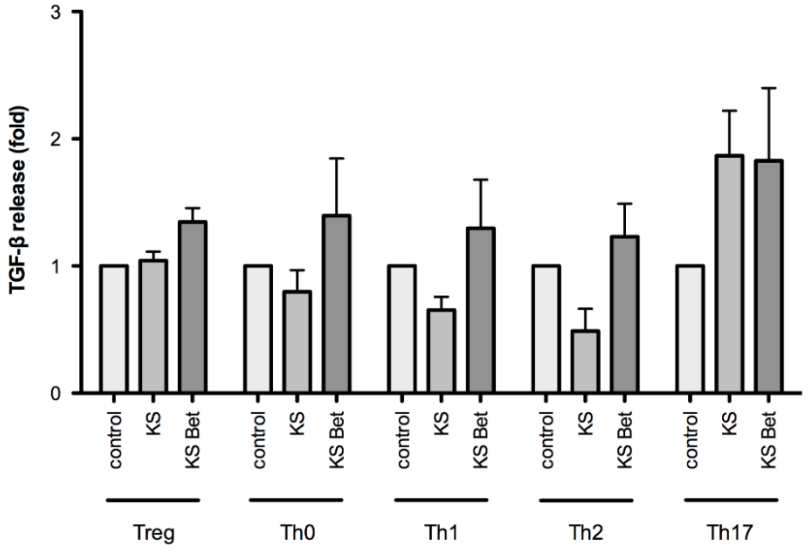


Figure 19: Impact of keratinocytes derived mediators on T cell TGF-β release. T cell subsets (Treg, Th0, Th1, Th2 and Th17) generated in 48 well plates were incubated for 72 h with supernatants derived from steady state keratinocytes (KS) or Bet APE treated keratinocytes (KS Bet). T cell TGF-β release was measured by ELISA. Data is normalized to corresponding medium control and shown as fold induction ± SEM; n = 4.

both, mediators from unstimulated and APE stimulated keratinocytes, exerted an inducing influence on T cell TGF-β release. Only observable in pg/ml was the fact, that Treg cells did not produce significantly more TGF-β than the other T cell lines (see Figure A1B).

In addition to TGF-β ELISA, **Multiplex** technique was used to check the influences on T cell cytokine release of IFN-γ, TNF-α, IL-13, IL-10, IL-22, IL-4 and IL-5. Multiplex data shown in figures 20 – 23 confirmed the results described in preceding passages and demonstrated once more that mediators derived from unstimulated keratinocytes can have a strong inhibiting effect on T cell cytokine release in nearly all cases.

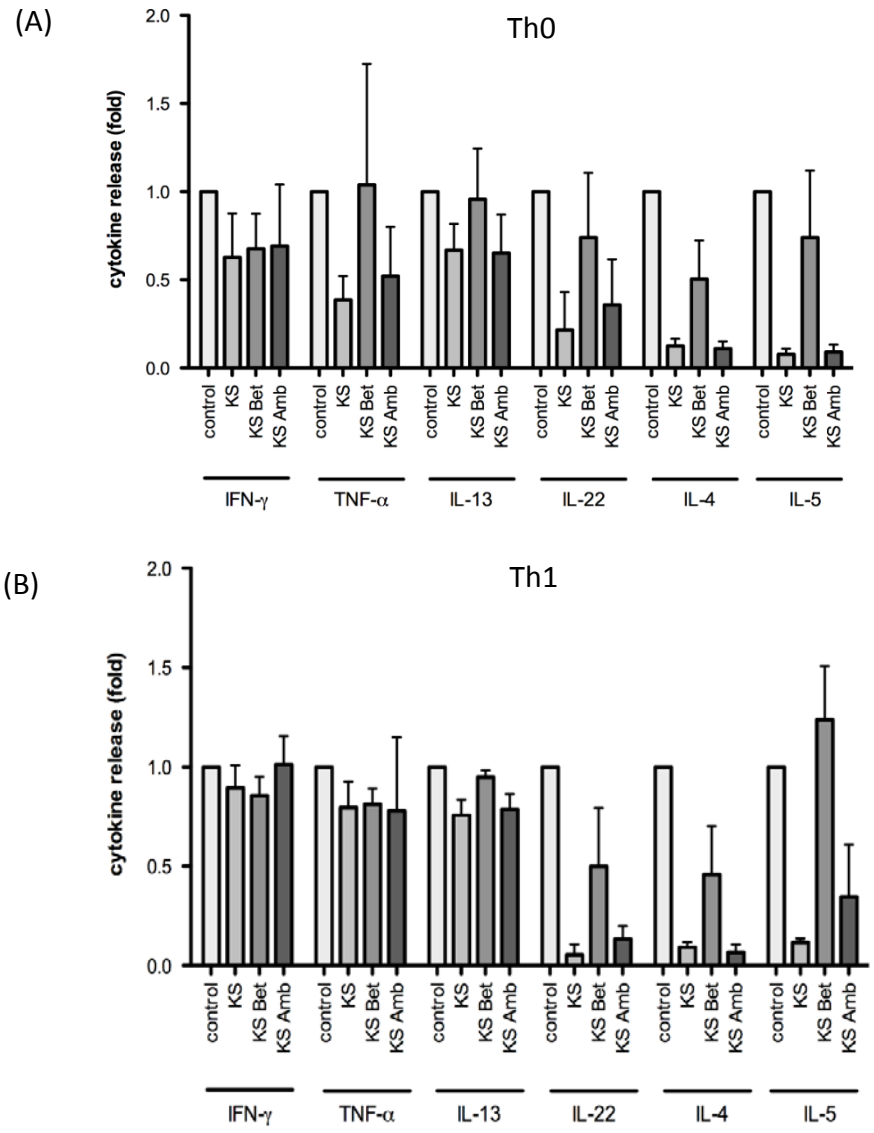


Figure 20: Impact of keratinocytes derived mediators on the cytokine release of Th0 and Th1 cells. (A) Th0 and **(B)** Th1 cells generated in 48 well plates were incubated for 72 h with supernatants derived from steady state keratinocytes (KS), Bet APE treated keratinocytes (KS Bet) or Amb APE treated keratinocytes (KS Amb). T cell cytokine release (IFN-γ, TNF-α, IL-13, IL-22; IL-4 and IL-5) was measured by multiplex technique. Data is normalized to corresponding medium control and shown as fold induction ± SEM; n = 4.

For Th0 and Th1 cells a clear decrease of IFN-γ, TNF-α, IL-13, IL-22, IL-4 and IL-5 secretion was observed with the latest three even been down-regulated to less than 20 % of control values. In comparison mediators of Bet APE stimulated keratinocytes could not achieve this strong inhibiting effect and hardly changed the cytokine release levels for Th0 and Th1 cells (Figure 20A and B). However, mediators derived from Amb APE treated keratinocytes repressed the cytokine release nearly as effective as the supernatants from unstimulated keratinocytes.

Treg cells were similar influenced by the keratinocyte supernatants as Th0 and Th1 cells, which was reflected by a general down regulation of IFN-γ, IL-10, TNF-α, IL-13 and IL-5 (Figure 21).

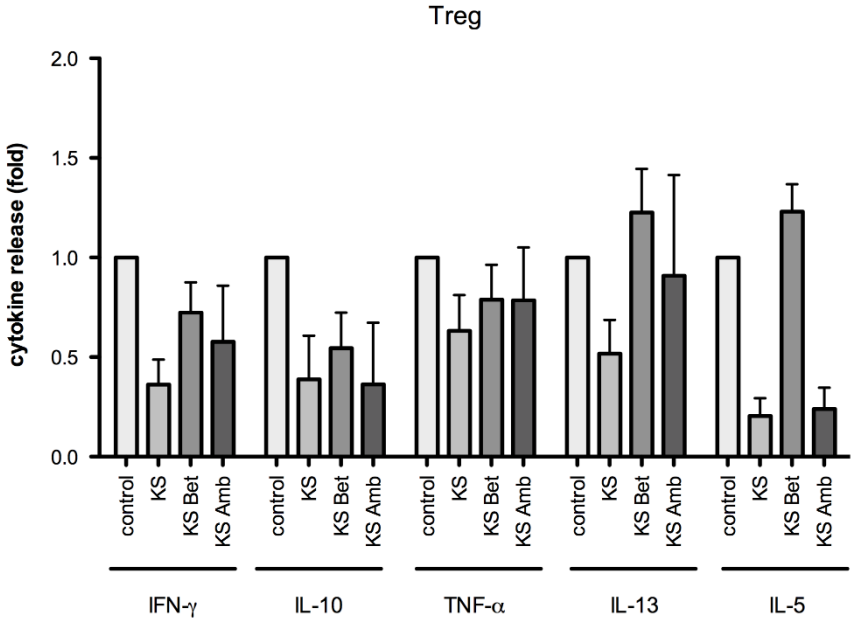


Figure 21: Impact of keratinocytes derived mediators on the cytokine release of Treg cells. Treg cells generated in 48 well plates were incubated for 72 h with supernatants derived from steady state keratinocytes (KS), Bet APE treated keratinocytes (KS Bet) or Amb APE treated keratinocytes (KS Amb). T cell cytokine release (IFN-γ, IL-10, TNF-α, IL-13 and IL-5) was measured by multiplex technique. Data is normalized to corresponding medium control and shown as fold induction ± SEM; n = 4.

In the Th2 subset the suppressing effects of the keratinocyte supernatants were extremely strong and diminished nearly all IL-10, IL-4 and IL-5 cytokine secretion (Figure 22). Besides this also IFN-γ, IL-13 and TNF-α release was clearly down-regulated in Th2 cells after incubation with keratinocyte derived factors. Furthermore, the presence of mediators from

Bet APE treated cells showed in the Th2 subset also an inhibiting effect, although it was far less pronounced than under steady conditions. In comparison to this Amb APE treated keratinocytes produced mediators with comparable inhibiting strength as observed for the mediators obtained under steady state condition.

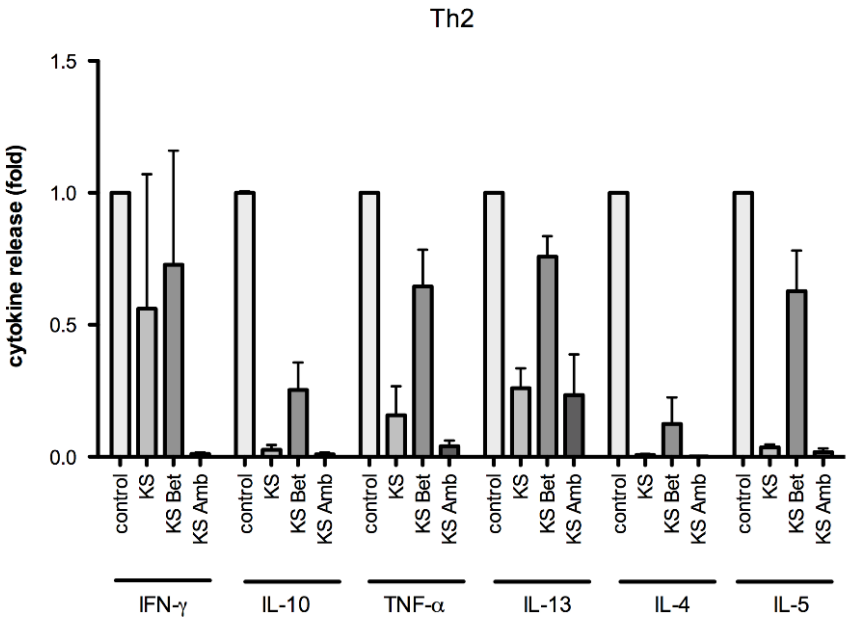


Figure 22: Impact of keratinocytes derived mediators on the cytokine release of Th2 cells. Th2 cells generated in 48 well plates were incubated for 72 h with supernatants derived from steady state keratinocytes (KS), Bet APE treated keratinocytes (KS Bet) or Amb APE treated keratinocytes (KS Amb). T cell cytokine release (IFN-γ, IL-10, TNF-α, IL-13, IL-4 and IL-5) was measured by multiplex technique. Data is normalized to corresponding medium control and shown as fold induction ± SEM; n = 4.

Results for Th17 cells shown in figure 23 were dominated by the induction of IL-17 release when the T cells were co-cultured with keratinocyte supernatants. This was true for all three conditions, unstimulated, Bet APE and Amb APE stimulated, although mediators derived from unstimulated and Amb APE stimulated keratinocytes were three times stronger in inducing IL-17 release than the mediators derived from Birch APE treated keratinocytes.

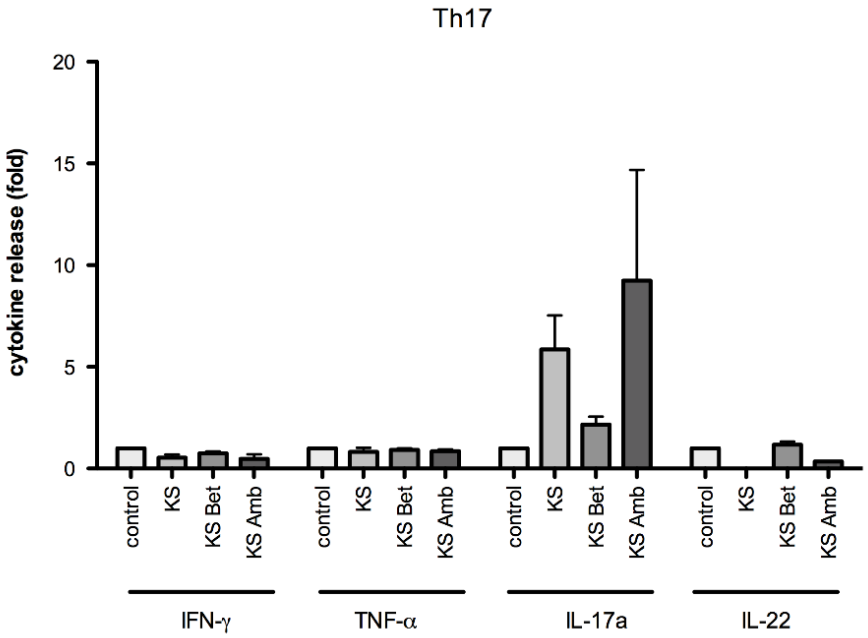


Figure 23: Impact of keratinocytes derived mediators on the cytokine release of Th17 cells. Th17 cells generated in 48 well plates were incubated for 72 h with supernatants derived from steady state keratinocytes (KS), Bet APE treated keratinocytes (KS Bet) or Amb APE treated keratinocytes (KS Amb). T cell cytokine release (IFN-γ, TNF-α, IL-17A and IL-22) was measured by multiplex technique. Data is normalized to corresponding medium control and shown as fold induction ± SEM; n = 4.

4.2.3 Influence of keratinocytes derived mediators on T cell mRNA expression

Since preceding parts covered only influences on the measureable cytokine release, following part shall present further information by testing effects of keratinocyte derived mediators on the mRNA expression in $CD4^+$ T cell subsets. Starting point for this analysis was the incubation of $CD4^+$ T cells with supernatants collected from a) unstimulated (steady state) keratinocytes, b) IFN-γ pre-stimulated keratinocytes (inflammatory type), c) Bet APE stimulated keratinocytes and d) Amb APE stimulated keratinocytes. The influence on the mRNA level of the transcription factors T-Bet, GATA-3, Foxp3, RORC2, and the cytokines IFN-γ, IL-4, IL-10, IL-17, IL-22 and TGF-β was determined by qPCR. Due to the large amount of data resulting from these experiments only selected results are shown in this passage. However, graphic charts representing additional date are shown in the attachment. This includes also data from experiments with IFN-γ pre-stimulation since they showed no notable difference to the results obtained from experiments with supernatants from unstimulated keratinocytes (see figures A5 - A9). Since all data was normalized to the house keeping gene elongation factor 1α (EF1α) and its own medium control by $2^{(-\Delta\Delta C_T)}$ method, the graphs are illustrating relative effects within one tested factor.

In line with the earlier described cytokine data qPCR results of T cells were also mainly dominated by inhibiting effects caused by the presence of keratinocyte derived mediators. As shown in figure 24 this inhibiting effect could be observed in **Th0 control cells** for the transcription factors T-Bet and GATA-3, which were both dampened in their expression by keratinocyte derived mediators. However, only mediators derived from unstimulated keratinocytes provoked for both, T-Bet and GATA-3, a decrease in the mRNA level. Mediators from Amb APE stimulated cells did not show any effect for T-Bet expression as mediators from Bet APE stimulated cells did not for GATA-3. In addition, the Amb APE treatment of keratinocytes produced mediators not effective in inhibiting IFN-γ and IL-4 mRNA expression although for the other two settings a clear suppression could be observed for these cytokines. Besides this also IL-22 mRNA levels were decreased in Th0 cells after co-culture with supernatants of unstimulated and Amb APE stimulated keratinocytes. However, mediators derived from Bet APE treated cells did not achieve any effect for IL-22. In contrast to the described inhibiting effects, the transcription factors RORC2 and Foxp3 as well as the mRNA levels of the cytokine IL-17 were positively affected by the presence of keratinocyte supernatants. Especially mediators derived from

unstimulated and Amb APE stimulated keratinocytes led here to an increase in the mRNA expression.

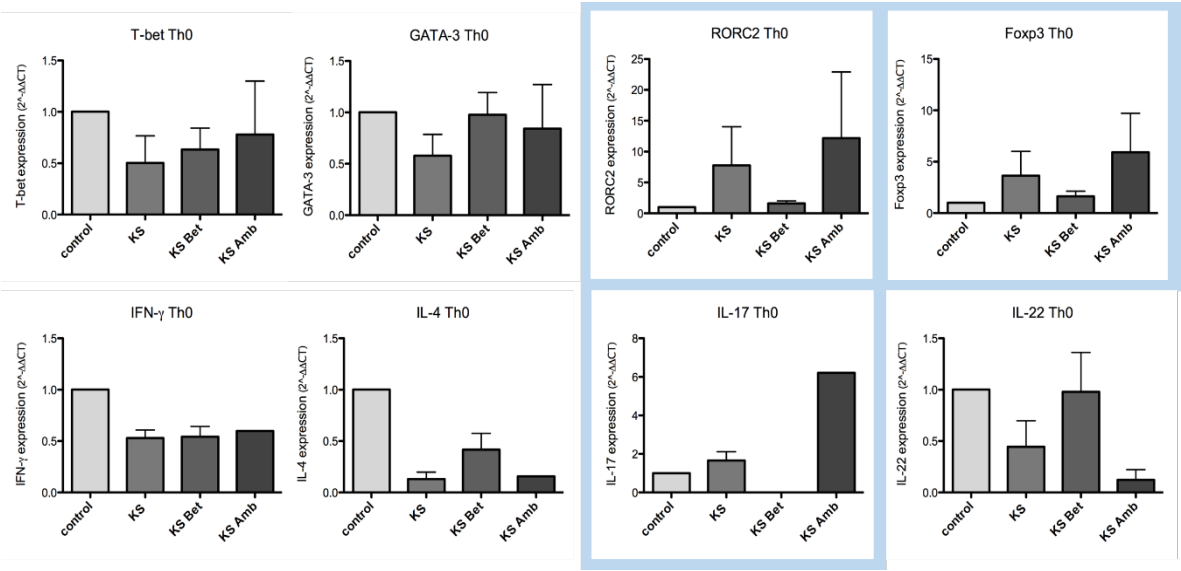


Figure 24: Effects of keratinocytes derived mediators on the mRNA expression of Th0 cells. Th0 cells generated in 48 well plates were incubated for 72 h with supernatants derived from steady state keratinocytes (KS), Bet APE treated keratinocytes (KS Bet) or Amb APE treated keratinocytes (KS Amb). T cell mRNA expression (T-bet, GATA-3, RORC2, Foxp3, IFN-γ, IL-4, IL-17 and IL-22) was analysed by qPCR. Data is normalized to house-keeping gene EF1 and to medium control by $2^{(-\Delta\Delta C_T)}$ method and shown ± SEM. n numbers for T-bet, GATA-3, RORC2 and Foxp3 are n = 4 for control, KS and KS Bet and n = 2 for KS Amb. IFN-γ: control and KS n = 4, KS Bet n = 3 and n = 1 for KS Amb. IL-4: control and KS n = 3, KS Bet n = 3 and n = 1 for KS Amb. For IL-17: control and KS n = 2, KS Bet n = 0 and n = 1 for KS Amb. IL-22: control n=4, KS n = 3, KS Bet n = 3 and n = 2 for KS Amb. Highlighted in blue = inducing effects of keratinocyte derived mediators on T cell mRNA levels.

Figure 25 illustrates the effects of keratinocytes derived mediators for the **Th1 subset**. As in Th0 cells an inhibiting effect could be observed for the key transcription factor T-Bet as well as for GATA-3, which were both dampened in their expression by keratinocyte derived mediators. For T-Bet expression levels this was true for all tested settings: supernatants from unstimulated, Bet APE and Amb APE stimulated keratinocytes. However, GATA-3, the Th2 cell assigned transcription factor, was not suppressed by mediators derived from Bet APE treated keratinocytes. In addition to these observations, Th1 cells showed decreased expression of IFN-γ and IL-22 mRNA with a down regulation of around 80 % in comparison to control. For provoking this inhibition keratinocyte supernatants from untreated or Amb APE treated cells were the most effective ones, assigning supernatants from Bet APE stimulated keratinocytes again a less inhibiting activity.

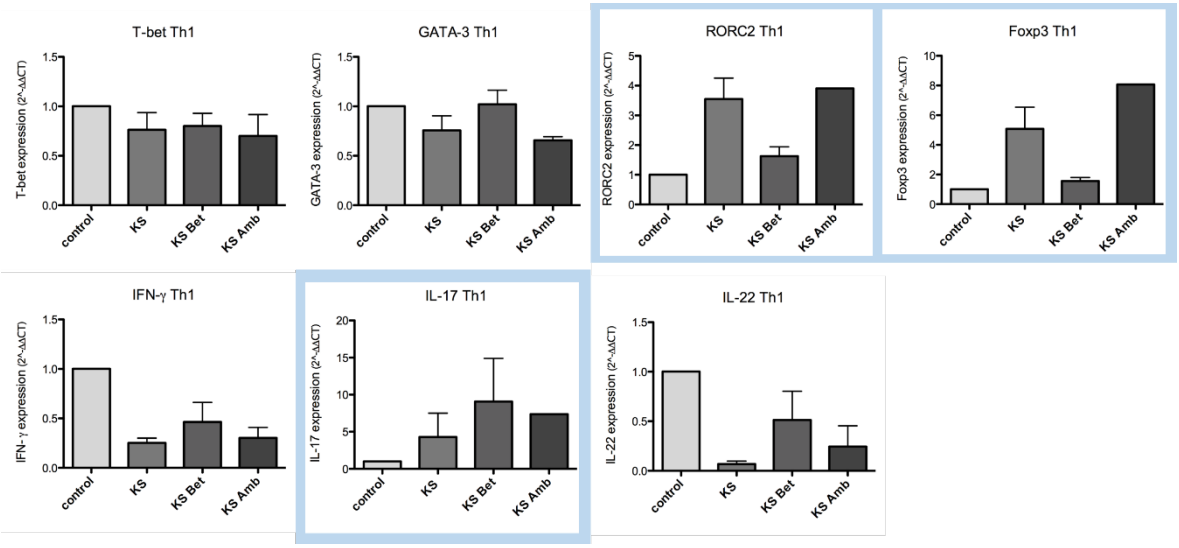


Figure 25: Effects of keratinocytes derived mediators on the mRNA expression of Th1 cells. Th1 cells generated in 48 well plates were incubated for 72 h with supernatants derived from steady state keratinocytes (KS), Bet APE treated keratinocytes (KS Bet) or Amb APE treated keratinocytes (KS Amb). T cell mRNA expression (T-bet, GATA-3, RORC2, Foxp3, IFN-γ, IL-17 and IL-22) was analysed by qPCR. Data is normalized to house-keeping gene EF1 and to medium control by $2^{(-\Delta\Delta C_T)}$ method and shown ± SEM. n numbers for T-bet, GATA-3, Foxp3, IFN- γ and IL-22 are n = 4 for control, KS and KS Bet and n = 2 for KS Amb. RORC2: control, KS and KS Bet n = 3 and n = 1 for KS Amb. For IL-17: control, KS and KS Bet n = 2 and n = 1 for KS Amb. Highlighted in blue = inducing effects of keratinocyte derived mediators on T cell mRNA levels.

Exceptions in the Th1 subset of the inhibiting effects of keratinocyte derived mediators were the mRNA levels of RORC2 and Foxp3. Here both, mediators from unstimulated and Amb APE stimulated cells, had a clear inducing effect compared to medium control. Only for mediators from Birch APE treated cells no effect on the expression level could be observed. However, mRNA levels for the cytokine IL-17 were induced in equal measure independently of the keratinocyte stimulation way.

For experiments performed with **Th2 cells** not all conditions could be tested due too low cell numbers and low RNA yield. Therefore, only changes in the mRNA levels in response to mediators derived from unstimulated and Bet APE stimulated keratinocytes were examined and are shown in figure 26. With the exception of RORC2, both, mediators derived from unstimulated and Birch APE stimulated cells, had a persistent inhibiting effect on the tested mRNA levels. The transcription factors GATA-3 and T-bet as well as the typical Th2 cytokines IL-4 and IL-10 were negatively affected in their mRNA expression. Strikingly supernatants of unstimulated keratinocytes were in all four cases most effective in

suppressing the mRNA levels in comparison to control without keratinocyte supernatant and also in comparison to supernatants derived from Bet APE treated cells.

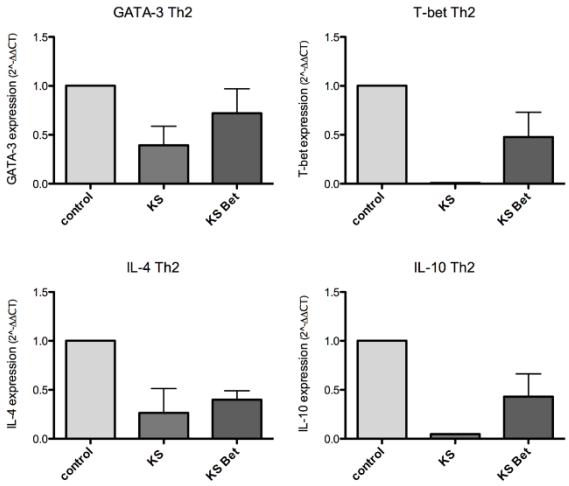


Figure 26: Effects of keratinocytes derived mediators on the mRNA expression of Th2 cells. Th2 cells generated in 48 well plates were incubated for 72 h with supernatants derived from steady state keratinocytes (KS) or Bet APE treated keratinocytes (KS Bet). T cell mRNA expression (GATA-3, T-bet, IL-4 and IL-10) was analysed by qPCR. Data is normalized to house-keeping gene EF1 and to medium control by 2^(-$\Delta\Delta C_T$) method and shown ± SEM. n numbers for GATA-3, T-bet and IL-4 are n = 4 for control and KS Bet and n = 2 for KS. For IL-10: n = 3 for control and KS Bet and n = 1 for KS.

Results for the **Th17 subset** were characterized by an induction of the transcription factors RORC2 and Foxp3 as well as the IL-17 cytokine mRNA levels (Figure 27). The inducing effect of the keratinocyte derived mediators, however, was restricted to these three factors and stands in contrast to the inhibiting effect on the mRNA expression of T-bet, IL-22, IFN-γ and IL-10. As observed in the other T cell subsets especially the mediators derived from unstimulated keratinocytes were most effective in changing the mRNA levels, whereas mediators from Bet APE stimulated keratinocytes had less or no effect.

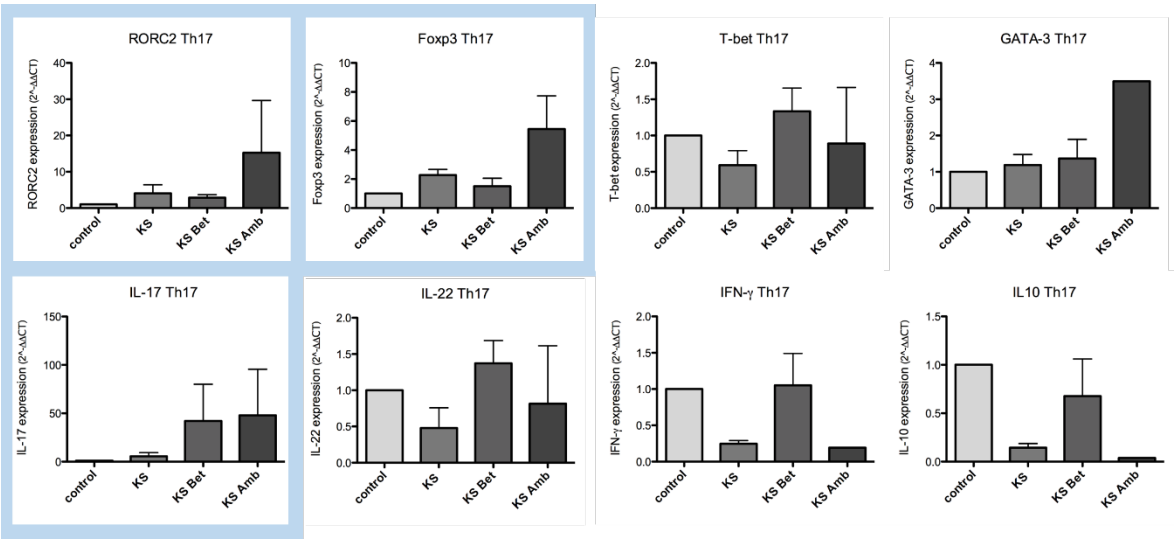


Figure 27: Effects of keratinocytes derived mediators on the mRNA expression of Th17 cells. Th17 cells generated in 48 well plates were incubated for 72 h with supernatants derived from steady state keratinocytes (KS), Bet APE treated keratinocytes (KS Bet) or Amb APE treated keratinocytes (KS Amb). T cell mRNA expression (RORC2, Foxp3, T-bet, GATA-3, IL-17, IL-22, IFN-γ and IL-10) was analysed by qPCR. Data is normalized to house-keeping gene EF1 and to medium control by $2^{(-\Delta\Delta C_T)}$ method and shown ± SEM. n numbers for RORC2, Foxp3, T-bet, IL-17 and IL-22 are n = 4 for control, KS and KS Bet and n = 2 for KS Amb. For GATA-3, IFN-γ and IL-10: control, KS and KS Bet n = 4 and n = 1 for KS Amb. Highlighted in blue = inducing effects of keratinocyte derived mediators on T cell mRNA levels.

Due to low cell number not all stimulation setups could be tested for the **Treg cell line**. Nevertheless, effects of mediators derived from unstimulated and Bet APE stimulated keratinocytes on the Treg mRNA expression could be investigated and analysed for several targets (Figure 28). For instance, inhibiting effects of the keratinocyte supernatants could be observed for the mRNA expression of the transcription factors T-bet and GATA-3 as well as for the cytokines IL-10, TGF-β and IFN-γ. In addition, supernatants from unstimulated keratinocytes also decreased the mRNA level of Foxp3, while supernatants from Bet APE treated keratinocytes did not show this tendency.

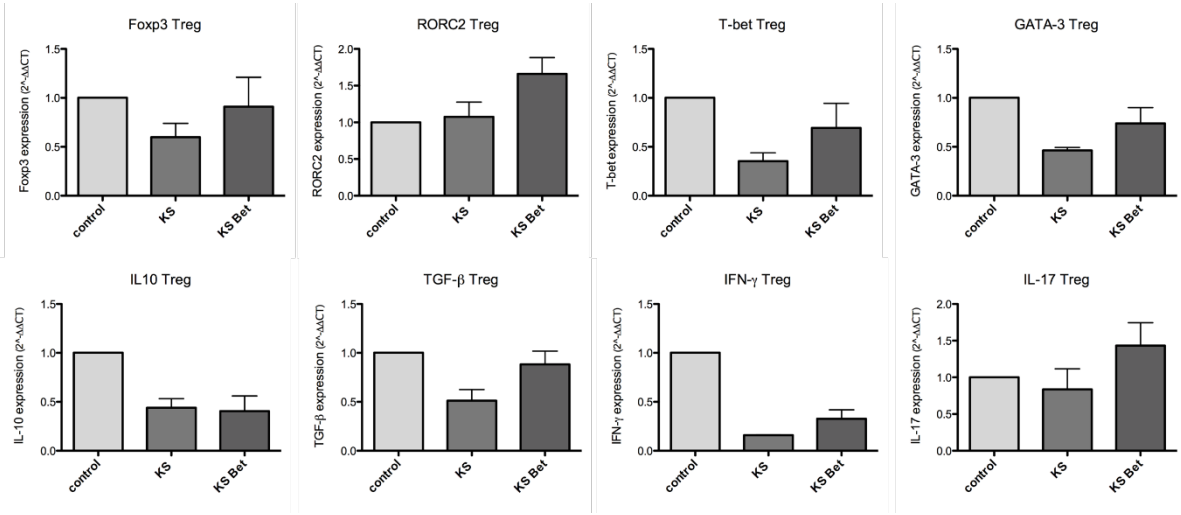


Figure 28: Effects of keratinocytes derived mediators on the mRNA expression of Treg cells. Treg cells generated in 48 well plates were incubated for 72 h with supernatants derived from steady state keratinocytes (KS) or Bet APE treated keratinocytes (KS Bet). T cell mRNA expression (Foxp3, RORC2, T-bet, GATA-3, IL-10, TGF-β, IFN-γ and IL-17) was analysed by qPCR. Data is normalized to house-keeping gene EF1 and to medium control by $2^{(-\Delta\Delta C_T)}$ method and shown ± SEM. n numbers for Foxp3, RORC2, TGF-β and IL-17 are n = 3. For T-bet, GATA-3, IL-10 and IFN-γ: control and KS Bet n = 3 and n = 2 for KS.

4.3 General effects of pollen derived mediators on keratinocytes

Aqueous pollen extract (APE) contains several immunologically active substances which can influence cells in their effector functions (Gilles *et al.*, 2009a). One example representing this phenomenon was already given in preceding passages of this study, showing that the influences keratinocytes exert on T cells via soluble mediators can change in response to APE. To gain deeper insight in this plant pollen provoked modulation, the following study parts focus on the effects APE can have on the skin´s epithelial barrier functions. For this purpose influences of APE were tested on cell morphology, cytokine release and mRNA expression of human primary keratinocytes.

4.3.1 Effects of APE on keratinocyte cell morphology

To test whether APE affects keratinocytes morphologically, cells were incubated for 3 h with *Ambrosia artemisiifolia* (Amb), *Betula pendula* (Bet) or *Pinus sylvestris* (Pin) APE in different concentrations. Cell shape and general appearance were subsequently checked under microscope.

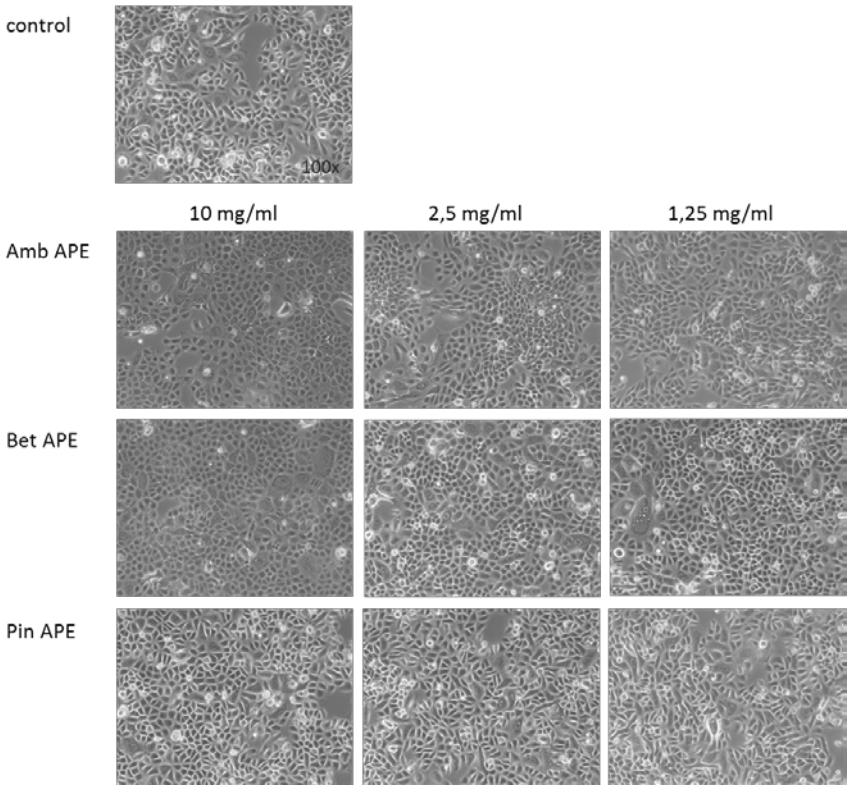


Figure 29: Effects of APE on keratinocyte cell morphology. Human primary keratinocytes were stimulated for 3 h with different concentrations (10 mg/ml, 2.5 mg/ml, and 1.25 mg/ml) of Amb, Bet and Pin APE. Changes in morphology were analysed by light microscopy, magnification factor = 100x. Data are representative for n = 2 experiments.

As figure 29 illustrates cells under medium control conditions were almond shaped and bright glowing. In contrast treatment with the highest concentration of Amb and Bet APE (10 mg/ml) resulted in cells more flattened, less shiny and more contracted. These effects became weaker with lower APE concentrations until the keratinocytes resembled control cells again. However, looking at the 2.5 mg/ml concentration panel, Amb APE seemed to maintain effects stronger than Bet APE since in direct comparison cells appeared for Amb APE less shiny and still more tightened in cell groups. Different from Amb and Bet APE the incubation with Pin APE provoked in no concentration visible effects on the keratinocytes morphology.

4.3.2 Effects of APE on keratinocyte cytokine release

Changings in the cytokine secretion of human primary keratinocytes in response to APE were analysed by ELISA for following targets: IL-8 (CXCL-8), CCL20 (MIP-3α), IP-10 (CXCL-10) and CCL5 (RANTES). Effects on the cytokine release of IL-1β, IL-1α as well as IL-18 are shown in part 4.4.

<u>Effects of APE on IL-8 release</u>

Release of the chemoattractant factor IL-8 was determined in response to several settings, among them also the comparison of different APE species (Amb, Bet, Phl and Pin APE) in different concentrations (Figure 30A and B).

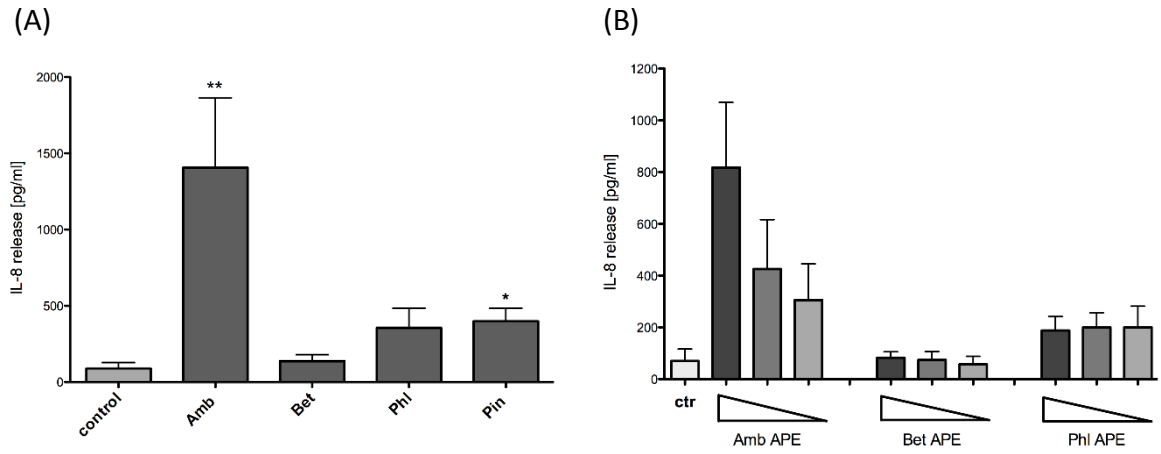


Figure 30: APE induces IL-8 release in keratinocytes. Human primary keratinocytes were stimulated for 3 h with APE and IL-8 release was measured by ELISA. **(A)** Comparison of IL-8 release provoked by different APE (10 mg/ml); Amb, Bet, and Phl APE n = 5; Pin n = 3. **(B)** IL-8 release in response to different APEs (Amb, Bet and Phl APE) in different concentrations (10 mg/ml, 5 mg/ml and 2.5 mg/ml); n = 4. Data are shown as mean ± SEM in pg/ml. * $p < 0.05$ and ** $p < 0.01$, Mann-Whitney test. ctr = control

Notably, Amb APE stimulation of the keratinocytes led after 3 h to the most prominent release of IL-8 in comparison to control and the other tested APEs. In detail, Amb APE enhanced the cytokine release around 10 fold, whereas Bet APE provoked nearly no effect and Phl and Pin APE only reached a threefold enhancement. The same effect could be observed when APEs were tested in descending concentrations (Figure 30B). Amb APE treatment with the highest concentration of 10 mg/ml caused a strong induction in IL-8 secretion which decreased concentration dependent. However, Bet APE showed no prominent enhancing effect on IL-8 release and also the effects of Phl APE were not strong enough to show a concentration dependence.

Time dependent analysis of IL-8 release shown in figure 31 revealed that IL-8 secretion started 3 h after APE stimulation to be enhanced in comparison to medium control. However, this effect was only observable for Amb APE stimulation (Figure 31A), which reached after 6 h even a significant induction of IL-8 release. Bet APE in contrast exerted far weaker effects than Amb APE and began only after 6 h of stimulation to differ from medium control in IL-8 levels (Figure 31B).

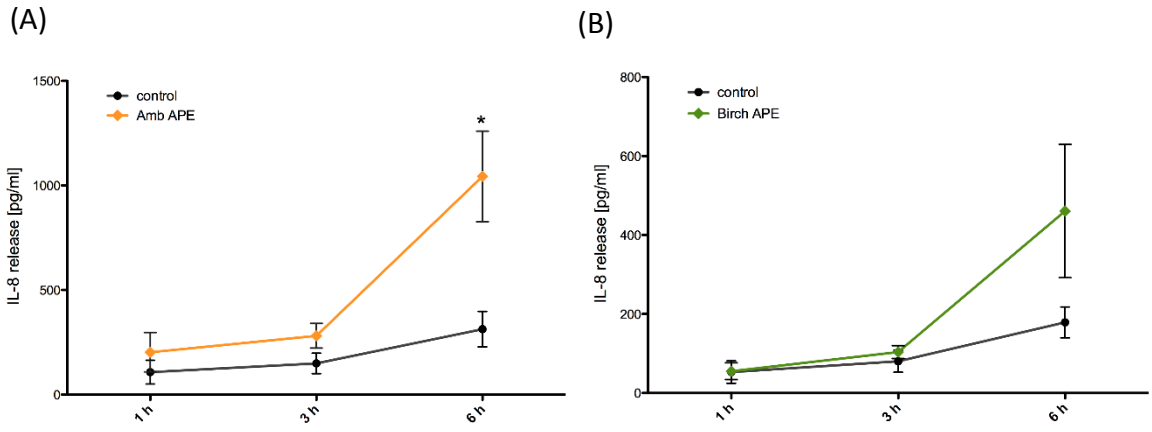


Figure 31: Time dependent effects of Amb and Bet APE on IL-8 release of keratinocytes. Human primary keratinocytes were stimulated for 1 h, 3 h and 6 h with Amb and Bet APE, respectively. IL-8 release was measured by ELISA. **(A)** IL-8 release in response to Amb APE (2.5 mg/ml) and to **(B)** Bet APE (10 mg/ml); n = 4. Data are shown as mean ± SEM in pg/ml. * $p < 0.05$, Mann-Whitney test.

To test if the observed enhancement of IL-8 release in response to APE is induced by a protein factor, keratinocytes were incubated with Amb APE samples comprising a > 3kDa fraction including proteins, a ≤ 3 kDa fraction free of protein and a heat inactivated protein free fraction (Figure 32). Compared to control all tested samples resulted in enhanced IL-8 levels, although the strongest induction could be observed in response to the heat inactivated fraction. The fractions > and ≤ 3 kDa were both less effective in provoking IL-8 release than total APE but among each other comparable in the intensity.

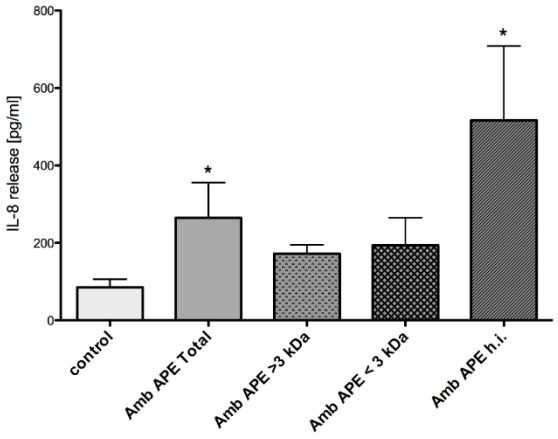


Figure 32: IL-8 release in response to Amb APE fractions. Human primary keratinocytes were stimulated for 3 h with Amb APE fractions and IL-8 release was measured by ELISA. n = 4. Data are shown as mean ± SEM in pg/ml. * $p < 0.05$ Mann-Whitney test. h.i. = heat inactivated.

Effects of APE on CCL20 release

Besides IL-8 the influence of APE on the secretion of another chemotactic cytokine, CCL20, was tested. As shown in Figure 33A keratinocytes treated 3 h with Amb and Phl APE induced a significant increase in CCL-20 release compared to unstimulated control. In contrast to Amb and Phl APE the extracts derived from birch showed only a minor tendency to induce CCL20 secretion and for Pin APE no difference to unstimulated control could be observed at all. Results of concentration dependence experiments (Figure 33B) confirmed the inducing effects for Amb APE and Phl APE. In addition, it could be shown once more that Bet APE provoked CCL20 release fell short in comparison to the release provoked by other tested APEs. For tests with protein free fractions of Amb APE a similar pattern as described before for IL-8 could be observed. Although it seems that keratinocytes stimulated with the protein containing fraction > 3kDa still have the tendency to induce CCL20, the same effect was observed for the treatment with heat inactivated Amb APE (Figure 33C).

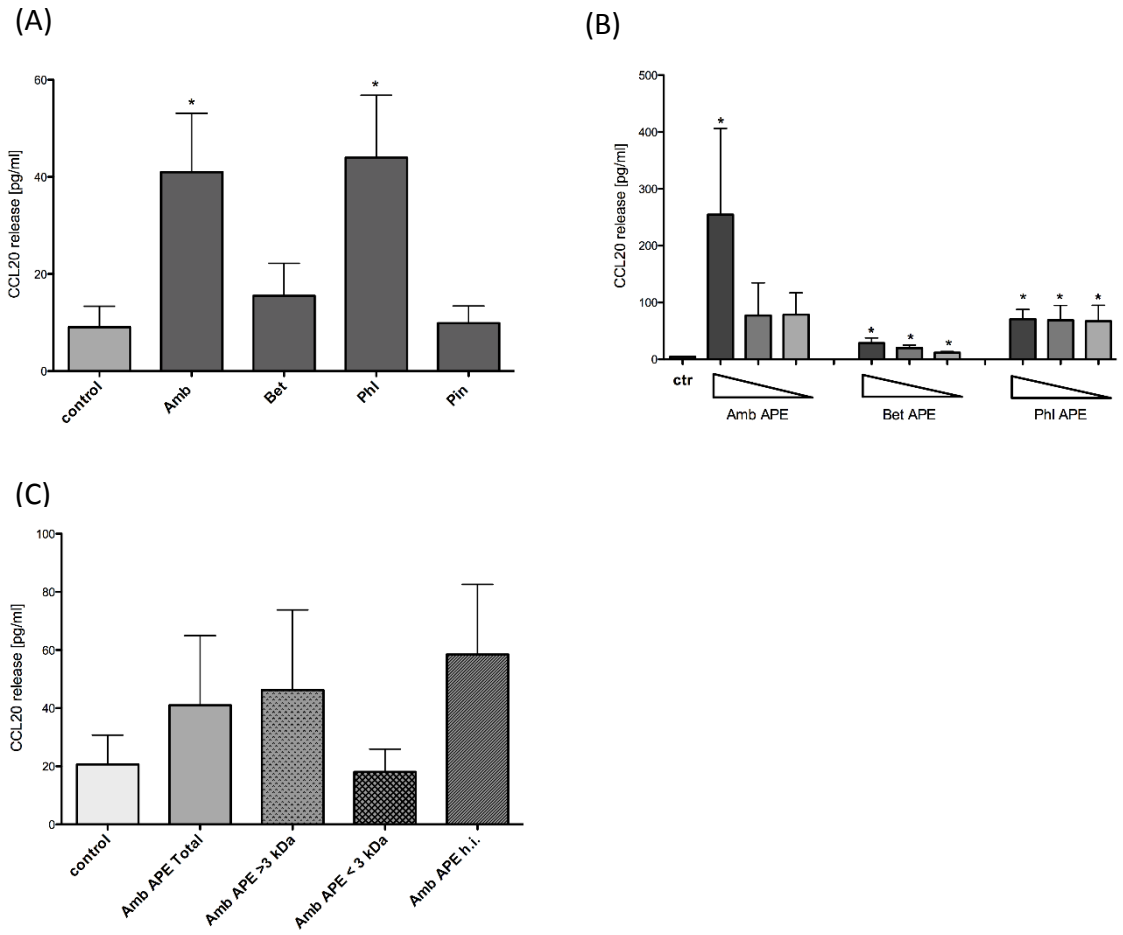


Figure 33: CCL20 release in response to APE stimulation. Human primary keratinocytes were stimulated with APE for 3 h and CCL20 release was measured by ELISA **(A)** Comparison of CCL20 release provoked by different APE (10 mg/ml); Amb, Bet, and Phl APE n = 5; Pin n = 3. **(B)** CCL20 release in response to different APEs (Amb, Bet and Phl APE) in different concentrations (10 mg/ml, 5 mg/ml and 2.5 mg/ml); n = 4. **(C)** CCL20 release in response to Amb APE fractions, n = 2. h.i. = heat inactivated. Data are shown as mean ± SEM in pg/ml. * $p < 0.05$, Mann-Whitney test.

Effects of APE on IP-10 and CCL5 release

In two pilot experiments the effect of different APE species (Amb, Bet and Phl APE) were tested regarding their potential to provoke the release of the pro-inflammatory chemokines IP-10 and CCL5 from human primary keratinocytes. As figure 34 demonstrates primarily Amb and Phl APE had an inducing effect on the IP-10 release measured 3 h after stimulation. Bet APE in contrast did not show this effect and even inhibited IP-10 secretion. Amb and Phl APE also provoked a pronounced induction of CCL5 release in the keratinocyte and even the stimulation with Bet APE led to an increase in CCL5 level, although it was lower when compared to the effect of the other APEs (Figure 34B). Data is shown from experiments with IFN-γ/TNF-α pre-stimulation since IP-10 and CCL5 is hardly produced by

keratinocytes under steady state conditions. However, the same tendencies could be observed without pre-stimulation, although effects were around 1000 x lower (data not shown).

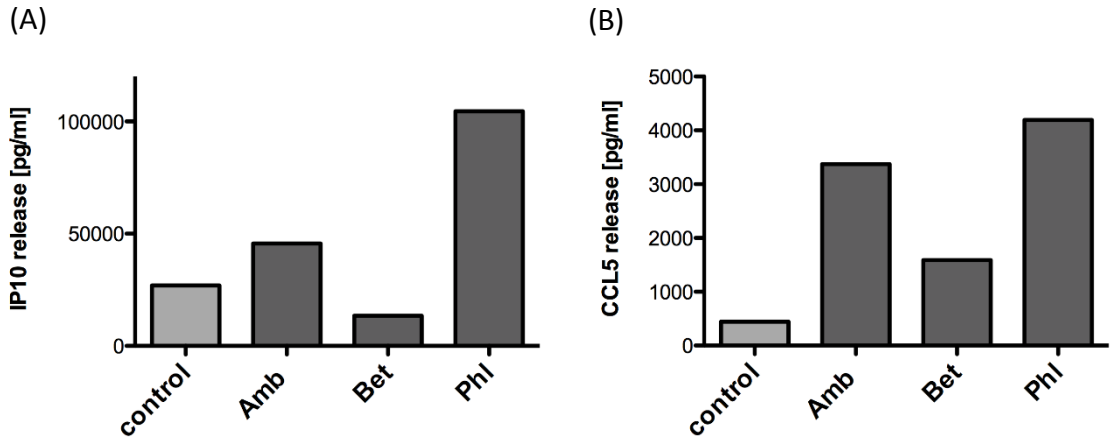


Figure 34: IP-10 and CCL5 release in response to APE stimulation. IFN-γ pre-stimulated human primary keratinocytes were incubated for 3 h with APE and **(A)** IP-10 and **(B)** CCL5 release was measured by ELISA; n = 1.

4.3.3 Effects of APE on keratinocyte mRNA expression

Besides morphology and cytokine release also the impact of APE on mRNA expression was checked for following targets. The filament-aggregating protein filaggrin, the Th2 response promoting cytokine TSLP, the protease activated receptor PAR2 and the hypoxia-inducible factor-1α (HIF-1α). Samples were taken either from 2D keratinocyte cell cultures or 3D skin models and were subsequently analysed by qPCR.

Results of filaggrin and TSLP mRNA expression in 3D skin models revealed that in case of filaggrin an upregulation of mRNA levels in response to Amb APE was observable, whereas TSLP mRNA levels did not change after 3 h of stimulation with Amb APE (Figure 35A and B). However, TSLP mRNA levels were enhanced after stimulation with Birch APE in 2D cultures of keratinocytes (Figure 35C).

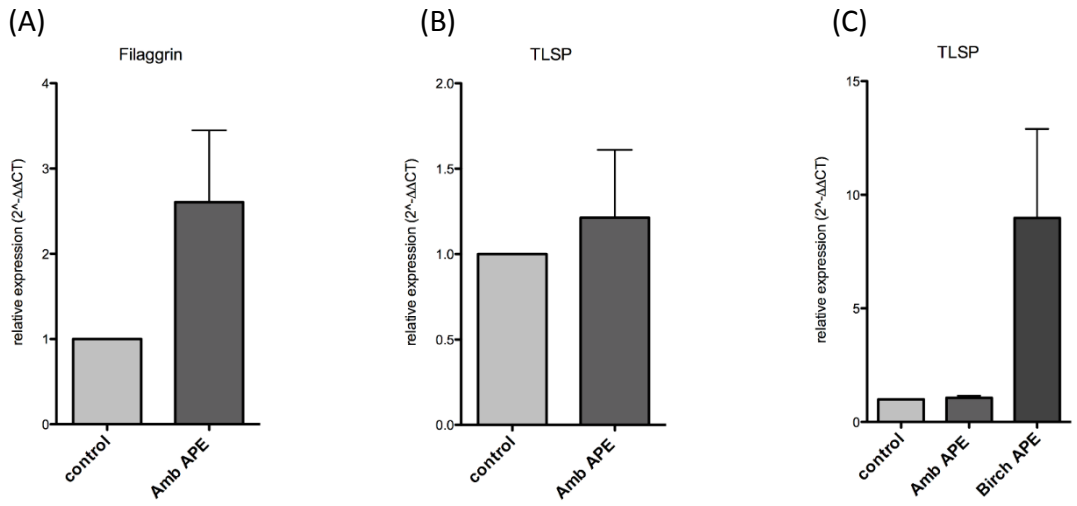


Figure 35: Influence of APE on Filaggrin and TSLP mRNA expression in human primary keratinocytes 3D skin models or 2D keratinocytes cell cultures were stimulated with 2.5 mg/ml of Amb APE for 3 h. RNA was isolated from the cells and cDNA was transcribed for qPCR analysis. (A) Filaggrin mRNA expression in 3D skin models. (B) TSLP mRNA expression in 3D skin model, n = 4. (C) TSLP mRNA expression in 2D culture, n=2. Relative gene expression was analysed by $2^{(-\Delta\Delta C_T)}$ method and results are shown as mean ± SEM.

In contrast to filaggrin and TSLP, the mRNA levels of PAR2 and HIF-1α showed in comparison to control only a very slight tendency to increase in response to Amb APE (Figure 36A and B).

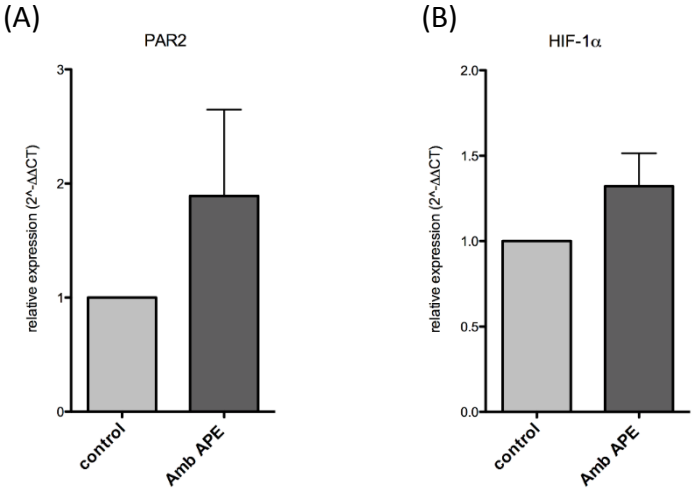


Figure 36: Influence of Amb APE on PAR2 and HIF-1α mRNA expression in human primary keratinocytes. 3D skin models or 2D keratinocytes cell cultures were stimulated with 2.5 mg/ml of Amb APE for 3 h. RNA was isolated from the cells and cDNA was transcribed for qPCR analysis. (A) PAR2 mRNA expression in 3D skin models, n = 3. (B) HIF-1α mRNA expression in 2D culture, n = 3. Relative gene expression was analysed by $2^{(-\Delta\Delta C_T)}$ method and results are shown as mean ± SEM.

4.4 Effects of APE on inflammasome effector mechanisms in keratinocytes

Inflammasome complexes are tightly associated with responses to environmental danger signals and react very fast with the activation of the pro-inflammatory cytokines IL-1β and IL-18. In the course of cell supernatant analysis for study part 4.3 it could be observed that in response to APE keratinocytes also release IL-1β. This finding led to the assumption that in human keratinocytes inflammasomes may be implicated in reactions to APE. To test this hypothesis, experiments regarding the potential of plant pollen substances to activate inflammasome mechanisms as well as experiments investigating additive effects of UV-B as a second environmental factor were conducted. To mimic physiological situation even better than in 2D cell cultures, a 3D skin model was established and used for stimulation experiments with APE as well.

4.4.1 Influence of pollen substances on IL-1β release

To proof the concept of inflammasome activation by plant pollen substances, IL-1β release in response to APE had to be confirmed. For this purpose human primary keratinocytes were stimulated either with Amb APE alone or in combination with a preceding IFN-γ/TNF-α treatment supporting the cytokine's pro-form production.

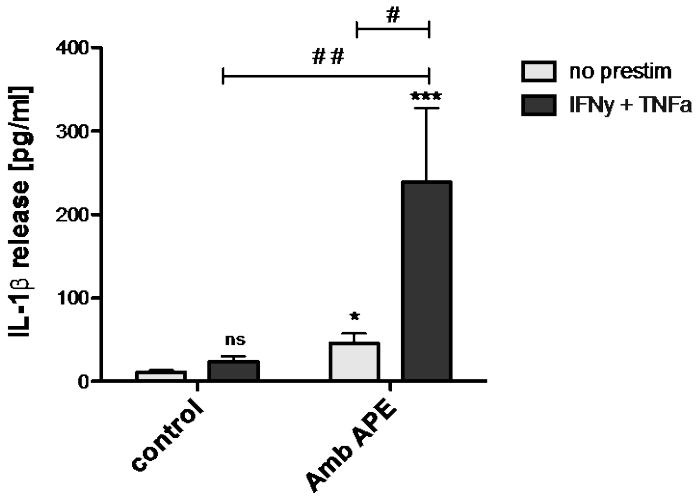


Figure 37: IFN-γ/TNF-α pre-stimulation of human primary keratinocytes enhances Amb APE provoked IL-1β release. Human primary keratinocytes were pre-stimulated with IFN-γ (300 U/ml) and TNF-α (100 ng/ml) for 18 h and subsequently incubated with Amb APE (2.5 mg/ml) for 6 h. IL-1β release was measured in cell supernatants by ELISA. Data are shown as mean ± SEM; n = 5. * and $^{\#}$ $p < 0.05$; ** and $^{\#\#}$ $p < 0.01$; *** and $^{\#\#\#}$ $p < 0.001$ for repeated measures ANOVA followed by Tukey post hoc analysis.

As shown in figure 37 ELISA data of cell supernatants revealed that IL-1β release was considerably enhanced when keratinocytes were stimulated with a combination of Amb APE and IFN-γ /TNF-α. However, in comparison to unstimulated control IL-1β release was also significantly increased in response to Amb APE alone. Therefore, pollen substances per se, although the cytokine release was more efficient in combination with IFN-γ /TNF-α pre-stimulation, are sufficient to induce IL-1β release in keratinocytes.

4.4.2 Influence of APE on inflammasome associated cytokine release

To investigate and to compare the potential of different pollen extracts to induce inflammasome associated cytokine release, human primary keratinocytes were stimulated with pollen extracts of following plants: *Ambrosia artemisiifolia* (Amb APE), *Betula pendula* (Bet APE), *Phleum pratense* (Phl APE) and *Pinus sylvestris* (Pin APE).

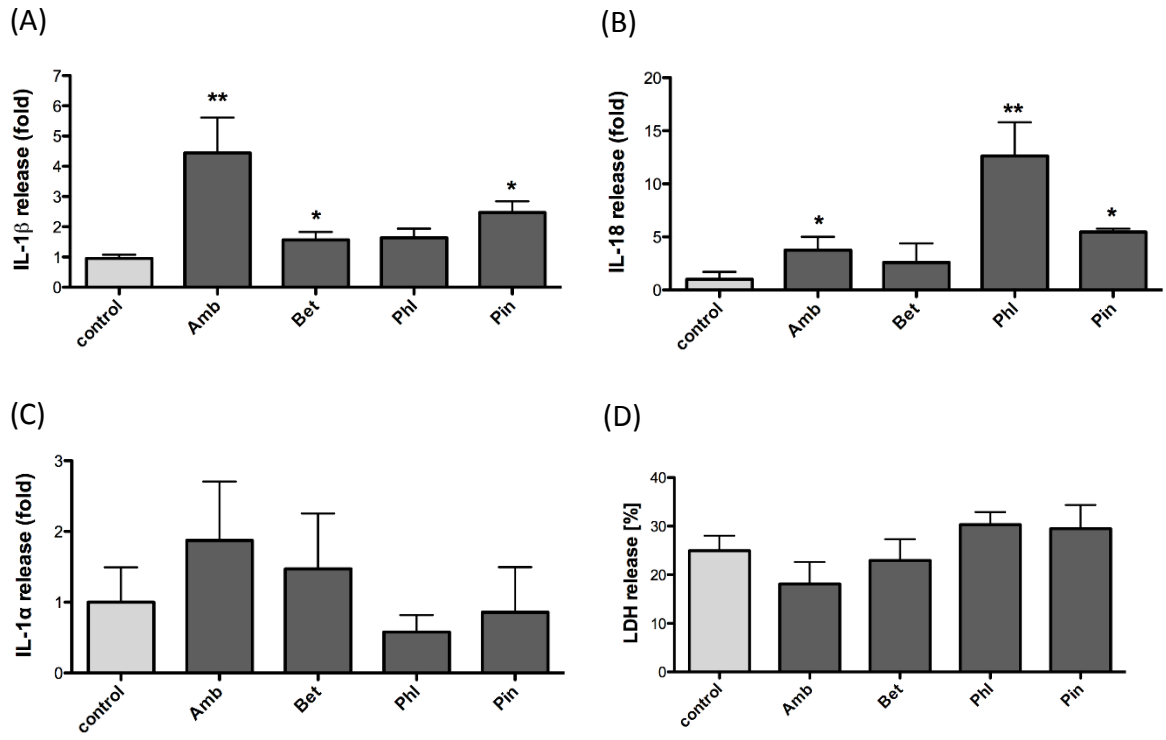


Figure 38: Effects of different APEs on cytokine release and cell death. Human primary keratinocytes were treated for 3 h with APEs of *Ambrosia artemisiifolia* (Amb), *Betula pendula* (Bet), *Phleum pratense* (Phl) or *Pinus sylvestris* (Pin) (10 mg/ml). **(A)** IL-1β **(B)** IL-18 and **(C)** IL-1α release was measured by ELISA and results were normalized to control mean values. Data represent n = 3 – 5. **(D)** Results of LDH assays shown as % of total cell death, n = 3 - 5. Data are shown as mean ± SEM with *p < 0.05; **p < 0.01; Mann Whitney test.

Results revealed that all tested extracts induced the secretion of both inflammasome hallmark cytokines, IL-1β and IL-18 (Figure 38). Comparing the different pollen extracts it could be observed that the most prominent effect for IL-1β release was achieved using the extract of A. *artemisiifolia*. Incubation with this pollen extract induced a 5.2 ± 1.3 fold increase in cytokine release in comparison to unstimulated control (Figure 38A). Although the other extracts, Bet APE, Phl APE and Pin APE showed weaker effects regarding IL-1β secretion they still induced a twofold increase. In contrast to IL-1β the release of IL-18 was most prominent in response to the extract prepared from P. *pratense* pollen (12.6 ± 3.2 fold induction) (Figure 38B). However, also the other tested pollen extracts led to enhanced IL-18 levels in the keratinocyte supernatant, e.g. in response to Amb APE IL-18 levels were increased by a factor of 3.8 ± 1.2 in comparison to control.

To see if plant pollen extracts also have an impact on IL-1α release, cell supernatants of APE treated keratinocytes were analyzed for IL-1α as well. Although lower in its fold induction than IL-1β, enhanced IL-1α secretion could be detected in the same pattern as seen for IL-1β (Figure 38C).

Since release of cytokines can also occur as a consequence of cell death, LDH assays were conducted to study whether the observed IL-1β, IL-18 and IL-1α secretion was independent of cell death. Results of this test confirmed that for cells treated with plant pollen extracts there was no significant difference in LDH release level in comparison to untreated control (Figure 38D).

Besides ELISA data also immunohistology data revealed an impact of Amb APE on inflammasome associated cytokine production. As shown in figure 39 Amb APE treatment of human primary keratinocytes led to enhanced levels of IL-18 protein.

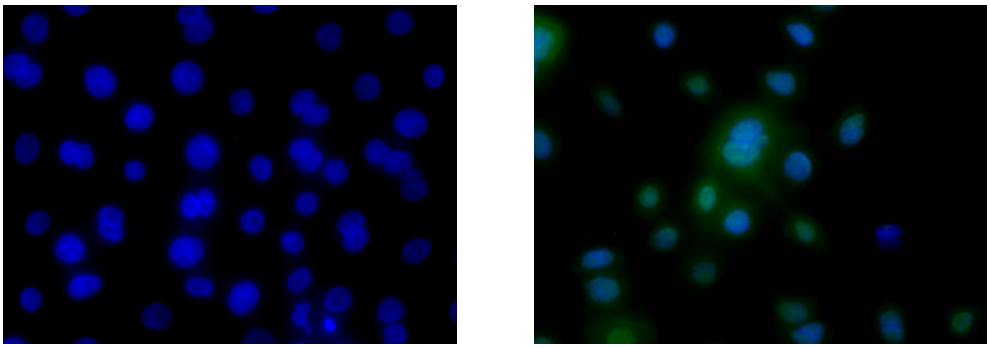


Figure 39: Amb APE stimulation induces IL-18 expression in human primary keratinocytes. Human primary keratinocytes were stimulated for 3 h with Amb APE (2.5 mg/ml) and subsequently fixed and stained with a FITC labelled antibody against human IL-18. Blue = DAPI, Green = FITC /IL-18. Left side = unstimulated control, right side = Amb APE stimulated cells.

4.4.3 Caspase-1 activation by APE

In order to be biologically active and released by the cell, IL-1β and IL-18 have to be cleaved by caspase-1, the effector protease of the inflammasome complex. Therefore, inflammasome activation in response to APE was additionally tested by Western Blot detecting the active caspase-1 subunit p20. Besides the release of IL-1β and IL-18 (Figure 38A and B) the protein level of caspase-1 p20 subunit was clearly enhanced after the stimulation with APE (Figure 40A). Furthermore, both extracts prepared from Bet APE and Amb APE increased the p20 level in the keratinocytes in the mean 1.5 times (Bet APE: 1.63 ± 0.19 and Amb APE: 1.54 ± 0.16 fold induction, n=6) (Figure 40B).

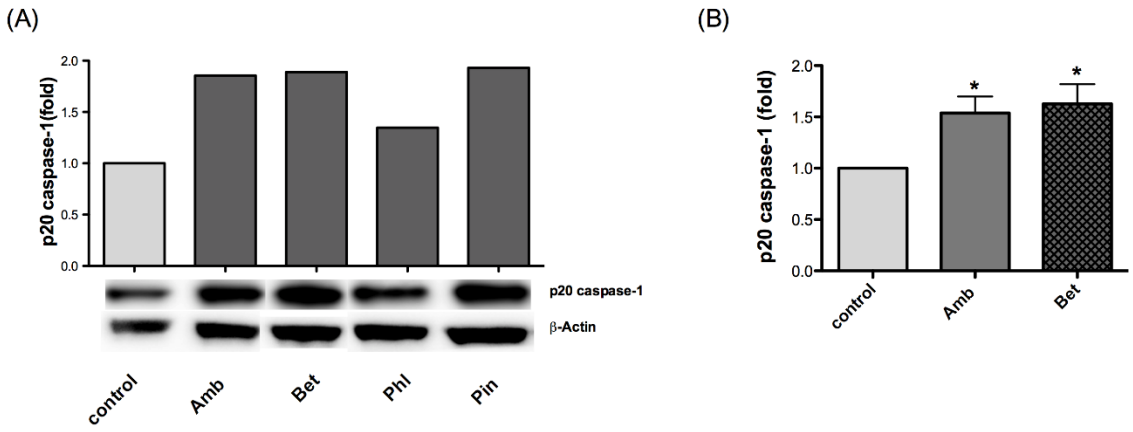


Figure 40: Aqueous pollen extract (APE) from different plant species induces inflammasome associated caspase-1 activation in keratinocytes. Human primary keratinocytes were treated for 3 h with APEs of *Ambrosia artemisiifolia* (Amb), *Betula pendula* (Bet), *Phleum pratense* (Phl) or *Pinus sylvestris* (Pin) (10 mg/ml). **(A)** Caspase-1 p20 protein levels detected in cell lysates by Western Blot; n = 1 with β-Actin as loading control **(B)** Caspase-1 p20 mean protein level in Bet and Amb APE stimulated keratinocytes after 4 h; n = 6. Data are shown as mean ± SEM with *p < 0.05; **p < 0.01; Mann Whitney test.

4.4.4 Concentration dependent effects of APE on inflammasome activation

Since immune reactions, especially to environmental factors, are often dependent on the concentration of the substance, experiments regarding the required APE concentration to provoke inflammasome associated mechanisms in keratinocytes were conducted.

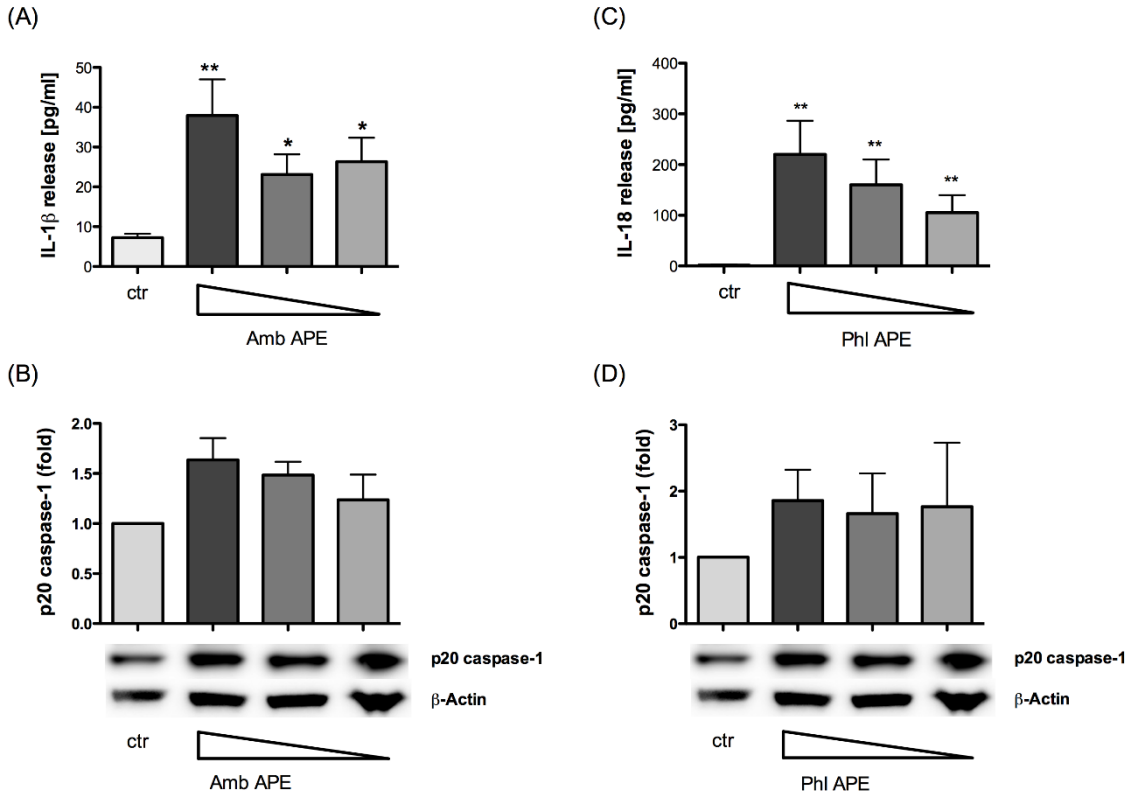


Figure 41: APE induces inflammasome activation in a concentration dependent manner. Human primary keratinocytes were stimulated with Amb or Phl APE in different concentrations. Analysis of caspase-1 activation was performed by Western Blot detecting caspase-1 p20 unit. Cytokine release was measured by ELISA. **(A)** IL-1β release (n = 5) and **(B)** caspase-1 p20 level (n = 2) after 3 h of Amb APE stimulation (10, 2.5 and 1.25 mg/ml. **(C)** IL-18 release (n = 5) and **(D)** caspase-1 p20 level (n = 2) after 3 h of Phl APE stimulation (10, 5 and 2.5 mg/ml). Data are shown as mean ± SEM. Western Blot data are normalized to β-actin loading control and ELISA data are shown in pg/ml. *p < 0.05; **p < 0.01; Mann Whitney test.

With Amb APE and Phl APE being the most effective extracts in inducing IL-1β and IL-18 release from human keratinocytes they were also used for further experiments in different concentrations. As shown in figure 41A Amb APE provoked the strongest response regarding IL-1β secretion when the highest concentration with 10 mg/ml was applied. Dose response related dilution of the pollen extract led to a decrease in the IL-1β level, although the two lowest concentrations 2.5 and 1.25 mg/ml still doubled the IL-1β release in comparison to untreated control. Besides the cytokine release, the concentration graded

Amb APE showed equivalent effects on the activation of caspase-1. Western Blots for the caspase-1 p20 subunit illustrate a concentration dependent activation and demonstrate that for APE concentrations as low as 1.25 mg/ml effects on inflammasome-associated proteins was still observable (Figure 41B). In line with the results for Amb APE also extracts prepared from P. *pratense* showed a strong concentration dependence regarding the IL-18 release (Figure 41C). However, the lowest APE concentration was still able to induce a significantly higher IL-18 secretion than measured in the unstimulated control. The same was true for the activation of caspase-1 by Phl APE (Figure 41D). Results of Western Blot analysis for the p20 subunit showed for all used concentrations an enhanced protein level, indicating that Phl APE can activate caspase-1 even at a low concentration.

4.4.5 Induction of IL-1β and IL-18 release by APE protein components

To identify the nature of the active components in aqueous plant pollen extracts causing the release of IL-1β and IL-18, Amb APE was fractionized in protein (≥ 3 kDa) and non-protein containing (< 3 kDa) fractions by filter centrifugation. In addition to these fractions, heat inactivated Amb APE was tested to study its effects on human primary keratinocytes.

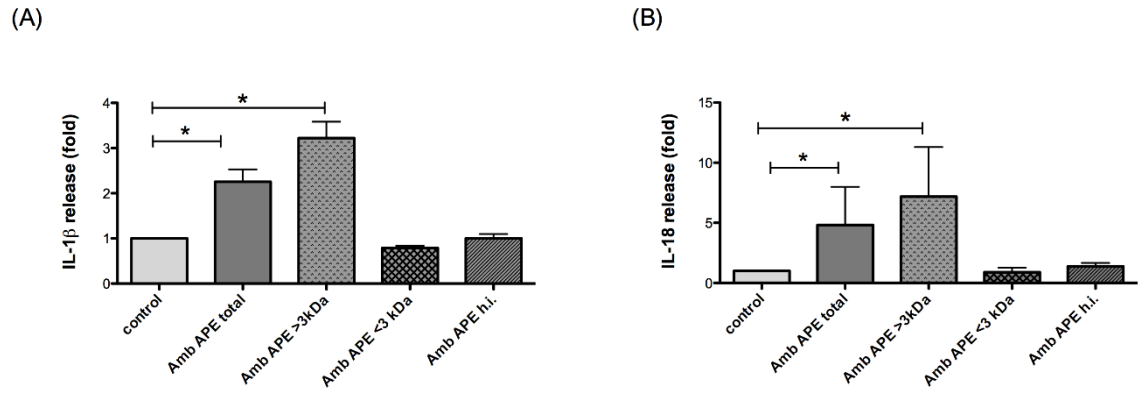


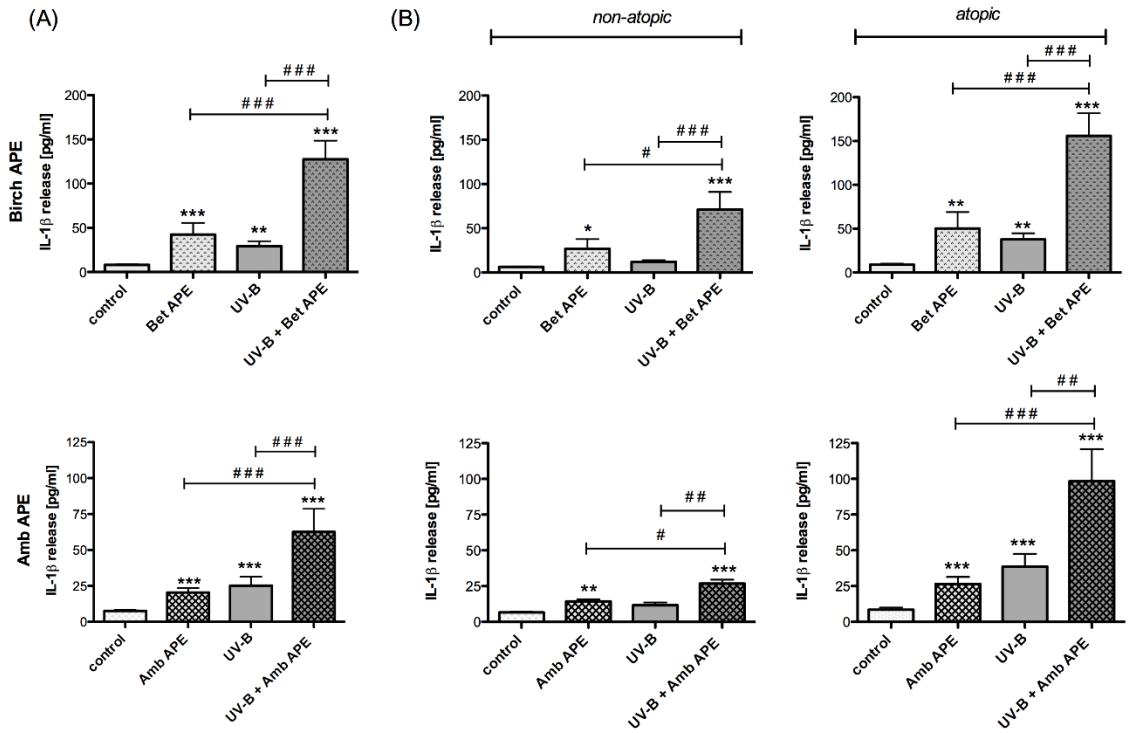
Figure 42: Protein components of APE are responsible for the induction of IL-1β and IL-18 release in keratinocytes. Human primary keratinocytes were stimulated for 4 h with protein containing fraction (>3kDa), non-protein fraction (<3kDa) and heat inactivated samples of Amb APE. **(A)** IL-1β and **(B)** IL-18 release was measured by ELISA. Data are shown as mean ± SEM; n = 4. *p < 0.05; Mann Whitney test.

ELISA data from these experiments revealed that regarding the IL-1β cytokine release only the protein containing fraction could reach the same effect as the total pollen extract (Figure 42A). Keratinocytes treated with the non-protein containing filtrate as well as cells treated with heat inactivated APE did not release more cytokines than unstimulated control

cells. The same effects were observed for the IL-18 release shown in figure 42B. As seen for IL-1β only the protein containing extracts provoked an IL-18 cytokine release suggesting a protein dependent mechanism for the inflammasome activation by pollen extracts. Cell death independence of these results was confirmed by LDH-Assay (data not shown).

4.4.6 Additive enhancement of IL-1β and IL-18 release by APE and UV-B

One of the best known inflammasome activators in human keratinocytes is UV-B irradiation. To test the strength of APE as an inflammasome activator in human primary keratinocytes and to compare its effects with the effects of UV-B light, experiments with both environmental factors were conducted. Since UV-B and contact to plant pollen is likely to occur at the same time during outdoor stays, also a combined stimulation of UV-B and APE was included in the test. For this purpose human primary keratinocytes were treated either with APE and UV-B alone or in combination. Cells treated with the combination were first irradiated with UV-B and directly afterwards stimulated with APE.



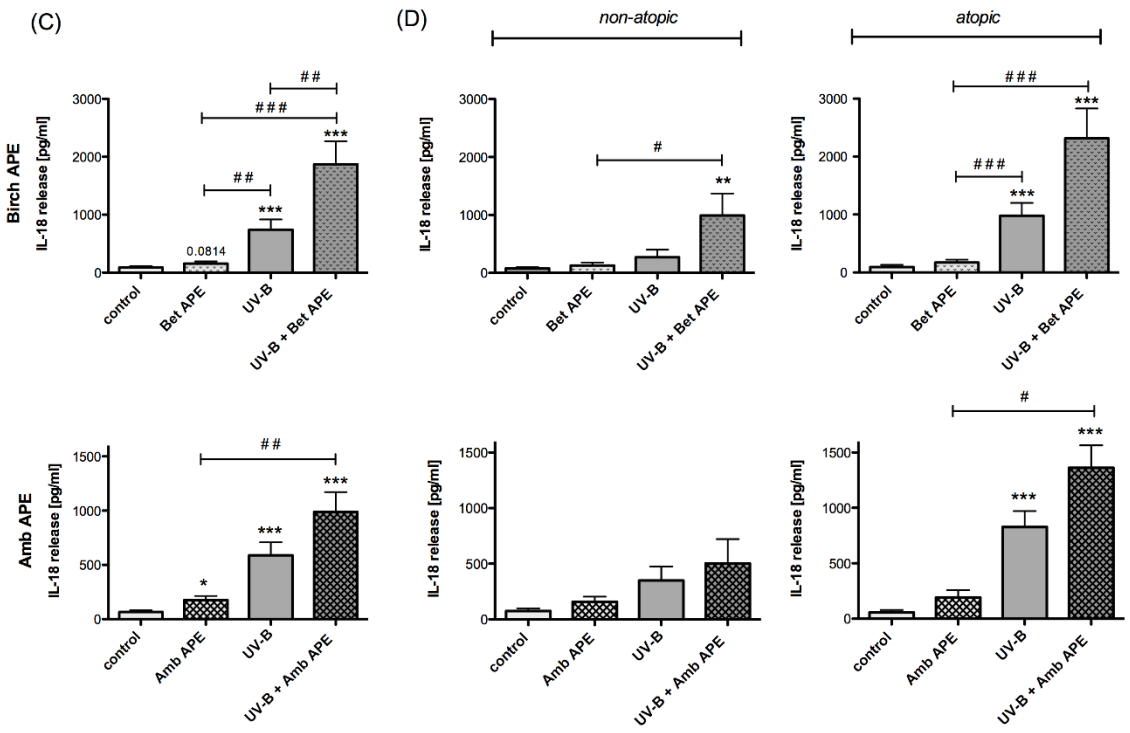


Figure 43: **APE and UV-B enhance the secretion of inflammasome associated cytokines in an additive manner and dependent on the donor's atopy status.** Human primary keratinocytes were treated for 4 h either with Amb or Bet APE (2.5 mg/ml and 10 mg/ml, respectively) alone or in combination with UV-B exposure (90 mJ/cm^2). Cytokine release was measured by ELISA and caspase-1 p20 unit was detected by Western Blot. **(A)** IL-1β release provoked by Bet APE (upper panel) and Amb APE (lower panel) and UV-B in all donors. **(B)** IL-1β release separated according to keratinocyte donor's atopy status: non-atopic (left) and atopic donors (right). **(C)** IL-18 release provoked by APE and UV-B in all donors, Bet APE upper panel and Amb APE lower panel **(D)** IL-18 release separated according to keratinocyte donor's atopy status: non-atopic (left) and atopic donors (right).
Data are shown as mean ± SEM. For Bet APE: A) – (D) n = 15 (n = 5 non atopic, n = 10 atopic). For Amb APE: (A) – (D) n = 10 (n = 5 non atopic, n = 5 atopic). * and $^{\#}$ $p < 0.05$; ** and $^{\#\#}$ $p < 0.01$; *** and $^{\#\#\#}$ $p < 0.001$ for one-way ANOVA followed by Tukey post hoc analysis.

Figure 43A illustrates that IL-1β release provoked by Bet APE (upper panel) as well as Amb APE (lower panel) reached levels comparable to the levels resulting after UV-B irradiation. Furthermore, the data show for the first time that a combination of APE and UV-B provoked a significant higher IL-1β release than one of the single factors induced. This was true for Bet APE (Bet APE: 42.45 ± 13.05 pg/ml, UV-B: 29.19 ± 5.67 pg/ml, UV-B/Bet APE: 127.65 ± 20.93 pg/ml) as well as for Amb APE (Amb APE: 20.38 ± 3.17 pg/ml, UV-B: 25.14 ± 6.23 pg/ml versus UV-B/AmbAPE: 62.60 ± 15.98 pg/ml). In addition, the difference between cells from pollen allergic donors and cells from donors without allergic diseases was studied. As demonstrated in Figure 43B both groups, non-atopic and atopic, showed in response to Bet (upper panel) and Amb APE (lower panel) significantly enhanced levels of IL-1β in the cell

supernatant in comparison to untreated controls. Furthermore, the additive effect with UV-B was maintained in cells of non-atopic donors as well as in cells of atopic donors. However, in most cases, cells from atopic donors showed higher IL-1β levels in the supernatant. In detail APE and UV-B tended to induce also in single use higher IL-1β release in atopic donors than in non-atopic donors. In addition, the combination of APE and UV-B resulted for Bet APE in a twofold and for Amb APE in a nearly three times higher IL-1β release in atopic donors than in non-atopic donors (Bet APE: 71.26 ± 19.82 pg/ml non-atopic versus 155.83 ± 27.28 pg/ml atopic; Amb APE: 26.8 ± 2.7 pg/ml non-atopic versus 98.4 ± 22.4 pg/ml atopic, $p < 0.001$) (Figure 43B). Analysis of the IL-18 levels in cell supernatants showed similar results as observed for IL-1β (Figure 43C). Both APE and UV-B induced significant IL-18 release in single use and had in combination with UV-B a strong additive effect (Bet APE: 156.46 ± 37.96 pg/ml, UV-B: 742.129 ± 176.05, UV-B/Bet APE: 1872.45 ± 396.16 ; Amb APE: 174.67 ± 38.90 pg/ml, UV-B: 588.52 ± 120.05 pg/ml versus UV-B/APE: 988.43 ± 182.28 pg/ml). However, in contrast to the results for IL-1β, UV-B was more effective in inducing IL-18 cytokine release than Bet or Amb APE in single use (Figure 43C). This effect was also observed when the experiments of atopic and non-atopic donor's cells were considered separately (Figure 43D). In addition, the separate analysis for IL-18 illustrates that cells from atopic donors may be more sensible to environmental factors than non-atopic cells. Besides releasing higher IL-18 levels in response to APE or UV-B, atopic cells showed a significant additive effect when compared to non-atopic cells.

4.4.7 Additive enhancement of caspase-1 activation by APE and UV-B

Regarding the activation of caspase-1, Amb APE and UV-B showed influences in single use as well as in combination (Figure 44).

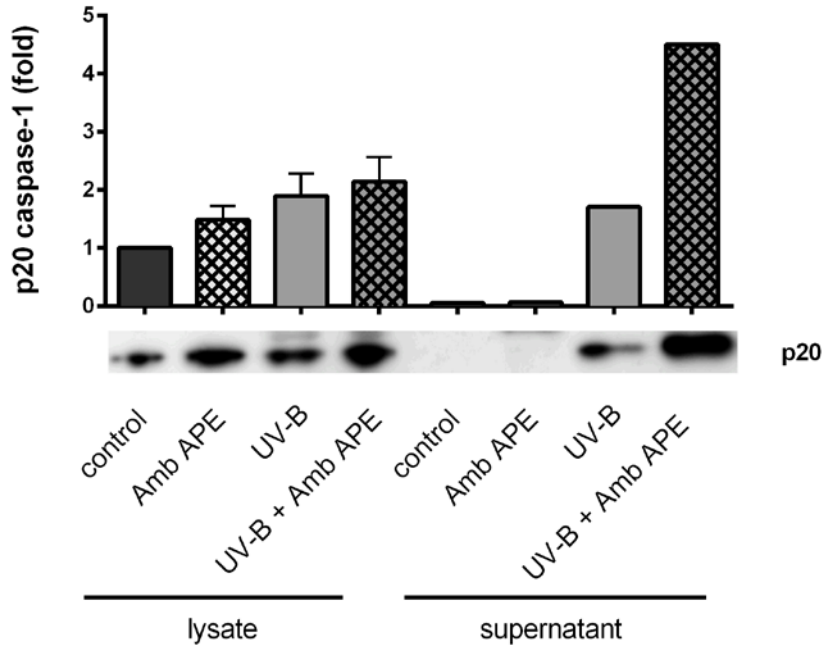


Figure 44: APE and UV-B enhance the intra- and extracellular level of active caspase-1. Caspase-1 p20 protein level in cell lysates (n = 4) and supernatants (n = 1) after treatment with Amb APE and UV-B.

Western Blot analysis demonstrated that Amb APE and UV-B alone induced the caspase-1 p20 subunit in comparable levels (Amb APE: 1.50 ± 0.25 versus UV-B: 1.75 ± 0.34 fold induction in comparison to control). In combination Amb APE and UV-B irradiation were even more effective than the single factor stimulation (2.01 ± 0.44 fold induction in comparison to control) (Figure 44). Since it is known that after its activation caspase-1 can also be released by the cells, Western blot analysis of keratinocyte supernatants was conducted (Figure 44). High protease content in the pollen extract made it difficult to obtain enough intact protein for a clear analysis. Nevertheless, in the case of UV-B alone and especially for the combination with Amb APE a strong p20 unit release occurred so that detection was possible. Results of this analysis showed that in comparison to control as well as in comparison to the single factors the release of p20 was enhanced when keratinocytes were treated with UV-B and Amb APE in parallel. This demonstrated again the additive effect of these two factors and emphasizes their potential regarding inflammasome activation in human primary keratinocytes.

4.4.8 Concentration dependent effects of APE and UV-B

Besides the general additive effects of UV-B described in preceding passages, a concentration dependent effect regarding IL-1β and IL-18 cytokine release as well as for caspase-1 activation could be demonstrated in combination with Bet APE (Figure 45). Using descending concentrations of Bet APE the IL-1β secretion (Figure 45A) and IL-18 (Figure 45B) from keratinocytes decreased steadily, whereas UV-B radiation contributed to an enhanced and more prominent effect. The same could be observed in Western Blot for caspase-1 activation showing increased levels of p20 unit especially for the combination of Bet APE with UV-B (Figure 45C).

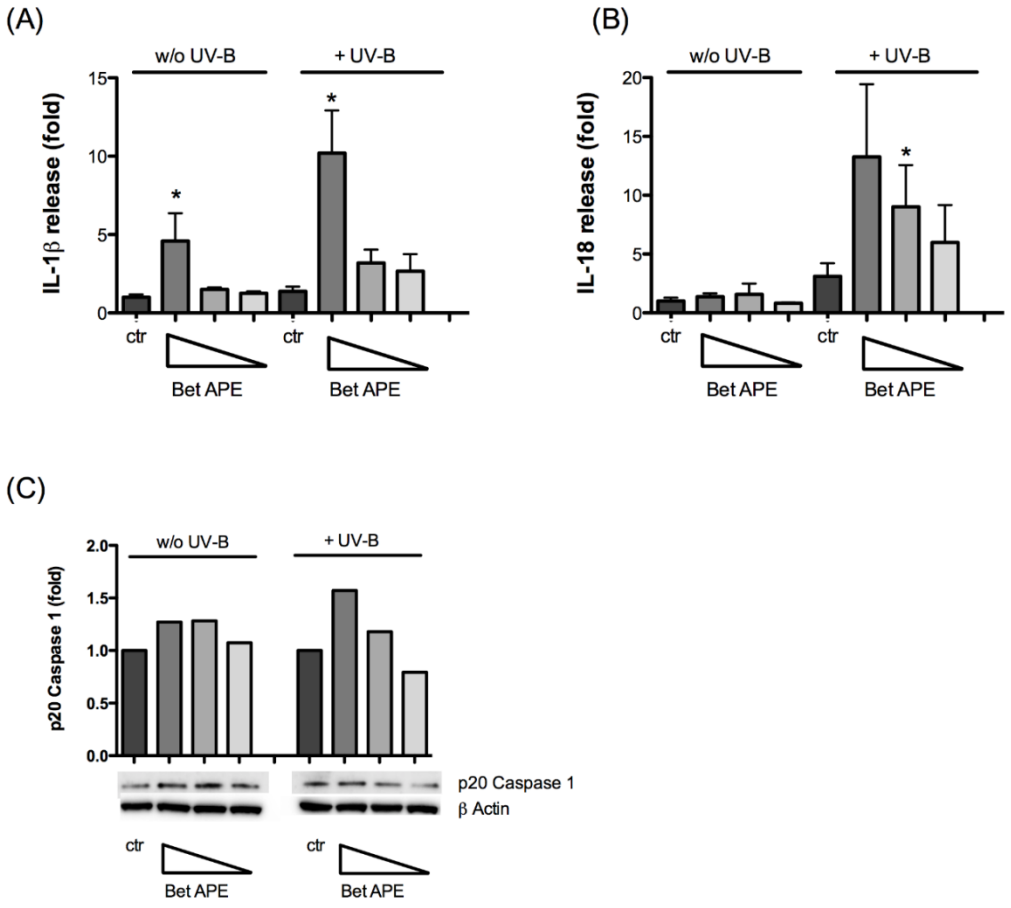


Figure 45: APE and UV-B enhance concentration dependent inflammasome associated cytokine release and caspase-1 activation. Human primary keratinocytes were stimulated with Bet APE in different concentrations and in combination with UV-B (90 mJ/cm^2). Analysis of caspase-1 activation was performed by Western Blot detecting caspase-1 p20 unit. Cytokine release was measured by ELISA. **(A)** IL-1β release, **(B)** IL-18 release (n = 5) and **(C)** caspase-1 p20 level (n = 1) after 3 h of Bet APE stimulation (10, 5 and 2.5 mg/ml). Data are shown as mean ± SEM. Western Blot data are normalized to β-actin loading control and ELISA data are shown as fold induction in comparison to control. *p < 0.05; **p < 0.01; Mann Whitney test. ctr = control.

4.4.9 Effects of APE on mRNA expression in 3D skin models

Influences of pollen extracts were not only tested in 2D culture system but also in 3D skin models mimicking the composition of the natural two layered system of the skin with a dermal and epidermal part. 3D cultures were checked for their expression of differentiation markers to guarantee a successful establishment of the model. Stainings for Involucrin (PE) and Keratin 14 (FITC) showed the typical distribution of Involucrin expressing cells in the upper epidermal part containing differentiated cells and Keratin 14 expression in the lower part representing basal keratinocytes (Figure 46).

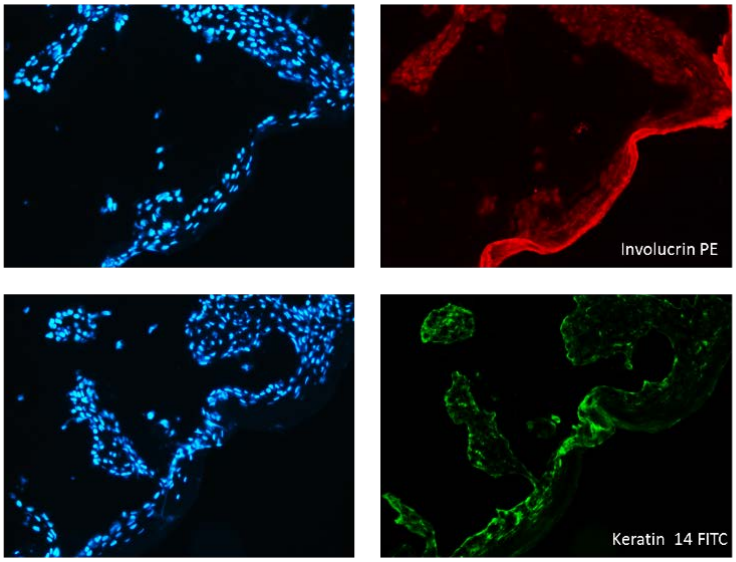


Figure 46: Expression of K14 and Involucrin in the established 3D skin model. Immunehistological staining was performed on 8 µm sections of a 3D skin model. K14 = green/FITC; Involucrin = red/PE and DAPI = blue.

For testing the impact of APE on the mRNA expression of inflammasome associated genes, Amb pollen extract was applied for 3 h on the top of fully differentiated 3D skin models. Afterwards RNA was isolated, transcribed into cDNA and qPCRs were conducted with primers for caspase-1, IL-1β, IL-18, AIM-2 and NLRP3. qPCR data showed for both cytokine pro-forms IL1-β (2.46 ± 0.8 fold induction) and IL-18 (2.03 ± 0.39 fold induction) an induction of mRNA expression after APE stimulation (Figure 47B and C). Furthermore, caspase-1 expression tended to be increased in cells of APE treated 3D skin models (Figure 47A). Concerning AIM-2 inflammasome a twofold induction of mRNA expression could be observed and also for NRLP3 a tendency to enhanced mRNA levels was observable (Figure 47D and E).

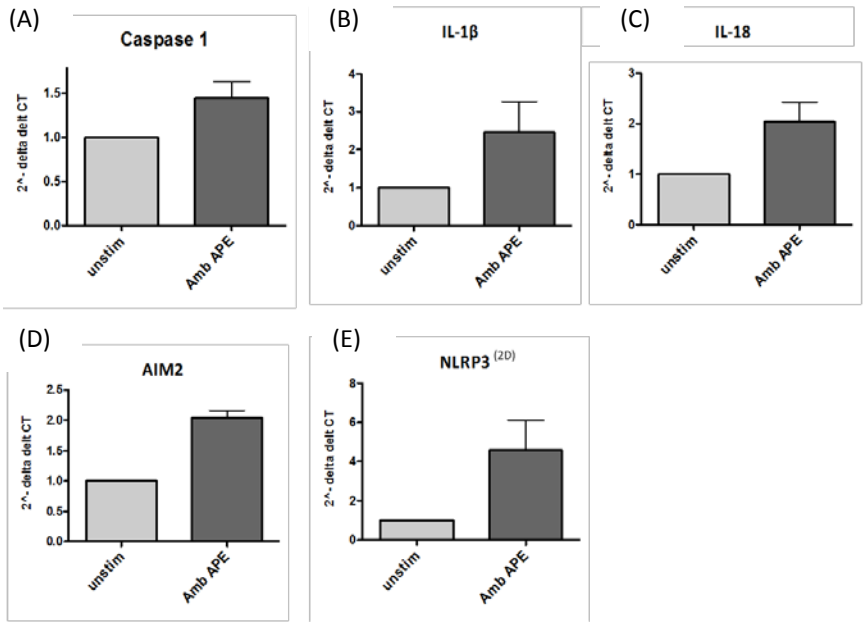


Figure 47: Amb APE influences the mRNA expression of inflammasome-associated genes. qPCR was conducted using samples of Amb APE stimulated 3D models and 2D cultures of keratinocytes. Relative gene expression was analysed by $2^{(-\Delta\Delta C_T)}$ method in reference to house-keeping gene EF1α. Relative gene expression is shown for **(A)** Caspase-1 **(B)** IL-1β pro-form **(C)** IL-18 pro-form **(D)** AIM2 and **(E)** NRLP3. Results are shown as mean ± SEM, n = 3.

4.4.10 Inhibition of caspase-1 function

Inhibition of caspase-1 as further proof for an inflammasome dependent APE response, was tested using the caspase inhibitor Ac-YVAD-cmk as well as siRNA knock down technique. Both treatments should lead in the end to inhibited release of the key cytokines IL-1β and IL-18 due to missing activation by caspase-1.

Due to time reasons only preliminary data could be obtained of these experiments giving first hints but also demanding further investigations. As shown in figure 48A and B caspase-1 inhibitor exerts effects on UV-B as well as UV-B + Bet APE treated cells by down-regulating IL-1β and IL-18 release. However, effects without UV-B treatment are missing so far and should be part of future experiments.

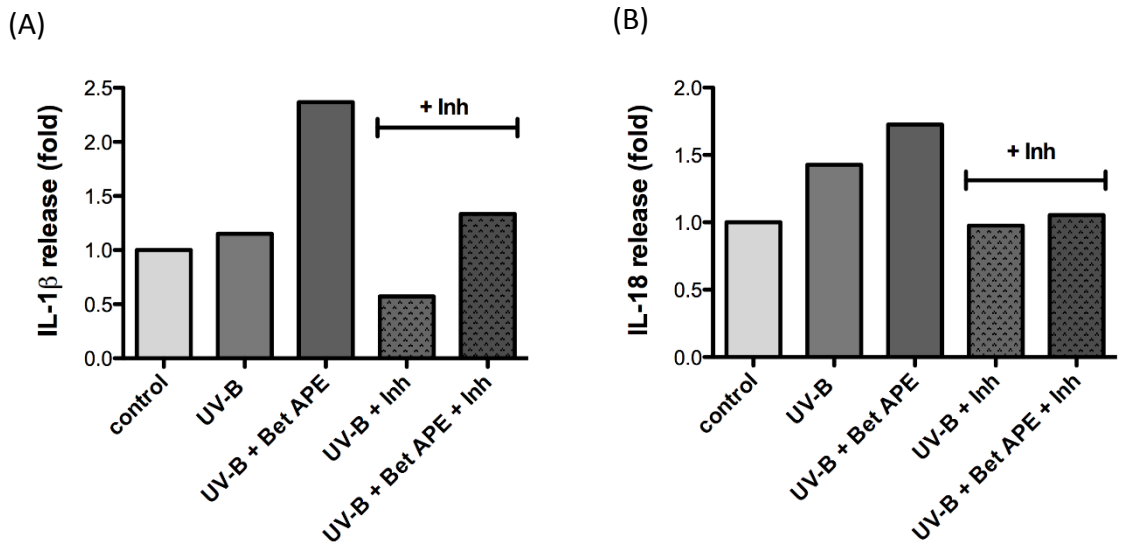


Figure 48: Caspase-1 inhibitor suppresses the inflammasome associated cytokine release after Bet APE and UV-B stimulation. Human primary keratinocytes were pre-treated for 45 min with caspase-1 inhibitor Ac-YVAD-cmk (100 µM) and subsequently stimulated with Bet APE in different concentrations and in combination with UV-B (90 mJ/cm^2). Cytokine release was measured by ELISA **(A**) IL-1β and **(B)** IL-18 release; n = 1.

siRNA experiments with the aim to knock down caspase-1 as well as AIM-2 mRNA translation in human primary keratinocytes were controlled by qPCRs. Figure 49 showing relative mRNA expression illustrates that knock down of caspase-1 as well as of AIM2 was successfully achieved.

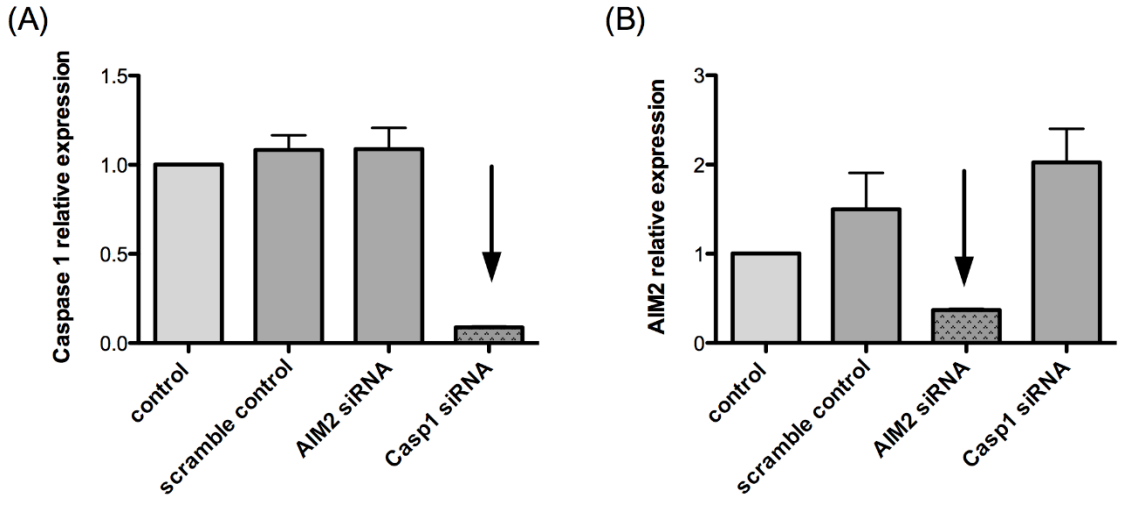


Figure 49: Inhibition of caspase-1 and AIM-2 expression by siRNA. Human primary keratinocytes were transfected with capsase-1 and AIM-2 target siRNA. Successful knock-down was checked by qPCR for corresponding mRNA. Relative expression of **(A)** capsase-1 and **(B)** AIM2 in transfected cells.

Samples treated with specific siRNA showed a clearly down-regulated expression of the target mRNA in comparison to control. However, first experiments with siRNA knock down followed by APE stimuulation did not show the expected down-regulation of IL-1β and IL-18 release in response to missing caspase-1 expression (data not shown). Therefore, further tests are necessary to solve experimental problems and for being able to draw a clear conclusion from the results.

4.4.11 Osmolarity Test

The possibility that APE induces osmotic stress triggering inflammasome activation, was ruled out by testing the change of osmolarity in the medium after adding the pollen extract. Results of this test showed that all pollen extracts used for the study did not provoke any changes in osmolarity (Figure 50).

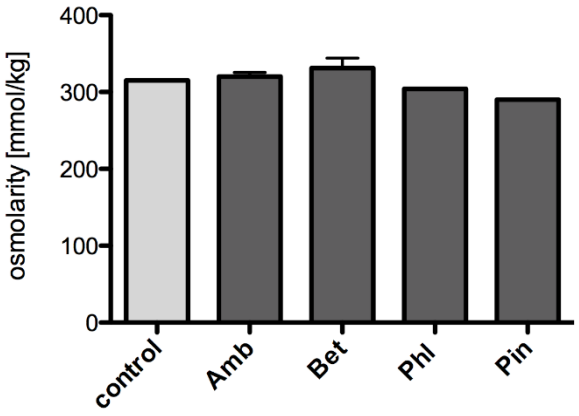


Figure 50: Addition of APE does not provoke changes in medium osmolarity. APEs (Amb, Bet, Phl and Pin) were mixed with DermaLife basal medium (c_{end} = 10 mg/ml) and changes in osmolarity compared to pure DermaLife basal medium were measured using osmometer technique. Data are shown in mmol/kg ± SEM and represent duplicate measurements of n different APEs mixes and control medium. Amb APE n = 5; Bet APE n = 4; Phl and Pin n = 1.

5 Discussion

Keratinocytes form a complex barrier which shields the human body from environmental insults and guarantees a balanced exchange of the skin with the outer world. In consequence keratinocytes receive important first-hand information which, however, has to be transmitted further on to other cells to ensure adequate responses. For instance immune reactions to pathogens or other external danger signals have to be orchestrated with immunce cells to protect the body from harm. Since T cells are known to be essential for the outcome of an immune reaction the crosstalk between keratinocytes and this cell type is of special interest. In contrast to former studies focusing mainly on the effects T cells can have on keratinocytes, the presented project investigated how keratinocytes can affect T cell effector functions. More precisely it was studied if soluble mediators derived from keratinocytes influence the plasticity of $CD4^+$ T cells and therefore may have the potential to skew immune responses in certain directions. Furthermore, effects of environmental factors, such as plant pollen and UV-B radiation, on keratinocytes in general and on their mediator production have been studied. This included also tests regarding the potential of plant pollen to activate the inflammasome in human primary keratinocytes. While experimental results have already been shown in detail in preceding passages the following discussion chapter provides interpretation and principal implication of the collected data in context to the current state of scientific knowledge.

5.1 Optimization of medium conditions for co-culture experiments

For in vitro culture the cell medium is the most important component of the culture environment, because it provides the necessary nutrients, growth factors and hormones for cell growth, and additionally regulates the pH and the osmotic pressure of the system (Yang and Xiong, 2012). Therefore, the culture medium has to be thoroughly adapted to each cell type and is an essential factor which has to be considered especially for co-culture experiments using different cells types.

In the current study co-culture experiments with the need to combine cell culture conditions for human primary keratinocytes and T cells were conducted. Since keratinocytes and T cells differ in their differentiation and growth behaviour they also have specific nutritional requirements. For instance, standard keratinocyte media provide

optimal conditions for adherent cells and support growth and proliferation in a well-adjusted Ca^{2+} concentration and pH range, especially when supplemented with factors like epidermal growth factor, insulin, hydrocortisone, transferrin and bovine pituitary extract (compare Table 19). T cell media, in contrast, are primarily designed to support the growth of cells in suspension and provide cell type optimized concentration of amino acids, vitamins, inorganic salts, and carbon sources (Yang and Xiong, 2012). To maintain both cell types during in vitro culture close to the physiological state from which they were isolated, several tests with different media were carried out in this study. Afterwards the resulting data were analysed to identify an appropriate medium for the generation of keratinocyte derived mediators and for their subsequent use in T cell cultures.

To exclude effects from supplemental factors in advance, only basal- and serum-free media were used in the experiments. Results revealed that both tested standard keratinocyte media (DermaLife and KGM 2) were not suitable for culturing T cells, since they changed the cytokine profile of the tested T cell subsets with a dominating shift to IL-22 production even in low concentration. Hence, it seems to be likely that the keratinocyte media contain factors influencing T cell differentiation and cytokine release, even in their basal formula. One candidate for this observed phenomenon might be the amino acid tryptophan whose metabolites e.g. tryptamine, are known inductors of the aryl hydrocarbon receptor (AHR) and therefore able to impact IL-22 as downstream product (Zelante *et al.*, 2014).

Since the results disqualified standard keratinocyte media for T cell culture, more testing became necessary to find alternative options and to gain further insights in the complexity of media influences on cell culture. For instance, it could be demonstrated that serum free AIM-V medium which was developed and optimized for culturing blood cells, is in its formulation not beneficial for keratinocyte growth. The observed cell death of keratinocytes in response to AIM-V medium might be due to an undersupply of necessary nutrition factors or due to high concentrations of inappropriate ingredients. In addition, M119, a medium very rich in vitamins and amino acids (Yang and Xiong, 2012), was shown to support cell death and to induce the expression of K14 indicating retention of the keratinocytes in their basal state. Furthermore, M199, just as RPMI and IMDM media which were initially designed to support the growth of lymphoid cells, induced high TNF-α release from keratinocytes under inflammatory conditions. Since TNF-α is released in response to danger signals (e.g. UV-B) and can also promote cell apoptosis (Bashir *et al.*, 2009; Yarosh

et al., 2000), the induction of this cytokine can be seen as a negative effect and therefore as a counterargument regarding the cytokine inducing media. Besides the effect on TNF-α release RPMI also impacted keratinocytes negatively in their expression of the differentiation marker involucrin. This might be an effect caused by the lack of $CaCl_2$ which is essential for keratinocyte differentiation but missing in RPMI basal medium (Yang and Xiong, 2012).

Besides the effects of cell culture media on keratinocytes, the influences of media on T cells were also tested. Crucial for the validation of the tested media was the impact on the T cell cytokine release because it is a hallmark effector function of T cells and also lineage defining (Eyerich and Zielinski, 2014). In general the data demonstrated that only DMEM did not dramatically influence the T cells in their cytokine release. Therefore, DMEM was chosen as medium to generate keratinocyte derived mediators. Nevertheless, it has to be considered for future studies that F12 was also relatively positively ranked, as presented in the summary Table 27. Hence a mixture of DMEM/F12 would also be thinkable and could be a good candidate for keratinocyte / T cell co-culture systems.

5.2 Influence of keratinocyte derived mediators on T cell effector functions

5.2.1 Influence of keratinocyte derived mediators on T cell proliferation

Besides danger signal recognition and initiation of cell activation, a rapid expansion of specific immune cells is another crucial factor for an efficient immune response. In the case of T cells, particularly for the naïve type, triggering of the TCR together with the associated CD3 and the costimulatory molecule CD28 is an important signal for inducing proliferation. However, T cells can also respond TCR independent to other factors and in consequence enhance their proliferation rate. For instance, cytokines like IL-2, IL-4, IL-7 and IL-15 have been shown to support T cell expansion (Geginat *et al.*, 2003). In addition, the signal protein Notch has been identified to be necessary for TCR-mediated activation of T cells and to regulate cell proliferation (Palaga *et al.*, 2003). Furthermore, negative regulation is possible as well and was demonstrated for instance for macrophage or DC derived indoleamine 2,3-dioxygenase which inhibits T cell proliferation (Hwu *et al.*, 2000). Thus, factors from other cells are able to influence T cell proliferation and by this impact indirectly immune responses.

To test if keratinocyte derived mediators also have an influence on the proliferation of $CD4^+$ T cell subsets, experiments were conducted that combined a αCD3/αCD28 based restimulation of differentiated T cells with an incubation in the presence of keratinocyte supernatants. H^3 incorporation assays revealed that no significant changes in the T cell proliferation were provoked by keratinocyte derived factors in comparison to negative control. Hence, this current study could not confirm the results of a former study claiming that keratinocytes can inhibit T cell proliferation via the secretion of TGF-β and PGE2 (Kopfnagel *et al.*, 2011). However, this contrariness might be due to different experimental set-ups which include the use of T cells in general instead of distinct $CD4^+$ T cell subsets and the co-culture with skin-equivalents or direct cell-cell culture instead of using keratinocyte supernatants. Taken together, the influence of keratinocyte derived mediators on the proliferation of T cells seems to be limited in the used experimental set-up and also independent of keratinocyte activation status.

5.2.2 Influence of keratinocyte derived mediators on T cell cytokine release

Cytokine release can be considered as one of the most important cell effector functions since it ensures the contact-independent communication with other cells and also allows

to influence many processes. For T helper cells the cytokine release is moreover a specific classification marker assigning the cell to a certain subgroup with distinct abilities and tasks (Eyerich and Zielinski, 2014). Nevertheless, plasticity between the single subtypes and changes in the cytokine pattern are possible, although they depend on the surrounding environment and specific signals (Geginat *et al.*, 2014).

5.2.2.1 Effects of mediators derived from untreated and IFN-γ treated keratinocytes

To check whether keratinocyte derived mediators are able to influence the cytokine release of Th cells and by this may impact T cell plasticity, different Th subsets were co-incubated with supernatants from human primary keratinocytes. Notable, the presence of keratinocyte derived factors mainly inhibited the cytokine release of the tested Th subsets. The sole deviation from this pattern was the induction of IL-17 in the Th17 lineage which is conform to former studies showing that soluble factors from human keratinocytes can provoke elevated IL-17 production in $CCR6^{+}CD4^{+}$ T cells (Muhr *et al.*, 2010).

In addition, it could be observed that mediators obtained from keratinocytes under inflammatory conditions often had the same tendencies as the mediators from unstimulated keratinocytes, however, this was not true for all settings. For instance, the inhibition of IL-10 and IL-4 as well as the induction of IL-17 was equally pronounced for both conditions, but for IFN-γ and IL-22 the keratinocyte mediators derived from the inflammatory set-up did not show the inhibitory capacity seen before with the mediators from unstimulated cells. Thus, pre-stimulation of keratinocytes with IFN-γ alters at least partly the effect of the mediators on the T cell cytokine release and seems to favour a Th1 response since it primarily suppresses the Th2 cytokines IL-4 and IL-10. This observation was not totally unexpected since inflammatory stimuli like IFN-γ are known to influence keratinocytes in several ways and therefore are also likely to change the composition and properties of the keratinocyte released mediator mix. For example IFN-γ stimulation of keratinocytes dramatically induces the production of IP-10, which is a known Th1 chemoattractant and therefore also supporting the development of a Th1 response (Groom and Luster, 2011). Taken together the results allow the assumption that the dominating dampening effect of keratinocyte derived mediators on T cell cytokine release is a general phenomenon and might help to keep T cell responses and unwanted reactions under control. Under steady state conditions this effect is probably mainly effective in the dermis

where $CD4^+$ T cells reside, but under inflammatory conditions $CD4^+$ T cell in the epidermis could also be a target. Furthermore, the loss of the inhibitory effect on T cell IL-22 and IFN-y release by mediators from IFN-y pre-stimulated keratinocytes may also be seen as an orchestrating instrument. For instance, keratinocytes activated by a danger signal could set the course for initiating an immune response by ceasing their inhibiting effects. Regarding the positive effect of keratinocyte derived mediators on the T cell IL-17 release, the assumption is obvious that keratinocytes support in this way the production of a cytokine which is constantly needed in moderate levels for AMP production. Since IL-17 is needed during steady state as well as under inflammatory conditions it makes sense that the same effect was observable when keratinocyte mediators from IFN-y pre-stimulations were used.

Although several influences of keratinocyte derived mediators on T cell cytokine release could be observed in this study, the question of the responsible factors remained. An answer to this question is far from trivial because the supernatants from keratinocytes comprise manifold mediators and molecules of different nature and probably also with time point depending effects. Nevertheless, a first step was taken in this study by testing if the “active” compounds in the keratinocyte supernatant are of protein or of non-protein nature. Interestingly, experiments with protein-free supernatants revealed again differences between the effect on T cell IL-17 release and the effect on the other tested cytokines. Thus, IFN-γ, IL-22 and IL-4 levels were decreased in response to total keratinocyte supernatant but the cytokine levels did not diminish when protein-free keratinocytes supernatants were used. In contrast, for IL-17 release, the presence of both protein containing and protein-free keratinocyte supernatants resulted in an increase of the cytokine level. This observation indicates that the responsible factor for the enhanced IL-17 production is a non-protein factor whereas a protein factor is pivotal for the inhibition of the other cytokines. With this result the claim made from Muhr et al., 2011 that IL-1, a protein factor, is essential for the induction of IL-17 in CD4 CCR6 T cells was not confirmable. However, the current results are consistent with another study (Peters *et al.*, 2013) showing an IL-1 independent induction of IL-17 in T cells in response to keratinocytes mediators.

With these experiments using protein depleted keratinocyte supernatants, some initial hints regarding the nature of the T cell influencing factors derived from keratinocytes could

be seen, but further investigation is clearly needed. This would comprise for example a complex fraction analysis by high pressure liquid chromatography (HPLC) and mass spectrometry followed by in vitro cell culture tests which could include the use of antibodies and antagonists against promising candidate factors.

5.2.2.2 Effects of mediators from APE treated keratinocyte on T cell cytokine release

Being the interface to the environment the skin is one of the most exposed organs to extrinsic factors. Among these factors are pollen with the capacity to provoke allergic reactions in sensitized patients, including exacerbation of atopic eczema rashes (Werfel *et al.*, 2015). One aim of the current study was to investigate how plant pollen extracts can change the effects of keratinocyte derived mediators on T cell effector functions. Based on experiments conducted with unstimulated and IFN-γ stimulated keratinocytes the set-up was extended by including the stimulation of keratinocytes with extracts from two known, highly allergenic pollen species: A. *artemiisfolia* and B. *pendula* pollen.

The first target of this test series regarding the impact of keratinocytes derived mediators on T cell cytokine release, was TGF-β1, a cytokine which controls several aspects of inflammatory responses: T cell differentiation, B cell isotype switching and also tolerance (Banchereau *et al.*, 2012). For instance, TGF-β1 is essential for the induction of Foxp3 in naive $CD4^+$T cells and is also a hallmark cytokine produced by Tregs. In combination with IL-1β, IL-6 and IL-23 it further induces the differentiation of naive T cells into pathogenic Th17 cells while inhibiting the generation of Th1 and Th2 cells (Banchereau *et al.*, 2012). Based on these facts it was assumed that TGF-β1 would be primarily released by the Treg cell line and also be most affected in this cell type by the presence of keratinocyte derived mediators. However, results showed that in comparison to the other tested Th subsets Tregs did not produce significantly more TGF-β1 and that the cytokine release with 100 - 200 pg/ml was in general quite low. This may indicate that despite good expression of Foxp3, the Treg cell line was not optimal differentiated or that the culture conditions negatively affected the TGF-β1 release. Nevertheless, some moderate effects could be observed, including the difference between mediators from unstimulated and Bet APE stimulated keratinocytes regarding the tendency to inhibit TGF-β1 release in Th1 and Th2 cells. Unstimulated keratinocytes produced factors with an inhibiting effect on TGF-β1 release but in contrast, Bet APE stimulated keratinocytes did not suppress cytokine release.

Therefore, one can assume that different factors or at least different factor concentrations are present in the two conditions. However, the fact that both tested keratinocyte supernatants did induce the production of TGF-β1 in Th17 cells shows that also similar effects are possible. Thus, the ensuing effect seems not only to be dependent on the composition of the keratinocyte supernatant but also on the Th subset they are incubated with.

For the cytokine data generated by multiplex technique a general analogy to preceding experiments with mediators from unstimulated keratinocytes could be observed. Thus, the previously observed inhibiting effect of the steady state keratinocyte supernatants on IFN-γ, IL-4, IL-10 and IL-22 could be confirmed and testing further cytokines like TNF-α, IL-5 and IL-13 even supplemented this data. Generally it became clear that independent of the Th subset, the cytokines most affected and suppressed by the presence of keratinocyte supernatants were IL-22 and the Th2 cytokines IL-4, IL-5 and IL-10. IFN-γ and TNF-α in contrast were often less inhibited which in combination with low Th2 cytokine release might support the creation of a Th1 dominated environment under steady state conditions. Furthermore, the induction of IL-17 in the Th17 subset could be reconfirmed, although this data has to be handled with care since the establishment of the Th17 cell line was not entirely clean and showed an IL-17 exceeding IFN-γ production. Nonetheless, it can be assumed that soluble factors from keratinocytes indeed foster IL-17 release selectively. Since IL-17 itself can also stimulate keratinocytes to produce IL-17 recruiting chemokine CCL20 (Harper *et al.*, 2009), this may be also part of a self-sustaining feedback loop.

For experiments with mediators derived from Bet and Amb APE stimulated keratinocytes the hypothesis was set that pollen substances might promote the generation of a Th2 favouring milieu comparable to the acute phase of AE. However, this hypothesis could not be confirmed since the mediators provoked alike the mediators form unstimulated keratinocytes a general downregulation of Th2 cytokine release. Nonetheless, this could be different in cells from atopic donors which haven´t been tested so far and also the change to shorter stimulation periods with APE could result in a different outcome. In the current setting, however, Amb APE influences were mostly comparable with the effects observed for mediators derived from unstimulated keratinocytes. Mediators generated under Bet

APE influence showed no or only less pronounced effects in comparison to control and the other conditions. This was true for the inhibiting effects as well as for the IL-17 inducing phenomenon. Therefore, it seems obvious that Bet and Amb APE act differently on keratinocytes with different soluble mediator production as consequence. This may be due to varying factors in the pollen extract but might also be due to time point dependent effects. Nevertheless, it is also striking that Amb APE treatment showed a very similar pattern to the experiments conducted with mediators derived from unstimulated keratinocytes. Again this could be of time point origin, for instance that after 24 h specific mediators produced in response to Amb APE are already degraded and only leave the "standard" mediators behind. For Bet APE, in contrast, the question remains if simply less of the effective mediators are present or if a different mediator mix is produced by the keratinocytes which could provoke T cell cytokine release results resembling the medium control. These questions, however, can only be answered by further investigations including enhanced n-numbers, deeper analysis of the keratinocytes derived mediators in steady state and in response to APE as well as the inclusion of cells from atopic donors.

5.2.3 Influence of keratinocyte derived mediators on T cell mRNA expression

Since lineage commitment and cytokine production of Th cells is strongly influenced by the presence and activation of specific transcription factors (TFs) the current study also investigated the influence of keratinocyte derived mediators on the mRNA expression of lineage defining TFs. In addition, the mRNA levels of the T cell subset hallmark cytokines were determined to check also here influences of keratinocyte supernatants and to compare the results with the collected cytokine protein data.

Notably, the mRNA expression of the tested TFs corresponded well with the expression of their assigned lineage cytokines and also the measured protein data. For example, T-bet, the master TF of Th1 cells, tended to be suppressed by the presence of steady state keratinocyte supernatants which was in accordance with the observed inhibition of IFN-γ mRNA expression and protein secretion. For GATA-3, the characteristic TF for Th2 cells, the same suppression tendency in response to keratinocyte supernatants could be demonstrated and matched nicely with low expression of the Th2 hallmark cytokine IL-4. Appropriately, the inhibition of GATA-3 and the cytokine IL-4 was most pronounced in the subsets which normally possess the highest expression of these factors, namely Th2 and

Th0 control cells. In addition, IL-22 mRNA levels were strongly suppressed in Th0, Th1 and Th17 cells, standing in line with the cytokine release results which have shown the same keratinocyte supernatant induced inhibiting effects.

As it was observed for the cytokine release, the mRNA data also presented candidates which were not affected negatively by keratinocyte derived mediators but instead supported in their expression. For example the induction of RORC2, the master TF of Th17 cells, and the IL-17 mRNA expression in Th17, Th1 and Th0 cells perfectly mirror the observation from the cytokine release data showing a positive impact of keratinocyte mediators on the IL-17 production. Moreover, positive effects on the expression of the Treg assigned TF Foxp3 were determined in Th0, Th1 and Th17 cells, but strikingly not in the Treg cell line. In contrast to Th0, Th1 and Th17 the Treg subset responded to the presence of keratinocyte derived mediators with a slight down-regulation of Foxp3 expression accompanied by a decreased expression of the Foxp3 and Treg associated cytokines TGF-β and IL-10. The reason for this phenomenon could be that Tregs expressing higher levels of Foxp3 than other T cell lines are in general less sensitive to further inductions or that the establishment of the Treg cell line has not been fully successful and therefore may not show representative results. However, the expression of Foxp3 and TGF-β was clearly enhanced in the Treg lineage control setting compared to other TF and cytokines so that a Treg phenotype had been assumed. However, to rule out all uncertainty regarding the Treg phenotype additional functional testing e.g. by conducting suppression assays would be necessary for future experiments.

Regarding the influence mediators from APE stimulated keratinocytes can have on the mRNA expression of Th cells, results similar to those of the cytokine release experiments could be observed. Amb APE keratinocyte supernatants (KS) showed the same results as the mediators from unstimulated keratinocytes, in all tested settings. Once again, Bet APE KS showed no or only far less pronounced effects on the T cell mRNA expression when compared to KS from Amb APE stimulated or unstimulated cells. This confirms the results from the protein data and shows again that despite these first striking results there is still need for further experiments clarifying the background and mechanisms of the observed effects.

In conclusion this study part showed that keratinocyte derived mediators do not only influence the cytokine production potential of Th cells but also have an impact on the

preceding mRNA expression (Figure 51). Thus, the observed influence on the cytokine secretion may have it roots in the effects on transcription factors and gene transcription itself.

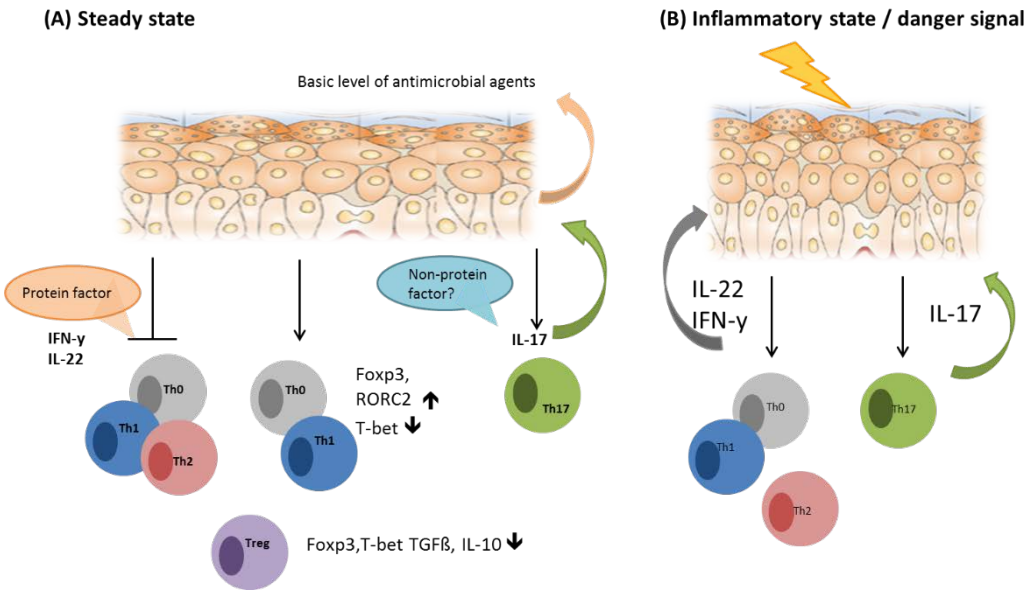


Figure 51: Influence of keratinocyte derived factors on Th cell subsets. (A) Observed effects under steady state conditions. **(B)** Observed results for the inflammatory state.

Taken together keratinocytes indeed seem to be able to create a microenvironment which has immune modulating functions and can influence T cell plasticity. All in all this became apparent by the identification of Th subset depending effects of keratinocyte derived mediators and most notably also by demonstrating an exceptional position of Th17 and its IL-17 release. In addition, experiments also gave insights into previously unknown effects e.g. the induction of Foxp3 in response to soluble mediators from keratinocytes. It should be noted as well that previous studies with keratinocytes and T cells mainly concentrated on the effects T cells can have on keratinocytes and not the other way round. Thus, the current study provides new information in a scarcely investigated research field and moreover tested the modulating effects pollen extracts can have on Th cell influencing keratinocyte derived mediators for the first time. To expand knowledge in this field further it would be worth considering the comparison of healthy and atopic donor cells and include experiments in 3D co-culture systems in order to study the crosstalk of keratinocytes and T cells in an even more physiological setting. Besides this the inclusion of DC cells in the

system or the impact on the newly described ILCs would be of interest and very promising to provide new information about immune response regulation in human skin.

5.3 General effects of APE on human primary keratinocytes

Pollen extracts are a complex mixture of proteins, lipids, carbohydrates and other molecules which are by far not all identified or investigated in their effects on human cells. Most of the previous studies concentrated on the allergens, their allerginicity and the associated mechanisms, but only few studies have chosen an integrated approach comprising the effects pollen factors can have in general on skin epithelium. Some studies in this field, however, already revealed that e.g. proteases can harm the skin barrier and that in general the dendritic cell phenotype can be influenced by APE as well as that certain compounds like adenosine can have immunomodulatory effects (Gilles *et al.*, 2012). Nonetheless, a clear picture of the effects pollen extracts can have directly on human keratinocytes is still missing. Thus, the current study provides new knowledge with the potential to be essential for the understanding of several mechanisms in the skin in response to plant pollen exposure.

For instance, the check for morphological changes in responses to different pollen extracts (Amb, Bet and Pin APE) revealed that Amb APE, the pollen extract from the ragweed plant, had the strongest influence. Cell shape and adherence distinctively changed and led to a different appearance in comparison to control cells. Although Bet APE provoked similar, albeit less pronounced results compared to Amb APE, Pin APE did not show any obvious effects indicating that there are indeed differences between the pollen species and their effects on keratinocyte morphology and growth behaviour visible by microscope. Nevertheless, the fact that only few studies have been carried out so far regarding the morphology of keratinocytes in response to environmental factors makes it difficult to draw clear conclusions. However, Watt et al. (1988) have shown that cell shape influences DNA synthesis and involucrin expression and concluded from their experiments that a rounded morphology acts as a signal to keratinocytes to stop dividing and to undergo terminal differentiation. This confirms that the cell shape indeed can influence mechanisms in the cell and probably the other way around as well. However, to put the observed results in a better context with distinct cell processes or functions, further experiments including

the check for differentiation marker expression and the analysis of adhesion molecules and tight junctions would be advisable.

Besides cell morphology the effects of APE on the release of pro-inflammatory cytokines and chemokines by keratinocytes were tested. For instance, IL-8 which is produced by a wide range of cell types after stimulation with IL-1α, IL-1β, IL-17, TNF-α, or TLR ligands was tested (Yeh *et al.*, 2001). IL-8 in general acts as a chemoattractant for neutrophils, NK cells, T cells, basophils and eosinophils and plays a role in several diseases, among them skin disorders like psoriasis. The binding of IL-8 to its receptors CXCR1 and CXCR2 leads to the activation of the transcription factor NF-κB but can also activate p38 MAPK followed by activator protein-1 (AP1) induction (Akdis *et al.*, 2011). From literature it is also known that the fungus Candida albicans can be an inductor of IL-8 in keratinocytes (Kano *et al.*, 2003) or that serine proteases in house dust mite can provoke an IL-8 secretion from human keratinocytes via protease activated receptor 2 (PAR-2) activation (Kato *et al.*, 2009). In addition, it was shown that timothy grass pollen extract can induce IL-8 release in human bronchial epithelia cells (Blume *et al.*, 2013), but for the combination of keratinocytes and pollen extract so far no distinct effects are described in literature. Thus, by testing IL-8 release the current study also gives new insights in the potential of pollen extract to provoke immune reactions in human primary keratinocytes. Stimulation experiments with different pollen species revealed that Amb APE primarily had the potential to induce high amounts of IL-8 from human primary keratinocytes. As it has been previously described for other stimulation factors and cells (Pearl *et al.*, 2001) IL-8 levels also peaked in the current set-up for keratinocytes around 6 h after stimulation. Strikingly, Bet APE had only a very weak potential to induce IL-8 and provoked in comparison to Amb APE and Phl APE only moderate IL-8 release. Therefore, the results seem to underline first that different APEs have different effects on human keratinocytes and second that the inflammatory provoking potential of Amb APE is higher than one of other APE species. Since IL-8 can be induced by IL-1 and TNF-α which are cytokines also released by keratinocytes in response to danger signals, an autocrine feedback loop might be possible. Notably, stimulation experiments with heat inactivated Amb APE and the fraction < 3kDa without protein still showed IL-8 inducing capacity. Therefore, it can only be assumed that in both fractions, protein and

non-protein, IL-8 stimulating mediators are present but no further conclusion can be drawn.

In addition to IL-8, keratinocytes can also release CCL20, a chemoattractant factor which has the capacity to interact with the chemokine receptor CCR6 to recruit IL-17A producing cells into the skin. For this reason CCL20 plays also an important role in the pathogenesis of psoriasis where it supports the IL-17 dominated immune reaction (Becher and Pantelyushin, 2012; Harper *et al.*, 2009). CCL20 can be induced in human keratinocytes for instance by UV-B radiation or house dust mite extracts but also in response to the cytokines IL-17, IL-22, TNF-α or IL-1α (Kennedy-Crispin *et al.*, 2012; Ko *et al.*, 2015; Peters *et al.*, 2013). During the current study it became clear that Amb APE and Phl APE have the ability to induce moderate CCL20 release as well. As it has already been the case for IL-8, Bet APE could not induce CCL20 release in high amounts at the tested 3 h time point and therefore was the least effective APE tested in this setting. This indicates that not all pollen species impact keratinocytes in their cytokine release in the same way and that Amb APE is indeed the one with the highest inflammatory potential.

Interestingly the same was true regarding the APE effects on the release of another pro-inflammatory mediator, the chemokine IP-10 (CXCL10) which is potently induced by IFN-γ in keratinocytes. Moreover, IP-10 is assigned a Th1 recruitment function as well as a role in several skin diseases, such as psoriasis, atopic dermatitis and contact dermatitis (Kanda *et al.*, 2007). Its production can be enhanced by the presence of IL-18, thus promoting infiltration of Th1 cells into skin and amplifying inflammation (Kanda *et al.*, 2007). In the current study, however, IFN-γ pre-stimulated keratinocytes only reacted after the stimulation with extracts from Amb or Phl with an increased IP-10 release. Bet APE in contrast did not show an inducing effect, although the low n number and time point effects must be ruled out as underlying reasons before a clear statement can be made on this point. Nevertheless, the previously observed effects of IL-8 and CCL20 release were quite similar so that a general lower potential of Bet APE in comparison to Amb and Phl APE to induce pro-inflammatory mediators in keratinocytes might be assumed. This assumption is supported by further preliminary data showing that CCL5 (RANTES) is also induced by APE stimulation and is most pronounced in response to Amb and Phl APE while Bet APE only provokes moderate induction. CCL5, like the other tested cytokines, is a chemotactic factor which plays an active role in recruiting leukocytes to inflammation sites. Together with

CCL20 it is assumed to mediate the migration of Langerhans cells in the skin (Ouwehand *et al.*, 2011). It also seems to be an essential factor in skin diseases since for example an overexpressed gene expression of CCL5 in AD skin lesions has already been demonstrated (Rebane *et al.*, 2012). Therefore, effects on CCL5 can also be of importance in skin immune responses and a regulation by APE may have an essential effect on the outcome.
Taken together plant pollen extracts indeed have effects on the production of pro-inflammatory cytokines by keratinocytes which was demonstrated for IL-8, CCL20, IP-10 and CCL5. Hence, APE can be considered as a clear danger signal for the skin epithelium and is without doubt worth to study for further effects. Interesting in this context would be for instance an investigation of the APE impact on the release of other cytokines like CCL17, CCL22, CCL26, GM-CSF, Galectin-9 or TGF-β as well as the influence of APE on the AMP production by keratinocytes. Moreover, the ability of APE to create a Th2 response promoting milieu in the skin, which may support allergic reactions against pollen, should be in focus of further investigations.

To collect further information about the impact of APE on human primary keratinocytes the effect on mRNA expression of selected genes (filaggrin, TSLP, PAR2 and HIF-1α) was tested. These experiments were partly conducted using three dimensional (3D) skin models to mimic a realistic situation with different epidermal layers. Regarding mRNA expression former studies have already shown that there are differences between 3D and 2D culture (Antoni *et al.*, 2015; Smith *et al.*, 2012). Hence, the system may be an essential factor regarding the outcome and relevance of a study. Especially for structural proteins like filaggrin the 3D skin environment is of importance since it resembles the composition of human skin much closer than flat 2D keratinocyte cultures (Antoni *et al.*, 2015). Filaggrin itself is important for the terminal differentiation of keratinocytes and is extensively processed to participate in the formation of the stratum corneum (Sandilands *et al.*, 2009). Loss of function mutations of filaggrin are highly associated with AE and also with enhanced IL-1 expression in the stratum corneum (Kezic *et al.*, 2012). Therefore, filaggrin was also an interesting candidate for the current study to test the influence of APE stimulation on keratinocytes and indeed showed a slight induction of mRNA expression in response to Amb APE. This may indicate that Amb APE actually strengthens the formation of the stratum corneum by upregulating filaggrin expression. However, functional protein

expression data in keratinocytes are missing on this topic and should be collected with the help of histological stainings to see if this effect might be true.
From other studies it is already known that stimulation with pollen extract enhances the transepithelial electrical resistance of bronchial epithelial cells giving a hint that not only filaggrin expression might increase but also the epithelial barrier tightens in response to pollen (Blume *et al.*, 2015).

Furthermore, the mRNA expression of the Th2 cytokine TSLP was analysed and showed striking differences between Amb and Bet APE stimulation. While Bet APE induced TSLP expression, Amb APE did not provoke elevated levels. However, the time point question might also be relevant here and further experiments would be necessary to verify this result. Nevertheless, the induction of TSLP mRNA by Bet APE could be a sign of a Th2 shift and a promotion of skin allergic reaction (Leyva-Castillo *et al.*, 2013). In addition, other factors like PAR-2 and hypoxia inducible factor 1 alpha (HIF-1α) were also considered to be of interest since recent publications demonstrated for example a link to TSLP induction (Jang *et al.*, 2013; Moniaga *et al.*, 2013) (Figure 52). PAR-2, a G-protein coupled receptor, is widely expressed in the skin and therefore also on its own a promising target for the proteases contained in APE. The receptor is activated by trypsin like serine proteases which can have an endogenous or exogenous source (Lee *et al.*, 2010). For instance, it has been shown that PAR-2 can be activated by proteases from house dust mite, fungi or cockroach indicating that also the serine protease rich extract from ragweed might be able to promote PAR-2 activation (Bagarozzi *et al.*, 1998; Lee *et al.*, 2010). Upon stimulation PAR-2 can lead to NF-κB activation and in consequence to enhanced IL-8 release (Hou *et al.*, 1998). Moreover, PAR-2 expression has been observed in increased levels in lesional skin of atopic eczema patients, suggesting that this receptor plays a role in inflammatory dermatosis (Komatsu *et al.*, 2007). In the murine system PAR-2 also has been shown to be involved in the Th2 mediated allergic inflammation via the induction of TSLP (Moniaga *et al.*, 2013; Yoo *et al.*, 2005). In the current study the response to Amb APE showed high variance between the single experiments but nonetheless a tendency to elevated PAR-2 mRNA. Since IL-8 secretion by keratinocytes can be a result of PAR-2 induction it would be worth testing if there is also a connection between the observed IL-8 release after Amb APE stimulation.
Jang et al. also could demonstrate a link to TSLP for the transcription factor HIF-1α which works via UV-B stimulation and the Jun N-terminal kinase (JNK) and extracellular signal-

regulated *kinases (*ERK) pathway (Jang *et al.*, 2013). HIF-1α itself is a major regulator of cellular adaption to low oxygen levels and has been shown in former studies to promote bactericidal activity of keratinocytes (Peyssonnaux *et al.*, 2008; Rangasamy *et al.*, 2011) Peyssonnaux 2008). Since the epidermal microenvironment is characterized by low O_2 availability HIF-1α is especially important in the skin and was additionally shown to be an essential regulator of keratinocyte differentiation and epidermal barrier function by regulating filaggrin expression (Wong *et al.*, 2015). In the current study HIF-1α expression was hardly effected by Amb APE stimulation making further experiments necessary to investigate if there is indeed no effect or if adapted experimental set-ups can promote more distinctive results.

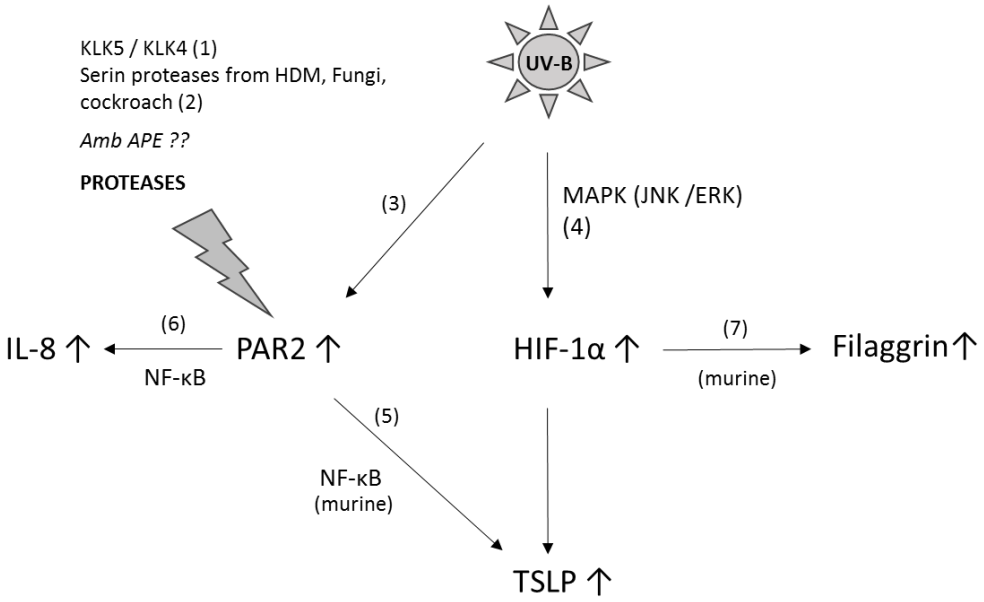


Figure 52: Consequences of PAR-2 and HIF-1α activation in the human skin by environmental factors. Information was collected and combined from: (1) Briot et al., 2009 (2) Lee et al., 2010 (3) Scott et al., 2001 (4) Jang et al., 2013 (5) Yoo et al., 2015 and Moniaga et al., 2013 (6) Hou et al., 1998 (7) Wong et al., 2015.

5.4 Effects of APE on inflammasome associated effector mechanisms

Previous studies regarding inflammasome activation focused mainly on cells, which are known for strong inflammasome responses, e.g. monocytes or dendritic cells. However, not much attention was paid to epithelial cells like keratinocytes, although they do express all necessary inflammasome components and are the first to be in contact with environmental substances (Yazdi *et al.*, 2010). Among the rare studies handling inflammasome activation in keratinocytes, the involvement in skin reactions e.g. in response to bee venom or *Staphylococcus aureus* has been described (Dombrowski *et al.*, 2012; Miller and Cho, 2011). In addition, asthma studies with lung epithelial cells and experiments with Der p 1 allergen and keratinocytes showed that inflammasome activation can also play a role in type I allergy (Dai *et al.*, 2011; Im and Ammit, 2014). Nonetheless, the contribution of inflammasome responses to skin diseases like atopic eczema is still controversially discussed and in the case of reactions to plant pollen simply not examined yet. Furthermore, it has been mostly disregarded that a combination of trigger factors may be more effective in provoking inflammasome reaction than individual factors. Therefore, the current study not only expands knowledge in this field but also shows for the first time an influence of pollen derived mediators on inflammasome activation in human primary keratinocytes as well as additive effects of UV-B treatment.

For experiments, different pollen species were selected, among them *A. artemisiifolia, B. pendula* and *P. pratense* pollen known for their high and *P. sylvestris* pollen known for its low allergenicity (Behrendt and Becker, 2001). Since the extracts of these pollen all induced IL-1β and IL-18 release, a general basic mechanism, which is independent of the pollen's allergenic potential, can be assumed. However, the level of cytokine release provoked by the extracts differed, indicating that the strength of the response is species dependent. In addition, a difference in the activation potential for IL-1β and IL-18 was observed for Phl APE. Regarding the fold induction, IL-18 levels were enhanced stronger than IL-1β levels. A possible explanation may be a particular factor in Phl APE supporting IL-18 maturation selectively. This kind of distinct licensing has been described before for murine dendritic cells in response to *Listeria monocytogenes* (Schmidt and Lenz, 2012) and may be helpful for fine tuning effects by changing the IL-1β / IL-18 proportion. Apart from this, it could be shown that Amb APE had the strongest overall effects regarding cytokine release. This would fit well to the hazardous role ragweed plant pollen is assigned to for triggering

immune reactions (Csillag *et al.*, 2010). Furthermore, Amb APE did not need an extra priming signal to induce cytokine release of the keratinocytes, although it was tested to be higher with an IFN-γ/TNF-α pre-stimulation. This confirms the already described phenomenon that keratinocytes possess pre-stored pools of precursor cytokines, probably due to the constitutive expression of IL-1β and IL-18 pro-forms (Contassot *et al.*, 2012). However, this phenomenon does not exclude the possibility that pollen extracts can also affect the priming part and cytokine pro-form production. A hint in this direction was given by qPCR results showing an induction of both IL-1β and IL-18 mRNA levels in keratinocytes stimulated with Amb APE. This suggests that APE can be a trigger for both signals, the priming part and the activation of caspase-1. Besides IL-1β and IL-18, IL-1α is another cytokine which is at least for its release partially dependent on casapase-1 activation (Gross *et al.*, 2012). Therefore, the increased secretion of IL-1α is a further indicator for an inflammasome involvement in the response to APE. In addition LDH assays confirmed the cell death independence of the observed effects.

The cytokine data is complemented by the induction of caspase-1 activation in keratinocytes after APE treatment. Extracts from all tested pollen provoked an enhanced level of active caspase-1, showing a common effect of the different pollen species. For Amb and Phl APE the level of active caspase-1 was even enhanced after stimulation with low concentrations of APE, which reflects the results observed for IL-1β and IL-18 release in the concentration-dependent experiments. Thus, APE also exerts effects in low concentrations suggesting that even a small amount of pollen can provoke measureable reactions. In addition, it can be concluded from the data that a protein source is responsible for these reactions since Amb APE free of protein and fractions < 3 kDa did not show any effects on IL-1β and IL-18 cytokine release. In consequence, this excludes low molecular substances of Amb APE as inflammasome activating factors in keratinocytes. Nevertheless, a long list of other candidates remains, among them enzymes like proteases or NADPH oxidases, which may contribute to inflammasome activation by proteolytic activity or by ROS production (Varga *et al.*, 2013).

Another key finding of this study is that Amb and Bet APE can induce IL-1β/ IL-18 cytokine release and the activation of caspase-1 in comparable levels as the known inflammasome activator UV-B radiation. This underlines the potential of pollen substances to induce

inflammatory responses and marks it as an effective danger signal. Moreover, the current study shows that a combination of the two factors, APE and UV-B, induces inflammasome activation in an additive manner. This suggests that UV-B may also contribute in vivo to an exacerbation of skin reactions developed in response to pollen contact. Especially interesting in this context is that filaggrin knock down in a human skin model was shown to enhance UV-B sensitivity, possibly indicating that AE patients suffering from filaggrin mutations might be even more susceptible for UV-B effects combined with pollen (Mildner *et al.*, 2010). Furthermore, Scott et al. demonstrated that UV-B irradiation enhances in keratinocytes the expression of PAR-2, a factor typically up-regulated in AE lesions (Scott *et al.*, 2001) (see figure 52). However, counter-arguments to the UV-B contribution regarding inflammatory responses can also be found in literature. For example, it was published that UV-B also has immunosuppressive effects and is clinically used in therapy for several skin diseases e.g. psoriasis or vitiligo (Totonchy and Chiu, 2014). Although these beneficial effects have to be taken in consideration, this does not weaken the proposed hypothesis. For example UV-B is applied for therapy only in low dosages of narrowband range and the benefit for AE patients remains, even among experts, controversially discussed. In fact some studies showed a positive effect of UV-B for chronic AE lesions but mostly only the combination with UV-A led to a significant improvement (Meduri *et al.*, 2007). In addition, acute flares seem to respond only to UV-A and not to UV-B (Garritsen *et al.*, 2014). Therefore, the positive effects that UV-B may have in low dosage on chronic skin lesions do not exclude the possibility that in acute reactions and higher dosages UV-B is contra productive and can act as an adjuvant factor. Moreover, UV-B radiation may even pave the way for pollen substances to penetrate the skin since it was not only shown to cause DNA damage and inflammation in higher dosages but also skin barrier disruption (Meduri *et al.*, 2007).

Notably the data also reveal differences between cells of atopic and non-atopic donors suggesting a stronger susceptibility regarding APE and UV-B treatment for atopic donors. This susceptibility may have many causes, among them differences in inflammasome associated gene expression or pathways, higher sensibility to proteases or also changes in the tight junctions system (Bivik *et al.*, 2013; Novak *et al.*, 2005; Ritter *et al.*, 2014; Takai and Ikeda, 2011). Nevertheless and despite contradictory studies regarding the role of inflammasomes in Th2 responses (Gurung *et al.*, 2015; Helmby and Grencis, 2004; Kawase

et al., 2003; Madouri *et al.*, 2015; Ritter *et al.*, 2014), the current data indicate that inflammasome activation may indeed play a role in skin reactions including the pathogenesis of atopic eczema. This is supported by mouse models demonstrating that an over-secretion of IL-18 from epidermal cells results in AE-like skin eruptions (Konishi *et al.*, 2002). Furthermore, characteristic flares of atopic eczema often occur in skin regions like neck and face which are exposed to air and therefore also easily accessible for pollen and UV-B (Hostetler *et al.*, 2010). Another study showed that increased daily sun exposure in a children's cohort was associated with poorly controlled eczema (Sargen *et al.*, 2014). Besides this, the inflammasome hallmark cytokines IL-1 and IL- 18 are known to control both Th1 and Th2 responses (Xu *et al.*, 2000). Thus, IL-1β and IL-18 are good candidates to exert influence on the biphasic disease course of AE. Especially IL-18 seems to be important since its Th1 supporting function in the presence of IL-12 switches to a Th2 directing manner when IL-12 is absent (Xu *et al.*, 2000). Therefore, pollen derived low molecular weight factors, which were shown in former studies to effectively suppress IL-12 production by dendritic cells (Gilles *et al.*, 2015; Traidl-Hoffmann *et al.*, 2005), can contribute to Th2 supporting milieu with the help of IL-18. In addition to the effects IL-1 and IL-18 can have on Th1 and Th2 cells, both cytokines can affect a multitude of other cells and mechanisms (Figure 53).

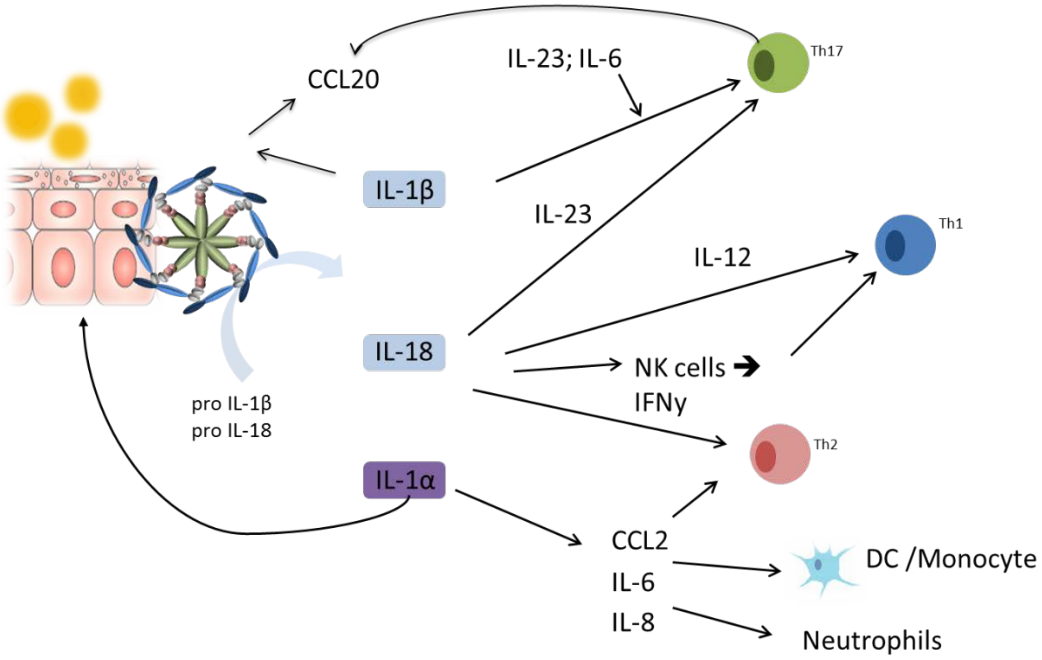


Figure 53: Possible autocrine and paracrine effects of the cytokines IL-1 and IL-18 after their activation.

For instance IL-1β is essential for the support of Th17 development and could promote by this also the IL-17 induced release of CCL20 by keratinocytes. IL-1 itself can also act in an autocrine feedback directly on keratinocytes as it has been described for example for S. aureus infections (Olaru and Jensen, 2010). Furthermore, IL-1α can promote the release of IL-6, IL-8 and CCL2 which can mediate chemoattraction of neutrophils or dendritic cells (Deshmane *et al.*, 2009; Hammond *et al.*, 1995). IL-18 in contrast can impact on NK cells and by stimulating their IFN-γ production contribute indirectly to the support of a Th1 response (Srivastava *et al.*, 2013). In murine and cell line studies it has also been shown that IL-1β and IL-18 can affect the stabilization of HIF-1α. Thus, an indirect influence on TSLP via HIF1-α and the known inductor UV-B (compare figure 52) could be another link to a Th2 response as it is observed in acute eczema lesions.

Besides this the current data showed that Amb APE can influence the mRNA expression of inflammasome associated genes. Although for IL-1β and IL-18 mRNA data is only representative for the cytokines´ pro-forms, the induction of caspase-1 and the inflammasomes AIM2 and NLRP3 is an additional sign for the ability of Amb APE to provoke an upregulation of inflammasome mechanisms. Unfortunately preliminary experiments concerning the inhibition of caspase-1 to proof ultimately the inflammasome dependence of the APE induced effects, were only partly successful. To overcome the suboptimal working caspase-1 antibody siRNA knock down experiments were started and knock down of caspase-1 and AIM2 gene was already successfully achieved. However, APE stimulation data is still missing and should be part of future experiments.

Taken together the results support the hypothesis that pollen influence the immunological barrier of the skin by triggering the inflammasome system of human keratinocytes on its own as well as by supporting the effects of UV-B irradiation. Thus, pollen themselves can provide a danger signal but also exert additive effects which may be important for the initiation and persistence of inflammatory allergic skin reactions.

6 Summary

This thesis project gives insight in the complex crosstalk between human skin epithelial cells and T helper cells and how this crosstalk can be influenced by environmental factors. It could be demonstrated that soluble factors derived from keratinocytes can distinctively influence Th cell subtypes in their cytokine production and therefore potentially support T cell plasticity. Moreover, it could be shown that contact to plant pollen alone or combined with UV-B light activated inflammasome mechanisms in keratinocytes.

In detail, the first study part revealed that the presence of keratinocyte derived mediators provoked in $CD4^+$ T cells an inhibition of the release of nearly all tested cytokines. This observation led to the assumption that in absence of any danger signal keratinocytes support a quiescent state of T cells. However, this effect became less prominent and more Th1 response oriented under inflammatory conditions. The induction of IL-17 release as exception of the observed inhibiting effect on other cytokines, indicated a clear preference of keratinocytes to support cytokine production important for skin homeostasis and basic defence mechanisms like the production of antimicrobial peptides. Furthermore, inducing effects on the Treg assigned transcription factor Foxp3 were observable for several Th subsets. This suggested an additional implication of keratinocyte derived factors in Treg development. Although the stimulation with plant pollen extracts did not provoke striking changes in the keratinocytes' ability to impact on T cell effector functions, direct influences of aqueous pollen extract (APE) on keratinocytes were clearly visible under different experimental set-ups in the second study part. Thus, APE of different plant species (A. *artemisiifolia*, B. *pendula*, P. *pratense* and P. *sylvestris*) not only changed cell morphology but also enhanced the release of chemotactic and pro-inflammatory cytokines (IL-8, CCL20, IP-10, CCL5) and had impact on the mRNA expression of several atopic eczema related targets (filaggrin, PAR2, TSLP). Depending on the pollen specie these effects were more or less pronounced but the role of the most effective one was clearly assigned to A. *artemisiifolia* pollen. Aside from this, it could be shown that APE also has the potential to activate inflammasome related mechanisms in human keratinocytes. In this context evidence could be provided that APEs induce IL-1β and IL-18 release and provoke activation of the inflammasome key protease caspase-1. Furthermore, it could be shown that this

effect is even stronger in combination with UV-B irradiation as additional environmental factor than in single exposure and that cells of allergic donors showed a tendency to be more sensitive to these triggers.

Taken together, this project provides new data in the field of cell crosstalk in the skin, including so far unknown individual effects on distinct Th subtypes. In addition, it identifies the combination of plant pollen and UV-B radiation as a risk factor for inflammatory skin reactions.

7 Zusammenfassung

Die vorliegende Arbeit untersucht die Interaktion von $CD4^+$ T Zellen und Keratinozyten im Allgemeinen wie auch unter dem Einfluss von Umweltfaktoren. Im Zuge dessen konnte gezeigt werden, dass Keratinozytenmediatoren die Zytokinsekretion verschiedener T helfer Zell - Subtypen beeinflussen und somit auch Effekte auf die T Zell Plastizität haben können. Des Weiteren aktivierte der Kontakt mit Pflanzenpollenextrakt allein oder in Kombination mit UV-B Strahlung in Keratinozyten Inflammasom Mechanismen und löste die Sekretion pro-inflammatorischer Botenstoffe aus.

Im Einzelnen wurde beobachtet, dass die von Keratinozyten produzierten löslichen Faktoren in der Lage sind Subtypen von T-Helfer Zellen spezifisch in ihrer Zytokinproduktion zu beeinflussen und somit potenziell die Plastizität von T-Zellen unterstützen. Die hierbei im Grundzustand beobachtete Sekretionshemmung fast aller getesteten T Zell Zytokine führte zu der Annahme, dass Keratinozyten über die Bildung bestimmter Mediatoren einen Ruhezustand der T-Zellen fördern können. Unter entzündlichen Bedingungen erschien dieser gehemmte Zustand jedoch weniger ausprägt und viel mehr in Richtung einer Th1 Antwort orientiert. Die aufgetretene Förderung einer Th17 Antwort deutet darauf hin, dass Keratinozyten vor allem die Produktion von Zytokinen unterstützen, die für den Erhalt der Hauthomöostase oder für Abwehrmechanismen wie z.B. die Bildung von antimikrobiellen Peptiden von Bedeutung sind. Ebenso konnte in mehreren T Zell Subtypen ein fördernder Effekt auf den typischerweise in regulatorische T-Zellen exprimierten Transkriptionsfaktor Foxp3 beobachtet werden, der auf eine Rolle der Keratinozyten in der Treg Entwicklung schließen lässt. Obwohl die Stimulation von Keratinozyten mit Pflanzenpollenextrakt keine ausgeprägte Änderung der Fähigkeit T-Zell Effektorfunktionen zu beeinflussen zur Folge hatte, zeigten sich dennoch unter anderen experimentellem Bedingungen direkte Einflüsse von APE auf die Keratinozyten selbst. So veränderten die Pollenextrakte verschiedener Pflanzenspezies (A. *artemisiifolia*, B. *pendula*, P. *pratense* und P. *sylvestris*) nicht nur die Zellmorphologie, sondern steigerten auch die Freisetzung von chemotaktischen und pro-inflammatorischen Zytokinen (IL-8, CCL20, IP-10, CCL5). Des weiteren übten die Pollenextrakte Einfluss auf die mRNA Expression von mehreren mit dem atopischen Ekzem im Zusammenhang stehenden Zielgenen aus (Filaggrin, TSLP, PAR2). In Abhängigkeit von

der Pollenart waren diese Effekte mehr oder weniger stark ausgeprägt, aber die Rolle des effektivsten Extrakts konnte klar dem des A. *artemisiifolia* Pollens zugewiesen werden. Abgesehen davon konnte ebenfalls gezeigt werden, dass Faktoren in den Pollenextrakten in der Lage sind, Inflammasomkomplexe in humanen Keratinozyten zu aktivieren. So führte der Kontakt mit APE sowohl zu einer Induktion der IL-1β und IL-18 Sekretion wie auch zur Aktivierung der Protease Caspase-1, dem Schlüsselelement des Inflammasomkomplexes. Diese Effekte verstärkten sich in Kombination mit UV-B Strahlung in additiver Weise und es konnte beobachtet werden, dass Zellen von allergischen Spendern die Tendenz besitzen empfindlicher und mit erhöhter Zytokinausschüttung auf diese Umweltfaktoren zu reagieren.

Zusammenfassend lässt sich somit sagen, dass diese Arbeit nicht nur neue Daten in dem Bereich der Zellkommunikation in der Haut bereitstellt sondern auch die Kombination aus Pflanzenpollen und UV-B Strahlung als Risikofaktor für entzündliche Hautreaktionen identifiziert.

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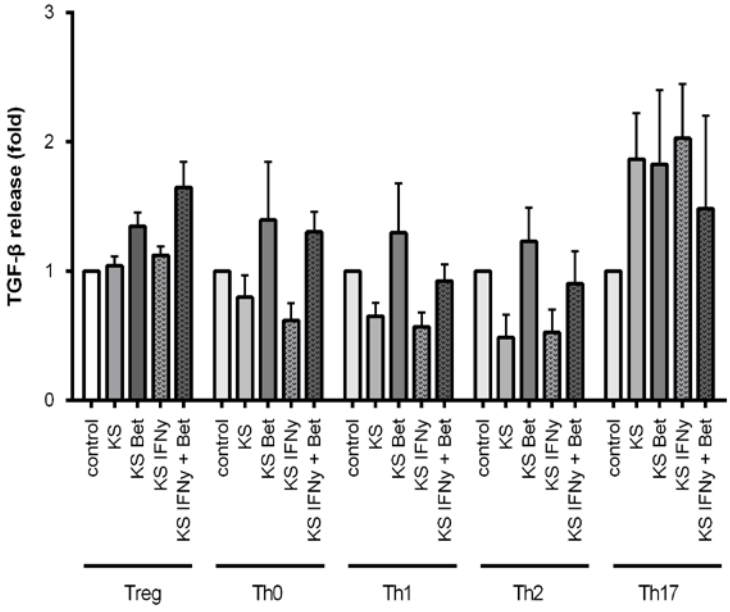
Appendix

Supplementary data for the result chapter 4.2.2.2:

(A)



(B)

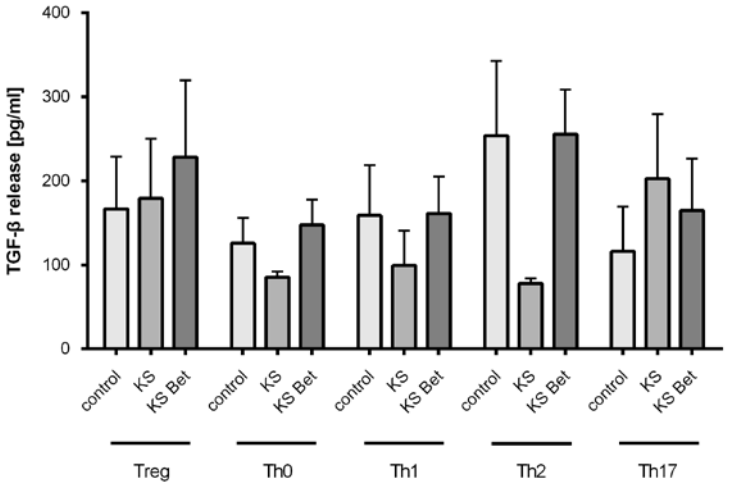


Figure A 1: Impact of keratinocytes derived mediators on T cell TGF-β release. **(A)** T cell subsets (Treg, Th0, Th1, Th2 and Th17) generated in 48 well plates were incubated for 72 h with supernatants derived from steady state keratinocytes (KS), Bet APE treated keratinocytes (KS Bet) or IFN-γ (300 U/ml) pre-stimulated keratinocytes (KS IFN-γ) alone or in combination with Bet APE treatment (KS IFN-γ Bet). TGF-β release was measured by ELISA. Data is normalized to corresponding medium control and shown as fold induction ± SEM. **(B)** T cell subsets (Treg, Th0, Th1, Th2 and Th17) generated in 48 well plates were incubated for 72 h with supernatants derived from steady state keratinocytes (KS) or Bet APE treated keratinocytes (KS Bet). TGF-β release was measured by ELISA and data is in pg/ml ± SEM. n = 4

Corresponding figure in the main manuscript: Figure 19

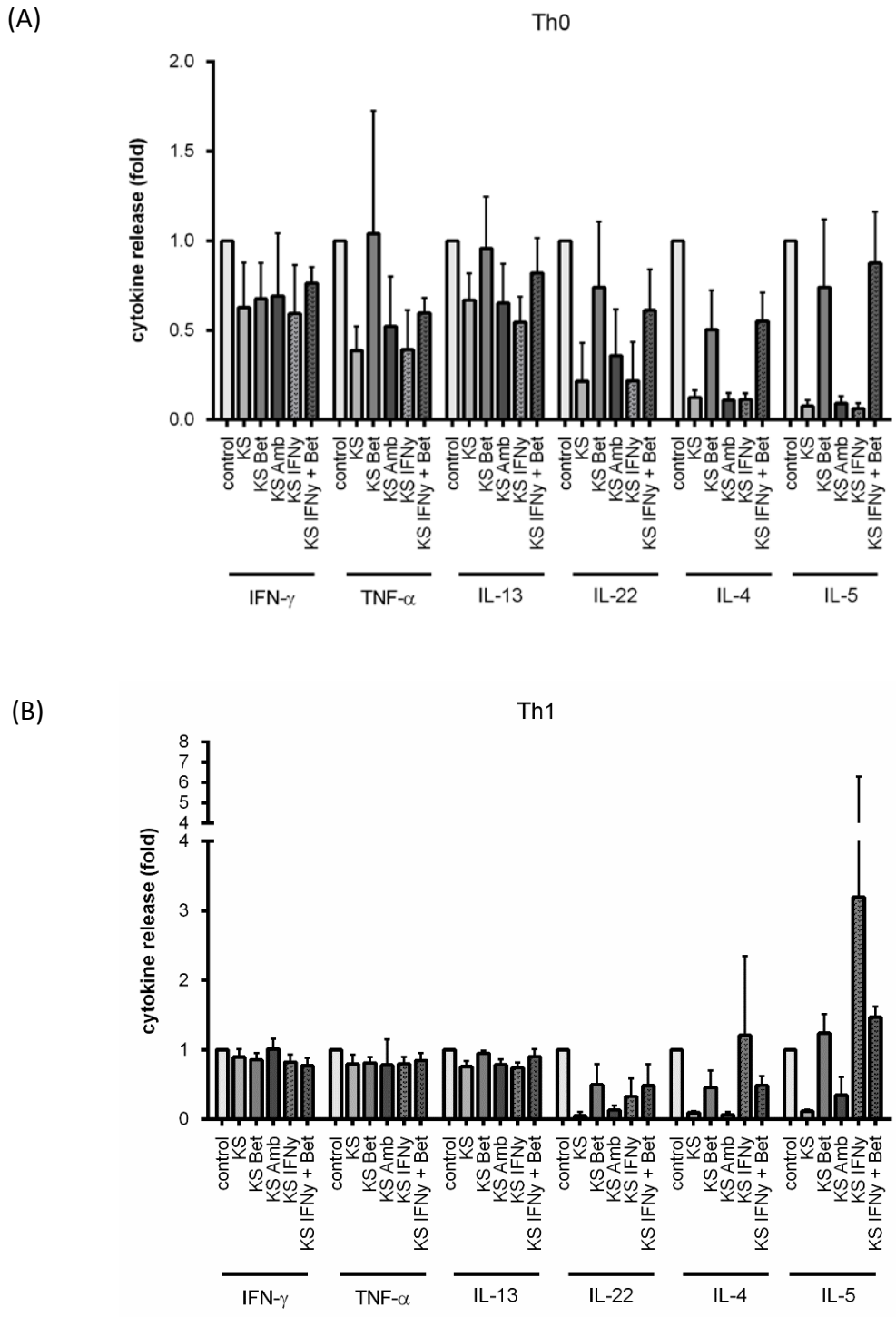


Figure A 2: Impact of keratinocytes derived mediators on the cytokine release of Th0 and Th2 cells. (A) Th0 and **(B)** Th1 cells generated in 48 well plates were incubated for 72 h with supernatants derived from steady state keratinocytes (KS), Bet APE treated keratinocytes (KS Bet) or IFN-γ (300 U/ml) pre-stimulated keratinocytes (KS IFN-γ) alone or in combination with Bet APE treatment (KS IFN-γ Bet). T cell cytokine release (IFN-γ, TNF-α, IL-13, IL-22; IL-4 and IL-5) was measured by multiplex technique. Data is normalized to corresponding medium control and shown as fold induction ± SEM; n = 4.

Corresponding figure in the main manuscript: Figure 20

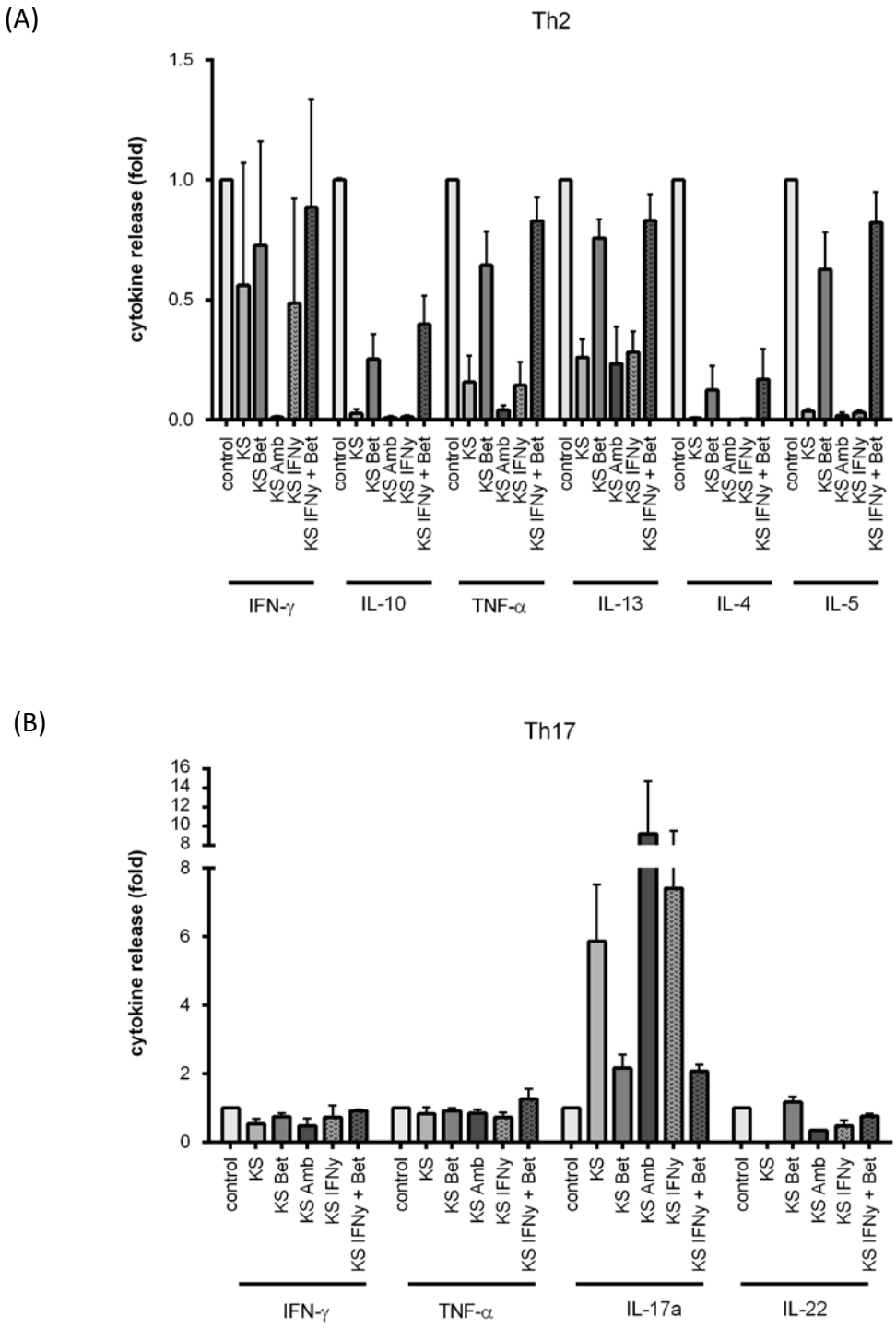


Figure A 3: Impact of keratinocytes derived mediators on the cytokine release of Th2 and Th17 cells. (A) Th2 and **(B)** Th17 cells generated in 48 well plates were incubated for 72 h with supernatants derived from steady state keratinocytes (KS), Bet APE treated keratinocytes (KS Bet) or IFN-γ (300 U/ml) pre-stimulated keratinocytes (KS IFN-γ) alone or in combination with APE treatment (KS IFN-γ Bet). T cell cytokine release was measured by multiplex technique. Data is normalized to corresponding medium control and shown as fold induction ± SEM; n = 4.

Corresponding figures in the main manuscript: Figure 22 and 23

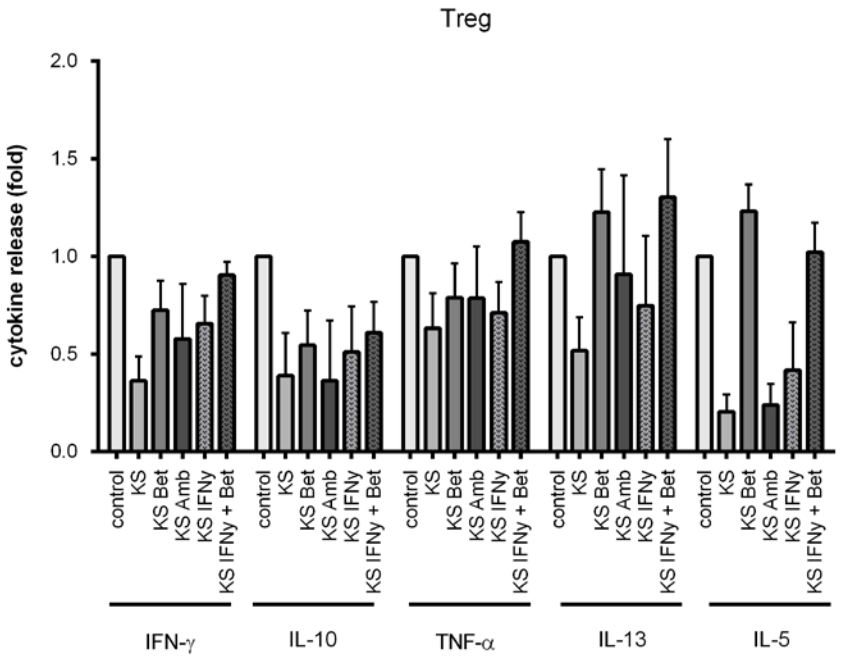


Figure A 4: Impact of keratinocytes derived mediators on the cytokine release of Treg cells. Treg cells generated in 48 well plates were incubated for 72 h with supernatants derived from steady state keratinocytes (KS), Bet APE treated keratinocytes (KS Bet) or IFN-γ (300 U/ml) pre-stimulated keratinocytes (KS IFN-γ) alone or in combination with Bet APE treatment (KS IFN-γ Bet). T cell cytokine release was measured by multiplex technique. Data is normalized to corresponding medium control and shown as fold induction ± SEM; n = 4.

Corresponding figure in the main manuscript: Figure 21

Supplementary data for the result chapter 4.2.3:

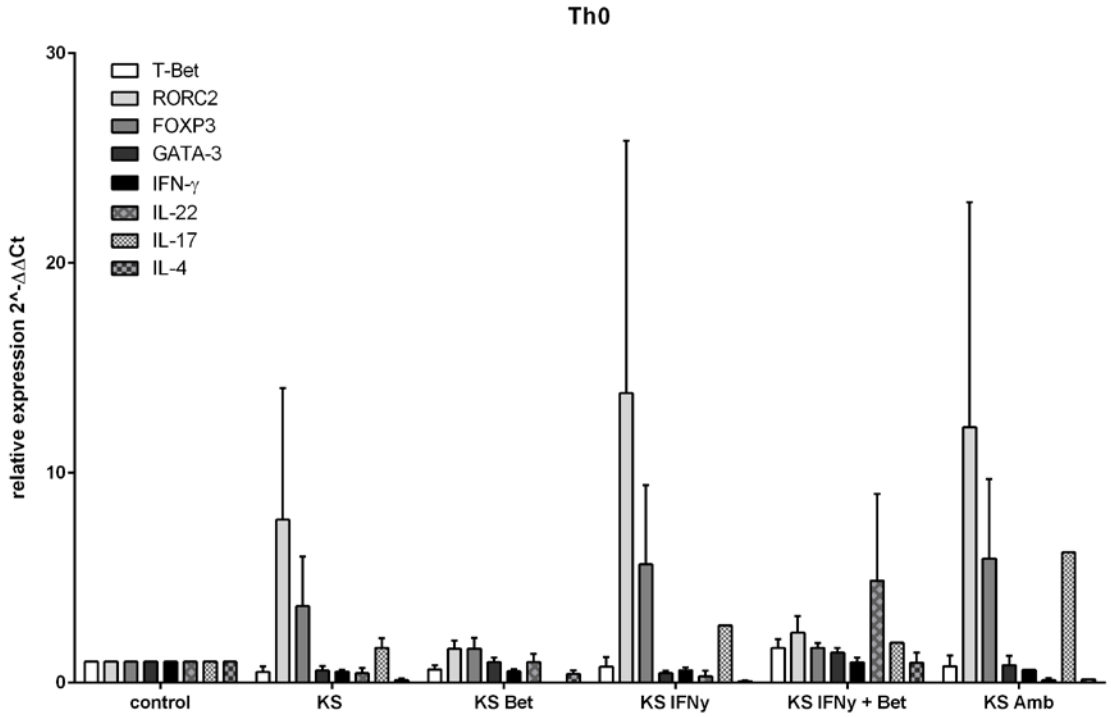


Figure A 5: Effects of keratinocytes derived mediators on the mRNA expression of Th0 cells. Th0 cells generated in 48 well plates were incubated for 72 h with supernatants derived from steady state keratinocytes (KS), Bet APE treated keratinocytes (KS Bet) or IFN-γ (300 U/ml) pre-stimulated keratinocytes (KS IFN-γ) alone or in combination with Bet APE treatment (KS IFN-γ + Bet), or Amb APE treated keratinocytes (KS Amb). T cell mRNA expression was analysed by qPCR. Data is normalized to house-keeping gene EF1 and to medium control by $2^{(-\Delta\Delta C_T)}$ method and shown ± SEM. n numbers for **T-bet, GATA-3, RORC2 and Foxp3** are n = 4 for control, KS and KS Bet; n = 3 for KS IFN-γ + Bet; n = 2 for KS IFN-γ and KS Amb. **IFN-γ**: control and KS n = 4, KS Bet and KS IFN-γ + Bet n = 3; n = 2 for KS IFN-γ and n = 1 for KS Amb. **IL-4:** control and KS n = 3, KS Bet and KS IFN-γ + Bet n = 3; n = 2 for KS IFN-γ and n = 1 for KS Amb. **IL-17**: control and KS n = 2, KS Bet n = 0 and n = 1 for KS IFN-γ + Bet, KS IFN-γ and KS Amb. **IL-22:** control n = 4, KS and KS IFN-γ + Bet and KS Bet n = 3; n = 2 for KS Amb KS IFN-γ.

Corresponding figure in the main mansuscript: Figure 24

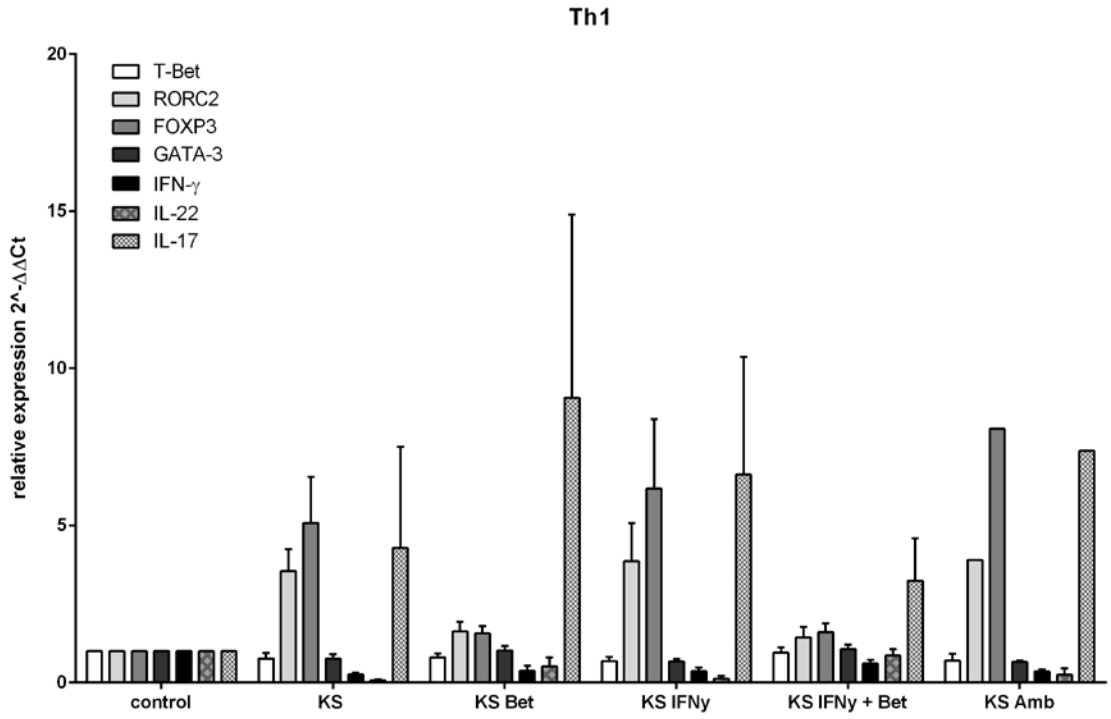


Figure A 6: Effects of keratinocytes derived mediators on the mRNA expression of Th0 cells. Th0 cells generated in 48 well plates were incubated for 72 h with supernatants derived from steady state keratinocytes (KS), Bet APE treated keratinocytes (KS Bet) or IFN-γ (300 U/ml) pre-stimulated keratinocytes (KS IFN-γ) alone or in combination with Bet APE treatment (KS IFN-γ + Bet), or Amb APE treated keratinocytes (KS Amb). T cell mRNA expression was analysed by qPCR. Data is normalized to house-keeping gene EF1 and to medium control by 2^(-ΔΔC_T) method and shown ± SEM. n numbers for **T-bet, GATA-3, Foxp3, IFN- γ and IL-22** are n = 4 for control, KS, KS Bet, KS IFN-γ and KS IFN-γ + Bet and n = 2 for KS Amb. **RORC2**: control, KS and KS Bet, KS IFN-γ and KS IFN-γ + Bet n = 3 and n = 1 for KS Amb. **IL-17**: control, KS, KS Bet, KS IFN-γ and KS IFN-γ + Bet n = 2 and n = 1 for KS Amb.

Corresponding figure in the main mansuscript: Figure 25

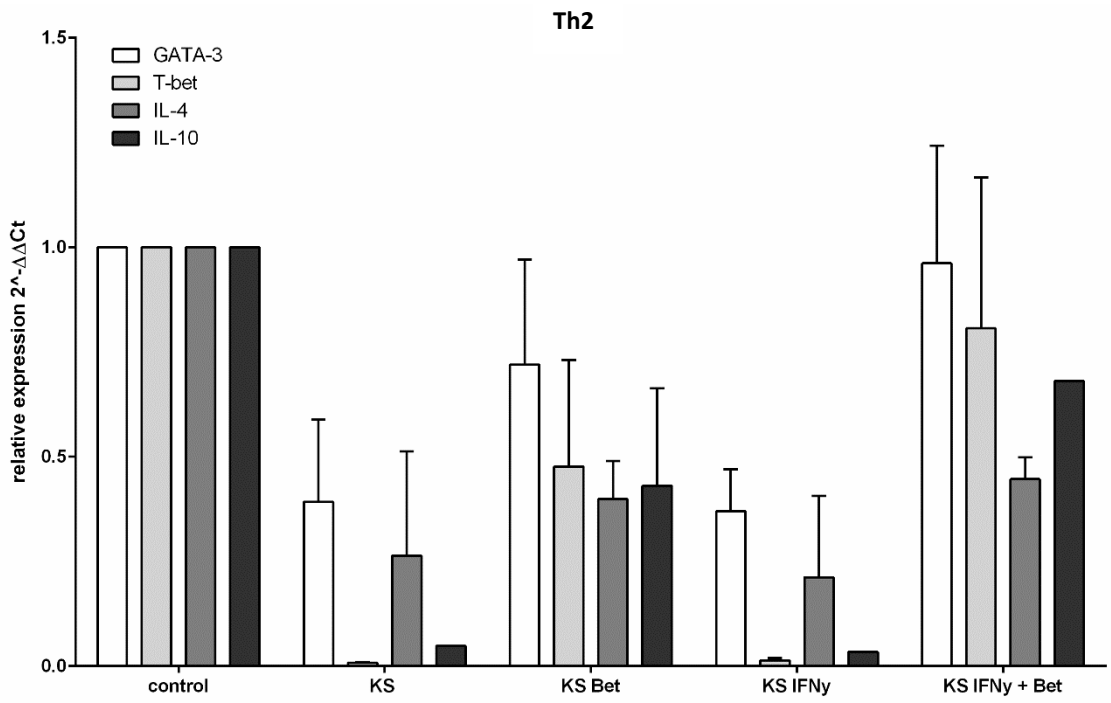


Figure A 7: Effects of keratinocytes derived mediators on the mRNA expression of Th2 cells. Th2 cells generated in 48 well plates were incubated for 72 h with supernatants derived from steady state keratinocytes (KS), Bet APE treated keratinocytes (KS Bet) or IFN-γ (300 U/ml) pre-stimulated keratinocytes (KS IFN-γ) alone or in combination with Bet APE treatment (KS IFN-γ + Bet). T cell mRNA expression was analysed by qPCR. Data is normalized to house-keeping gene EF1 and to medium control by $2^{(-\Delta\Delta C_T)}$ method and shown ± SEM. n numbers for GATA-3, T-bet and IL-4 are n = 4 for control and KS Bet; n = 3 for KS IFN-γ + Bet and n = 2 for KS and KS IFN-γ. For IL-10: n = 3 for control and KS Bet and n = 1 for KS, KS IFN-γ and KS IFN-γ + Bet.

Corresponding figure in the main mansuscript: Figure 26

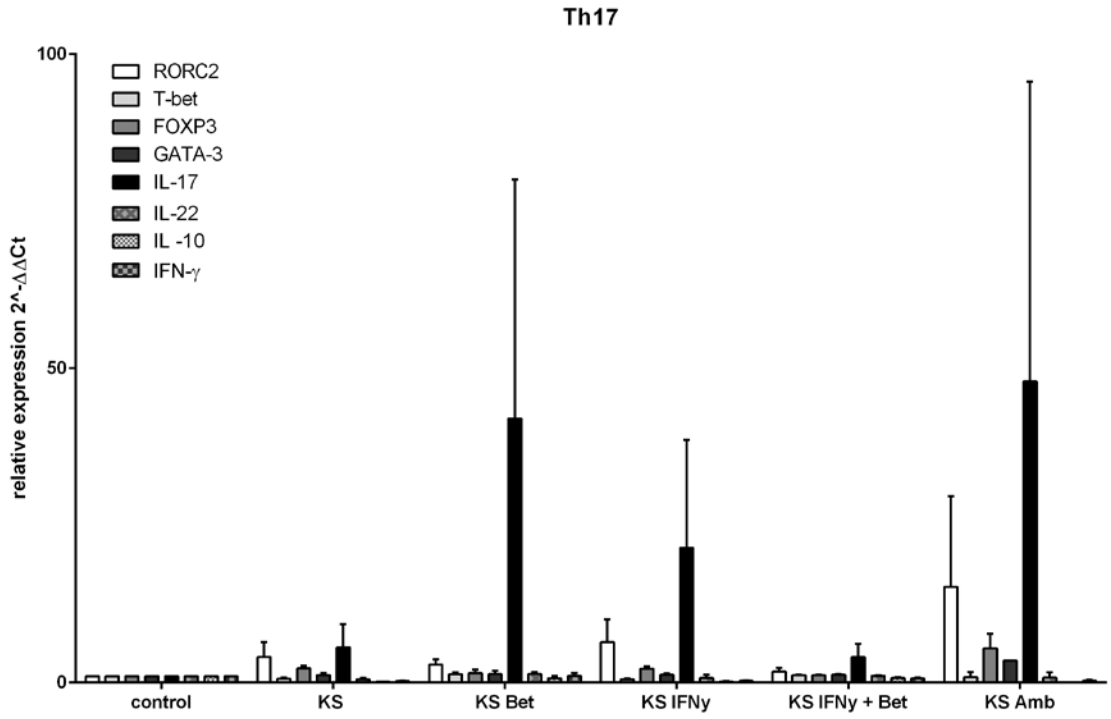


Figure A 8: Effects of keratinocytes derived mediators on the mRNA expression of Th17 cells. Th17 cells generated in 48 well plates were incubated for 72 h with supernatants derived from steady state keratinocytes (KS), Bet APE treated keratinocytes (KS Bet) or IFN-γ (300 U/ml) pre-stimulated keratinocytes (KS IFN-γ) alone or in combination with Bet APE treatment (KS IFN-γ + Bet), or Amb APE treated keratinocytes (KS Amb). T cell mRNA expression was analysed by qPCR. Data is normalized to house-keeping gene EF1 and to medium control by $2^{(-\Delta\Delta C_T)}$ method and shown ± SEM. n numbers for RORC2, Foxp3, T-bet, IL-17 and IL-22 are n = 4 for control, KS, KS Bet, KS IFN-γ and KS IFN-γ + Bet and n = 2 for KS Amb. For GATA-3, IFN-γ and IL-10: control, KS, KS Bet, KS IFN-γ and KS IFN-γ + Bet n = 4 and n = 1 for KS Amb.

Corresponding figure in the main mansuscript: Figure 27

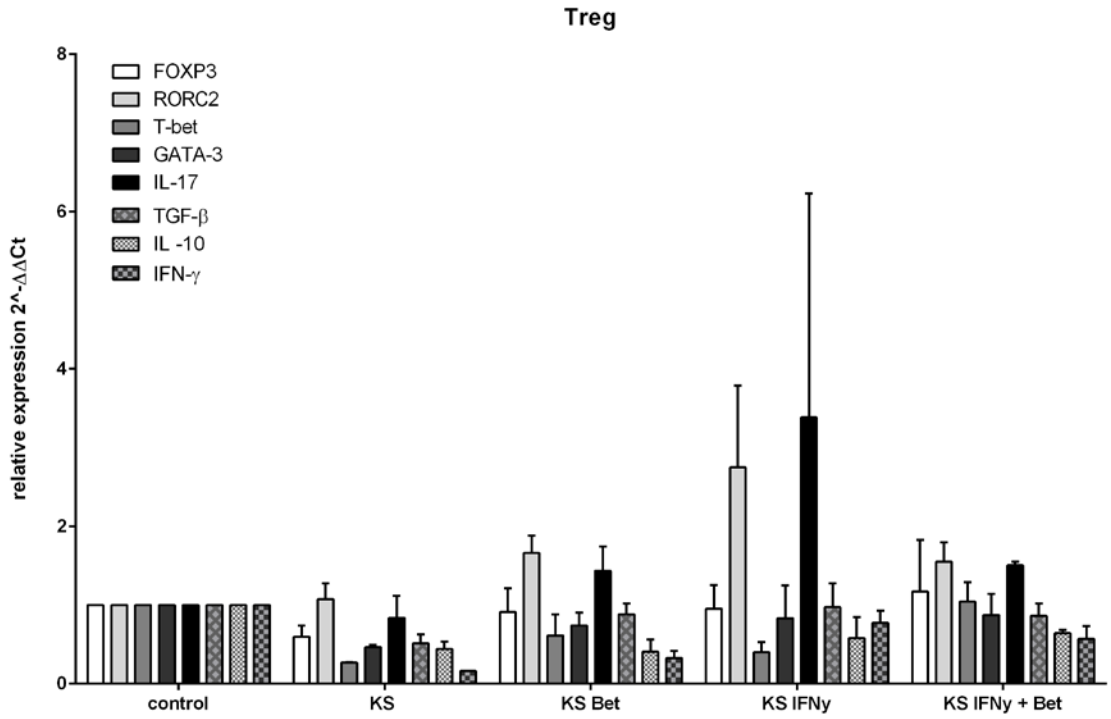


Figure A 9: Effects of keratinocytes derived mediators on the mRNA expression of Treg cells. Treg cells generated in 48 well plates were incubated for 72 h with supernatants derived from steady state keratinocytes (KS), Bet APE treated keratinocytes (KS Bet) or IFN-γ (300 U/ml) pre-stimulated keratinocytes (KS IFN-γ) alone or in combination with Bet APE treatment (KS IFN-γ + Bet). T cell mRNA expression was analysed by qPCR. Data is normalized to house-keeping gene EF1 and to medium control by $2^{(-\Delta\Delta C_T)}$ method and shown ± SEM. n numbers for Foxp3, RORC2, TGF-β and IL-17 are n = 3. For T-bet, GATA-3, IL-10 and IFN-γ: control and KS Bet n = 3 and n = 2 for KS and KS IFN-γ.