

THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

Investigation of the Role of Endocannabinoid Tone in the
Biology of Selected Non-Neuronal Skin Cells

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LIST OF ABBREVIATIONS

AA	Arachidonic acid	DAGL	Diacylglycerol lipase (2-AG synthesizing enzyme)
2-AG	2-arachidonoylglycerol (one of the most studied endocannabinoids)	DAMP	Damage-associated molecular pattern
ACEA	Arachidonyl-2'-chloroethylamide (synthetic cannabinoid)	DAPI	4',6-diamidino-2-phenylindole-dihydrochlorid (nuclear counter stain)
AD	Atopic dermatitis	eCB	Endocannabinoid
AEA	N-arachidonylethanolamine ("anandamide"- one of the most studied endocannabinoids)	ECS	Endocannabinoid system
		EGF	Epidermal growth factor
		EMT	Endocannabinoid membrane transporter
AM251	1-(2,4-Dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-1-piperidinyl-1H-pyrazole-3-carboxamide (CB ₁ inverse agonist)	FAAH	Fatty Acid Amide Hydrolase (endocannabinoid degrading enzyme)
		FBS	Fetal bovine serum
AML	Antimicrobial lipid	FcεRI	High-affinity IgE receptor (receptor for the Fc region of IgE, constitutively expressed on MCs)
AMP	Antimicrobial peptide		
cAMP	Cyclic adenosine monophosphate	FITC	Fluorescein isothiocyanate
CB₁	Cannabinoid receptor type 1	HDF	Human dermal fibroblast
CB₂	Cannabinoid receptor type 2	GPCR	G protein-coupled receptor
CBD	(-)-cannabidiol	HF	Hair follicle
CBC	(-)-cannabichromene	MC_T	Human mucosal-type mast cell
CBDV	(-)-cannabidivarin	Ig	Immunoglobulin E
CBG	(-)-cannabigerol	IL	Interleukin
CBGV	(-)-cannabigerovarin	KC	Keratinocyte
CBN	Cannabinol	KO	Knockout
CD	Cluster of differentiation	LOX	Lipoxygenase
c-Kit	Mast cell growth factor receptor, also known as CD117	LPS	Lipopolysaccharide
CLR	C-type lectin receptor	LTA	Lipoteichoic acid
CMF	Calcium and magnesium free	MA	Methanandamide (synthetically created FAAH-resistant chiral analog of anandamide)
COX-2	Cyclooxygenase-2	MAGL	Monoacylglycerol lipase
CRH	Corticotropin-releasing hormone	MAPK	Mitogen-activated protein kinase
CTS	Connective tissue sheath	MC	Mast cell
CYP	Cytochrome enzyme P450	NAPE	N-acyl phosphatidylethanolamine (the main precursor of AEA)
DAB	3,3'-diaminobenzidine		
DAG	Diacylglycerol		

NAPE-PLD	N-acyl phosphatidylethanolamine specific phospholipase D (can catalyze the synthesis of AEA)	SCF	Stem cell factor (a cytokine, which acts as the endogenous activator of c-Kit/CD117)
NAT	N-acyltransferase	SCR	Scrambled oligos (negative control of siRNA)
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells complex (transcription factor that plays a key role in regulating inflammation)	SG	Sebaceous gland
NGF	Nerve growth factor	SLP	Sebaceous lipid synthesis
NHEK	Primary human epidermal keratinocytes	SP	Substance P
NLR	NOD-like receptor	SR141716	Rimonabant (selective CB ₁ receptor blocker)
NOS	Inducible nitric oxide synthase	TdT	Terminal deoxynucleotidyl transferase
OEA	Oleoylethanolamide (monounsaturated analogue of AEA)	TH	T helper cell
ORS	Outer root sheath (covers the inner root sheath and hair shaft of the hair follicle)	THC	(-)- Δ^9 - <i>trans</i> -Tetrahydrocannabinol (the principal psychoactive constituent of <i>Cannabis sativa</i>)
PAMP	Pathogen-associated molecular pattern	THCV	(-)- Δ^9 -tetrahydro-cannabivarin
PBS	Phosphate-buffered saline	TLR	Toll-like receptor (class of proteins that play a key role in the innate immune system)
PEA	Palmitoylethanolamide (“endocannabinoid-related” substance)	TNB	Tris-NaCl-blocking buffer
PPAR	Peroxisome proliferator-activated receptor	TNFα	Tumor necrosis factor alpha (inflammatory cytokine)
PPRE	PPAR response elements	TNT	Tris-NaCl-Tween buffer
PRR	Pattern recognition receptors	TRI	Total RNA isolation reagent
RT	Room temperature	TRIB3	Tribbles homolog 3
RLR	Retinoic acid-inducible -I-like receptor	TRP	Transient receptor potential ion channel superfamily
RXR	Retinoid X receptor (a type of nuclear receptor that is activated by 9-cis retinoic acid)	TSA	Tyramide signal amplification

1. INTRODUCTION

In the last few years, the endocannabinoid system (ECS) has emerged as an important regulator of human skin biology. It has been shown that various cellular components of the skin can express different endocannabinoid (eCB) ligands, receptors, and enzymes that play a significant role in the maintenance of skin homeostasis (Bíró et al., 2009; Pucci et al., 2011; Oddi and Maccarrone, 2014; Oláh et al., 2017).

Our work group has also been actively studying the functions of this cutaneous ECS: from its different members within various components of human skin to the associated molecular pathways (Telek et al., 2007; Czifra et al., 2012, Oláh et al., 2014; 2016a; 2016b). One of our main research interests is how the functional ECS influences the physiology of different cutaneous non-neuronal cell types in health and disease.

We have provided the first evidence that functionally active ECS elements can be found on human sebocytes and sebaceous lipid production (SLP) of these cells can be regulated by the administration of certain eCBs (Dobrosi et al., 2008; Oláh et al., 2014; 2016b; 2017). However, there was no information about the expression of the enzyme apparatus involved in the metabolism of these eCBs, or about the role of the local eCB tone created by these enzymes.

Recently, ECS was also shown to be potential regulator of mast cell's (MCs) (Cerrato et al., 2010; Cantarella et al., 2011; De Filippis et al., 2013), but its exact role in the biological processes of these cells still has to be clarified. This is especially true for primary human MCs, more specifically, for human skin MCs *in situ*.

Therefore, within the confines of this thesis we investigate the role of eCB tone in the regulation of the above mentioned two important non-neuronal cells of the skin, namely the sebocytes and MCs.

2. OVERVIEW OF LITERATURE

2.1 Key aspects of the biology of the integumentary system

Human skin is one of the largest organs of the body with its 1.8 m² (average adult). It serves not only as a well-structured protection shield against various external agents, but it also plays a crucial role in thermoregulation and water balance (Matsui et al., 2015; Varkey et al., 2015). Furthermore, it produces several hormones (e.g., vitamin D; Nejati et al., 2013), stores a variety of substances (e.g., lipids), regenerates its integrity during wound healing (Evans et al., 2013; Kezic and Jakasa, 2016), and also allows to detect temperature, pressure, touch, vibration, and pain (Arda et al., 2014).

2.1.1 Skin histology

To carry out all aforementioned tasks, human skin has a multilayer structure that consists of three major parts: epidermis, dermis, and subcutis (**Fig. 1A**; McLafferty et al., 2012; Wong et al., 2016).

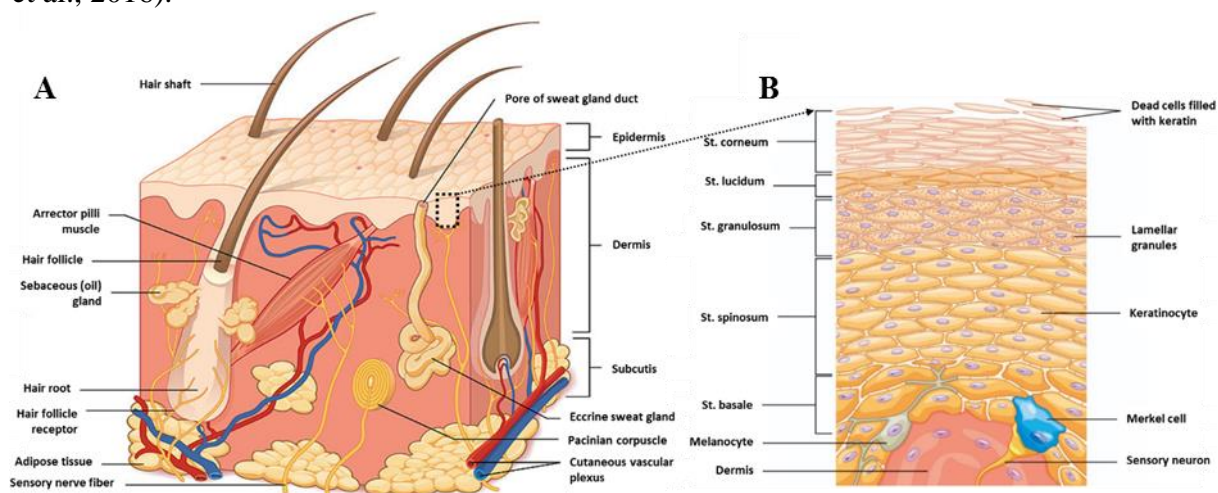


Fig. 1: Human skin histology

(A) Structure of human skin and (B) layers of epidermis.

(adapted from: https://upload.wikimedia.org/wikipedia/commons/4/4f/501_Structure_of_the_skin.jpg and https://en.wikipedia.org/wiki/File:502_Layers_of_epidermis.jpg, accessed at: 16.01.2017)

Epidermis. The outermost layer, i.e. epidermis is the main element of physical defense. Depending on its anatomical location, it is composed by up to five layers named *stratum basale*, *stratum spinosum*, *stratum granulosum*, *stratum lucidum*, and *stratum corneum* (**Fig. 1B**; Arda

et al., 2014; Matsui and Amagai, 2015). Predominant resident cells in these layers are the keratinocytes (KCs). These cells go through a well-determined and highly Ca^{2+} -dependent differentiation and keratinization process while moving suprabasally, until they are finally shed from the surface (Arda et al., 2014).

In addition to KCs, the epidermis also hosts a number of other cells: melanocytes produce the primary pigment called melanin, Langerhans cells, which are a type of dendritic cell, and Merkel cells which serve as mechanoreceptors (Moll et al., 2005; Cichorek et al., 2013).

Dermis. Directly beneath the *stratum basale* lies the dermis (**Fig. 1A**) with its two layers: the superficial one called the papillary region under which is the reticular dermis. The whole dermis is rich in different elastin and collagen fibers, providing flexibility to the entire organ. Most of the skin-associated appendages such as hair follicles (HFs), sebaceous- (SGs) and sweat glands are located in the dermis (Montagna et al., 1952; Hussain et al., 2013).

Subcutis. The lowermost layer of the skin is the subcutis (**Fig. 1A**), also known as hypodermis, composed mainly of adipocytes, fibroblasts, different fibers, nerves, blood, and lymphatic vessels. The thickness of the subcutis varies among individuals, but its main task remains the same: insulating the body against temperature changes (Arda et al., 2014).

2.1.2 Skin as a barrier

Skin implements its multiple tasks by acting as a physical, chemical and biological barrier (Proksch et al., 2008; Natsuga et al., 2014).

Physical barrier. Major part of the skin's physical barrier resides in the epidermis, more specifically in the *stratum corneum* (**Fig. 1B**). This layer consists of corneocytes that are tightly surrounded by extracellular lipid matrix (Candi et al., 2005; de Koning et al., 2012). Corneocytes are the final result of the differentiation of KCs, also called cornification. During this process their plasma membrane undergoes a complete transformation, resulting in a special

insoluble structure – the so-called cornified envelope (Candi et al., 2005; de Koning et al., 2012; Eckhart et al., 2013; van Shemden and Bouwstra, 2016).

Lipid regions of *stratum corneum* contain three main lipid classes: cholesterol, free fatty acids, and ceramides. They form well-structured layers (lamellae) consisting of repeating lamellar and lateral phases. In this way, *stratum corneum* is able to regulate water diffusion, as well as the permeation of chemicals and toxins (Cui and Schlessinger, 2015; van Smeden and Bouwstra, 2016; Brander, 2016). This protective lipid layer is complete with the oily, waxy product of SGs (sebum) that is excreted to the skin surface (Pappas, 2009; De Luca and Valacchi, 2010).

Chemical barrier. Even though bacteria, fungi, and viruses pose a constant danger to the human body, they are rarely able to cause infections thanks to the chemical barrier of the skin. As part of this natural shield, several antimicrobial peptides (AMPs) and antimicrobial lipids (AMLs) – exerting broad antimicrobial effects – are produced. These bioactive compounds are able to kill pathogens in a relatively short period of time, for example, by attacking their membrane integrity or by disrupting the synthesis of their nucleic acids (Mendez-Vilas et al., 2013; Cui et al., 2015; Clausen and Agner, 2016).

Biological barrier. Not all microbes are harmful: many of them are advantageous, and live on the skin surface, providing a microbial barrier. These fungal (e.g., *Malassezia spp*) and bacterial (e.g., *Staphylococcus epidermidis*) microorganisms are able to take actions against pathogens, which represent a major threat for the human body (Littman and Pamer, 2011; Grice and Segre, 2011; Gallo and Nakatsuji, 2011).

2.1.3 Biology of sebaceous glands (SGs)

Role of SGs are often underappreciated with respect to skin homeostasis and immunity. The vast majority of research studies have exclusively addressed their ability to produce lipids, since latter has a crucial role in the pathogenesis of acne – one of the most common chronic skin diseases (Kurokawa et al., 2009; Das and Reynolds, 2014; Moradi Tuchayi et al., 2015).

Besides the lipid production, SGs are also important in stem cell biology, cellular differentiation, skin aging, and inflammatory skin processes (Hinde et al., 2013; Zouboulis et al., 2014).

Histology of SGs. SGs (**Fig. 2A-B**) can be found throughout the whole skin surface except the palms of the hands and soles of the feet. These exocrine glands can be located independently (e.g., in the eyelids and lips), but are most often associated with HFs. The latter, together with the arrector pili muscles and the SGs, form the so-called pilosebaceous unit (Makrantonaki et al., 2011; Hinde et al., 2013).

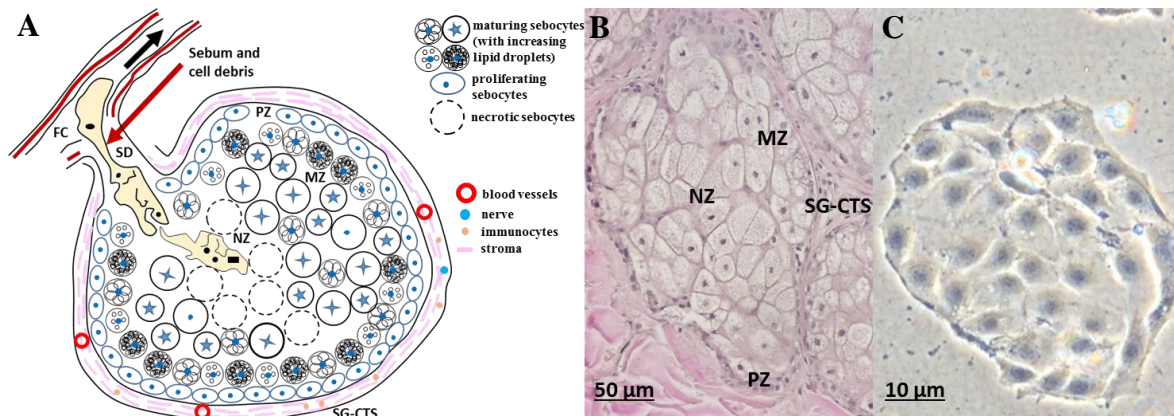


Fig. 2: Histology of human SG and SZ95 cells

(A) Schematic representation of a SG lobe (based on Hinde et al., 2013 and Turksen, 2015). (B) Image of hematoxylin and eosin stained human SGs (own figure). (C) Light microscopy image of SZ95 sebocytes (taken by Nóra Dobrosi). FC: follicular canal, MZ: maturation zone, NZ: necrosis zone, PZ: peripheral zone, SD: sebaceous duct, SG-CTS: sebaceous gland connective tissue sheath, black arrow: direction of sebum flow from sebaceous duct to skin surface.

SGs are surrounded by a dense stroma that is rich in collagen and fibroblasts. Most SGs consist of a series of lobes (acinar structure), which are connected to a main duct that is directly linked to a follicular canal. Within a SG lobe (**Fig. 2A-B**), highly proliferative, undifferentiated sebocytes typically lie along the peripheral layer. Those cells that are detached from the aforementioned layer move towards the center, while going through a well-regulated differentiation process (maturation zone). When reaching the center, the nuclei of the cells start to shrink, until they become completely disintegrated and their lipid droplets released (degeneration zone). The entire process is called holocrine secretion, at the end of which the

secretory product contains not only lipids, but also cell debris (Makrantonaki et al., 2011; Hinde et al., 2013; Fischer et al., 2017).

Sebum production and its function. One of the most important tasks of the sebocytes is to continuously produce sebum in order to lubricate the skin surface. Sebum of sebocytes mainly contains neutral lipids (di- and triglycerides, free fatty acids, cholesterol esters, cholesterol) and some special components such as squalene and wax esters (Zouboulis et al., 2014). The composition of sebum differs based on the age, and whether the SGs are healthy or affected by a disease. As this oily substance is secreted, it moves through a tubular system into the infundibular part of the HFs that is connected and opened to the external surface of the body (**Fig. 2A**). Functions of SGs are well-controlled by several factors (e.g., sex steroids, growth factors, and neuropeptides), just like the composition of sebum (e.g., by peroxisome proliferator-activated receptors [PPARs], histamine, etc.) (Thiboutot, 2004; Tóth et al., 2011b; Zouboulis et al., 2014; Zouboulis et al., 2016, Szöllősi et al., 2017).

When physiological secretion of sebum does not work properly (under or over production), diseases such as common acne, seborrhea, and dry skin syndrome can develop (Tóth et al., 2011b; Hinde et al., 2013; Moradi Tuchayi et al., 2016). Moreover, the specific composition of sebum is also able to regulate the diversity patterns of cutaneous microbiota. Alteration of this microbial community may contribute to the pathogenesis of several skin disorders, including atopic dermatitis (AD) (De Luca and Valacchi, 2010; Grice, 2014).

Immunological role of SGs. It is assumed that SGs are also actively involved in the processes of immunological host defense. Sebocytes are armed with pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs), cluster of differentiation (CD) 1, and CD14, which can recognize several dangerous pathogens. When stress factors are detected, the expression of pro-inflammatory cytokines, as well as the level of neuropeptides increase in the sebocytes, leading to inflammation. In addition, these multifaceted cells produce several AMPs (e.g., β -defensins, cathelicidin) and AMLs (e.g., monounsaturated fatty acids) further

supporting the physical and immunological barrier functions of the skin (Makrantonaki et al., 2011; Zouboulis and Makrantonaki, 2014).

Pathophysiology of SGs. Diseases affecting SGs are common dermatological disorders such as acne, seborrhea, and rosacea, which have all a high occurrence within the general population (Zouboulis et al., 2014; Piraccini and Alessandrini, 2014). Among them, acne is the most frequent, which affects approximately 85% of young adults aged 12–25 years (Lynn et al., 2016). The following multiple factors result in its development: increased sebum production, androgen activity, inflammation, follicular hyperkeratinization, and the action of *Cutibacterium* (formerly called *Propioibacterium*) acnes within the sebaceous follicle (Zouboulis et al., 2014).

Unfortunately, present therapeutic treatments of illnesses associated with SG disorders face various obstacles: most of them cause various systematic side effects (e.g., itch, hepatic impairment, and anemia) and can disrupt the homeostatic function of SGs (Tripathi et al., 2013; Zouboulis et al., 2014). Therefore clinical medicine has a great demand for therapies that can supply better solutions to these problems.

Available SG model systems. For better understanding the pathophysiology of SG-associated disorders and their multiple factors, the development of experimental SG models is needed. Unfortunately, complex culture systems that would provide a way to examine the functions of intact SGs within their natural tissue environment are still not available (Zouboulis et al., 2014; Szöllősi et al., 2017). So far, to study the biology of SGs, mostly *in vitro* cell lines have been used. Even though the primary cell lines show natural sebocyte characteristics, they also face some major limitations, such as short sustainability (from 3 to 6 passages) and a need for large number of donors. One solution for overcoming these challenges was to create immortalized sebocyte cell lines, such as SZ95, SEB-1, and Seb-E6E7 (Zouboulis et al., 1999; Thiboutot et al., 2003; Lo Celso et al., 2008).

SZ95 cell line. Among the immortalized sebocyte cell lines, the first and the best characterized one is the SZ95 (**Fig. 2C**). It was created by the group of Zouboulis by transfecting facial sebocytes with SV-40 large T antigen (Zouboulis et al., 1999). Compared to normal human sebocytes, SZ95 cells show similar phenotyp, as well as functional characteristics, and are proven to be stable even at high passages. Furthermore, SZ95 sebocytes go through a differentiation process, during which, they are able to accumulate lipid droplets. Important differentiation markers such as keratin 7 and specific proteins are also well-detectable (Xia et al., 2009). This cell line has been used for many years in our research group to investigate the biology of SGs (Dobrosi et al., 2008; Tóth et al., 2011b; Géczy et al., 2012; Oláh et al., 2014, 2016b).

2.1.4 Skin as an immune organ

In addition to the protective barrier functions, defending the host against various threats, skin provides a massive immune surveillance function consisting of two main sub-systems: the innate and the adaptive immunity (Kupper and Fuhlbrigge, 2004; Nestle et al., 2009; Bangert et al., 2011).

Innate immunity primarily provides a fast, but non-specific and rather short-term protection. This in-born system consists of numerous skin sentinels, such as epidermal predominant resident- (e.g., KCs and sebocytes) and innate immune cells (e.g., Langerhans cells, macrophages, mast cells [MCs] and dendritic cells) (Nestle et al., 2009; Bangert et al., 2011; Pasparakis et al., 2014). Most of these cells express several PRRs, with which they can recognize pathogen- (PAMPs), and damage-associated molecular patterns (DAMPs). TLRs, C-type lectin (CLRs), NOD-like (NLRs), and retinoic acid-inducible gene-I-like (RLRs) receptors are well-known sub-groups of PRRs (de Koenig et al., 2010; Suresh and Moser, 2013; Skabytska et al., 2014).

Compared to innate immunity, the responses of adaptive immune system are slower, but more efficient and more specific. Its most important tasks are (i) to distinguish the self and non-

self-antigens (ii) to develop such responses that will remove pathogens or pathogen-infected cells, (iii) and to create immunological memory, which helps to recognize pathogens, which have already been encountered once earlier and faster (Nestle et al., 2009; Bangert et al., 2011; Panelius and Meri, 2015).

2.1.5 Biology of cutaneous mast cells (MCs)

MCs are one of the most important effector cells of cutaneous immune system. Within the skin, they play a pivotal role in many physiological functions such as host defense, wound healing, tissue remodeling, and hair growth. MCs participate in development of several diseases that are mostly associated with allergic and cutaneous inflammatory actions (Navi et al., 2007; Metz et al., 2008; Kennelly et al., 2011).

Origin and development. After Paul Ehrlich first described MCs in 1878 (Ehrlich, 1878) these immune cells were only thought to be a basic component of the connective tissue for nearly a century (Ghably et al., 2015). Later on, it became clear that MCs are bone-marrow derived cells, which develop from pluripotent hematopoietic cells (CD34+), the same precursors of megakaryocytes, erythrocytes, and myeloblasts.

After leaving the bone marrow, immature MCs circulate in the vascular system. Due to chemotactic signals they predominantly migrate into connective or mucosal tissues, where – due to the specific local tissue milieu and to the presence of certain factors (e.g., stem cell factor [SCF], neural growth factor [NGF], and interleukins [IL]) – they complete their maturation process (Galli et al., 2008; Krystel-Whittemore et al., 2016). Indeed, binding of SCF to its receptor, i.e. c-Kit (also known as CD117), is a key stimulus of MC proliferation, differentiation, maturation, and survival. In addition, it can also increase certain MC responses such as secretion and release of mediators (Ito et al., 2012; Dahlin and Hallgren, 2015).

It is also important to point out that undifferentiated MC progenitors can also be deposited in tissues, namely the stroma of rodent and human HFs, more specifically within their connective tissue sheath (CTS). From these precursors, mature MCs can also be generated *in*

situ in the absence of bone marrow (Kumamoto et al., 2003; Ito et al., 2010). CTS-MCs have a major role in the regulation of hair cycle (Paus et al., 2008; Liu et al., 2013).

Classification and physiology. MCs have a characteristic appearance thanks to their high number of metachromatic granules (50-200/cell), which contain various mediators (Wernersson and Pejler, 2014; Krystel-Whittemore et al., 2016). Based on the neutral protease composition and/or anatomical location, two subsets of MCs can be distinguished in both humans and mice (**Fig. 3**): the mucosal types (MC_T or MMCs respectively) and the connective tissue specific ones (MC_{TC} or CTMCs respectively). MC_{TS} are mainly localized in the epithelium of the lung and in the gastrointestinal tract, and their granules mainly contain tryptase. MC_{TCS} such as cutaneous, peritoneal, and intestinal submucosal MCs, mostly release granules rich in chymase, carboxypeptidase, and a cathepsin G-like proteinase. Heparin is produced by all types, with the exception of MMCs (Galli et al., 2011; Krystel-Whittemore et al., 2016). Recently, a new type of MC classification has also been presented, according to which MCs are able to switch from a pro-inflammatory to an immunosuppressive phenotype (Harvima and Nilsson, 2011; Frenzel and Hermine, 2013; Chan et al., 2013).

Resting MCs have no or very little “basal” activity, but once activated, these distinctive granular cells are able to release mediators depending on the nature, and strength of the activation stimuli (**Fig. 3**, Galli et al., 2011; Metcalfe et al., 2016).

MCs are triggered by allergens, which are associated with immunoglobulin (Ig) E and bind to their high affinity IgE receptor (FcεRI). However, G protein-coupled receptors (GPCRs), as well as PRRs such as TLRs, and NLRs can also trigger the release of granules and strengthen specific immunological responses (Abraham and St. John, 2010; Galli et al., 2012; de Sousa Junior et al., 2015). The secretion of vesicles can also be caused by the products of the complement system (C3a and C5a), neuropeptides (substance P, [SP]), tumor necrosis factor alpha (TNFα), and SCF (Fureder et al., 1995; Huber et al., 2013).

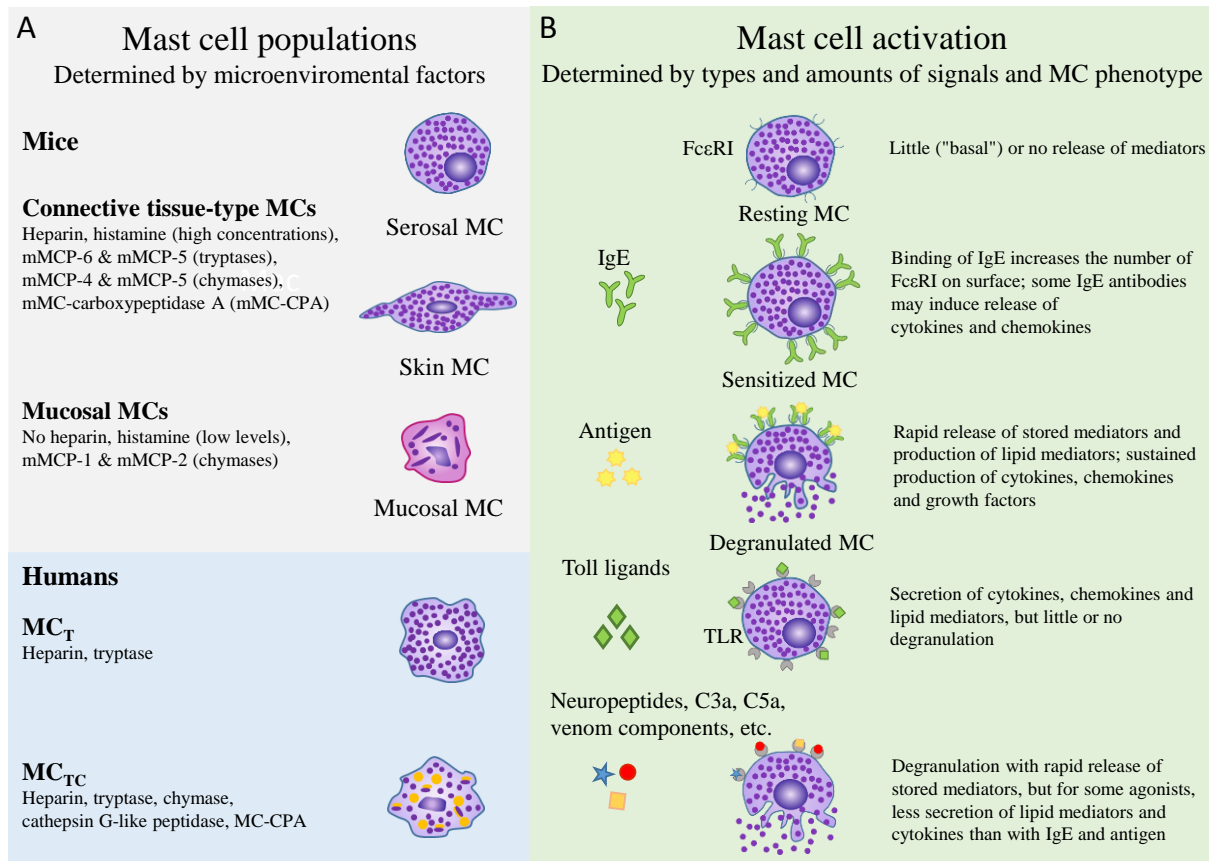


Fig. 3: Summary of MC subpopulations and the type of their activations

(A) Classification: MCs are heterogeneous in morphology and functions. They can be subcategorized into populations based on their anatomical location/and or the content of granules, both in humans (bottom left) and mice (top left). (B) Activation: Resting MCs can already release small amounts of different mediators (top right). The slight activation of the cells can be induced through their IgE receptors. When an antigen binds to these sensitized forms, MCs get immediately activated, and they rapidly release their stored mediators through exocytosis (degranulation). Furthermore, MCs express many surface receptors (bottom right) that can be triggered by signals that are independent from IgE. MC: mast cell, MC_T: mast cell containing mainly tryptase, MC_{TC}: mast cell containing both tryptase and chymase, MCP: different fractions of mice MC proteases, FcεRI: high-affinity IgE receptor, IgE: immunoglobulin E, TLR: Toll-like receptor (modified from Galli et al., 2011).

Activation of the cells can induce regulated exocytosis (degranulation) of pre-formed mediators (i.e., biogenic amines, serine and other proteases, proteoglycans, cytokines, lysosomal enzymes, and growth factors) and constitutive exocytosis of *de novo* synthesized substances (i.e., inducible nitric oxide synthase [NOS], phospholipid metabolites, and prostaglandin D2, **Fig. 4**).

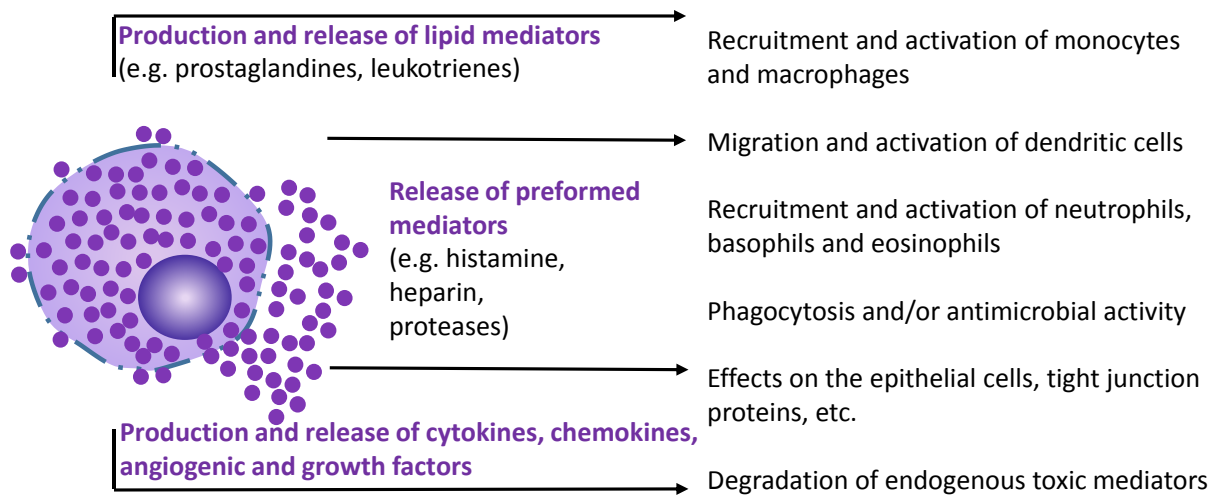


Fig. 4: Effects of MC degranulation

Preformed and *de novo* synthesized MC mediators regulate various physiological processes (modified from Metz and Maurer, 2009).

Short-term responses associated with MC degranulation include the activation of rapid anaphylactic and allergic reactions, as well as the activation of innate immune and inflammatory responses (i.e., recruitment of leukocytes to sites of infection, cytokine upregulation, and vasodilatation). Long-term responses, instead, involve primarily wound repair and tissue remodeling (Abraham and St. John, 2010; Galli et al., 2011; Urb and Sheppard, 2012; da Silva et al., 2014).

Experimental examination of MCs. Isolation and maintenance of MCs have been challenging for several reasons (Kulka and Metcalfe, 2010; Radinger et al., 2010). Firstly, there are only a low number of MCs in their resident tissues, and it is hence relatively difficult to obtain them in reasonably large quantities. Secondly, methods with multiple purification processes can act as serious stress resulting in a poor yield and sometimes altered properties of the cells. Finally, the maturation process of MCs is strictly dependent on their local tissue milieu (Rao and Brown, 2008; Stone et al., 2010; Gilfillan et al., 2011). Thus, the reproduction of these conditions is also critical.

Most of the MC studies have been carried out on cell lines and models of rodent origin. Most widely used primary MC cultures are mouse and rat bone marrow-derived cells, since they are easy and cost-effective to culture (Jensen et al., 2006; Meurer et al., 2016). Mouse bone

marrow-derived cells represent a more “mucosal-like” phenotype. Therefore, researchers have developed a technique in which mouse peritoneal cell-derived MCs are cultured in the presence of recombinant murine-SCF. This resulted in serosal-type mouse MCs that respond to IgG dependent signals through their Fc-epsilon receptor IIIA (FcεRIIIA or CD16a) (Malbec et al., 2007; Kovarova, 2013). Rat basophilic leukemia cells (RBL-2H3) are also available. These cells are frequently applied because they are physiologically similar to MCs, and have high stability in tissue culture (Passante et al., 2009; Passante and Frankish, 2009).

Most of the human MC experiments were performed by using *in vitro* derived MCs, from normal hematopoietic progenitors (CD34+ or CD133+ cells), purified from umbilical cord or peripheral blood. Cord blood derived cells are relatively immature and are characterized by segmented nucleus and lower number of granules (Moon et al., 2003; Theoharides et al., 2006), compared to the relatively matured peripheral blood types (Saito et al., 2006; Bandara et al., 2015). Currently, there are four human MC lines available: HMC-1, LAD-1, LAD-2 and LUVA cells. Despite the fact that HMC-1 cells do not express FcεRI, and contain no chymase and little tryptase, they are the only one that grow continuously, therefore they are frequently used (Nilsson et al., 1994; Guhl et al., 2010). LAD-1 and LAD-2 are leukemia–lymphoma cell lines, which have variable reproduction time, their phenotypic properties are not stable in culture, but they are FcεRI+/c-Kit+ and can release β-hexosaminidase (Drexler and MacLeod, 2003; Kirshenbaum et al., 2003; Guhl et al., 2010). LUVA cells have metachromatic granules that contain tryptase, but they are growth factor independent, and in long-term culture the cells lose their FcεRI positivity (Laidlaw et al., 2011).

Control of MC activation. As previously mentioned, the activation of MCs is primarily important because it initiates the process of inflammation, through which the homeostatic balance can be maintained. The inappropriate operation of MCs, such as the excessive degranulation and increase in the number of cells, may often be the cause of several unwanted symptoms, such as itch, flush, and even anaphylaxis (Molderings et al., 2011; Frieri et al.,

2013). Nowadays, the incidence of the inflammatory and allergic diseases that are associated with MC dysregulation (i.e., AD, allergic asthma, and rheumatoid arthritis) are continuously increasing and represent a major challenge (Marshall, 2004; Stone et al., 2010; Galli et al., 2008). For this reason, much effort has been spent in exploring how to prevent and/or control the pathological actions of these immune cells. To date, several pharmacons have been developed against excessive MC activation and mediator secretion, e.g., H1 histamine receptor antagonists, membrane stabilizers (e.g., cromolyn and ketotifen), protease and chymase inhibitors (e.g., the tryptase inhibitor APC 2059), as well as drugs targeting SCF and/or c-Kit (e.g., anti-SCF antibodies) (Jensen et al., 2007; Finn and Walsh, 2013; Molderings et al., 2016).

The endogenous controls that limit excessive activation and numbers of MCs within healthy human tissues have been studied much less, thus their identification remains a major challenge for translational MC medicine. The behavior of MCs is tightly controlled by those regulatory cues that emanate from their local tissue environment. Under cell culture conditions these microenvironmental factors are missing. Therefore, human MCs are best analyzed under *in situ* conditions. In human system, MC-rich human skin is particularly suitable for such investigations (Weber et al., 2003) that has recently become a relatively easily accessible organ, especially as the “by-product” of routine plastic surgeries (e.g., facelift operations). As already mentioned, mature MCs can be differentiated from MC precursors, notably in the CTS of murine and human HFs, without the bone marrow being present. Thus, we hypothesized potent regulatory mechanisms that help to prevent the excessive increase in the number of MCs by controlling the intracutaneous maturation from the resident progenitors within the human skin, more specifically within the HFs’ CTS.

Psychological stress can trigger the hypothalamic–pituitary–adrenal axis and sensory nerves in the skin. This results in the release of neuroendocrine and neural mediators that are greatly involved in a number of MC functions such as immune defense, wound healing, and hair growth (Kumamoto et al., 2003; Arck et al., 2005; Rao and Brown, 2008; Harvima and

Nilsson, 2012). These factors (e.g., corticotropin-releasing hormone [CRH], neuropeptides, neurotrophins, and α -melanocyte-stimulating hormone) can lead to the activation of MCs, which then secrete such pro-inflammatory mediators that can stimulate sensory C-fibers. In addition, the maturation process of skin MCs, including CTS-MCs is also tightly regulated by stress-associated mediators such as CRH (Papadopoulon et al., 2005; Ito et al., 2010) and SP (Peters et al., 2007). Thus, the healthy, human adult scalp HF with its MC-rich CTS is a relevant human model system for studying primary skin MCs, their progenitors and endogenous controls within a precisely defined mesenchymal compartment *in situ*.

Recent research has revealed new opportunities regarding possible neuroendocrine controls of MCs. Among them, ECS seems to be a promising candidate, as it has been shown to possess anti-inflammatory attributes in both the human body and the skin (Karsak et al., 2007; Maccarrone et al., 2015; Oláh et al., 2017; Oláh and Bíró, 2017). Although there are already studies on ECS as a potential neuroendocrine regulator of these immune cells (De Filippis et al., 2008; Cantarella et al., 2011; Cerrato et al.; 2010), the exact function of this complex signaling system still remains unclear, especially with respect to the control of the human skin MCs.

2.2 Endocannabinoid system (ECS)

The plant *Cannabis sativa* has been used for medicinal and recreational purposes for thousands of years, but its main psychoactive constituent, the (-)-*trans*- Δ^9 -tetrahydrocannabinol (THC), was isolated only in the early '40s (Adams et al., 1940; **Fig. 5**). This was the first stepping stone in discovering that the human body is also able to produce cannabinoid-like compounds. These endocannabinoids (eCBs), together with their specific receptors and metabolic enzymes, form the so-called ECS. Over the past couple of decades, this complex lipid signaling network has emerged as one of the most remarkable regulatory systems involved in establishing and maintaining a variety of physiological processes, including among others appetite, pain sensation, and memory (Di Marzo, 2006; Mechoulam et al., 2014; Maccarrone et al., 2015; Oláh et al., 2017; Solymosi and Kőfalvi, 2017).

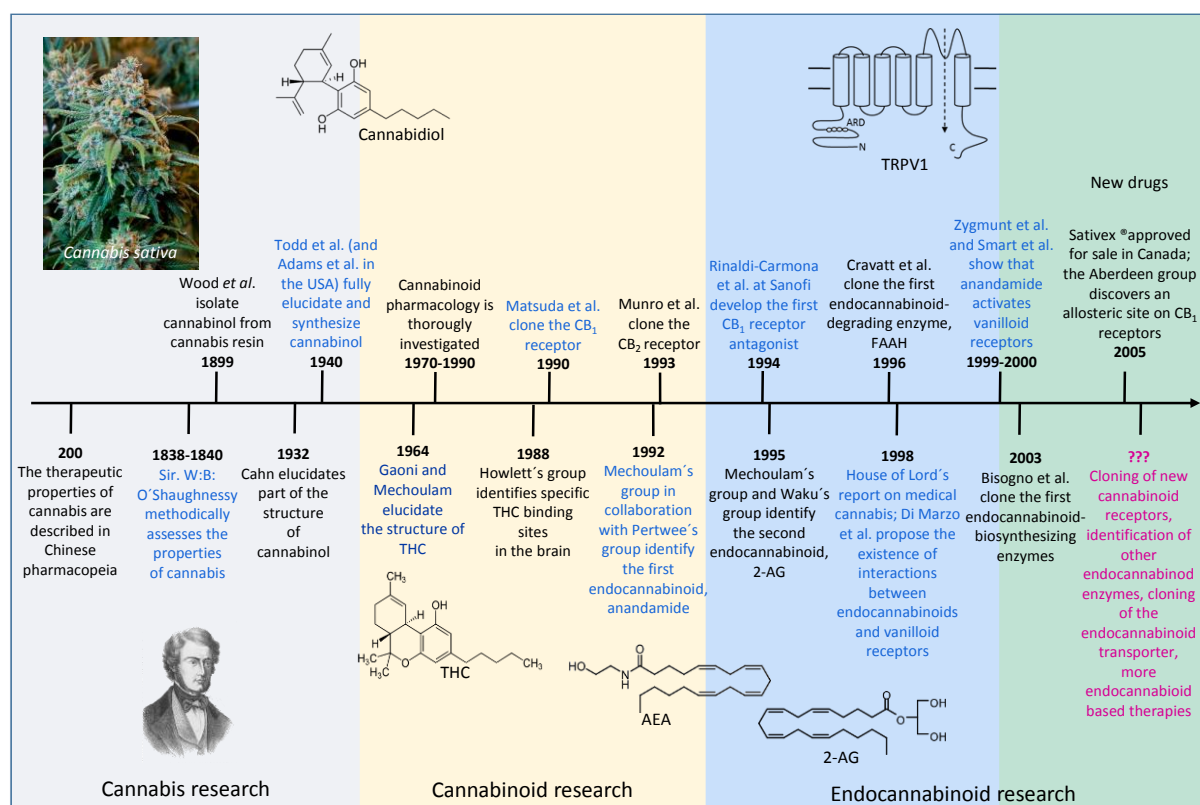


Fig. 5: Key points in the timeline of cannabis, cannabinoid, and eCB research

A summary about the outstanding milestones within the ECS research field: from the isolation and characterization of the first (endo)cannabinoids and receptors, to more recent studies on therapeutic research. AEA: anandamide, 2-AG: 2-arachidonoylglycerol, THC: (-)-*trans*- Δ^9 -tetrahydrocannabinol, TRPV1: transient receptor potential vanilloid 1, FAAH: fatty acid amide hydrolase (modified from Di Marzo, 2006).

2.2.1 Structure of ECS

In spite of the fact that the first cannabinoid compounds were already described in 1899 (Wood et al., 1899; Di Marzo et al., 2006), scientists were only able to get a more accurate picture about their mechanism of action much later, when in 1988 the first cannabinoid receptors were identified (**Fig. 5**, Devane et al., 1988). This step was followed not only by the discovery of additional ligands and their targets, but also of an enzymatic apparatus that is actively involved in the metabolism of cannabinoid compounds. As a result, we now have a more comprehensive view on the structure and functionality of the ECS.

2.2.1.1 Cannabinoid ligands

Cannabinoids are a group of chemical compounds that act through the activation of cannabinoid receptors. These specific ligands are arranged in three different categories: plant-derived cannabinoids, a.k.a. phytocannabinoids, eCBs, and synthetic analogues (Fraguas-Sanchez et al., 2016; Wiley et al., 2016).

The *Cannabis sativa* plant contains more than 500 biologically active compounds, out of which more than 100 are considered to be cannabinoids (Solymosi and Köfalvi, 2017). These secondary metabolites are produced in the so-called trichomes that are located on the female *Cannabis* plants. These microscopic formations have been evolutionarily developed to protect the host against herbivores, insects, and fungal attacks. A trichome consists of a stalk and a head, in which the actual production of the compounds takes place. Potency of the active ingredients within these glandular outgrowths depends on the life cycle of the plant, the soil, and the method of cultivation (Mahlberg and Kim, 2004; Potter, 2014; Andre et al., 2016).

Even though the first identified cannabis-derived chemical product was cannabinal ([CBN]; Wood et al., 1899); the scientific community has rather focused its attention on THC, the principal psychoactive constituent of the *Cannabis sativa*, since the beginning of its characterization. Being a lipid soluble substance, THC is very quickly absorbed and distributed within the human body. This psychotropic phytocannabinoid has a greater influence on key

functions like motor- and cognitive skills, as well as memory, emotions, appetite, and it may induce analgesia. Its effects are mainly mediated through the so-called “classical” cannabinoid receptors (i.e. CB₁ and CB₂, see below) (Pertwee, 2008; Di Iorio et al., 2013; Dinis-Oliveira, 2016). Synthetic, purified THC is currently available on the market under the international nonproprietary names dronabinol (Marinol[®], Solvay Pharmaceuticals) and nabilone (marketed as Cesamet[®]) as a means to reduce the adverse reactions of chemotherapy, i.e., nausea and vomiting (Lam and Frost, 2014; May and Glode, 2016). The oral mucosal spray Sativex[®], which also contains this terpeno-phenolic compound, is applied as complementary therapy in multiple sclerosis treatment (Feliu et al., 2015). Despite its beneficial properties, medical use of THC is still under debate, due to its known short-term unwanted effects (e.g., temporary memory loss, anxiety, and hallucinations), as well as its unknown long-term side effects on the brain (Carlini, 2004; Volkow et al., 2014).

Unsurprisingly, recent research efforts aim to develop cannabinoid therapies with the least amount of undesirable secondary effects. Unlike THC, (-)-cannabidiol (CBD) is not psychoactive, but has several therapeutic benefits such as anti-inflammatory, anti-epileptic, anti-psychotic, anti-convulsive, and anti-cancer effects (Devinsky et al., 2014; Burstein, 2015; Leo et al., 2016). According to the most recent data, CBD has low affinity for the classical cannabinoid receptors, even though its presence at low concentration ranges is already sufficient to antagonize them (Pertwee, 2008). In addition, it has also been reported to modulate novel cannabinoid-, but also certain glycin-, serotonin- and adenosine receptors (Campos et al., 2012). Although up to now most CBD experiments have been carried out using animal models, there is an increasing amount of clinical work testing its beneficial effects on human patients. GW Pharmaceuticals has a leading role within these attempts. It has not only launched the aforementioned Sativex[®] (which next to THC also contains CBD in an equal amount), but also successfully completed the penultimate phase of clinical trials for their new CBD containing medication, called Epidiolex[®]. This drug has been mainly developed to treat children with

severe epileptic symptoms (e.g., Dravet syndrome), but there are also running tests to examine its effectiveness in schizophrenic disorders (Rosenberg et al., 2015; Russo, 2016; Ligresti et al., 2016). Moreover, it should also be noted that CBD containing topical formulations are now being investigated in appropriate clinical trials to assess its efficiency in acne (successfully completed phase Ib, ongoing phase II) and in AD (successfully completed phase Ib, recruiting for phase II), while the phase Ib trial involving patients suffering from psoriasis is scheduled to the second part of the year (Spleman et al., 2018; Botanix Pharmaceuticals; <https://www.botanixpharma.com/>).

Cannabinoids, more precisely eCBs, can also be synthesized by humans and animals. Among them, N-arachidonylethanolamine (AEA), also known as anandamide, is so far one of the most studied. The name of this endogenous analogue of THC has Sanskrit origins: ānanda means “bliss, delight” (Self, 1999). As the origin of the name suggests, AEA is able to influence the pleasure-reward system and appetite, but it also takes part in the regulation of pain and sleeping cycle. Recent studies have also indicated its possible role in hormonal balance, reproductive systems, cancer development, and memory functions (Di Marzo et al., 2004; Pacher et al., 2006; Wiley et al., 2016).

Another well studied eCB is 2-arachidonoylglycerol (2-AG). This full agonist of the classical cannabinoid receptors can be found in relatively high levels in the central nervous system. 2-AG has an impact on the regulation of appetite, the immune system, neuroprotection, and pain (Sugiura et al., 2006; Luchicchi and Pistis, 2012).

Besides “classical” eCBs, the growing group of “eCB-like” mediators should also be mentioned. A large part of this group are endogenous analogues, such as 2-arachidonoylglycerol-ether and virodhamine. In addition to these eCB-like mediators, there are several other compounds that have been shown to exert cannabimimetic actions. The best known representatives of this group are N-acylethanolamines (such as oleoylethanolamide [OEA], palmitoylethanolamide [PEA], linoleoylethanolamide) and monoacylglycerols (such as

2-oleoylglycerol, 2-palmitoylglycerol, and 2-linoleoylglycerol) (Hansen and Diep, 2009; Kleberg et al., 2014).

In recent years, several synthetic phyto-, and eCB analogues have been created for therapeutic purposes. These man-made alterations have functional similarities to THC: for example, they are able to activate the same receptors in the body in an even stronger manner (Di Marzo et al., 2004; Fraguas-Sanchez et al., 2016). It has also been shown that certain orthosteric and allosteric cannabinoid ligands may act in a different manner on several signaling pathways via the same receptor complex by the selective activation of different intracellular effectors. This mechanism is called functional selectivity or biased agonism. The main goal of this selective activation is to strengthen desired and suppress adverse effects of ECS (Diez-Alarcia et al., 2016; Ibsen et al., 2017).

2.2.1.2 Classical and novel pharmacological targets of eCB ligands

Cannabinoids can exert their effects on target tissues throughout the whole body via specific receptors. To date two GPCRs have been identified as “classical” targets of the cannabinoid ligands, namely cannabinoid receptor type 1 (CB₁) and cannabinoid receptor type 2 (CB₂). The effects of THC are mostly the outcome of its engagement with CB₁ that is mainly, but not exclusively, expressed in the central nervous system (Mackie, 2005a; Galve-Roperh et al., 2013; Di Marzo et al., 2015). In contrast, CB₂ rather appears within the organs of the immune system (Galiegue et al., 1995; Atwood et al., 2010), and affects biological processes such as immune responses (Ashton et al., 2007).

Both receptors can activate different pathways via inhibitory G proteins, resulting in the inhibition of adenylate cyclase and decrease of the cyclic adenosine monophosphate (cAMP) levels, in parallel to the stimulation of the mitogen-activated protein kinase (MAPK) (Galve-Roperh et al., 2013; Ibsen et al., 2017). CB₁ also modulates a number of ion channels such as voltage gated Ca²⁺ and some type of K⁺ channels, thus in this way can also regulate the release

of neurotransmitters from the pre-synaptic membranes of neurons (Ameri, 1999; Dalton et al., 2009).

CB₁ can form complexes with e.g., dopamine, adenosine, angiotensin, δ opioid, orexin-1 receptors, and even with CB₂, which itself can connect with CXCR4 chemokine receptor or GPR55 (Mackie, 2005b; Hudson et al., 2010; Oláh et al., 2017). These heteromers usually show characteristics of the coupled parent receptors, but may also induce unusual pharmacological effects.

Compounds can evoke a number of cannabinoid-like effects without activating the classical cannabinoid pathways. Endogenous lipid metabolite of AEA was shown to induce cell migration via the activation of GPR18 (Alexander S.P., 2012; McHugh et al., 2012). Despite its low homology with the classical CBs, GPR55 has also emerged as a putative CB receptor based on its ability to respond to various cannabinoid ligands. Among others, THC, 2-AG, OEA have been described as its agonists (Godlewski et al., 2009). Another recently deorphanized receptor, GPR119, was also found to interact with several “eCB-like” or “eCB-related” compounds that mostly belong to the N-acylethanolamine family, e.g., OEA, PEA and linoleylethanolamine (Brown, 2007, Hassing et al., 2016).

Besides the aforementioned metabotropic receptors, researchers have found possible interactions between the ECS and a number of transient receptor potential (TRP) channels. The vanilloid TRPV1 is known to be one of the best cellular targets for cannabinoid ligands, especially for AEA, but it can also be activated by THC and CBD (Pertwee, 2006). Cannabinoids are also able to exert their effects through TRPV2 (Pertwee, 2006), TRPV3, TRPV4 (Fian et al., 2007; Mergler et al., 2011; Fian et al., 2007; De Petrocellis, 2012), the melastatin type TRPM8 (Caterina, 2014), and the ankyrin type TRPA1 (Jordt et al., 2004; Nilius et al., 2012).

Scientists investigated the potential for phyto-, endo- and synthetic cannabinoid compounds to activate PPARs. This nuclear receptor family has 3 isoforms: PPAR α , δ , and γ .

They heterodimerize with the retinoid X receptor (RXR), which binds to a specific peroxisome proliferator hormone response elements (PPREs) along the DNA, resulting in changes on the transcription of various genes (Michalik et al., 2006). In particular, they are taking part in the regulation of lipid-, carbohydrate-, and protein metabolism, as well as inflammation, cellular differentiation, and neuroprotection (Guan and Breyer, 2001; Berger et al., 2005; Robinson et al., 2009). In connection with the ECS, PPAR δ is the least investigated. This nuclear receptor was shown to be able to modulate CB₁ expression in adipocytes and it might have an important role in the emergence of cardiovascular diseases associated with obesity (Yan et al., 2007). AEA can potentiate both PPAR α and PPAR γ , but, for example, the two main phytocannabinoids (THC and CBD) have higher preference to the latter. Furthermore, PPAR α can be triggered by novel endogenous ligands such as PEA and OEA (Lo Verme et al., 2005; O'Sullivan, 2016).

2.2.1.3 Metabolism of eCB ligands and its therapeutic modulation

eCB metabolism is based on the biological necessity of arising from their lipid moiety. In general, these ligands act near the site of production (paracrine and autocrine ways) and their degradation takes place within a relative short period of time (Bisogno and Maccarrone, 2013).

The best way to outline the general metabolism of eCB ligands is probably an overview of the two best studied eCBs' (AEA and 2-AG) synthesis and degradation processes. The main precursor of AEA is *N*-arachidonoyl-phosphatidylethanolamine (NAPE). *N*-acyl phosphatidylethanolamine-specific phospholipase D (NAPE-PLD) and *N*-acyltransferase (NAT) can catalyze the release of AEA either in a short and direct way (Di Marzo, 2006; Muccioli, 2010, Blankman et al., 2013), or through alternative pathways (Sun et al., 2004; Liu et al., 2006). 2-AG is also produced via different processes, in which the common point is the catalyzation of the production of 2-arachidonate-containing diacyl-glycerols (DAGs). This step can be achieved either with the contribution of specific enzymes such as *sn*-1-selective α or β

DAG lipases, phospholipases, and phosphatases, or via the dephosphorylation of the intermediate arachidonoyl-lysophosphatidic acid (Di Marzo, 2006; Muccioli, 2010).

The fatty acid amide hydrolase (FAAH) enzyme is responsible for the degradation of fatty acid amides, showing greater preference to AEA. Under specific conditions, it is also able to hydrolyze 2-AG, which is mostly degraded by monoacylglycerol lipase (MAGL), (α/β)-hydrolase-6 and -12 (Di Marzo, 2008). In addition to FAAH and MAGL, the inducible cyclooxygenase-2 (COX-2) is also able to transform 2-AG and AEA into prostaglandins, causing their oxidation. Furthermore, these two endogenous ligands have been proven to be good substrates also for 12- and 15- lipoxygenases (LOXs), as well as for certain cytochrome P450 (CYP) enzymes (Burstein et al., 2000; Woodward et al., 2008; Urquhart et al., 2015).

Setting the appropriate levels, or tone, of eCB compounds can offer novel therapeutic perspectives in various diseases. Therefore, ECS research is currently focusing on the modification of its main metabolic enzymes. Catalytic activity of FAAH is usually inhibited in a concentration-dependent manner. The irreversible cyclohexylcarbamic acid 3'-carbamoylbiphenyl-3-ylester (URB597) is one of the best characterized FAAH inhibitors so far (Aghazadeh Tabrizi et al., 2015). For example, it has been shown to potentiate the actions of AEA and thus attenuate acute, neuropathic, and inflammatory pain via the indirect activation of CB receptors. Furthermore, FAAH is also able to relieve the pain-associated symptoms of osteoarthritis (Jayamanne et al., 2006; Schuelert et al., 2011).

Before being degraded, cannabinoids are removed from their activation site and diffuse back (most probably via facilitated diffusion) into the appropriate cells (Chicca et al., 2012). However, to date, the details of these processes have not been fully clarified. It is assumed that a yet uncharacterized specific eCB membrane transporter (EMT) might be responsible for the reuptake (and, in some cases maybe for the release too) of these lipid molecules (Di Marzo, 2008). In addition, recent studies have shown that caveolae-related endocytosis, FAAH-driven facilitated diffusion, different cytoplasmic AEA-binding proteins, and intracellular

compartments are also considered to be part of these specific membrane transports (Oddi et al., 2009; Nicolussi and Gertsch, 2015).

Although the exact mechanism of the reuptake of eCBs still remains an open question, there were several attempts to create and examine selective inhibitors of these cellular actions. The two main groups of these developed drugs are called aromatic acylamide derivatives (e.g., AM404, VDM11, OMDM-1, 2, UCM707, and AM1172) and carbamoyltetrazoles (e.g., LY2183240; Di Marzo, 2008). So far, experiments involving these selective inhibitors have been carried out using animal (mostly rodent) models (Moore et al., 2005; La Rana et al., 2006; Mitchell et al., 2007). The outcome of these studies were positive regarding the regulation of pain, behavior, and neurological functions (Chhatwal et al., 2005; Bortolato et al., 2006; Braidà et al., 2007). For example, AEA reuptake inhibitors were shown to attenuate conditioned fear (Braidà et al., 2007) and neuropathic pain (Lau and Vaughan, 2014).

2.3 The “*c(ut)*annabinoid” system

Functional members of ECS are widely represented in the skin and play an important role in various regulatory mechanisms, both under physiological and pathological conditions (Bíró et al., 2009; Roelandt et al., 2012; Caterina et al., 2014). Both resident and non-resident skin cells have also been shown to express eCB receptors and enzymes (Bíró et al., 2009; Caterina et al., 2014). With such a versatile presence, the effects of ECS on epidermal homeostasis have come under scrutiny (Oddi and Maccarrone, 2014). This chapter shortly summarizes the distribution and the effects of ECS in skin, together with its therapeutic relevance in various skin diseases.

2.3.1. Role of ECS in the biology of the epidermal barrier

The interest in the role of ECS in the control of epidermal physiology and of the skin barrier was fueled by the identification of various ECS components and their key regulatory functions connected to the proliferation, differentiation, and survival of KCs (Oddi and

Maccarrone, 2014). For example, AEA was shown to influence not only the differentiation of primary human epidermal (NHEK) and immortalized human KCs (HaCaT), but also the assembly of cornified envelope in a CB₁-dependent manner (Maccarrone et al., 2003; Pasquariello et al., 2009; Bíró et al., 2009). Further research has revealed that blockage of cornified envelope formation has been the result of the insufficient expression of proteins and enzymes, which are essential for final transformation of KCs to corneocytes (Paradisi et al., 2008; Pucci et al., 2011; Wondrak, 2016). CBD and (-)-cannabigerol (CBG; another non-psychotropic phytocannabinoid) have also been shown to suppress the expression of certain KC differentiation markers such as keratin 1, 10, involucrin, and transglutaminase 5 (Pucci et al., 2012). Using knockout (KO) mice models, Roelandt et al. have investigated whether and how the lack of CB₁ and CB₂ influences the cutaneous barrier functions. Interestingly, the absence of these receptors caused opposing effects on the duration and quality of epidermal recovery, on proliferation and differentiation of KCs, and on the structure of lipid bilayers (Roelandt et al., 2012).

In line with the aforementioned results, our research group has also found evidence supporting the role of ECS in basic biological processes of KCs. In particular, we have shown that the activation of CB₁ on hair matrix KCs by AEA and THC was able to induce intraepithelial apoptosis, causing potent hair growth-inhibitory actions through the apoptosis-driven premature catagen regression of HFs (Telek et al., 2007). Lastly, we have also demonstrated that AEA is able to modulate epidermal cell proliferation and survival through a Ca²⁺ dependent, joint CB₁-TRPV1 pathway (Tóth et al., 2011a). Last, but not least we have shown that the CB₁-selective agonist ACEA has not only been able to reduce the excessive proliferative activity of the KCs, but also the expression of keratin 6 and keratin 16, i.e. two cytokeratins, which are pathologically upregulated in psoriasis (Ramot et al., 2013).

Expression of several “novel” cannabinoid receptors playing an important role in the regulation of cutaneous homeostasis have also been proven on KCs. For example, members of

TRPV family that influence the construction of *stratum corneum*, cell junctions, and Ca^{2+} homeostasis of KCs (Sokabe and Tominaga, 2010; Kida et al., 2012) are also highly represented. Furthermore, all three PPARs have also been identified on KCs, of which PPAR α is considered to have the most important role in the development of the epidermal barrier (Rosenfield et al., 2000; Sertznig and Reichrath, 2011; Lima Ede et al., 2013).

Based on the aforementioned observations, possible eCB-based therapies might be useful for the future treatments not only of hyperproliferative skin disorders with impaired epidermal barrier defects such as psoriasis (Wilkinson and Williamson, 2007), but also in diseases that are associated with uncontrolled growth of abnormal skin cells, e.g., cutaneous carcinomas (Blazquez et al., 2006; Chakravarti et al., 2014).

2.3.2. Role of ECS in the biology of non-KC skin cells with focus on sebocytes

Just like KCs, melanocytes also express “classical” and other cannabinoid receptors, e.g., CB₁, CB₂, and TRPA1 (Pucci et al., 2012). It has been shown that cannabinoid ligands can increase their melano- and mitogenesis, as well as their dendricity via CB₁ (Pucci et al., 2012), however, these effects may well be context-dependent (Magina et al., 2011). Moreover, ECS has also emerged as a possible future therapeutic target in the treatment of the most dangerous type of skin cancer: melanoma (Blazquez et. al, 2006; Scuderi et al., 2011; Magina et al., 2011).

Dermal fibroblasts are also an important components of skin’s connective tissue and are mainly responsible for cutaneous integrity and wound healing. Several lines of evidence point to the fact that ECS plays a role in the development of extreme fibroblast proliferation that leads to the development of fibrotic skin diseases such as dermal fibrosis and scleroderma (Marquart et al., 2010; Balistreri et al., 2011; Lazzerini et al., 2012; Gonzalez et al., 2012).

Our laboratory has investigated the possible role of the cutaneous cannabinoid system on maintenance and functions of different skin associated glands. By using NCL-SG3 eccrine sweat gland cells, we have shown that certain prototypic eCBs can reduce the viability of these

secretory cells, next to the stimulation of their various cytoskeleton protein expression and lipid production via a CB₁/CB₂/TRP independent manner (Czifra et al., 2012).

We have especially focused on the exploration of the possible regulatory effects of the ECS on human SGs. We have demonstrated that important eCBs such as AEA and 2-AG are continuously produced in these exocrine glands. In addition, we have observed that these locally generated substances were able to increase the cellular differentiation through the activation of CB₂, thus induction of lipid production of human immortalized SZ95 sebocytes (Dobrosi et al., 2008). Using the same cell line and full-thickness human skin organ cultures, we have shown that CBD was able not only to decrease the lipogenesis of sebocytes via the activation of TRPV4, but also to reduce their proliferation. Besides the lipostatic and anti-proliferative effects, CBD exerted anti-inflammatory actions by inhibiting the p65 NF- κ B signaling through the activation of the A_{2A} adenosine receptor, which enhances the level of cAMP leading to the upregulation of tribbles homolog 3 (TRIB3) (Oláh et al., 2014). These results have particular importance in relation to possible therapeutic treatment of acne vulgaris. By its multifactorial nature, cure of this cutaneous disorder should be directed at correcting most symptoms at the same time (Williams et. al, 2012). Based on our findings, CBD with its wide-ranging effects may indeed be a promising medical agent for acne's treatment. Importantly, this assumption is now further supported by the data of a recent phase Ib clinical trial (Spleman et al., 2018).

Most recently, we have also studied the putative anti-acne effects of further non-psychoactive phytocannabinoids, such as (-)-cannabichromene (CBC), (-)-cannabidivarin (CBDV), CBG, (-)-cannabigerovarin (CBGV) and (-)- Δ^9 -tetrahydrocannabivarin (THCV). We have found that CBG and CBGV may have potential in the treatment of dry skin syndrome, whereas the other compounds, especially THCV, may be useful in the treatment of acne. Since they have also exerted remarkable anti-inflammatory actions, their use in the management of cutaneous inflammation could also be efficient (Oláh et al., 2016a; Oláh and Bíró, 2017).

With the above mentioned experiments we have provided the first evidence that certain functionally active ECS elements can be found on human SGs and that their lipid production can be stimulated by the administration of AEA or 2-AG. Even so, we still do not know much about the expression of the enzyme apparatus involved in synthesis and degradation of eCBs, nor about the role of local eCB tone created by these enzymes in human SGs. Thus, this thesis explores the expression of major members of ECS *in vitro* in human sebocytes, as well as *in situ* in human skin. We also investigate whether the pharmacological modulation of eCB homeostasis is able to regulate SLP.

2.3.3. Role of ECS in the immune responses of skin with focus on MCs

Over the past years, the possible immunoregulatory effects of ECS have been widely studied, especially after it became clear that this signaling system may play an important role in the development of various allergic and inflammatory diseases, such as AD, and chronic urticaria (Oddi and Maccarrone, 2014; Chiurchiu et al., 2015; Chiurchiu, 2016; Oláh et al., 2017).

The main immunoregulatory effects exerted by ECS in skin mostly affect the viability, proliferation, maturation, phagocytic actions, cytokine and chemokine release of cutaneous immune cells such as KCs, Langerhans cells, intraepithelial lymphocytes, dermal dendritic cells, cutaneous memory T cells and MCs (Oddi and Maccarrone, 2014).

As already mentioned, ECS has a high potential as a neuroendocrine control system in the regulation of MCs, although most details of possible interactions between them are still hidden (De Fillipis et al., 2008; Bíró et al., 2009; Cantarella et al., 2011). *In vitro* results, which have been published so far on how cannabinoid receptor stimulation affects rodent and human MC lines are contradictory (Gramberg et al., 2003; Samson et al., 2003; Small-Howard et al., 2005). For example, when the possible differential roles of CB₁ and CB₂ in cultured MCs have been investigated, agonists of these receptors (e.g., methanandamide [MA], a synthetic FAAH-resistant AEA analogue) were found to prevent the IgE activation of RBL-2H3 cells (Samson

et al., 2003). Even though this effect was abolished with the co-administration of the CB₁ antagonist/inverse agonist AM281, this compound alone was shown to have no effect on former cell activation (Samson et al., 2003). MA has also been reported to inhibit IgE mediated MC degranulation, while checking the potential anti-inflammatory effects of CB₁-mediated cAMP elevation on primary murine bone marrow-derived MCs (Small-Howard et al., 2005). Also using the RBL-2H3 cell line, Granberg et al., have shown that cannabimimetic PEA significantly inhibited the IgE-dependent antigen-induced serotonin release of the cells, while AEA was ineffective. Furthermore, they have also found that both 2-AG and MA enhanced the MC activation effect of the antigen-induced beta-hexosaminidase and serotonin release (Granberg et al., 2001). Unlike AEA, PEA was also shown to behave as an endogenous agonist for MCs' CB₂ (Facci et al., 2005). Giudice et al. have reported that CBD, unlike synthetic cannabinoids, could trigger the activation of RBL-2H3 cells (Giudice et al., 2007). Phytocannabinoids, such as THC and (-)- Δ^8 -*trans*-tetrahydrocannabinol, caused histamine release of rat peritoneal MCs via a cannabinoid receptor-independent manner. This was not the case when using eCBs and eCB related ligands (Bueb et al., 2001). When assessing the role of MC mediators on migration of cervical cancer cells (SW756), the supernatants of these cells were shown to increase the activation of LAD-2 cells, whose effects was then inhibited by cannabinoids acting through CB₂ (Rudolph et al., 2008).

3. GOALS

Main questions regarding the possible role of the endocannabinoid system as a neuroendocrine regulator on mast cells:

- 1.) Is the human hair follicle organ culture a clinically relevant model to study the role of endocannabinoid system in skin mast cells under physiological conditions?
- 2.) Do the resident mast cells situated in the connective tissue sheath of human hair follicles express functionally active CB₁?
- 3.) If so, how does the stimulation/inhibition of CB₁ affect the behavior of these mast cells?

To further investigate the role of the endocannabinoids in connection with the biology of sebaceous glands, we primarily aimed to answer the followings questions:

- 1.) Are the major endocannabinoid synthesizing and degrading enzymes expressed in human sebocytes *in vitro* and *in situ*?
- 2.) How does the modulation of endocannabinoid tone affect the biology of human sebocytes?

4. MATERIALS AND METHODS

4.1 Reagents

MC experiments. AEA, ACEA, AM251, SP and compound 48/80 were ordered from Sigma Aldrich (St. Louis, MO, USA). 5-carboxytetramethylrhodamine-conjugated AM251 (“Tocrifluor T1117”) was purchased from Tocris Bioscience (Bristol, UK). ACEA and AM251 were dissolved in DMSO, AEA in ethanol, SP in methanol, while compound 48/80 in filtered distilled water. Control cultures were always treated with an appropriate amount of vehicles.

SG experiments. VDM11 ((5Z,8Z,11Z,14Z)-N-(4-hydroxy-2-methylphenyl)-5,8,11,14-eicosatetraenamide) and AM404 (N-(4-Hydroxyphenyl)-5Z,8Z,11Z,14Z-eicosatetraenamide) together with AEA were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). URB597 (cyclohexylcarbamic acid 3'-(aminocarbonyl)-[1,1'-biphenyl]-3-yl ester) and UCM707 ((5Z,8Z,11Z,14Z)-N-(3-Furanylmethyl)-5,8,11,14-eicosatetraenamide) were obtained from Tocris Bioscience (Bristol, UK) and Cayman Chemical Company (Ann Arbor, MI, USA). [ethanolamine-1-³H]-AEA (60 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO, USA); whereas the manufacturer of γ -irradiated LPS from *Escherichia coli* 026:B6 was from Sigma Aldrich (St. Louis, MO, USA). LPS was dissolved in filtered distilled water. The solvent of all other compounds was absolute ethanol (Sigma Aldrich, St. Louis, MO, USA), except for the [³H]-AEA uptake assay, where UCM707 was dissolved in DMSO. Control cultures were always treated with an appropriate amount of vehicles.

4.2 Cell and organ cultures

4.2.1 Human hair follicle (HF) -organ culture

Normal micro-dissected anagen VI HFs were isolated from occipital and temporal scalp skin, obtained from 8 healthy individuals (aged: 49-72 years) undergoing routine facelift

surgery, according to the method previously described (Telek et al., 2007; Ito et al., 2010). Samples were provided by Dr. Wolfgang Funk (Clinic Dr. Kozlowski & Dr. Funk, München, Germany). Human tissue collection and handling were performed according to the Helsinki guidelines, following Institutional Research Ethics approval (University of Lübeck, Germany; No.: n. 06-109, 18-07-06) after obtaining written informed consent. In total, 414 micro-dissected HF_s were used and cultured for specified times in 24-multiwell plates at 37°C in 5% CO₂ and 95% air in William's E Medium (Biochrom, Cambridge, UK). Latter's supplements were the following: insulin 10 µg/ml (Sigma Aldrich, St. Louis, MO, USA), hydrocortisone 10 ng/ml (Sigma Aldrich, St. Louis, MO, USA), 2 mmol L-glutamine (Invitrogen, Carlsbad, CA, USA) and 1% antibiotic mixture (Thermo Fisher Scientific, Waltham, Massachusetts, USA). To adapt to the culture conditions, HF_s were incubated overnight, and appropriate treatments were first administered on the other day, except for the CB₁ knockdown experiments (for details, see in section 4.7). Medium was changed every second day, while treatments were performed daily.

For the organ culture with highly CB₁-selective, fluorescent AM251 analogue Tocrifluor T1117 (Tocris Bioscience, Bristol, UK), the said compound was applied at 1 µmol/l concentration within the medium for 1 day. For the experiments with MCs secretagogues, HF_s were first treated with AEA (30 µM) or ACEA (30 µM) for 24 hrs. Afterwards, either SP (100 pM) or compound 48/80 (10 µg/ml) were added to the medium, and in the presence of AEA and ACEA were cultured for an additional day (all substances were purchased from Sigma Aldrich, St. Louis, MO, USA). Isolation was done by Koji Sugawara, Claudia Cremling, and Nóra Zákány.

4.2.2 Culture of human outer root sheath (ORS) KCs

ORS is the outermost epithelial layer of the HF. Predominant cells of this multilayered tissue site are undifferentiated KCs (ORS-KCs). These cells play an important role in epidermal

regeneration (wound repair) and they might also be involved in the hair cycle (Limat and Hunziker, 1996). Since ORS-KCs are relatively easy to access and maintain in cell culture, they are frequently used. Most often they are obtained from plucked anagen HFs (Limat et al., 1995; Borb  r   et al., 2011).

Isolation and culturing procedures were performed as previously described (Borb  r   et al., 2011). Briefly, anagen HFs went through a 1 hr enzymatic digestion (0.2% trypsin, 0.1% ethylenediaminetetraacetic acid in calcium and magnesium free [CMF] phosphate-buffered saline [PBS], all purchased from Sigma Aldrich, St. Louis, MO, USA) at 37  C. ORS-KCs were then collected by centrifugation (1000 rpm, 10 min) and then seeded in culture medium, which contained 3:1 mixture of Dulbecco's modified Eagle medium (supplemented with L-glutamine, Na-pyruvate, 4.5 g/L glucose) and Ham's F12 (supplemented with 10[V/V]% Fetal Clone II [Hyclone, HyClone Laboratories Inc, Logan, Utah], 5   g/ml insulin, 0.4   g/ml hydrocortisone, 2.43   g/ml adenin, 2 nM triiodothyronine, 0.1 nM cholera toxin, 10 ng/ml human epidermal growth factor [EGF], 1 mM ascorbyl-2-phosphate, 100 IU/ml penicillin G, and 25   g/ml gentamycin; all from Sigma Aldrich, St. Louis, MO, USA), both purchased from Invitrogen, CA, USA. Finally, ORS-KCs were seeded on mitomycin-treated (Sigma Aldrich, St. Louis, MO, USA) human dermal fibroblast (HDF) feeder-layer.

For HDF isolation, de-epidermized human skin dermis samples were placed into a digestive enzyme solution: 8 ml fetal bovine serum FBS (final concentration 20%; Invitrogen, Carlsbad, CA, USA), 32 ml DMEM (high glucose; Invitrogen, Carlsbad, CA, USA), 100 mg collagenase I (0.25%; Invitrogen, CA, USA), penicillin/streptomycin (1%, Invitrogen, Carlsbad, CA, USA), 20 mg DNase I (final concentration 0.05%; Sigma Aldrich, St. Louis, MO, USA). After 24 hrs of incubation at 37  C, the digested dermis pieces were further disrupted by suspension, and then they were placed into a 50 ml centrifuge tube. The digestion process was stopped by HDF culture medium (10% FBS and 1% penicillin/streptomycin

containing DMEM; all from Invitrogen, Carlsbad, CA, USA). When after centrifugation (10 min, 1000 rpm), supernatant could easily be removed, cells were cultured for 3 days without changing the medium. When supernatant could not be removed so easily, the HDF medium was changed very carefully the day after. Cells were then further cultured until they reached approx. 80% confluence. Proliferation of the cells then was stopped by mitomycin C solution (0.4 mg/ml in CMF-PBS containing 100 µg/ml gentamycin; Sigma Aldrich, St. Louis, MO, USA) during an overnight incubation at 37°C, 100% humidity and 5% CO₂. The next day, the medium was removed, and cells were washed with sterile CMF-PBS and then harvested in trypsin-EDTA solution (0.1% Trypsin-0.2% EDTA in CMF-PBS containing 100 µg/ml gentamicin). Trypsin was inactivated by using HDF medium. Cells were then centrifuged (10 min, 1000 rpm) and resuspended in culture medium, and finally were seeded in Petri dishes (25,000 cells/35 mm-Petri dish). Two days after the procedure, HDFs were ready to be used as feeder layer for ORS-KCs.

Primary human material was collected after obtaining written informed consent, adhering to the Helsinki Declaration, and after obtaining permission from the Institutional Research Ethics Committee and Government Office for Hajdú-Bihar County (document IDs: IX-R-052/01396-2/2012, IF-12817/2015, IF-1647/2016, and IF-778-5/2017). The experiments were supervised by Balázs István Tóth and Tamás Bíró.

4.2.3 Culture of human SZ95 sebocytes

Human immortalized SZ95 sebocytes, originated from human facial SGs (Zouboulis et al., 1999), were grown in Sebomed® Basal Medium (Biochrom, Berlin, Germany) supplemented with 10 (V/V)% FBS (Life Technologies, Carlsbad, CA, USA), 1 mM CaCl₂, 5 ng/ml human EGF (Sigma Aldrich, St. Louis, MO, USA), and MycoZap™ Plus-CL (1:500; Lonza, Basel, Switzerland). The medium was changed every second day, and cells were

passed once they reached 60-70% confluence. Experiments were run by Attila Oláh, Erika Takács, Andrea Aranyász, and Nóra Zákány.

4.3 Staining protocols

4.3.1. Cryo and paraffin section preparation for stainings

After the adequate treatment time, cryo samples were embedded in cryo matrix (Cryochrom Embedding Medium, Thermo Shandon, Pittsburg, PA, USA) and were frozen in liquid nitrogen. From these specimens, 6 μ m thick cryosections were cut using a Leica 3500 cryostat (Leica Microsystems, Wetzlar, Germany). Sections were then placed onto Superfrost slides (Menzel GmbH & Co KG, Wetzlar, Germany), and were kept at -80°C, until they were used for different stainings.

Samples for paraffin embedding were placed into tissue processing cassettes and were fixed after the adequate treatment times for 24 hrs in 4% phosphate buffered formalin solution: 6.5 g Na_3PO_4 , 4.0 g $\text{NaH}_2\text{PO}_4 \times \text{H}_2\text{O}$, 100 ml of 40% formalin, and 800 ml distilled water. pH 7.4 was set by 0.1 M sodium hydroxide, and the solution was finally completed up to 1000 ml. All ingredients were purchased from Merck, Kenilworth, NJ, USA. Fixed samples then went through a dehydration process in an embedding machine (Tissue Processor Leica ASP300 S, Leica Microsystems GmbH, Wetzlar, Germany), using the following program:

I. phase at 40°C: 2 hrs 4% formalin; 30 min 70% ethanol; 1 hr 80% ethanol; 1 hr 96% ethanol I; 1 hr 96% ethanol II; 1 hr 100% ethanol I; 1,5 hrs 100% ethanol II; 1,5 hrs 100% ethanol III; 1 hr xylol I; 1 hr xylol II.

II. phase at 60°C: 30 min paraffin I; 30 min paraffin II; 1 hr paraffin III; and 1 hr paraffin IV.

At the end of the process cassettes were placed into a bath of paraffin dispenser (Tissue-Tek® TEC™ 5 Tissue Embedding Console Model 4715, Sakura Finetek, Torrance, CA, USA). Specimens were then placed into metal molds and were fixed with paraffin into the desired position. After cooling the molds, samples were removed and stored until sectioning. Before

the staining procedures, sections (4 μ m) from blocks were cut using Leica RM 2255 microtome (Leica Microsystems) and devolved on SuperFrost plus slides (Menzel GmbH & Co KG, Braunschweig, Germany).

Right before each staining procedure, sections were deparaffinized in xylol (4 times, 5 min), then were immersed 4-5 times into a series of descending alcohol dilutions (100%, 96%, 70%, 50%) (Merck, Kenilworth, NJ, USA), and finally were re-hydrated in distilled water. Experiments were run by Koji Sugawara and Nóra Zákány. Sections were prepared by Motoko Sugawara, Nóra Zákány, and Koji Sugawara.

4.3.2 MC histochemistry I: Leder's esterase staining

By using chloroacetate esterase, this cytochemical stain is able to selectively label MCs, whose granules will appear in deep pink and reddish colors (all necessary ingredients were provided from Merck, Kenilworth, NJ, USA). To perform the staining, first a Sörensen working solution was made, which consisted of a mixture of 32.18 ml stock A (2.73 g Na_2HPO_4 :250 ml distilled water) and 7.2 ml stock B (2.27 g KH_2PO_4 :250 ml distilled water). Right before the staining procedure, nitrosylated pararosanilin was compiled from 150 μ l of the previously prepared and filtered pararosanilin (0.5 g pararosanilin, 20 ml distilled water, and 2.5 ml concentrated hydrochloric acid) and from 150 μ l 4 (V/V)% NaNO_2 solutions. Final incubating medium was composed from the filtered mixture of 10 mg naphthol-ASD chloroacetate, 1 ml N,N dimethylformamide, 35 ml Sörensen working solution, and 200 μ l of nitrosylated pararosanilin.

After deparaffinization, the slides were placed into the incubating solution for 40 min at room temperature (RT). This was followed by a 5 min tap water wash and hematoxylin counterstain (approximately 1-2 min, RT). After the repeated tap water wash, slides were dehydrated via an ascending series of ethanol dilutions (70%, 96%, and 100%; Merck, Kenilworth, NJ, USA), were cleaned in xylol (Merck, Kenilworth, NJ, USA), and finally were

mounted with Eukitt® Quick-hardening mounting medium (O. Kindler GmbH & Co. Freiburg, Germany). Experiments were performed by Nina Biedermann, Nóra Zákány, and Koji Sugawara.

4.3.3 MC histochemistry II: alkaline-Giemsa staining

Alkaline-Giemsa solution stains granules of MCs dark blue to violet, while the background cytoplasm to pale blue. Sections were first deparaffinized and they were then placed into a diluted Giemsa solution (30 ml in 100 ml distilled water; Merck, Kenilworth, NJ, USA) for 20 min. The differentiation was achieved in 0.1% to 1% acetic acid and finished in absolute ethanol. This was followed by 2 times wash in xylol (10 min, RT). Finally, the slides were mounted with synthetic resin Eukitt (Eukitt, O. Kindler & Co, Freiburg, Germany). Experiments were run by Koji Sugawara and Claudia Kremling.

4.3.4 c-Kit (CD117), CB₁, tryptase, chymase, and FcεRIα immunostaining

For the detection of the aforementioned molecules, Tyramide Signal Amplification (TSA™) technology was applied (Perkin Elmer, Boston, USA). Cryosections were fixed in 1% paraformaldehyde in PBS (1.8 g NaH₂PO₄, 8 g NaCl, 1000 ml distilled water, pH 7.2 adjusted with 1 N sodium hydroxide; all purchased from Merck, Kenilworth, NJ, USA) for 10 min, followed by a 5 min ethanol-acetic acid (2:1) postfixation at -20°C. After PBS washes (3 times, 5 min, RT), primary antibodies with the following dilutions were applied during an overnight incubation at 4°C: rabbit-anti human CD117 at 1:1000 (Cell Marque Corporation, Rocklin, CA, USA), rat anti-mouse CD117 at 1:5000 (BD Biosciences, San Jose, CA, USA), rabbit anti-human CB₁ at 1:400 (Cayman Chemical Michigan, USA, or Santa Cruz, CA, USA), mouse anti-human FcεRIα at 1:1000 (OriGene – Acris Antibodies, Rockville, USA), mouse anti-human chymase at 1:1000 (Abcam, Cambridge, UK), and mouse anti-human tryptase at 1:5000 (Abcam, Cambridge, UK) in Tris-NaCl-blocking buffer (TNB: 0.1 M TRIS-HCl, pH 7.5, 0.15 M NaCl 0.5% Blocking Reagent; Merck, Kenilworth, NJ, USA). The following day,

biotinylated secondary goat antibodies were used against mouse or rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) 1:200 in TNB for 45 min at RT. After Tris-NaCl-Tween buffer (TNT: 0.1 mol/l Tris-HCl, 0.15 mol/l NaCl, 0.05[V/V]% Tween 20, 7.5 pH was adjusted with 1 N HCl; all purchased from Merck, Kenilworth, NJ, USA) wash, streptavidin horseradish peroxidase was administrated at 1:100 in wash buffer, for 30 min at RT. The reaction was amplified by fluorescein isothiocyanate (FITC) tyramide amplification reagent (1:50, solved in the provided amplification diluent, 5 min, RT). After repeated TNT wash, slides were counterstained with 4',6-diamidino-2-phenylindole-dihydrochlorid ([DAPI], Boehringer Mannheim, Germany) for 1 min, and then covered with Fluoromount-G (Southern Biotechnologies, Birmingham, USA). Primary antibodies were omitted as negative control for the unspecific binding of the secondary antibodies. Experiments were made by Claudia Kremling, Koji Sugawara, and Nóra Zákány.

4.3.5 c-Kit/CB₁ double immunostaining

TSA method was applied according to section 4.3.4. After fixation, cryosections were incubated with the aforementioned c-Kit (CD117) antibody (1:1000 [Cell Marque Corporation, Rocklin, CA, USA] in TNB, overnight at 4°C), that was followed by the application of biotinylated goat anti-rabbit IgG (1:200 in TNB, 45 min, RT; Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Streptavidin-conjugated horseradish peroxidase (1:100 in TNT, 30 min, RT) was then administered and slides were finally treated with FITC (1:50 in amplification diluent, TSA kit, 5 min, RT). After careful wash with TNT buffer, sections were incubated overnight with rabbit anti-human CB₁ antibody (Santa Cruz, CA, USA) at 4°C, followed by the application of goat biotinylated antibody against rabbit IgG (1:200 in TNB, 45 min, RT; Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Slides were then treated with streptavidin-conjugated horseradish peroxidase (1:100, 30 min, TSA kit), and finally with tetramer rhodamine-conjugated tyramide (1:50, 5 min, TSA kit). After the final

wash, sections were counterstained with DAPI for 1 min and mounted with Fluoromont-G. Primary antibodies were omitted as negative control for the unspecific binding of the secondary antibodies. Experiments were performed by Koji Sugawara and Nóra Zákány.

4.3.6 c-Kit/Tocrifluor T1117 double immunostaining

HF_s were treated with 1 μ M Tocrifluor T1117 for 24 hours. Following appropriate sample collection, cryosections were fixed in 1% paraformaldehyde in PBS (see section 4.3.4) for 10 min, followed by a 5 min ethanol-acetic acid (2:1) post-fixation at -20°C, using washing steps with PBS in between. To saturate the endogenous peroxidase activity, the slides were placed into a blocking solution (3[V/V]% H₂O₂ in PBS) for 15 min at RT. After washing the slides three times for 5 min with TNT, the endogenous avidin-biotin were blocked (15-15 min RT, Blocking Kit Vector Laboratories, Burlingame, USA), and sections were then incubated with the primary rabbit-anti human c-Kit antibody diluted in antibody diluents (1:1000; DCS; Innovative Diagnostik-Systeme, Hamburg, Germany) overnight at 4°C. After washing (3 times, TNT, 5 min, RT), slides were stained with secondary antibody (biotinylated goat anti-rabbit IgG; 1:200 in DCS, 45 min at RT). Sections were then washed and incubated with streptavidin-conjugated horseradish peroxidase (1:100, 30 min, TSA Kit). Finally, FITC-conjugated tyramide was applied (1:50, 5 min, RT, TSA Kit). Primary antibodies were omitted as negative control for the unspecific binding of the secondary antibody. Experiments were performed by Koji Sugawara, Nóra Zákány, and Claudia Kremling.

4.3.7 c-Kit/Ki67 double immunostaining

To examine the number of c-Kit⁺ actively proliferating cells, double immunostaining was performed. After the c-Kit TSA staining (see section 4.3.4), mouse anti-human Ki67 antibody at 1:20 or rat anti-mouse Ki67 at 1:100 (both from DAKO, Santa Clara, CA, USA) were used. After PBS wash, rhodamine goat anti-mouse or goat-anti-rat IgG secondary antibodies (Jackson Immuno Research, West Grove, PA, USA) were applied at 1:200 for 45

min. After counterstaining with DAPI, slides were mounted with Fluoromount-G™ (Thermo Fisher Scientific, USA). Primary antibodies were omitted as negative control for the unspecific binding of the secondary antibody. Experiments were performed by Koji Sugawara and Nóra Zákány.

4.3.8 Ki67/TUNEL double immunostaining

Double Ki67/TUNEL (“terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling”) immunostaining was performed to evaluate the number of proliferating and apoptotic cells within the HFs (Telek et al, 2007). Cryosections were first fixed in 1% paraformaldehyde in PBS (see section 4.3.4), then in ethanol/acetic acid (2:1, 10 min, -20°C). Afterwards, they were labeled with a digoxigenin-deoxyUTP (ApopTag Fluorescein In Situ Apoptosis Detection Kit, Intergen, Purchase, NY, USA) in the presence of terminal deoxynucleotidyl transferase (TdT). This was followed by an overnight incubation with mouse anti-human Ki67 antigen (DAKO, Santa Clara, CA, USA) as described above. TUNEL+ cells were visualized by an antidigoxigenin FITC-conjugated antibody (ApopTag kit), whereas Ki67 was detected by a rhodamine-labeled goat anti-mouse antibody (Jackson Immuno Research, West Grove, PA, USA). Finally, sections were counterstained with DAPI (1 µg/ml). The number of Ki67 and TUNEL positive cells were counted in defined areas in the CTS of HFs and were normalized to the number of total (DAPI) cells. Primary antibody and TdT enzyme were omitted as negative control. Experiments were performed by Koji Sugawara and Nóra Zákány.

4.3.9 c-Kit/TUNEL double immunostaining

This staining was applied to check the ratio of the apoptotic c-Kit+ MCs. The c-Kit TSA method (see section 4.3.4) was followed by a TUNEL reaction (see section 4.3.8). Primary antibody and TdT enzyme were omitted as negative control. Experiments were made by Koji Sugawara and Nóra Zákány.

4.3.10 SCF immunostaining

To identify the SCF immunoreactivity of the organ-cultured human HFs and isolated human ORS-KCs, an indirect immunofluorescence method was used. The anti-human SCF primary antibody (OriGene – Acris Antibodies, Rockville, USA) was diluted in PBS (1:20) and slides were incubated overnight at 4°C. As a secondary antibody, FITC-conjugated (rhodamine for ORS-KCs) goat anti-mouse IgG at 1:200 in PBS was applied for 45 min. As a positive control, an intact human scalp skin sample was used. Primary antibodies were omitted as negative control for the unspecific binding of the secondary antibody. Experiments were performed by Koji Sugawara.

4.3.11 Immunohistochemistry to explore expression pattern of members of ECS

For immunohistochemical investigation of NAPE-PLD, FAAH, MAGL (Novus Biologicals, Littleton, USA), and DAGL β (Bioss Inc., Massachusetts, USA), three formalin fixed paraffin embedded skin samples (rich in SGs) were used, all diagnosed as trichilemmal cyst in Gyula Kenézy University Hospital (formerly known as Gyula Kenézy County Hospital), Debrecen, Hungary. To examine the expression pattern of DAGL α (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) in SGs, three post-mortem frozen skin samples obtained from the vertex were used.

Next, 4 μ m thick, serial sections of paraffin-embedded and cryo-embedded tissues were cut. Cryostat samples were then fixed in acetone (10 min, -20°C), whereas formalin fixed sections were deparaffinized and prepared for heat-induced antigen retrieval. FAAH, MAGL, and DAGL β epitopes were retrieved in 11 mM citrate buffer (pH 6) using pressure cooker on full pressure (2 min). NAPE-PLD was retrieved in EnVision FLEX Target Retrieval Solution High pH (DAKO, Santa Clara, CA, USA) in water bath (95 °C, 30 min). After blocking the endogenous peroxidase activity (3 [V/V]% H₂O₂, 10 min, RT) in both type of sections, primary antibodies diluted in 1% Bovine Serum Albumin were applied at RT (Sigma Aldrich, St. Louis,

MO, USA): NAPE-PLD (mouse, clone: 5F7, 1:75, 60 min), FAAH (rabbit polyclonal, 1:20, 60 min), MAGL (mouse, clone: 2B11, 1:100, 60 min), DAGL α (rabbit polyclonal, 1:100, 30 min), and DAGL β (rabbit polyclonal, 1:50, 30 min). After this step, either EnVision FLEX Labeled polymer-HRP anti-rabbit or anti-mouse System (DAKO, Santa Clara, CA, USA) were used (30 min, RT) with 3,3'-diaminobenzidine ([DAB]; DAKO, Santa Clara, CA, USA) visualization techniques. Lastly, slides were counterstained with hematoxylin and mounted in permanent mounting medium (Histolab, Göteborg, Sweden).

To support the validity of stainings, samples with known antigen expression pattern were used as positive controls. To verify the specific staining of NAPE-PLD and MAGL, renal tubes were stained (expected positivity in case of NAPE-PLD: membranes of the proximal tubules based on The Human Protein Atlas <http://www.proteinatlas.org/ENSG00000161048-NAPEPLD/tissue>; expected positivity in case of MAGL: nuclei in all tubules). To check the specific expression of FAAH, testis samples were used (expected positivity: cytoplasmic staining in Leydig cells and in testicular tubules). In case of DAGL α (expected positivity: dendritic tree of Purkinje cells) and DAGL β (expected positivity: synaptic areas) cerebellum was applied as a positive control. Negative controls were obtained by omitting the primary antibodies. Experiments were performed by Ágnes Pór and Ilona Kovács.

4.4 Microscopy

For fluorescence and light microscopy the following microscopes were used in case of experiments with MCs: Keyence Biozero-8000 and Keyence Biozero-8100 microscopes (Keyence Corporation, Biozero-8000, Higashi-Nakajima, Osaka, Japan), in combination with Nikon lenses (Japan). Images were analyzed with Biozero Image Analyzer Software, Version 2.5.

For transmission electron microscopy an electron microscope JEM-1200EXII, JEOL, (Tokyo, Japan) was used. The sample preparation was performed as follows: organ-cultured

human scalp HFs were immersed in a solution containing 2 (g/100 ml)% paraformaldehyde, 2.5 (g/100 ml)% glutaraldehyde, and 0.025 (g/100 ml)% CaCl_2 in 0.1 M sodium cacodylate buffer (pH 7.4), and were then fixed. Afterwards, the samples were placed into 1 (V/V)% osmium tetroxide in the same buffer. The specimens were dehydrated in a gradient series of ethanol, immersed in propylene oxide, and embedded in plastic resin. Thin and thick sections were cut with a Leica Ultra UCT (Leica, Wetzlar, Germany). Thick sections (1 μm) were prepared for an alkaline-Giemsa histochemistry. Thin sections (70 nm) were stained with uranyl acetate as well as lead citrate. Experiments were made by Daisuke Tsuruta.

High magnification images of c-Kit and CB_1 double positive cells were taken by using laser scanning confocal microscopy (Fluoview 300, Olympus Tokyo, Japan) running Fluoview 2.1 software (Olympus). The thickness of the slides were identical with the ones that were used for fluorescence and light microscopy (6 μm).

For immunohistochemistry of SGs and their positive controls Nikon Eclipse E600 microscope (Nikon, Tokyo, Japan) was applied.

4.5 Determination of the number of MCs

During the evaluation processes, MCs were identified as degranulated, when five or more extracellularly situated metachromatic granules could be histochemically detected at 400x magnification, using fluorescence/light microscopy (**Fig. 6A-C**). The number of degranulated and total CTS-MCs were counted along the HFs per visual field (bright field), in at least 13 fields per HF (a minimum of 2 donors and 6 HFs/treatment group). MCs within human and mouse skin samples were counted per visual field, which individually means at least 13 fields per sample (a minimum of 2 donors). Counting was performed by Nóra Zákány and Koji Sugawara.

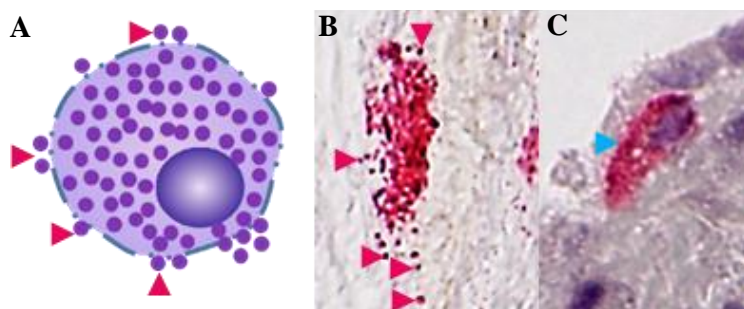


Fig. 6: Mast cell counting

(A-B) A MC was defined as degranulated when five or more extracellularly situated metachromatic granules (red arrows) could be histochemically detected at 400x magnification. (C) Non-degranulated MC (blue arrow).

4.6 Signal intensity measurements

Signal intensity of the stainings was evaluated by semiquantitative analysis, using NIH Image J software (National Institute of Health, Bethesda, Maryland) (Meyer et al. 2008; Bertolini et al. 2013, Harries et al. 2013). Measurements were performed by Nóra Zákány and Koji Sugawara.

4.7 CB₁ knockdown *in situ*

The following reagents were used during the process: human CB₁ siRNA (SC-39910), control (scrambled oligos, SCR), siRNA transfection reagent (sc-29528), and siRNA transfection medium (Santa Cruz Biotechnology Inc., Santa Cruz, USA). Experiments were performed according to the manufacturer's protocol and as previously described (Fig. 7; Samuelov et al., 2012). Freshly isolated HF's were placed into cold William's E medium right before the knockdown procedure. CB₁ specific siRNA (2.5 μ l), siRNA transfection reagent (2.5 μ l) or control siRNA (2.5 μ l) was mixed in 500 μ l transfection medium per well (24 well plate). After careful wash, 3 HF's were placed into each well for 6 hrs, at 37°C in a CO₂ incubator. When the specified time had elapsed, medium was changed to normal supplemented William's E medium. Cryo-embedding was performed 24 hrs after the transfection. Experiments were performed by Nóra Zákány and Koji Sugawara.

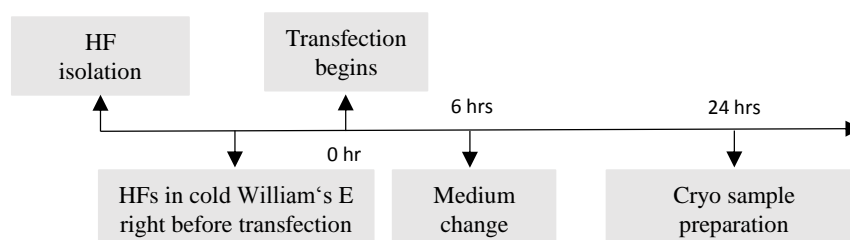


Fig. 7: Timeline with the main steps of hair follicle transfection *in situ*
HF: hair follicle.

4.8 Tryptase immunoassay

Organ-cultured human scalp HF^s were treated with CB₁ siRNA (24 hrs) according to the previous paragraph. The level of tryptase was measured by a fluorescent enzyme immunoassay in the supernatants by using a commercial assay from Phadia (ImmunoCap Tryptase, Uppsala, Sweden). The principle of the assay is based on a monoclonal antitryptase capture antibody that specifically binds tryptase. After washing, β -galactosidase-labeled antitryptase antibody was added. Bound complexes were stained by the conversion of 4-methylumbelliferyl-b-D-galactoside. The fluorescent signal was correlated with the amount of tryptase. Experiments were run by Koji Sugawara.

4.9 CB₁ knockout (KO) mice

CB₁ gene was inactivated by targeted disruption within MPI2 embryonic stem cells, using a non-receptor sequence during homologous recombination. The resulting mice were mated more than 13 times with C57BL/GJ animals, and therefore considered to be congenic (Járai et al., 1999; Zimmer et al., 1999). CB₁^{+/-} mice were generated with the crossing of wild-type (CB₁^{+/+}) and KO mice (CB₁^{-/-}). Experiments were performed by Anne Zimmer and Andreas Zimmer.

4.10 RNA isolation, reverse transcription and quantitative “real-time” polymerase chain reaction (Q-PCR)

Q-PCR was performed on an ABI Prism 7000 sequence detection system using the 5' nuclease assay as previously described (Telek et al., 2007; Ramot et al., 2010). Total RNA was isolated from HF^s using a total RNA isolation reagent (TRI) and digested with recombinant RNase-free DNase-1 according to the manufacturer's protocol. After isolation, 1 μ g of total RNA was reverse-transcribed into cDNA by using High Capacity cDNA kit following the manufacturer's protocol. PCR amplification was performed by using specific TaqMan[®] Gene Expression Assays (assay IDs: Hs00241497_m1 for human SCF; Hs00174092_m1 for IL-1 α ;

Hs00174097_m1 for IL-1 β ; Hs00985639_m1 for IL-6; Hs00174103_m1 for IL-8; Hs00174128_m1 for TNF- α ; Hs00419593_m1 for NAPE-PLD; Hs00391374_m1 for DAGL α ; Hs00373700_m1 for DAGL β , Hs00155015_m1 for FAAH; and Hs00200752_m1 for MAGL) using TaqMan universal PCR master mix protocol. When working with sebocytes, as internal control, transcripts of 18S RNA or cyclophilin A (peptidylprolyl isomerase A, [PPIA]) were determined (assay IDs: Hs99999901_s1 and Hs99999904_m1, respectively), whereas in our MC studies glyceraldehyde 3-phosphate dehydrogenase (GAPDH; ID: Hs99999905_m1) β -actin (ACTB; ID: Hs99999903_m1) and PPIA were applied.

The amount of transcripts was normalized to the control genes using Δ CT method. Finally, when indicated, results were further normalized to the expression of the vehicle control ($\Delta\Delta$ CT method). All reagents and detection systems were purchased from Applied Biosystems/Life Technologies, Carlsbad, CA, USA. Experiments were performed by Balázs István Tóth, Andrea Aranyász, and Attila Oláh.

4.11 Determination of intracellular lipids

For quantitative measurement of sebaceous (neutral) lipid content, 20000 cells/well were seeded and cultured in 96-well “black-well/clear-bottom” plates (Greiner Bio-One, Frickenhausen, Germany) in quadruplicates, and were treated with compounds for 48 hrs. Next, supernatants were discarded, and after two PBS washes, 100 μ l of a 1 μ g/ml Nile Red (Sigma Aldrich, St. Louis, MO, USA) solution in PBS was added to each well. The plates were then incubated at 37°C for 20 min, and fluorescence intensity was measured on FlexStation 3 multi-mode microplate reader (Molecular Devices, San Jose, CA, USA). Results are expressed as percentage of the relative fluorescence units in comparison with the vehicle controls using 485 nm excitation and 565 nm emission wavelengths. Experiments were performed by Nóra Zákány, Erika Takács, Andrea Aranyász, Arnold Markovics and Attila Oláh.

4.12 Determination of cellular viability

To determine the viability of cells, the conversion of tetrazolium salt MTT (Sigma Aldrich, St. Louis, MO, USA) to formazan by mitochondrial dehydrogenases was measured. Cells were seeded in 96-well plates (20,000 cells/well) in quadruplicates, and were treated as indicated for 48 hrs. This step was followed by a 2 hrs incubation with 0.5 mg/ml MTT solution. Finally, the concentration of formazan crystals (as an indicator of the number of viable cells) was measured colorimetrically at 565 nm by using FlexStation 3 multi-mode microplate reader (Molecular Devices, San Jose, CA, USA). Results were expressed as percentage of vehicle controls regarded as 100%. Experiments were performed by Nóra Zákány, Erika Takács, Andrea Aranyász, Arnold Markovics and Attila Oláh.

4.13 Determination of apoptosis

Reduction of the inner mitochondrial transmembrane potential is an initial mark and critical turning point during the mechanism of apoptosis (Green and Reed, 1998; Susin et al., 1998). To determine the mitochondrial membrane potential of SZ95 sebocytes 1,1',3,3',3'-hexamethylindodicarbo-cyanine iodide containing MitoProbe™ DiIC₁(5) Assay Kit (Life Technologies, Carlsbad, CA, USA) was used. Cells (20,000 cells/well) were cultured in 96-well “black-well/clear-bottom” plates (Greiner Bio-One, Kremsmuenster, Austria) in quadruplicates and were treated for 48 hrs. Supernatants were then discarded, cells were incubated for 30 min with DiIC₁(5) working solution (50 µl/well), then washed with PBS. The fluorescence of DiIC₁(5) was measured at 630 nm excitation and 670 nm emission wavelengths using FlexStation 3 multi-mode microplate reader (Molecular Devices, San Jose, CA, USA). Relative fluorescence values were expressed as percentage of vehicle controls regarded as 100%. Carbonyl cyanide m-chlorophenyl hydrazone ([CCCP], Life Technologies, Carlsbad, CA, USA), dissolved in the DiIC₁(5) working solution, was applied (1:200, 30 min) as positive control. Experiments were performed by Nóra Zákány, Erika Takács and Attila Oláh.

4.14 Determination of necrosis

To detect the necrosis of cells SYTOX Green staining (Life Technologies, Carlsbad, CA, USA) was used. The dye is not able to cross intact cell membranes, but it easily penetrates into necrotic cells with ruptured plasma membranes. Cells were cultured in 96-well “black-well/clear-bottom” plates (Greiner Bio-One, Kremsmuenster, Austria), and treated as indicated for up to 48 hrs. Cells were then washed with PBS and the culture medium was replaced with SYTOX Green (1 μ M dye; 30 min at 37°C), whose fluorescence intensity was measured at 490 nm excitation and 520 nm emission wavelengths using FlexStation 3 multi-mode microplate reader (Molecular Devices, San Jose, CA, USA). Lysis buffer (1:100 in the SYTOX Green working solution for 30 min; Life Technologies, Carlsbad, CA, USA) was used as a positive control. Relative fluorescence values were expressed as percentage of positive control regarded as 100%.

Due to their spectral properties, DilC₁(5) and SYTOX Green dyes were always administered in the same time, allowing us to assess necrotic and early apoptotic processes of the same cultures. Selective decrease of DilC₁(5) intensity indicated mitochondrial depolarization (i.e., the onset of early apoptotic processes), whereas an increase of SYTOX Green staining intensity revealed necrotic cell death. Experiments were performed by Nóra Zákány, Erika Takács and Attila Oláh.

4.15 Determination of cytokine release (enzyme-linked immunosorbent assay, ELISA)

SZ95 cells were seeded in Petri-dishes (500,000 cells/dish, d=15 mm) in 1.5 ml culture medium. Treatments were done on the following day as indicated. The released amounts of IL-1 α , IL-1 β , IL-6, IL-8, and TNF α were determined, using the collected supernatants, according to the manufacturers’ protocols (IL-1 β , IL-6, IL-8, and TNF α : BD Pharmingen, Franklin Lakes, NJ, USA; IL-1 α : R&D Systems, Minneapolis, MN, USA). In case of IL-6 and IL-8, supernatants were diluted (1:50), while in the case of IL-1 α , IL-1 β , and TNF α undiluted

supernatants were used. Experiments were performed by Nóra Zákány, Arnold Markovics and Attila Oláh.

Supernatant of human ORS-KCs culture (details of ethical approval can be found in section 4.2.2) were harvested and kept at -80°C until the immunoassay was performed. To measure the SCF level of the samples, Quantikine Human SCF ELISA Kit (R&D Systems, Minneapolis, MN, USA) was used according to the manufacturer's protocol. Wild-type and $\text{CB}_1^{-/-}$ mice skin was kindly provided by prof. Andreas Zimmer (University of Bonn, Germany). Experiments were performed by Arno Kromminga.

4.16 Western blotting

Cells were harvested in lysis buffer (20 mM Tris-HCl, pH 7.4, 5 mM EGTA, 1 mM 4-(2-aminoethyl) benzensulphonyl fluoride, and protease inhibitor cocktail diluted in 1:100 (all from Sigma Aldrich, St. Louis, MO, USA). To measure their protein content, a modified BCA protein assay (Pierce, Rockford, IL, USA) was used. Proteins of samples were separated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Samples were loaded with equal amount of protein (40 μg) per lane into a 10% Mini Protean TGX gel (Bio-Rad, Hercules, CA, USA). They were then transferred onto nitrocellulose membranes by using Trans-Blot[®] Turbo[™] Nitrocellulose Transfer Packs and Trans-Blot Turbo[™] System (both from Bio-Rad, Hercules, CA, USA). The membranes were then incubated overnight at 4°C with specific primary antibodies (1:200 in 5% milk containing PBS), namely mouse-anti-human FAAH, rabbit-anti-human NAPE-PLD, MAGL, and DAGL α (all from Abcam, Cambridge, UK), or goat-anti-human DAGL β (Santa Cruz, CA, USA). After this step, the membranes were exposed to horseradish peroxidase-conjugated rabbit IgG Fc segment-specific antibodies (developed in goat, 1:1000 in 5% milk containing PBS; Bio-Rad, Hercules, CA, USA). To detect the probes that were labeled and bound to the protein of interest, a SuperSignal[®] West Pico Chemiluminescent Substrate enhanced chemiluminescence kit (Pierce Biotechnology,

Waltham, USA) and KODAK Gel Logic 1500 Imaging System (Eastman Kodak Company, Kodak, Tokyo, Japan) were used. Experiments were performed by Nóra Zákány, Andrea Aranyász, and Attila Oláh.

4.17 [³H]-AEA uptake assay in SZ95 sebocytes

Cellular uptake of AEA in SZ95 sebocytes was measured by using an established multi-phase assay protocol previously described for different cell types (Nicolussi et al., 2014; Rau et al. 2016). Briefly, 1×10^6 SZ95 cells per sample were incubated (15 min, 37°C) with either the vehicle control, DMSO (5 µL), or the reference AEA uptake inhibitor, UCM707 (5 µL, 10 µM final; Cayman Chemicals, Ann Arbor, Michigan, USA) in 500 µL RPMI medium (Sigma Aldrich, St. Louis, MO, USA), at 37°C in AquaSil™ silanized screw-cap Eppendorf tubes. After this step, a mixture of [*ethanolamine*-1-³H]AEA (0.5 nM, American Radiolabeled Chemicals, Inc. St. Louis, USA) and unlabeled AEA (final 100 nM) was added and they were further incubated for 15 min. To stop the uptake process, the samples were put on ice following a rapid centrifugation at 800 x g for 5 min at 4°C. Supernatants were collected separately and the cell pellets were washed and resuspended using 500 µL ice-cold PBS containing 1% BSA (fatty acid-free, Sigma Aldrich, St. Louis, MO, USA). The washing solution was also kept, and after phase separation (1:1 CHCl₃:MeOH, ice cold, 1 ml, 10000 x g), was mixed with the organic phase, extracted from the primary supernatant resulting as the extracellular phase. The aqueous phase was collected apart. Cell pellet was resuspended in 250 µL PBS. All phases were transferred into scintillation tubes and mixed with Ultima Gold scintillation cocktail (Perkin Elmer, Switzerland) after liquid scintillation (Thermo Fisher Scientific, USA) counting using a Packard Tri-Carb 2100 TR beta counter. Data are reported as mean values of n=3 independent experiments performed in triplicates. Experiments were performed by Simon Nicolussi and Jürg Gertsch.

4.18 Determination of eCB levels

In order to determine eCB levels, cells were seeded in Petri dishes (5 million/5 ml culture medium) and were cultured with VMD11 or vehicle (5 ml medium/dish) for 24 hours. Afterwards, equal amount (i.e., 5 ml) of ice-cold methanol was added to each Petri dish. Cells and their supernatants together were then harvested and immediately stored at -80°C for further analysis.

Samples were first homogenized in a solution that contained 5 pmol of [^2H] $_8$ -AEA and 50 pmol of [^2H] $_5$ -2-AG, [^2H] $_4$ -PEA and [^2H] $_2$ -OEA. Afterwards, by open-bed chromatography the lipid-containing organic phase was pre-purified on silica gel (Bisogno et al., 1997), and analyzed by liquid chromatography-tandem mass spectrometry ([LC-APCI-MS], Marsicano et al., 2002). AEA, 2-AG, PEA, and OEA contents (pmol) were normalized per mg of extracted lipids. Experiments were performed by Fabiana Piscitelli and Vincenzo Di Marzo.

4.19 Assessment of FAAH-activity

After counting the cells, supernatants were removed, and sebocytes were harvested with trypsin. These samples were then transferred to -80°C until further analysis. As a first step, cell pellet extracts were incubated in 50 mM Tris-HCl, for 30 min at 37°C with [^{14}C]AEA (2 μM) and with VDM11, URB597, and UCM707. [^{14}C]Ethanolamine produced from [^{14}C]AEA hydrolysis was measured by scintillation, counting of the aqueous phase after extraction of the incubation mixture with 2 vol. of $\text{CHCl}_3/\text{CH}_3\text{OH}$ 2:1 (by vol.). Experiments were performed by Marco Allarà and Vincenzo Di Marzo.

4.20 Statistical analysis

MC experiments. Data were analyzed using GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA). The following tests were applied: Student's two tailed *t*-test for unpaired samples or Mann-Whitney *U*-test. *p* < 0.05 values were regarded as significant. Data are expressed as mean \pm SEM.

SG experiments. Data were analyzed by Origin Pro Plus 6.0 software (Microcal, Northampton, MA, USA), using Student's two tailed, unpaired *t*-test and $p < 0.05$ values were regarded as significant differences. Graphs were plotted using Origin Pro Plus 6.0 software (Microcal, East Northampton, USA).

5. RESULTS

5.1. eCBs limit excessive MC maturation and activation in human skin

5.1.1 Human CTS-MCs express CB₁

Our group previously identified that human scalp HF together with its MC-rich CTS express CB₁, while CB₂ was not detectable (Telek et al., 2007). Based on this intriguing finding, we aimed to investigate whether CB₁ is also expressed on those MCs that are directly located in the HF's CTS. To do so, c-Kit/CB₁ double immunofluorescent staining was applied. c-Kit (CD117) is an important stem/MC growth factor, which is suitable to identify MCs, even at different maturity stages (Ito et al. 2010; Valent et al., 2010; Gilfillan et al., 2011a). Importantly, HF CTS is free from c-Kit⁺ melanocytes, therefore, c-Kit marks exclusively MCs (Ito et al., 2010; Peters et al., 2007). Double immunofluorescence staining confirmed that around 75.5% of the CTS-MCs that showed c-Kit positivity, also expressed CB₁. This was the case in organ-cultured human HF (Fig. 8A-B), and in human isolated scalp skin (Fig. 8C).

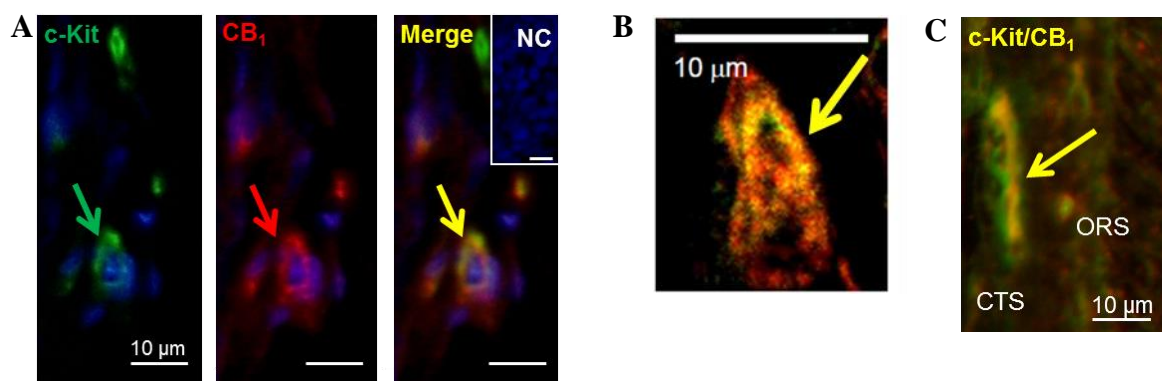


Fig. 8: CB₁ expression on MCs in organ cultured human HF and isolated scalp skin

(A) Immunofluorescent staining for CB₁ (red) and c-Kit (green) within human HF. (B) High magnification picture of c-Kit/CB₁ double positive cells within organ cultured human HF and (C) isolated scalp skin by laser scanning confocal microscopy (yellow arrows denote the double positivity, scale bars mark 10 μm). NC: negative control, ORS: outer root sheath, CTS: connective tissue sheath.

Our next step was to probe whether these CB₁ complexes are also functionally active, and if they are available for pharmacological modulation. Employing, a highly CB₁-selective ligand, the fluorescent AM251-derivative “Tocrifluor T1117” (1 μ M; Daly et al., 2010), we performed c-Kit/Tocrifluor double immunofluorescence microscopy. This showed that after 24 hrs of incubation, the CB₁-selective ligand was able to directly bind to those CTS-MCs, which also showed co-expression of c-Kit marker, demonstrating that CB₁ receptors of CTS-MCs can be targeted by AM251 even in intact HF*s in situ* (**Fig. 9**).

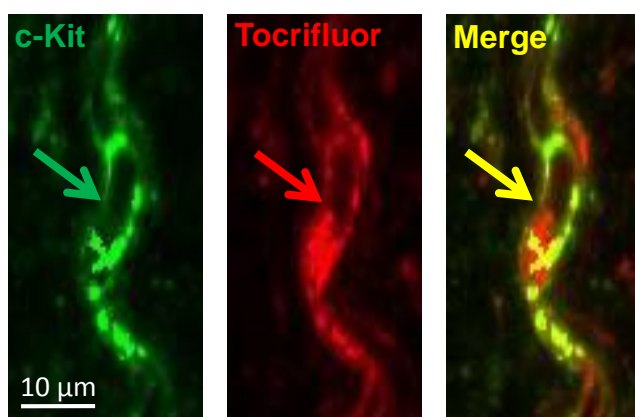


Fig. 9: CB₁s display functional and specific binding activity in CTS-MCs

Double immunofluorescent staining for c-Kit (green) and Tocrifluor (red) in organ cultured human HF*s* by laser scanning confocal microscopy. The yellow arrow denotes the double positivity (scale bars mark 10 μ m).

5.1.2 CB₁ inverse agonism increases the number and degranulation of CTS-MCs

In the next step, we wished to assess whether eCB AEA (30 μ M), CB₁ specific agonist ACEA (30 μ M), or selective CB₁ antagonist/inverse agonist AM251 (1 μ M) would modulate the biology of CTS-MCs. To do so, the aforementioned compounds (individually and in combination) were applied in the culture medium for 1 day. After preparation of cryosections, Leder's esterase immunohistochemistry was performed (**Fig. 10A**). Specified doses of AEA and ACEA had no significant impact on the detectable CTS-MCs' number and activity (**Fig. 10B-G**). By contrast, AM251 significantly increased both the number and degranulation rate of the cells (**Fig. 10A-G**). These effects, with the co-administration of AEA and/or ACEA, were completely abolished (**Fig. 10B-G**). As expected based on previous studies (Ito et al., 2010), we found spontaneously degranulated MCs within the vehicle control group as well (**Fig. 10F-G**). When comparing the histochemical analysis of un-manipulated human skin and freshly

isolated HFs, we found that the percentage of the activated MCs was significantly higher in latter case (**Fig. 10H**). This was most probably due to the process of microdissection, during which a certain number of MCs degranulated.

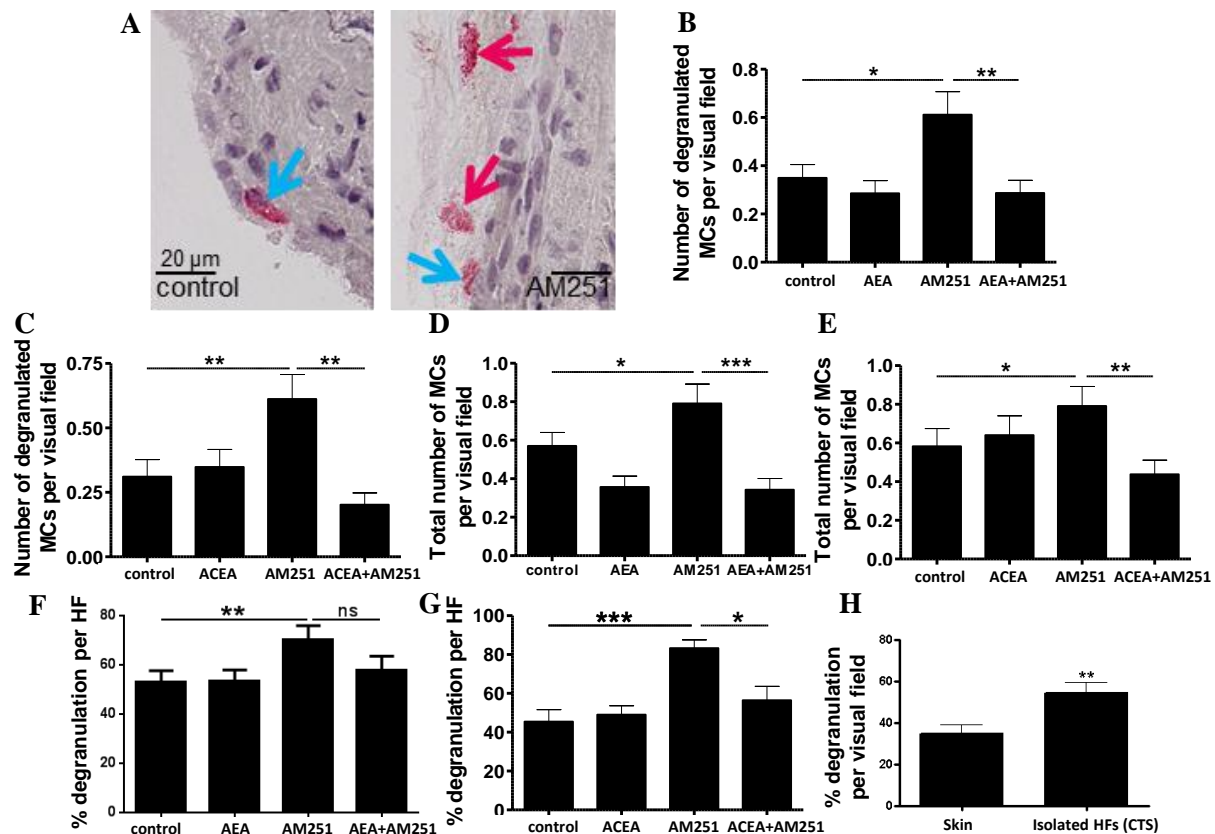


Fig. 10: The effects of CB₁ (inverse) agonist(s) on the number and degranulation rate of CTS-MCs
 Leder's esterase histochemistry. (A) Within the control HF the blue arrow points to a non-degranulated CTS-MC. Within the AM251 treated HF both non-degranulated (blue arrow) and degranulated (red arrow) cells can be seen (scale bars mark 20 μ m). The number of degranulated (B-C) and total CTS-MCs (D-E) per visual field, and their percentage of degranulation (F-G) after 24 hrs treatment with AEA (30 μ M), ACEA (30 μ M), AM251 (1 μ M) or their combination. (H) Percentage of degranulated CTS-MCs within HFs of intact human scalp skin and isolated human scalp HFs using Leder's esterase histochemistry. * p <0.05, ** p <0.01, *** p <0.001, mean \pm SEM. AEA and ACEA: CB₁ agonist, AM251: CB₁ receptor antagonist/inverse agonist.

Results of alkaline-Giemsa histochemistry by high-resolution electron microscopy (**Fig. 11A-B**) independently confirmed that antagonism of CB₁ enhanced the degranulation of CTS-MCs. Transmission electron microscopy was used to check whether there is morphological difference between control and AM251-treated MCs (**Fig. 11C**). In the control group, MCs were well preserved, monomorphonuclear cells (**Fig. 11C, left**), while in the AM251-treated group (**Fig. 11C, right**) they showed a typical ultrastructural morphology of degranulated human MCs, such as granules outside of the cell membrane, granule-granule fusions, and an

empty cavity in the area bounded by the former membrane (Drovak et al., 1988; Wernersson and Pejler, 2014).

This means that the degranulation of human skin MCs can be enhanced by abrogating homeostatic CB₁-signaling.

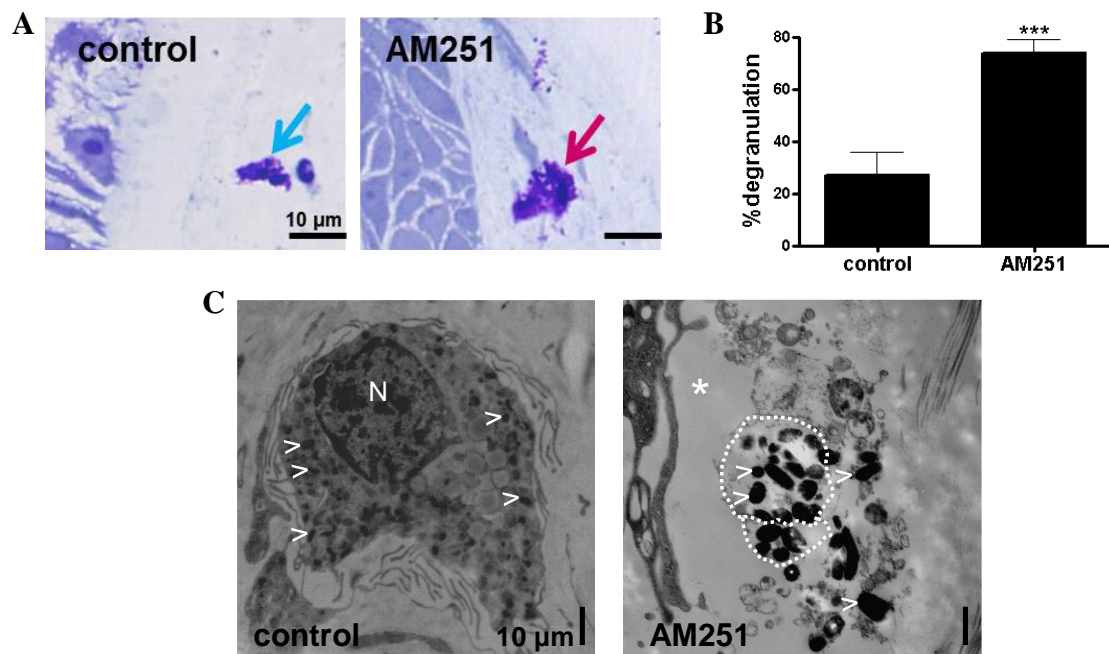


Fig. 11: CB₁ blockade significantly increases the degranulation of CTS-MCs *in situ*

(A) High-resolution light microscopy of alkaline-Giemsa histochemistry (scale bars mark 10 μm) and (B) its statistical analysis. Blue arrow denotes a non-degranulated MC, while red arrow a degranulated one. (C) Transmission electron microscopy images of MCs within control and AM251-treated human HF (scale bars mark 1 μm). MC in the control group (bottom left) is well preserved, rounded in shape with a complete intact cell membrane, densely packed with granules (white arrowheads). AM251-treated MC (bottom right) shows typical signs of degranulation, such as granules outside of the cell shape (white arrowheads), granule-granule fusions (dashed lines demarcated areas) and an empty cavity in the area bounded by the former membrane (white star). ***p < 0.001, mean ± SEM. AM251: CB₁ receptor antagonist/inverse agonist, N: nucleus.

5.1.3 CB₁ inverse agonism induces maturation, but not proliferation, of CTS-MCs

It was next examined how the stimulation/inhibition of CB₁ signaling affects the number of c-Kit⁺ CTS-MCs. This growth factor receptor is mostly expressed by MCs within the human skin mesenchyme and it is often used for the immunohistological detection of both mature and immature MC populations (Peters et al., 2007; Stone et al., 2010; Ito et al., 2010). Using quantitative immunohistomorphometry, we found that in case of CB₁ inverse agonism, the number of c-Kit⁺ CTS-MCs was significantly elevated *in situ* (Fig. 12A-C). In addition, we have observed that the number of Ki67⁺/c-Kit⁺ (Fig. 12D-E) and TUNEL⁺/c-Kit⁺ cells (Fig.

12F) did not significantly change after AM251 treatment. We found that c-Kit⁺ CTS-MCs were mostly negative for Ki67, both in control and AM251 treated groups. This indicates that CB₁ stimulation-caused increase in the number of c-Kit⁺ CTS-MCs may not be the result of the stimulation of the resident MC's proliferative and/or anti-apoptotic processes.

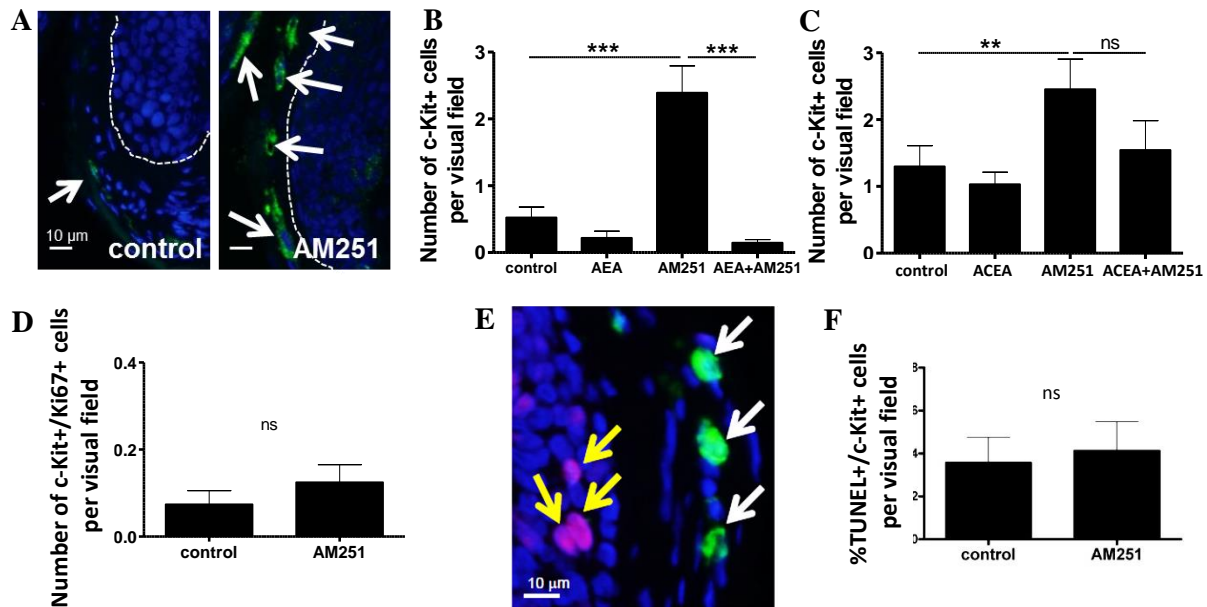


Fig. 12: Differentiation and proliferation of CTS-MCs in organ cultured HF

(A) c-Kit immunostaining (white arrows) in organ cultured HF. (B-C) Quantitative immunohistomorphometry of c-Kit⁺ cells and (D) Ki67⁺/c-Kit⁺ cells in organ cultured HF. (E) Representative image on which yellow arrows denote Ki67⁺ proliferating hair matrix KCs, while white arrows denote c-Kit⁺/Ki67⁻ cells. (F) Quantitative immunohistomorphometry of c-Kit/TUNEL double⁺ cells. ** $p < 0.01$, *** $p < 0.001$. Mean \pm SEM, scale bars mark 10 μ m. ACEA: CB₁ agonist; AEA: non-selective CB₁ agonist; AM251: CB₁ receptor antagonist/inverse agonist; ns: not significant.

We next investigated the expression of different antigens, such as tryptase, chymase, and high affinity IgE receptor alpha (Fc ϵ RI α), which were characteristically identified on matured MCs (Scherthaner et al., 2005; Stone et al., 2010; Valent et al., 2010; Gilfillan et al., 2011). Using the inverse agonist AM251, just as in the aforementioned experiments, the number of tryptase⁺, chymase⁺ and Fc ϵ RI α ⁺ MCs were significantly increased within the HF, as shown on **Fig. 13A-C**.

From these results we assumed that the abrogation of homeostatic CB₁ signaling first may stimulate the differentiation of resident, immature c-Kit⁻ MC progenitors into c-Kit⁺ cells, rather than the proliferation/apoptosis of the mature ones. These newly c-Kit⁺ cells could then

differentiate into fully mature tryptase+/chymase+/FcεRIα+ MCs. Under the given experimental conditions, the assumption that these cells could migrate here from other sources, such as blood or bone marrow, can be excluded - even though, the possibility of their *in utero*/postnatal migration from the bone marrow cannot be ruled out.

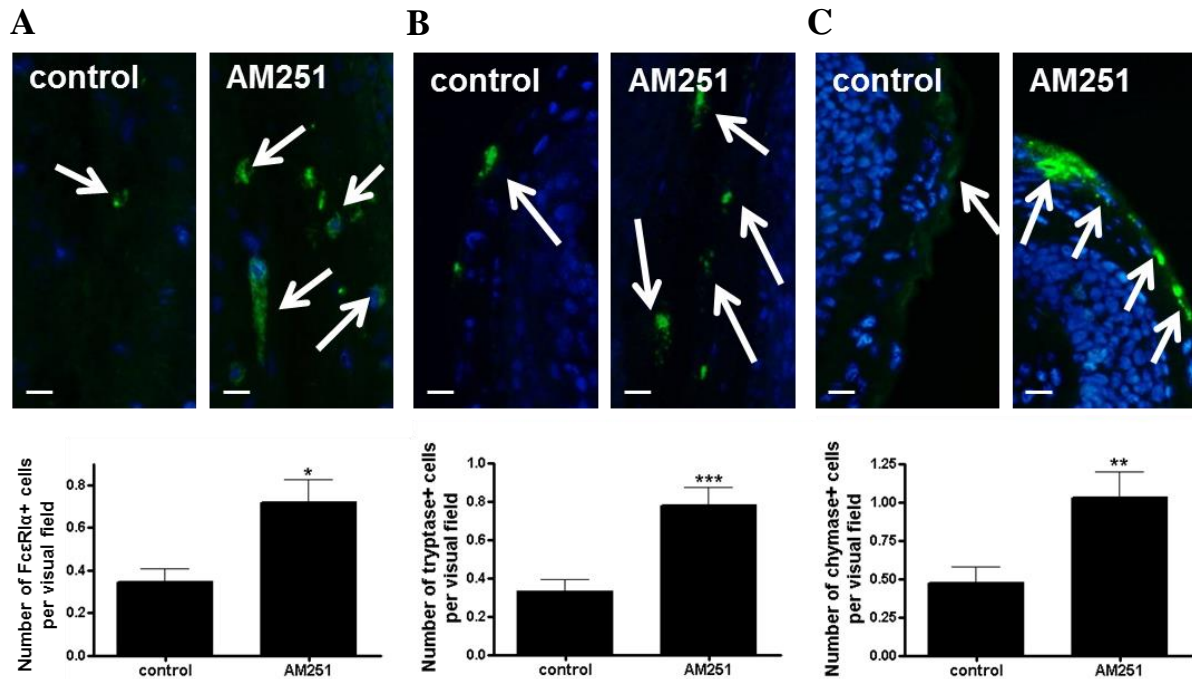


Fig. 13: AM251 increases the number of FcεRIα+, tryptase+ and chymase+ cells

(A) FcεRIα+, (B) tryptase+ and (C) chymase+ CTS-MCs are pointed by the white arrows. Below the representative images the respective graphs are shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, mean \pm SEM, scale bars mark 10 μ m. AM251: CB₁ antagonist/inverse agonist.

This further suggests that the observed CB₁ inverse agonism by AM251 can primarily stimulate maturation and differentiation of immature MC progenitors into c-Kit+ MCs. This means that constitutive CB₁ stimulation might have an important regulatory role in preventing the extreme intracutaneous maturation of MCs.

5.1.4 CB₁ gene knockdown is feasible in organ-cultured human HFs

To further assess the effects observed previously, we attempted to abrogate eCB signaling through CB₁. To do so, standard siRNA technology was applied. Based on the performed CB₁ immunohistochemistry, gene silencing in human HF was successful since the

receptor immunoreactivity was significantly reduced in the CB₁ siRNA treated HF (Fig. 14A-D).

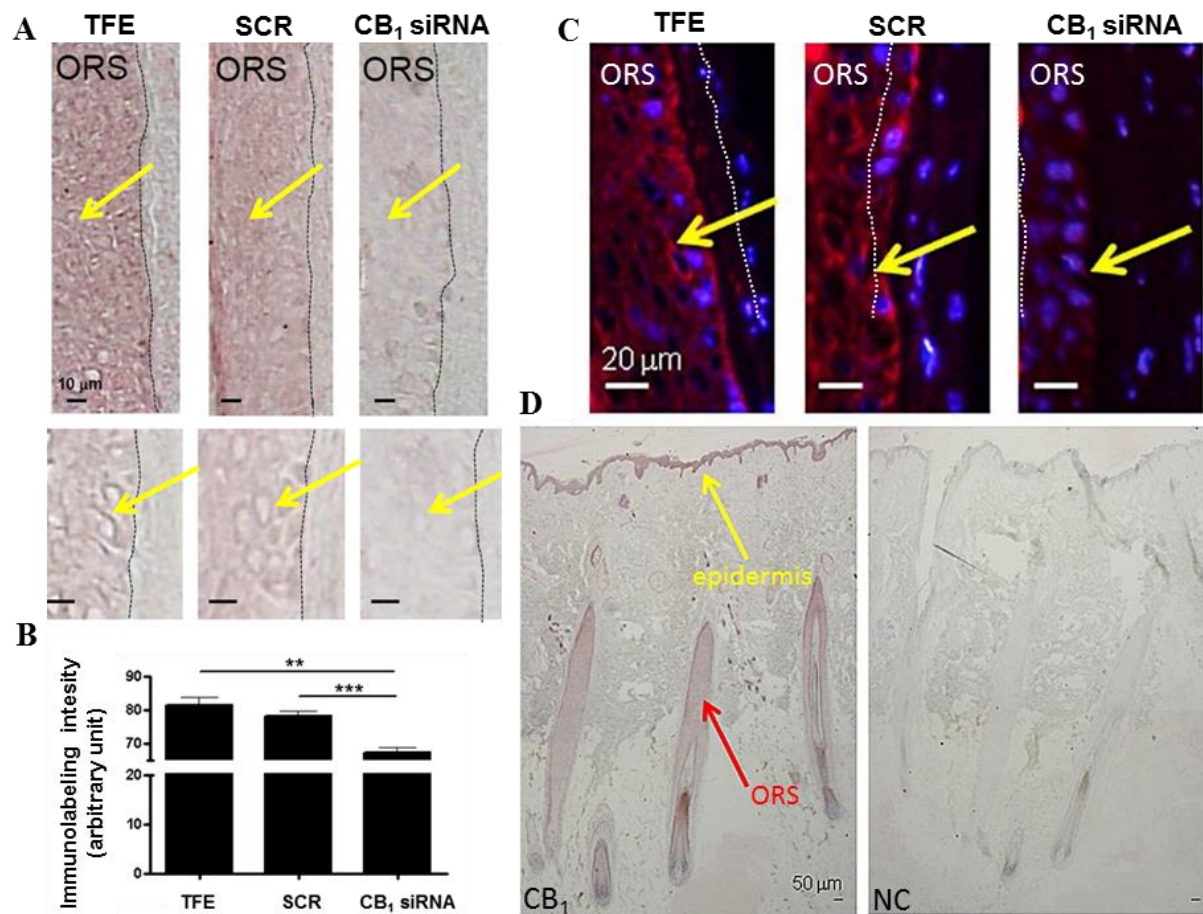


Fig. 14: CB₁ gene knockdown decreases CB₁ expression *in situ*

(A) Representative pictures of CB₁ immunohistochemistry after gene silencing. The yellow arrows denote the immunoreactivity of CB₁+ ORS-KCs. Lower panels show the higher magnifications (scale bars mark 10 μm). (B) Quantitative analysis of relative CB₁ immunoreactivity. (C) Representative pictures of CB₁ immunofluorescence (yellow arrow) *in situ* (scale bars mark 20 μm). (D) Representative images of CB₁ immunohistochemistry with intact human scalp skin sample (left) and negative staining control (right). Positive CB₁ immunoreactivity in the epidermis (yellow arrow) and ORS (red arrow, scale bars mark 50 μm). ***p* < 0.01, ****p* < 0.001, mean ± SEM. TFE: transfection reagents treated HF, SCR: scrambled RNA-transfected HF, CB₁ siRNA: CB₁-silenced HF, ORS: outer root sheath.

5.1.5 Number and activation of human CTS-MCs *in situ* is controlled by continuous homeostatic CB₁ activity

With the help of the knockdown experiments, we were able to identify that lower CB₁ expression caused a significant increase in the number and degranulation of CTS-MCs, similarly to the effects of AM251. Indeed, using Leder's esterase staining, we observed more mature and degranulated MCs within the CTS of HF transduced with CB₁ siRNA, compared

to the scrambled oligos (**Fig. 15A-C**). In addition, number of c-Kit⁺ cells (**Fig. 15D-E**) and the c-Kit immunoreactivity within the CTS (**Fig. 15F**) significantly increased in the CB₁-silenced HF_s.

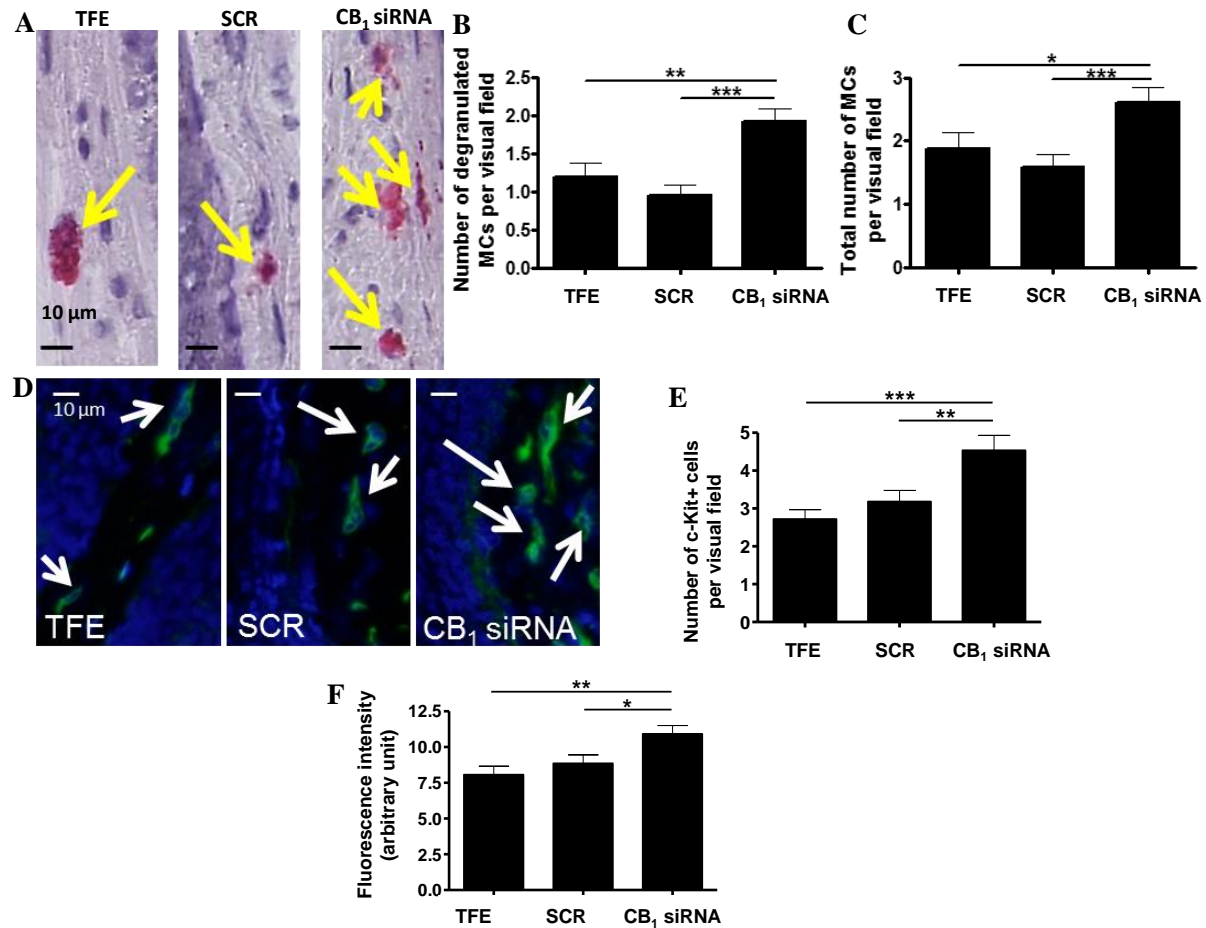


Fig. 15: Effects of CB₁ knockdown on the number and the degranulation activity of CTS-MCs

(A) Leder's esterase histochemistry (yellow arrows) and (B-C) quantitative histomorphometry. (D) c-Kit immunostaining (white arrows), (E) quantitative immunohistomorphometry, and (F) immunoreactivity. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, mean \pm SEM, scale bars mark 10 μ m. TFE: transfection reagents treated HF_s, SCR: scrambled siRNA treated HF_s, CB₁ siRNA: CB₁-silenced HF_s.

As in the case of Leder's esterase and c-Kit stainings (**Fig. 15A-F**), results of tryptase immunohistology also showed an increased number of mature, degranulated CTS-MCs in the CB₁ siRNA-transfected HF_s (**Fig. 16A-B**). Next to this, tryptase fluorescence intensity was reduced in the intra- (**Fig. 16C**), but enhanced in the intercellular space (**Fig. 16D**) following CB₁ gene silencing. This leads to the conclusion that this most abundant secretory granule-derived serine proteinase was actively released only after CB₁ knockdown. The fact that CB₁ gene silencing did not significantly change the tryptase concentration of the culture medium

(Fig. 16E) can easily be explained by the tryptase's strong binding activity to collagen that is represented in the CTS region of the HFs. By carefully observing the microscopic images, indeed we noted that many extracellular tryptase+ granules were situated in the collagen rich CTS, especially within CB₁ knockdown HFs (Fig. 16F). CB₁ gene silencing did not significantly increase the number of the Ki67+ cells, thus had no major effect on the proliferation activity of the CTS- MCs *in situ* (Fig. 16G).

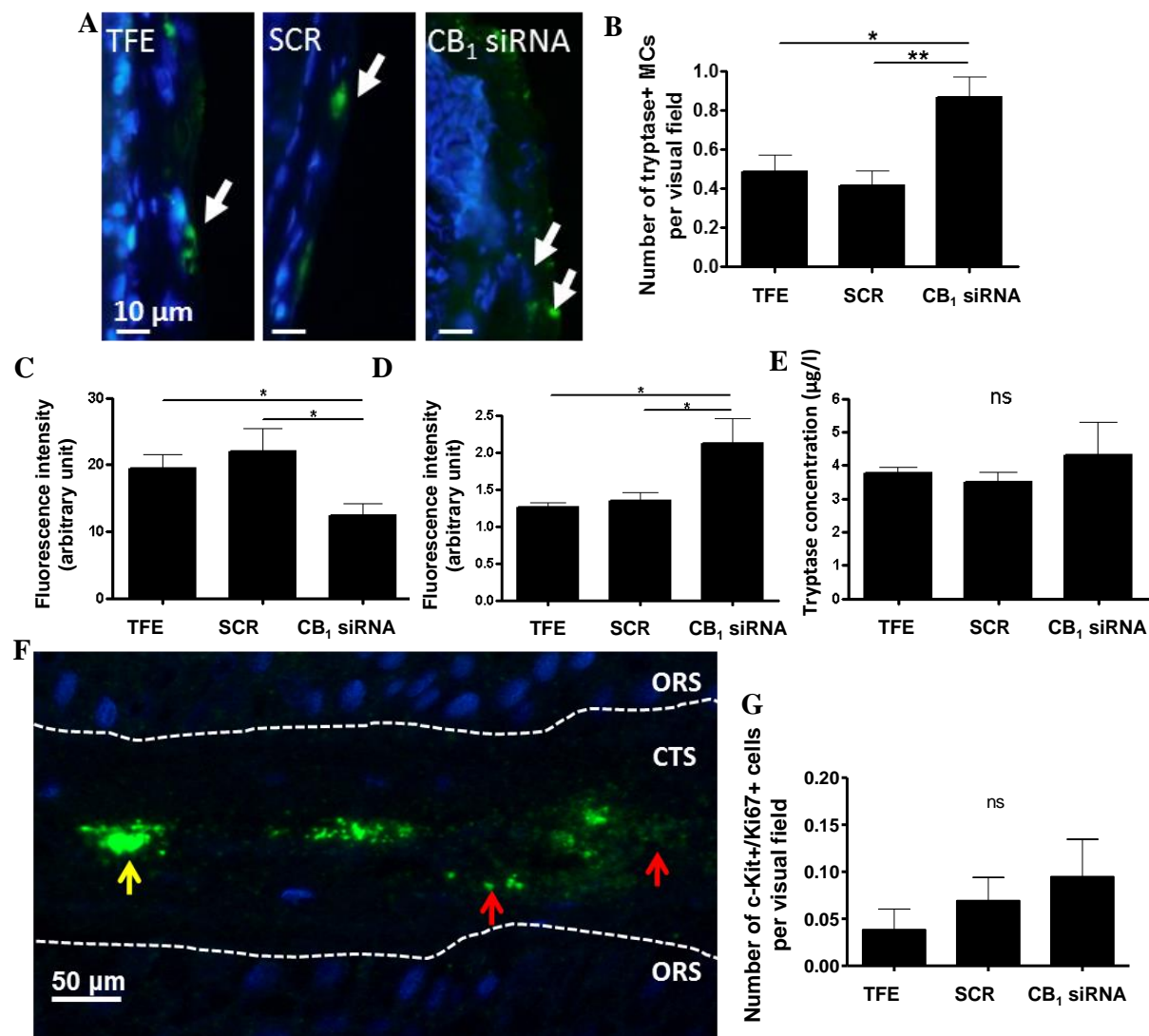


Fig. 16: CB₁ knockdown affects the number and immunoreactivity of tryptase positive MCs

(A) Representative images of tryptase immunohistology (white arrows, scale bars mark 10 μm). (B) Quantitative immunohistomorphometry of (B) tryptase+ cells, (C) intracellular tryptase immunoreactivity, and (D) of intercellular tryptase immunoreactivity. (E) Statistical analysis of tryptase levels in the HF organ-culture medium. (F) Representative image of tryptase immunohistology in CB₁ siRNA-treated HFs. Yellow arrow denotes tryptase+ intracellular immunoreactivity, while red arrows the intercellular tryptase immunoreactivity (scale bars mark 50 μm). (G) Quantitative immunohistomorphometry of c-Kit/Ki67 double+ cells. * $p < 0.05$, ** $p < 0.01$, mean \pm SEM. TFE: transfection reagent treated HFs, SCR: scrambled siRNA treated HFs, CB₁ siRNA: CB₁-silenced HFs, ORS: outer root sheath, CTS: connective tissue sheath, ns: not significant.

The above results suggest that locally produced eCBs in the CTS of human HFs might continuously stimulate CB₁, through which they keep c-Kit expression, maturation, and activation of MCs at a relatively low baseline level. CB₁ knockdown data further confirmed this concept, according to which the CB₁ inverse agonism stimulates the maturation of very immature, not yet c-Kit⁺, resident MC precursors *in situ*. Later these cells become first c-Kit⁺, after which they differentiate into mature tryptase⁺/chymase⁺/ FcεRIα⁺ MCs.

5.1.6 Excessive activation of MCs is controlled by CB₁

It is well known that MCs play a crucial role in the development of many inflammatory and allergic diseases, in addition to the defense processes of innate immunity (Navi et al., 2007; Metz et al., 2008; Stone et al., 2010; Kennelly et al., 2011). Thus, it would be very important to understand those physiological operations that can prevent unwanted activation of MCs. Therefore, we next investigated whether CB₁ stimulation is able to abrogate the MC activator effects of two classical MC secretagogues. We applied SP (100 pM) and compound 48/80 (10 μg/ml), with the combination of AEA (30 μM) or ACEA (30 μM). We found that both compounds were able to inhibit degranulation, hence abrogating the effects of the two MC secretagogues (**Fig. 17A-B**). Pharmacological stimulation of CB₁ thus prevents the excessive activation of human skin MCs *in situ*.

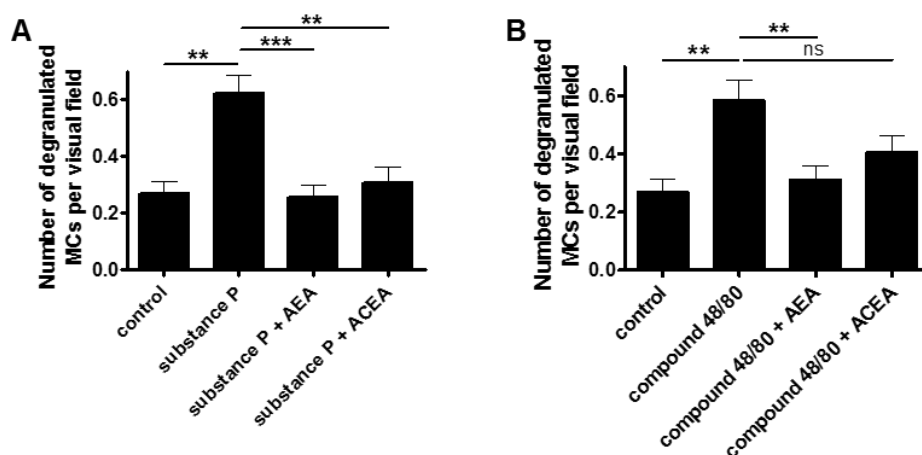


Fig. 17: CB₁ stimulation effectively counteracts excessive CTS-MC activation provoked by MC secretagogues

(A) Number of degranulated MCs after SP (100 pM) or (B) compound 48/80 (10 μg/ml) treatment in organ cultured human HFs (Leder's esterase histochemistry, after 48 hrs). ** $p < 0.01$, *** $p < 0.001$, mean \pm SEM, ns: not significant.

5.1.7 Effects of CB₁ inverse agonism on MCs are partly SCF dependent

As shown above, CB₁ is expressed along the epithelium of human HFs. Importantly, this area has been shown to be an important source of SCF (Ito et al., 2010). This major cytokine that operates via activating c-Kit, is a key element of MCs' maturation and proliferation (Ito et al., 2010, Maurer et al., 2003). Therefore, we next investigated whether CB₁ agonism/antagonism would also be able to increase the number of CTS-MCs via the indirect regulation of intrafollicular SCF expression and release of CB₁+ epithelial cells.

We showed that AM251 (1 μ M) significantly enhanced SCF not only at the mRNA (**Fig. 18A**), but also at the protein level (**Fig. 18B-C**) in organ cultured human HFs (as a positive control for SCF immunohistochemistry, intact human scalp skin sample was used [**Fig. 18D**]). When this CB₁ inverse agonist was applied in combination with ACEA (30 μ M), the previously observed SCF upregulation was abrogated (**Fig. 18A, C**). Additionally, by analyzing the culture medium of primary ORS-KCs, we showed that the SCF concentration was also increased *in vitro* when treating the cells with AM251 (**Fig. 18E**). When checking the SCF immunoreactivity after application of eCB ligands, a much higher fluorescence intensity was detected in the membrane of AM251 treated cells compared to the control group (**Fig. 18F**). 60% of AM251 treated ORS-KCs showed the above-described staining pattern, while this ratio was only 36.8% in case of the untreated cells. These results also indicate that the abrogation of the CB₁-mediated signaling might lead to an indirect SCF upregulation within the epithelium of human HFs. This suggests that ECS is able to adjust the production of this important growth factor.

To further study the indirect effect of CB₁ inverse agonism on CTS-MCs that is developed via the elevation of SCF secretion within the HF epithelium, the following experiment was performed: we applied AM251 (1 μ M) together with an SCF-neutralizing antibody (1 μ g/ml) within the culture medium (24 hrs). When comparing the number of c-Kit+

CTS-MCs between control and treated groups, we observed that the CB₁ inverse agonism-induced, previously described cell growth effect was partially, yet significantly reduced (**Fig. 18G**).

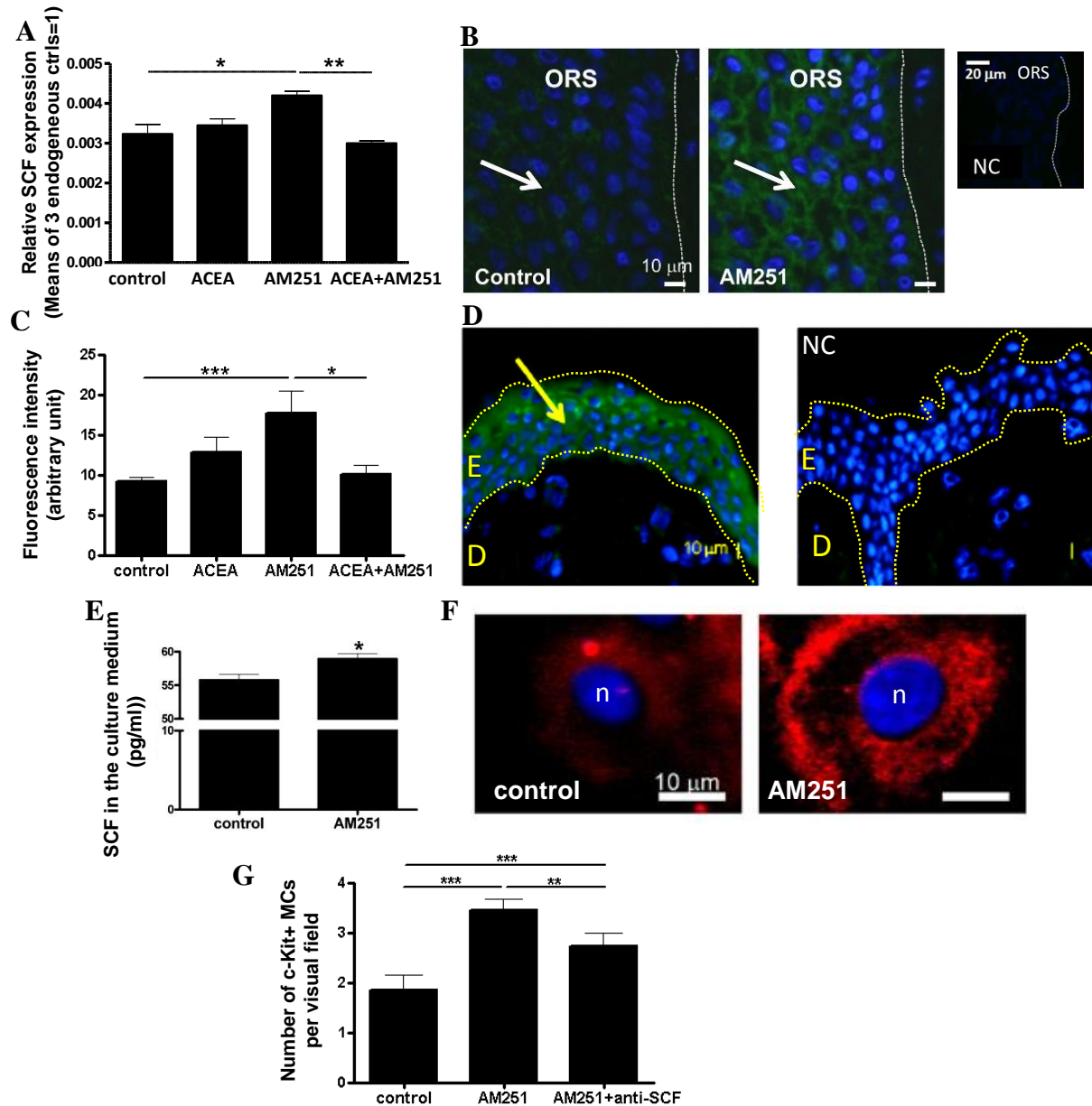


Fig. 18: Abrogation of homeostatic CB₁ signaling increases SCF expression and release

(A) Q-PCR analysis for SCF in HFs (24 hrs). (B) SCF immunostaining (white arrows-positive immunoreactivity) in organ cultured HFs. (C) Quantitative analysis of SCF immunoreactivity in HFs. (D) Indirect SCF immunofluorescence (yellow arrow-positive immunoreactivity) images of intact human scalp skin sample. (E) SCF level in the culture medium of ORS-KCs. (F) SCF immunostaining in ORS-KCs. (G) Quantitative immunohistomorphometry of c-Kit⁺ cells in organ-cultured HFs with AM251 (1 μM) and/or SCF-neutralizing antibody (1 μg/ml). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, mean \pm SEM (scale bars mark 10 and 20 μm). ORS: outer root sheath, E: epidermis, D: dermis, NC: negative control, n: nucleus.

5.1.8 Degranulation and maturation of CTS-MCs are increased in CB₁ KO mice

After the detailed *in situ* studies, we finally aimed to investigate how the modulation of CB₁ signaling affects the biology of CTS-MCs *in vivo*, using CB₁ KO mice (Zimmer et al., 1999; Karsak et al., 2007). In the experiments, age- and hair cycle-matched specimens were examined. As expected, c-Kit⁺ CTS-MCs showed CB₁ negativity in CB₁^{-/-} animals (**Fig. 19**).

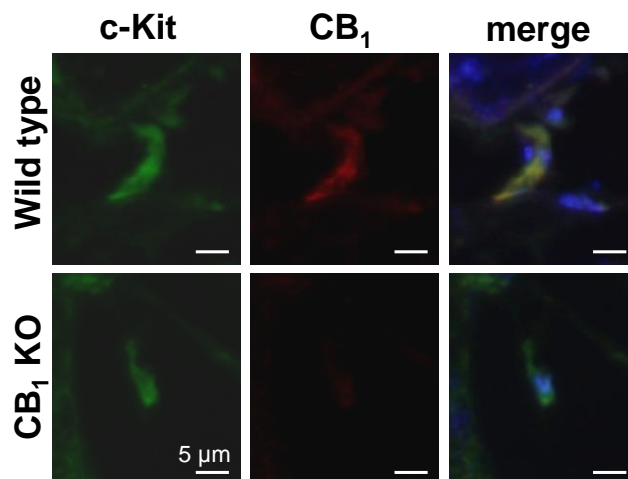


Figure 19: CB₁ expression in c-Kit⁺ CTS-MCs

CB₁ expression in c-Kit⁺ (green) MCs of CB₁ (red) KO mice is reduced compared to wild type mice. Double immunohistology for c-Kit and CB₁ (yellow) in both wild type and CB₁ KO mice (postnatal day 32). Scale bars mark: 5 μm

When comparing the subcutaneous CTS of the test animals, we found that not only the total number of MCs (**Fig. 20A-B**), but also the number of c-Kit⁺ cells (**Fig. 20C**) was significantly increased within CB₁ KO mice. This is in line with our organ cultured human HF data. Furthermore, the degranulation of MC activity was also significantly enhanced after CB₁ silencing (**Fig. 20D**).

There was no significant difference in the proliferation of MCs as revealed by comparing the number of Ki67⁺/c-Kit⁺ CTS-MCs in the control and KO mice (**Fig. 20E**). This indicates that, just like *in situ*, CB₁ stimulation *in vivo* also acts primarily on the maturation and activation of murine MCs, rather than on their proliferation. Thus, to control unwanted maturation and activation of MCs, continuous CB₁ stimulation also *in vivo* is needed.

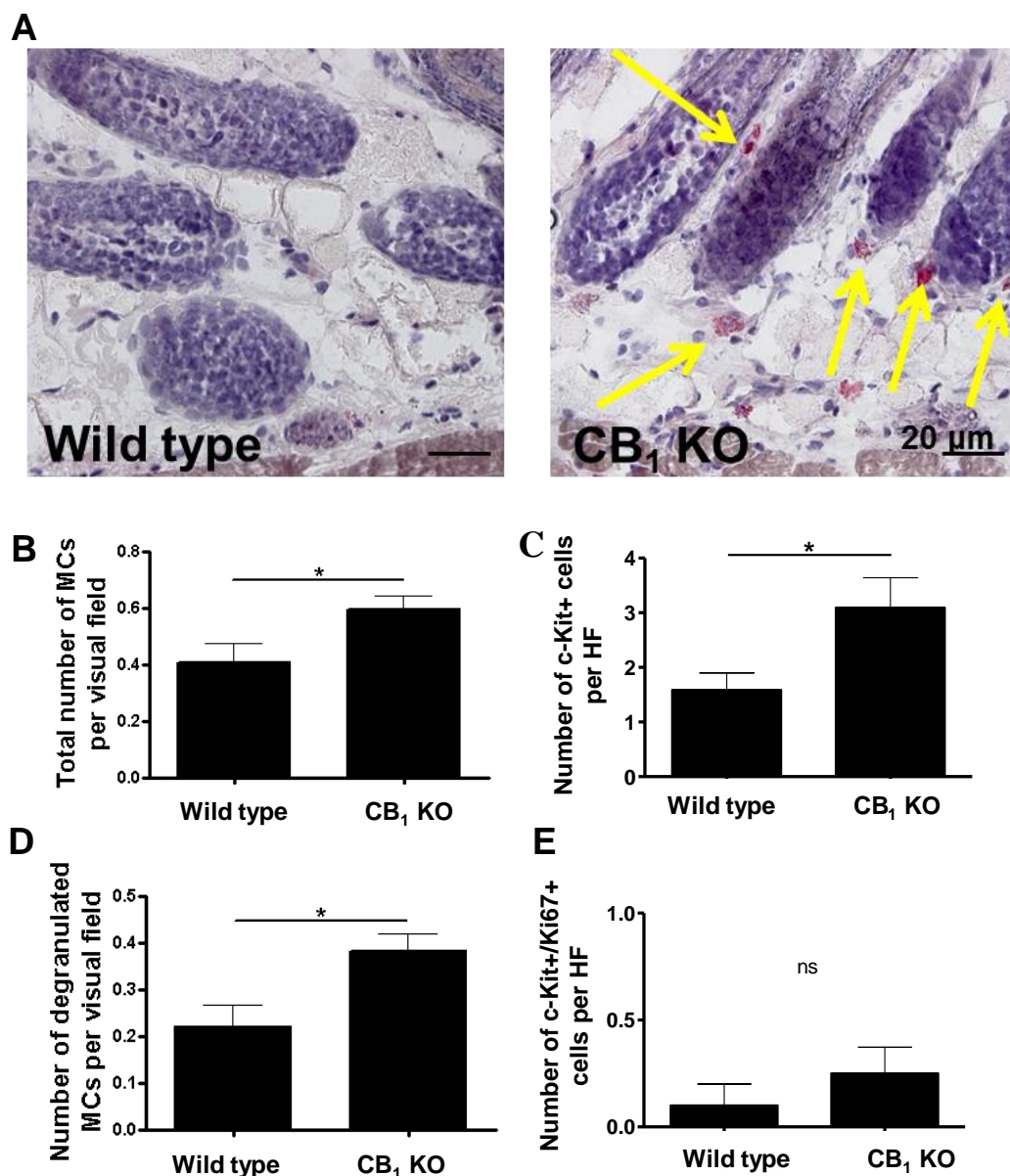


Fig. 20: Comparison of the CTS-MCs' number, degranulation and proliferation between wild type and CB₁ KO mice

(A) Representative pictures of Leder's esterase staining, yellow arrows denote CTS-MCs. (B) Quantitative histomorphometry of total number of CTS-MCs per visual field. (C) Quantitative immunohistomorphometry of the number of c-Kit+ cells per HF. (D) Quantitative histomorphometry of the number of degranulated CTS-MCs per visual field. (E) Quantitative immunohistomorphometry of the number of c-Kit/Ki67 double+ cells per HF. * $p < 0.05$, mean \pm SEM (scale bars mark 20 μ m). ns: not significant, KO: knockout, HF: hair follicle.

5.2 eCB tone regulates human sebocyte biology

5.2.1 Major elements of ECS' enzyme apparatus are present in cultured human sebocytes, as well as in human SGs *in situ*

We began our investigations by checking whether the most significant enzymes involved in the metabolism of AEA (NAPE-PLD and FAAH) and 2-AG (DAGL α and β , as well as MAGL) are expressed in human, immortalized SZ95 sebocytes. Our results showed that this was the case for all aforementioned enzymes both at the mRNA and protein level (**Fig. 21A-D**), regardless of the cells confluency. To further strengthen these data, we also checked the expression of the above enzymes *in situ* in human skin (**Fig. 22**). Optimized staining protocols were established for all enzymes. **Fig. 23** shows the appropriately labeled positive controls for DAGL α and β (human cerebellum), for NAPE-PLD and MAGL (human kidney), as well as for FAAH (human testicles).

In line with our *in vitro* data, the expression of all above enzymes was also confirmed in human SGs *in situ*, except for DAGL α , whose expression was questionable (**Fig. 22**), when comparing it with the neighboring endogenous positive control sweat glands (Czifra et al., 2012).

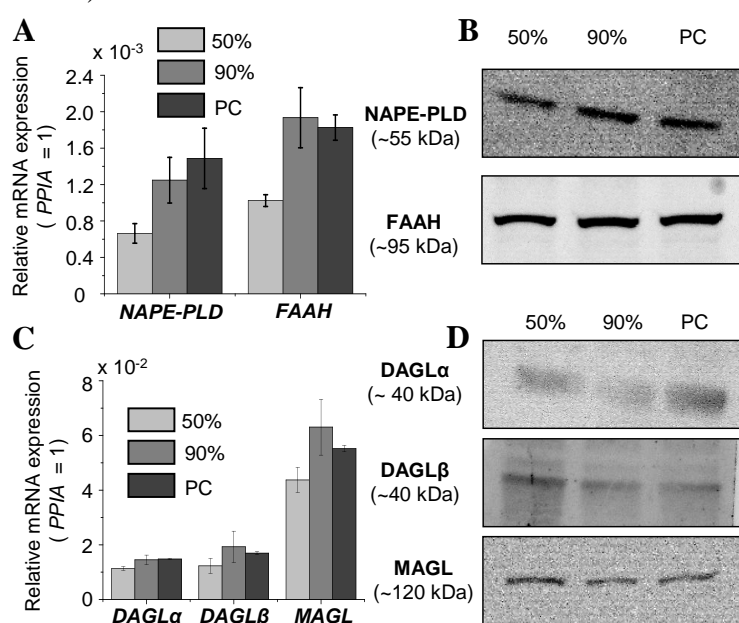


Fig. 21: Expression of major ECS enzymes in SZ95 sebocytes

(**A**, **C**) SZ95 sebocyte samples were collected at different confluencies for Q-PCR experiments. mRNA expressions of NAPE-PLD, FAAH, DAGL α and β , as well as MAGL were normalized to the expression of peptidylprolyl isomerase A (PPIA) of the same samples. Results are expressed as mean \pm SD of three determinations. (**B**, **D**) Western blot analyses of lysates of SZ95 sebocytes. PC: post-confluent culture, DAGL: diacylglycerol lipase, FAAH: fatty acid amide hydrolase, MAGL: monoacylglycerol lipase, NAPE-PLD: *N*-acyl phosphatidylethanolamine-specific phospholipase D.

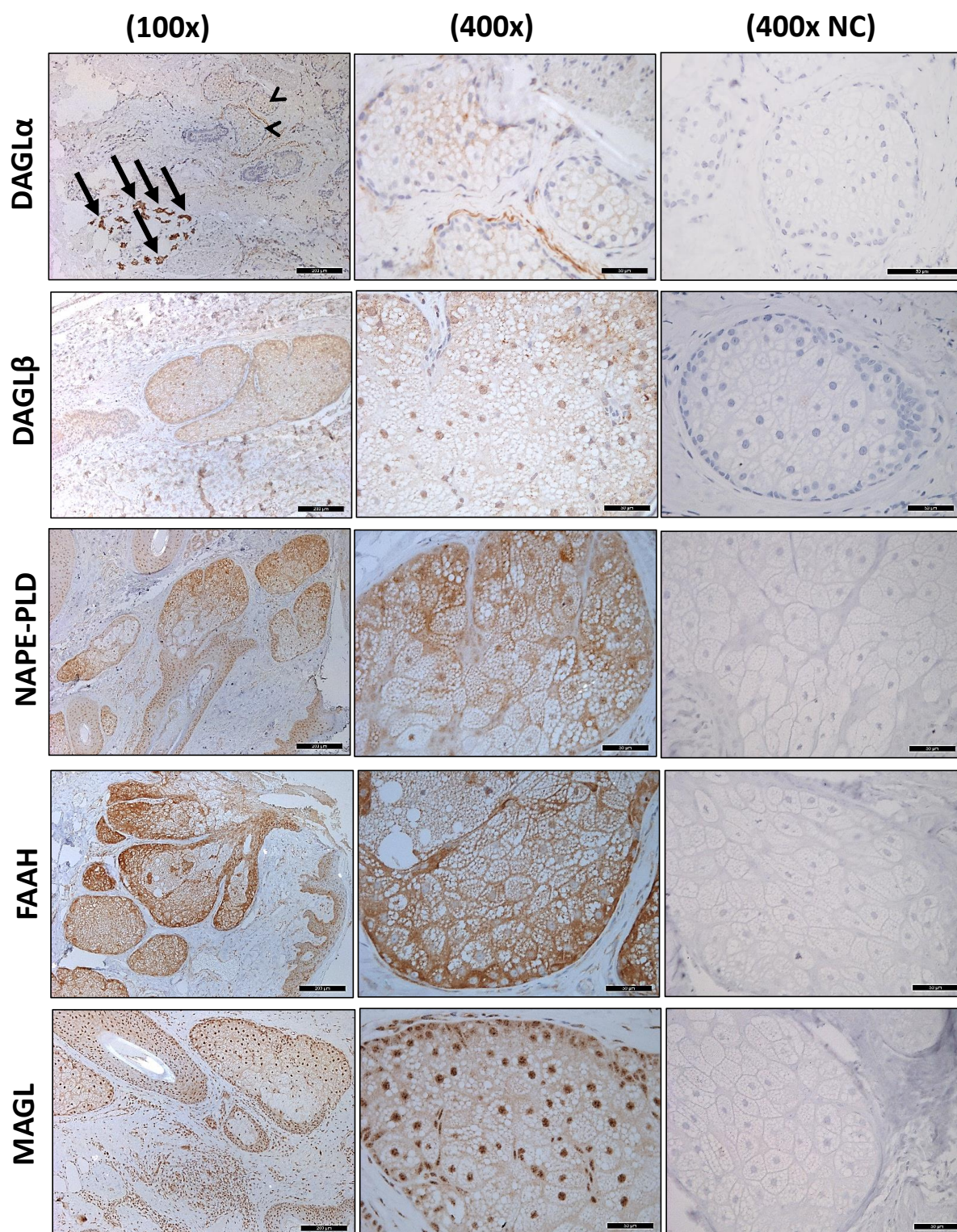


Fig. 22: Expression of major eCB-metabolizing enzymes in human SGs *in situ*

3,3'-diaminobenzidine (DAB; brown color) was used for chromogenic detection, whereas nuclei were counterstained by hematoxylin (blue color). Original magnifications: 100x (left column), 400x (middle and right columns); scale bars: 200 μ m (left column) and 50 μ m (middle and right columns). Arrows indicate sweat glands (endogenous positive control for DAGL α), whereas arrowheads mark SGs on the same image. Negative controls (right column) were obtained by omitting the primary antibody in all cases. DAGL: diacylglycerol lipase, FAAH: fatty acid amide hydrolase, MAGL: monoacylglycerol lipase, NAPE-PLD: N-acyl phosphatidylethanolamine-specific phospholipase D, NC: negative control.

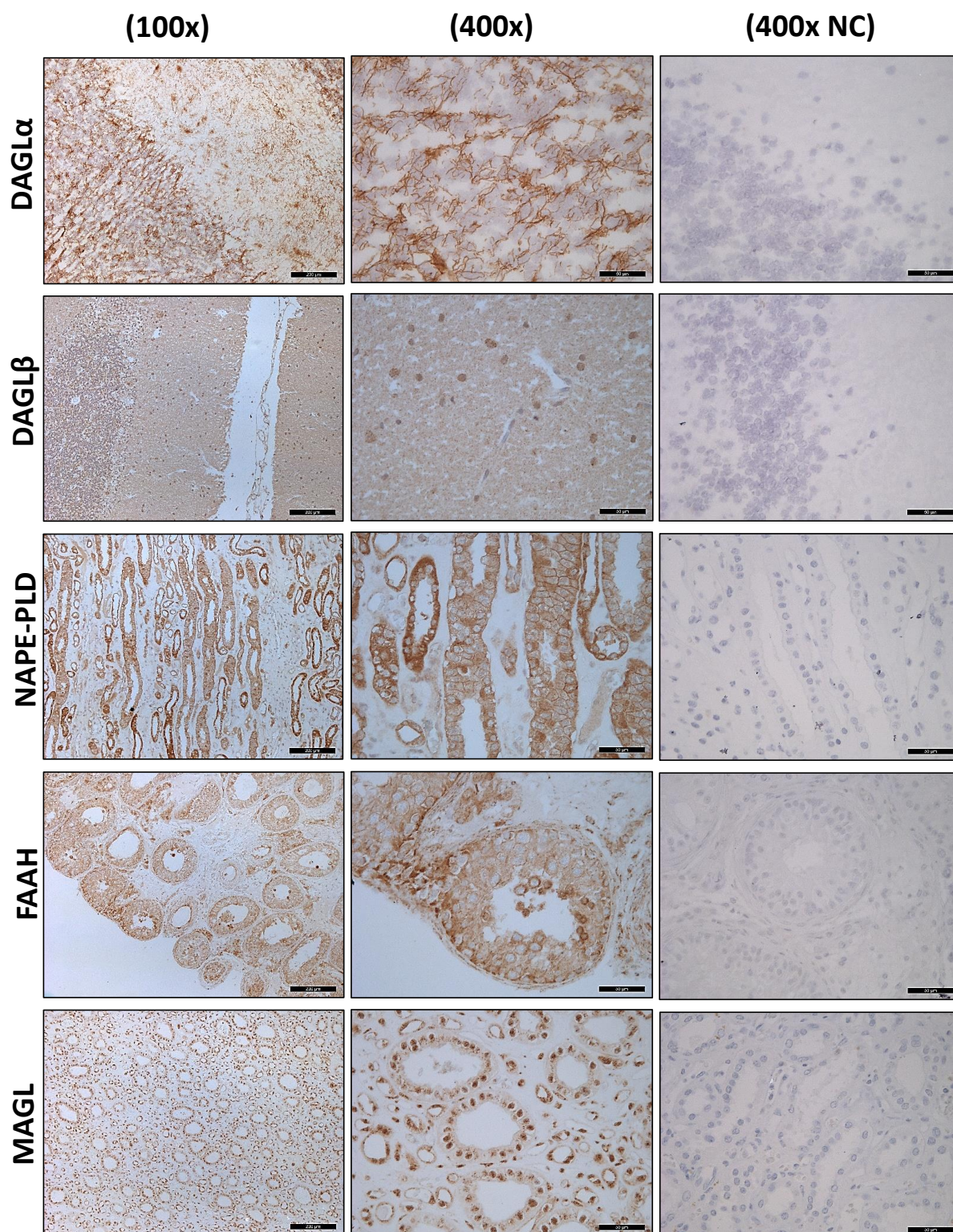


Fig. 23: Optimization of histological methods-labeling of the positive control tissues

3,3'-diaminobenzidine (DAB; brown color) was used for chromogenic detection, whereas nuclei were counterstained by hematoxylin (blue color). Original magnifications: 100x (left column), 400x (middle and right columns); scale bars: 200 μ m (left column) and 50 μ m (middle and right columns). Arrows indicate sweat glands (endogenous positive control for DAGL α), whereas arrowheads mark sebaceous glands on the same image. Negative controls (right column) were obtained by omitting the primary antibody in all cases. DAGL: diacylglycerol lipase, FAAH: fatty acid amide hydrolase, MAGL: monoacylglycerol lipase, NAPE-PLD: N-acyl phosphatidylethanolamine-specific phospholipase D, NC: negative control. DAGL α and β : cerebellum, FAAH: testicle, MAGL and NAPE-PLD: kidney.

5.2.2 eCB uptake of sebocytes can be inhibited pharmacologically

According to above results, SGs may be able to produce and degrade eCBs. Since EMTs that can be involved in such processes so far have not been identified, we are only able to investigate their possible role with functional measurements.

In an effort to check whether the uptake of AEA by the putative EMT can be inhibited pharmacologically in human sebocytes, we have measured the uptake of radiolabeled [^3H]AEA into SZ95 cells. By using a selective EMT-inhibitor UCM707 (10 μM , 15 min) (Lopez-Rodriguez et al. 2003; Rau et al., 2016), we observed that the amount of intracellular [^3H]AEA significantly decreased, while its extracellular level increased, when comparing it to the vehicle control, and, as a consequence of the inhibited uptake, FAAH-mediated degradation to [^3H]ethanolamine was also reduced. Taken together, these results indicate that UCM707 could significantly block the eCB uptake process (**Fig. 24**).

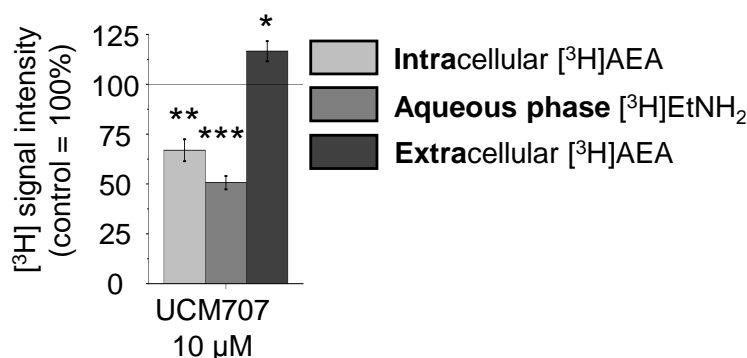


Fig. 24: Inhibition of the putative EMT in human sebocytes

Measurement of [^3H]AEA transport. Results are expressed in the percentage of the vehicle control (100%, solid line) as mean \pm SEM of three independent experiments, each run in triplicate. * p <0.05, ** p <0.01, *** p <0.001. URB597: reference FAAH-inhibitor, VDM11 and UCM707: EMT-inhibitors.

Theoretically, reduced AEA uptake can be the result of both the inhibition of the EMT and the inhibition of FAAH (i.e. the enzyme creating and maintaining the driving force for the uptake (Chicca et al., 2012; Nicolussi et al., 2014). To challenge this hypothesis, we assessed how the activity of FAAH changes in SZ95 sebocytes when using two different EMT-inhibitors. UCM707 and VDM11 are widely used to block cellular uptake of eCBs (De Petrocellis et al., 2000; Lopez-Rodriguez et al., 2003). Their effects were compared to URB597 that is a reference FAAH-inhibitor (Mor et al., 2004).

Our results showed that FAAH activity expressed by SZ95 sebocytes is constitutive, although its level is relatively low (2.32 ± 0.25 pmol/min/mg protein; $n=10$). IC_{50} values were above 25 μ M for both EMT-inhibitors, whereas the IC_{50} of URB597 was below 100 nM (**Table 1**). This means that none of the aforementioned inhibitors were able to inhibit the function of FAAH in a relevant way. Overall, our results convincingly suggest that VDM11 can block the eCB uptake in sebocytes and that this inhibition is likely to occur without influencing FAAH activity.

Compounds	Inhibition % (n=6)
URB597 0.1 μ M	66.1 ± 5.2
VDM11 5 μ M	22.0 ± 1.9
VDM11 10 μ M	20.9 ± 4.5
VDM11 25 μ M	-8.5 ± 0.8 (stimulation)
UCM707 5 μ M	4.9 ± 0.4
UCM707 10 μ M	26.4 ± 4.1
UCM707 25 μ M	20.1 ± 3.5

Table 1: Comparative assessment of FAAH-inhibitory capabilities of selected FAAH- and EMT -inhibitors on human SZ95 sebocytes
URB597: reference FAAH-inhibitor, VDM11 and UCM707: EMT-inhibitors.

5.2.3 EMT of human sebocytes facilitates the transport of eCB ligands to their site of degradation

Next, we aimed to further assess the eCB transport process of SZ95 cells. To do so, cells were treated with vehicle or VDM11 (De Petrocellis et al., 2000) for 24 hrs, and their eCB content was analyzed by LC-APCI-MS. Our results showed that concentration of AEA was significantly increased in the VDM11-treated samples (**Fig. 25A**), while the enhancement of 2-AG level was not significant (**Fig. 25B**). Next to AEA and 2-AG, we have also performed experiments with two eCB-like substances, namely PEA and OEA. We found that VDM11 increased (OEA) or tended to increase (PEA) their concentrations (**Fig. 25C-D**). In summary, our data demonstrated that homeostatic eCB and eCB-like mediator signaling in human sebocytes can be altered by the selective EMT inhibitor, VDM11.

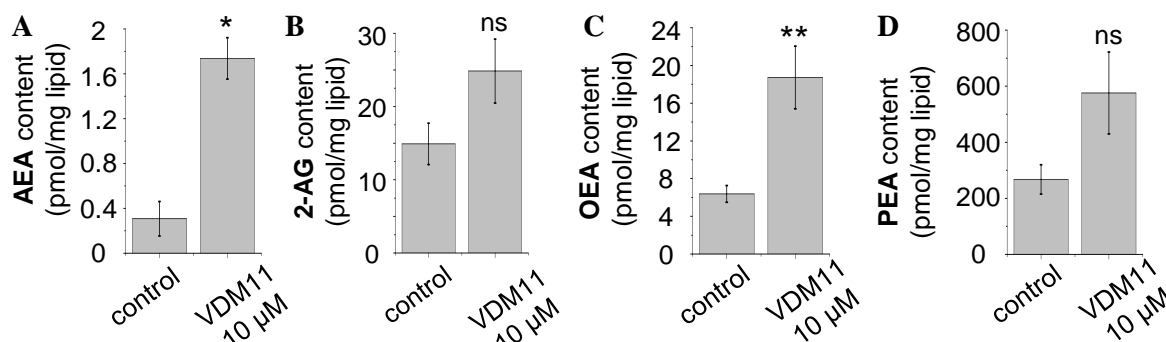


Fig. 25: Determination of classical and novel eCB content in SZ95 sebocytes

Assessment of AEA (A), 2-AG (B), OEA (C) and PEA (D) level by LC-APCI-MS. Results are expressed as mean±SEM of 3-4 independent cultures. * $p<0.05$, ** $p<0.01$. ns: not significant, VDM11: EMT-inhibitor.

5.2.4 EMT-inhibitors mimic lipogenic action of direct eCB-treatment, whereas selective FAAH-inhibition does not affect SLP

Previously, we have shown that SLP of eCB-treated sebocytes was dramatically elevated (Dobrosi et al., 2008; Oláh et al., 2014; Oláh et al., 2016b). Thus, in the next step, we aimed to investigate whether and how the EMT-controlled eCB-tone plays a role in regulating the functions of SGs. To do so, we first checked the viability of sebocytes in the presence of VDM11 using MTT-assay. Importantly, we found that, up to 10 µM, VDM11 can be used without the risk of cytotoxicity (48 hrs, **Fig. 26**).

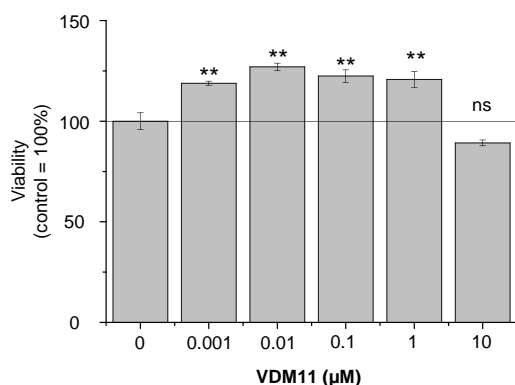


Fig. 26: Up to 10 µM, VDM11 did not decrease viability of human sebocytes

Viability of SZ95 sebocytes was monitored by MTT-assay following 48-hr treatments. Results are expressed in the percentage of the vehicle control (100%, solid line) as mean±SEM of four independent determinations. One additional experiment yielded similar results. ** $p<0.01$, difference compared to the vehicle control. ns: not significant, VDM11: EMT-inhibitor.

In order to investigate the effects of VDM11 on SLP (that is the most characteristic biological function of human sebocytes), fluorescent Nile Red assay was applied. Our results showed that VDM11 (5-10 µM, 48 hrs) significantly increased SLP, although in a modest way, which was far exceeded by direct AEA-treatment (**Fig. 27**).

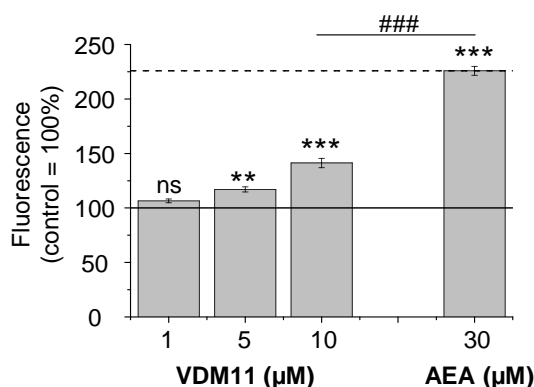


Fig. 27: Sebaceous lipid production of SZ95 cells

SLP of SZ95 sebocytes was monitored by Nile-Red assay following 48-hr treatments. Results are expressed in the percentage of the vehicle control (100%, solid line) as mean±SEM of four independent determinations. One additional experiment yielded similar results. ** $p < 0.01$, *** $p < 0.001$ difference compared to the vehicle control. ### $p < 0.001$, as indicated. ns: not significant, AEA: anandamide, VDM11: EMT-inhibitor.

This was also the case when using a non-cytotoxic concentration (10 μM, 48 hrs; **Fig. 28A-B**) of another well-known EMT-inhibitor, namely AM404 (Beltramo et al., 1997; Nicolussi and Gertsch 2015).

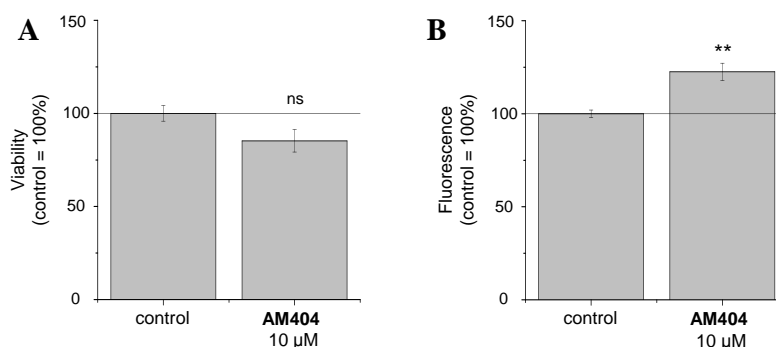


Fig. 28: Similar to VDM11, AM404 also promotes sebaceous lipid production

(A) Viability of SZ95 sebocytes was monitored by MTT-assay following 48-hr treatments. (B) SLP of SZ95 sebocytes was assessed by Nile Red assay following 48-hr treatments. Results are expressed in the percentage of the vehicle control (100%, solid line) as mean±SEM of four independent determinations. One additional experiment yielded similar results. ** $p < 0.01$, difference compared to the vehicle control. ns: not significant, AM404: EMT-inhibitor.

This further suggests that the inhibition of EMT may cause an increase in the lipid synthesis of sebocytes. Even though both above inhibitors could mimic the lipogenic actions of AEA (Dobrosi et al., 2008), they could not compete with the efficiency of direct AEA-treatment in elevating the SLP of the sebocytes (**Fig. 27**).

As mentioned above, it has previously been shown that not only the putative EMT, but also FAAH might be inhibited by eCB uptake inhibitors in a concentration-dependent way (Beltramo et al., 1997; Nicolussi and Gertsch 2015; Chicca et al., 2017). Although the results of our experiments clearly showed that SZ95 sebocytes have very low constitutive FAAH-activity, and that selective EMT-inhibitors, namely UCM707 and VDM11 can be used without

causing a substantial FAAH-inhibition (**Table 1**), we decided to assess the effects of the reference FAAH-inhibitor URB597 (Mor et al., 2004). Contrary to the results obtained with VDM11 and AM404, non-cytotoxic concentrations of URB597 could not significantly influence SLP (48 hrs; **Fig. 29A-B**). Such lack of effect by URB597 is most likely due to the cells' very low level of FAAH activity. This suggests that inhibition of eCB uptake enhances SLP, and this lipogenic effect of the EMT-inhibitors is independent of FAAH-activity.

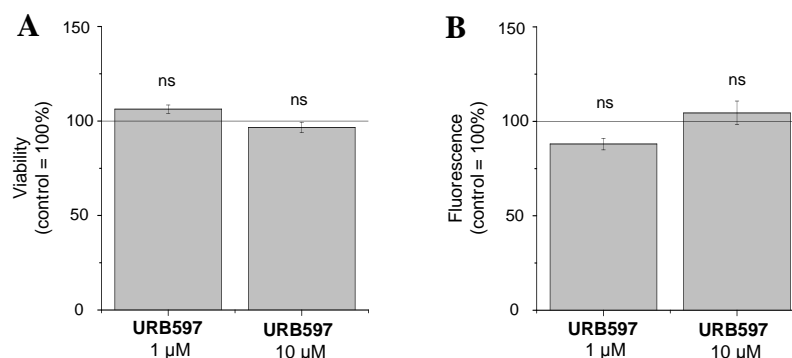


Fig. 29: In contrast to the effects of EMT-inhibitors, URB597 does not influence SLP

(A) Viability of SZ95 sebocytes was monitored by MTT-assay following 48-hr treatments. (B) SLP of SZ95 sebocytes was assessed by Nile Red assay following 48-hr treatments. Results are expressed in the percentage of the vehicle control (100%, solid line) as mean±SEM of four independent determinations. One additional experiment yielded similar results. ns: not significant, URB597: reference FAAH-inhibitor.

5.2.5 Co-administration of VDM11 does not further increase lipogenic action of direct

AEA treatment

Next, we investigated the combined effects of AEA and VDM11 on SLP of human sebocytes. This experiment revealed that VDM11 was unable to further potentiate the AEA-induced SLP of human sebocytes (**Fig. 30**), even at the highest investigated concentration (10

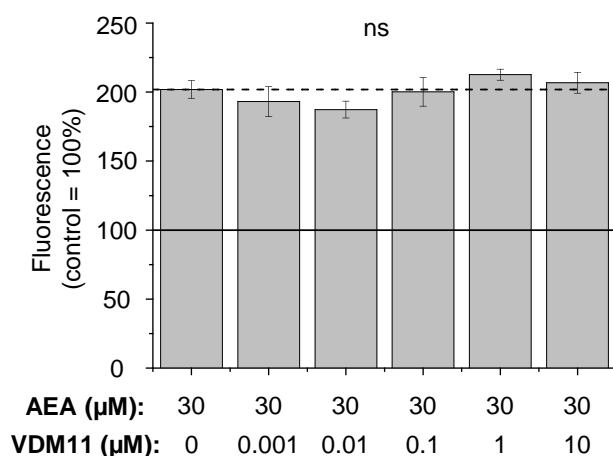


Fig. 30: VDM11 does not further increase lipogenic actions of AEA

SLP of SZ95 sebocytes by Nile Red assay following 48-hr treatments. Results are expressed in the percentage of the vehicle control (100%, solid line) as mean±SEM of four independent determinations. One additional experiment yielded similar results. SLP induced by AEA treatment is marked with the dashed line. ns: not significant, AEA: anandamide, VDM11: EMT-inhibitor.

μM). This suggests that the pro-lipogenic cannabinoid signaling by AEA ($30 \mu\text{M}$) was most probably activated to the maximum extent possible.

5.2.6 Up to $10 \mu\text{M}$, VDM11 does not evoke apoptosis in human sebocytes

Right after the level of SLP increases, sebocytes start differentiating, which leads to their apoptotic cell death (Dobrosi et al., 2008; Tóth et al., 2011b; Zouboulis et al., 2014; Fischer et al., 2017). We have demonstrated that inhibition of EMT was able to mildly enhance the lipid synthesis of sebocytes (Fig. 27, 28B), therefore, we aimed to check whether it also induces their early apoptosis.

We found that the most effective lipogenic concentration of VDM11 ($10 \mu\text{M}$, 48 hrs) slightly reduced the mitochondrial membrane potential of the cells (primary sign of apoptosis), although not significantly (Fig. 31). This shows that during the investigated period of time obvious pro-apoptotic effects have not developed.

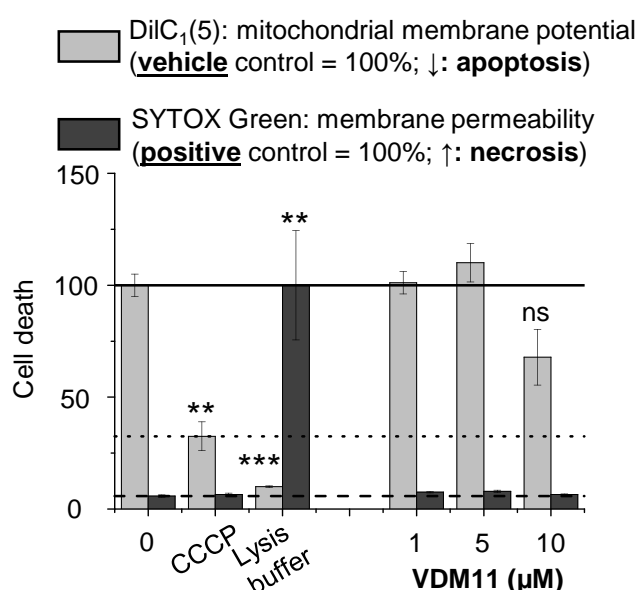


Fig. 31: Apoptotic and necrotic effects of VDM11 on SZ95 sebocytes

The most effective lipogenic concentration of VDM11 tends to decrease mitochondrial membrane potential. Apoptotic and necrotic cell death of SZ95 sebocytes was investigated by DilC₁(5)-SYTOX Green double labeling following the indicated 48-hr treatments. Selective decrease of DilC₁(5) intensity indicates the development of “pure” apoptotic processes, whereas simultaneous increase of SYTOX Green level points to necrosis. Data are expressed as mean \pm SEM of four determinations. ** p <0.01, *** p <0.001 values were regarded as significant differences. ns: not significant, CCCP: apoptosis positive control, lysis buffer: necrosis positive control, VDM11: EMT-inhibitor.

5.2.7 VDM11 interferes with the LPS-induced pro-inflammatory response of human sebocytes

A slight increase in the level of homeostatic SLP would be highly desirable when treating pathologically dry skin. Based on our intriguing findings, in such therapies EMT-

inhibitors like VDM11 might be advantageous. Since skin dryness and cutaneous inflammation often occur together (Zouboulis and Boschnakow, 2001; Zampeli et al., 2012; Kim et al., 2014; Mischo et al., 2014; Shi et al., 2015), we also wanted to examine whether and how VDM11 affects the immune properties of human sebocytes.

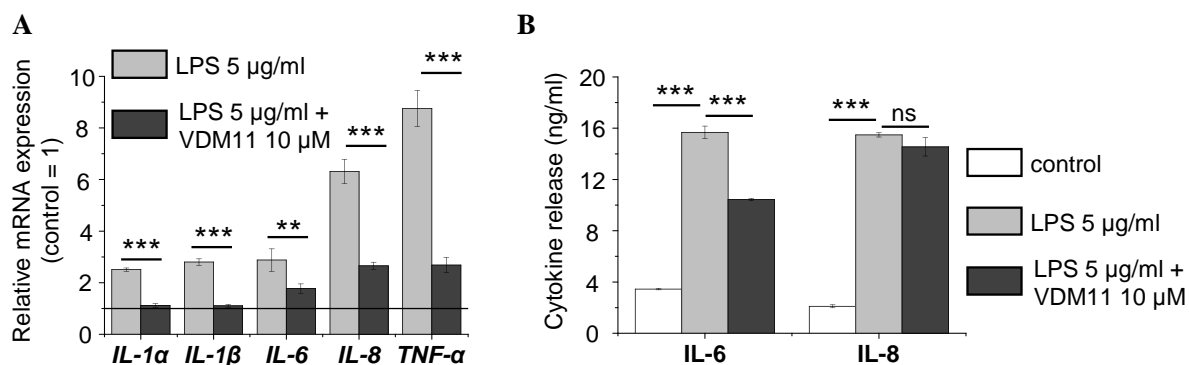


Fig. 32: Anti-inflammatory actions of VDM11 on SZ95 sebocytes

(A) Q-PCR analysis of SZ95 cells following 3-hr LPS-treatment. Data are presented by using $\Delta\Delta CT$ method regarding 18S RNA-normalized mRNA expressions of the vehicle control as 1 (solid line). One additional experiment yielded similar results. (B) IL-6 and IL-8 ELISA following LPS treatment with or without VDM11 treatment (24 hrs). Two additional experiments yielded similar results. Data are expressed as mean \pm SD of three determinations. ** p <0.01, *** p <0.001. LPS: lipopolysaccharide, IL: interleukin, TNF- α : tumor necrosis factor α , ns: not significant.

To induce pro-inflammatory cytokine production, lipopolysaccharide (LPS; 5 µg/ml; 3 hrs) was used (Oláh et al., 2016a). With the joint application of VDM11 (10 µM), the LPS-induced expression of IL-1 α , IL-1 β , IL-6, IL-8, and TNF- α of human sebocytes was effectively suppressed (**Fig. 32A**). In addition to this, by using ELISA assay, VDM11 was showed to significantly decrease the LPS-induced release of IL-6, and tended to reduce the amount of IL-8 (24 hrs; **Fig. 32B**). The concentration of TNF α , IL-1 α , and IL-1 β did not or barely reached the detection limit of the respective ELISA assays.

6. DISCUSSION

ECS is not only meaningful in the maintenance of homeostatic skin balance, but also in the development of different pathological skin conditions. Our research group has studied in details the function and regulatory role of this “*c(ut)annabinoid*” system, with a special focus on the non-neuronal skin cells. Within the frame of this doctoral thesis, we have investigated the effects of ECS on human skin MCs, and further examined its role within the biology of sebocytes.

6.1 ECS is involved in the regulation of cutaneous MC biology

Overall, our results show for the first time that the activation/degranulation, as well as the maturation of normal human skin type of MCs from their resident progenitor cells is negatively controlled via CB₁-mediated signaling *in situ*. We have also found that in parallel with the “direct” effects of CB₁ inverse agonism, there are “indirect” actions that operate through the regulation of HF-epithelium-produced SCF *in situ*.

This thesis has also clarified some previous rodent and human *in vitro* conflicting results in connection with CB₁ signaling and MCs mentioned in section 2.3.3, and helps us for better understanding the role of ECS within the biology of these immune cells. Previously described contradictory results could be explained by different approaches. One the one hand, not providing certain micro-environmental factors that are known to strongly influence the biology of MCs could eventuate such findings. In the current case, by using the HF organ culture, human skin MCs were examined under more “physiological” *in vitro* circumstances. On the other hand, it may also be possible that by using the current model system, the continuous eCB production within the HF-CTS was ensured by the present cells, whereupon there was an actual process that could be inhibited. While in contrast in the exclusive MC or MC-like (e.g., RBL-2H3) cell cultures there was no such eCB tone.

In addition, we found that CB₁ signaling regulates the epithelial SCF secretion (**Fig. 18A-F**); thus, in this way might also be responsible for limiting the cutaneous (over)production of this cytokine in general. Indeed, when neutralizing SCF by a specific blocking antibody, the stimulatory effect of CB₁ inverse agonism on the maturation of MCs from their progeny was almost completely abrogated (**Fig. 18G**). Importantly, the fact that the applied SCF-neutralizing antibody could not fully inhibit SCF activity suggests that the effects of AM251 are only partially dependent on the regulation of the epithelial SCF production (“indirect effects”), and at least some parts of it are mediated via “direct” actions on the MCs. Moreover, the results of the performed CB₁/c-Kit (**Fig. 8A-C**) and Tocrifluor (**Fig. 9**) immunostainings also suggest that the “direct” effects of the CB₁ inverse agonist work in parallel to the “indirect” ones (i.e. regulation of SCF secretion in human HF-KCs in a CB₁ dependent manner, **Fig. 33**).

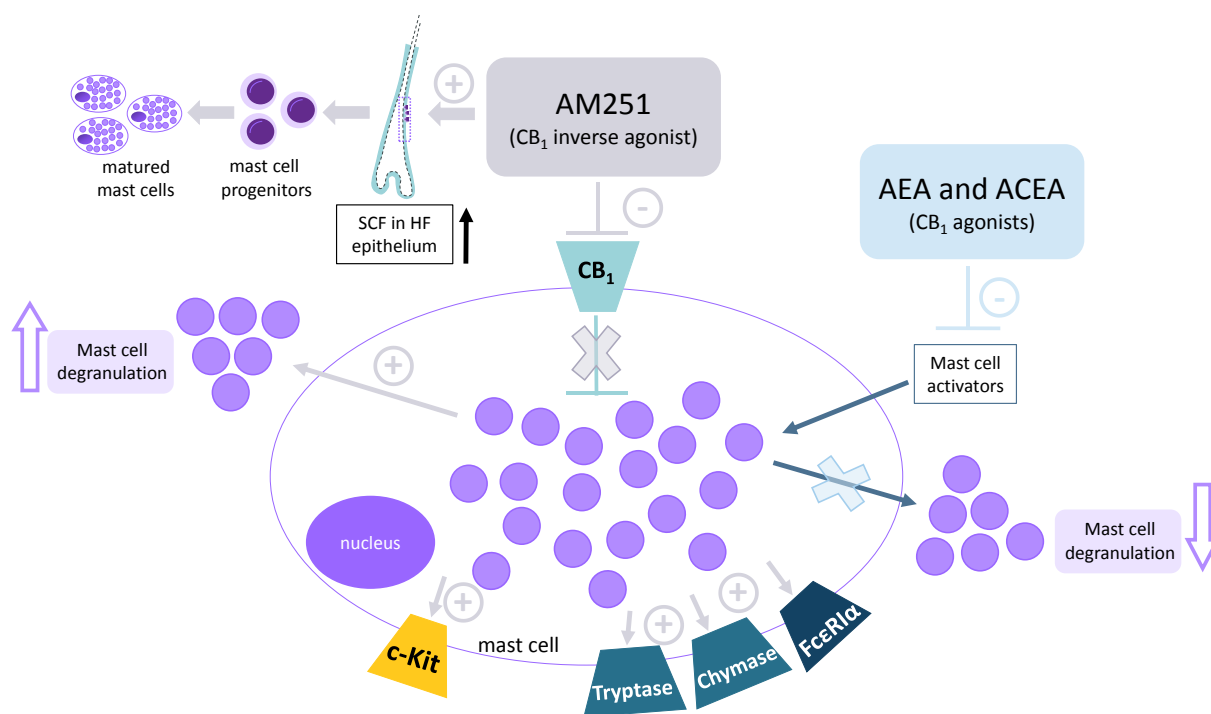


Fig. 33: CB₁ limits excessive MC activation and maturation from MC progenitors

eCBs control (i) activation of human skin MCs, (ii) their maturation from resident progenitor cells, and (iii) regulate SCF expression in human HF epithelium via CB₁, which, in turn, contributes to the above effects. SCF: stem cell factor, HF: hair follicle.

Overall, these data point to the involvement of ECS in the adjustment of epithelium-produced SCF within the HFs, through a “tonic” CB₁ stimulation. With the abrogation of CB₁

signaling, this SCF-control might be discontinued. In this way, the increased level of this cytokine might induce then the intracutaneous maturation process of MCs from immature, resident progenitor cells within the CTS (Kumamoto et al. 2003; Ito et al., 2010). In addition to this, our findings highlight not only how significant the connection between CB₁ signaling and SCF biology is, but also draws the attention to the importance of epithelial-mesenchymal interactions in the biology of human skin MCs in general.

Our work has also emphasized how relevant is the CTS of rodent vibrissae and human HFs for immature MC precursors, and that the differentiation of these cells into their mature forms is under a homeostatic eCB control. CB₁ mediated signaling has already been shown to be able to regulate the proliferation and/or cell death of fast-proliferating hair matrix KCs (Telek et al., 2007) and certain neuronal cell populations (Viscomi et al., 2010). In the present case however, it principally seems to influence the maturation and activation processes of CTS-MCs.

Our corresponding *in vivo* murine skin data (**Fig.19, 20A-E**) further confirms the above described findings regarding the regulation of skin MCs via CB₁, but also how substantial the use of human HF organ-culture is. It provides an unconventional, but physiologically and clinically relevant model system for investigating the biology of primary skin MCs *in situ*. In addition, it is also relatively cheap and easily accessible. Besides the possibility to evaluate certain effects in both qualitative and quantitative manner, HF organ culture has also allowed us to execute mechanistically informative methods. With e.g., the anti-SCF and gene knockdown *in situ* experiments, the range of the applicable technologies were broadened. Thus, this model system may expand and well complement the already available methods for studying MCs. The already existing organ-cultures, e.g., human skin assay (Lu et al., 2007; Seeliger et al., 2010), together with this methodology might be suitable for further investigation the possible use of selective CB₁ agonists, e.g. in the therapy of allergic and chronic inflammatory skin disorders.

It is important to note that we have also examined whether CB₁ stimulation/blockade would also affect other MC populations, namely MC_{TS} (Sugawara et al., 2013). To do so, we used human nasal polyp organ culture. These noncancerous outgrowths within the nose and/or sinuses were shown to be an excellent surrogate tissue for much more difficult-to-obtain human bronchial mucosa. Our data provided the first evidence that normal MC_{TS} also use CB₁-mediated signaling to limit not only their degranulation, but also their maturation from resident progenitor MCs *in situ*. Similar to HF-CTS MCs, the observed effects of pharmacologic or transcriptional CB₁ blockade included both direct and indirect (i.e. SCF-mediated) components. These results further underscore the key role of ECS in human MC physiology. Furthermore, they confirm the importance of CB₁-mediated signaling as an endogenous, “tonic” control system that can avoid excessive MC maturation and degranulation, similar to CTS- and human skin MCs.

In order to better understand the possible link between ECS and MCs, first some more general pieces of information have to be clarified regarding these cells. For example, why high number of MCs are located in specific tissues of the body (e.g., skin, blood vessel, lungs, etc.), especially in the ones that are in direct contact with the external environment? What are their exact physiological functions there? These are only 2 open questions from the ones that still need to be answered by the scientific community. It is assumed that the MC clusters might provide some low-level of immune protection and privilege for the above mentioned tissue sites (Waldmann, 2006; Meyer et al., 2008). In light of that defined regions of HFs have already been proven to maintain such function (Paus et al., 2005), thus the possible immune inhibitory role of perifollicular MCs in preventing of unwanted MC growth and functionality in human skin is even more remarkable. The tonic, inhibitory “eCB tone” described in this thesis, may represent such process. Its targeted modulation might therefore be therapeutically relevant in the treatment of diseases where the apoptosis of MCs is required, e.g., mastocytosis, MC-

associated tumors (Theoharides and Conti, 2004; Soucek et al., 2007; Huang et al., 2008; Ekoff and Nilsson, 2011).

6.2 Assessment of the role of ECS in human SGs

The ECS has recently been recognized as a new and important regulator of the biology of human SGs (Stander et al., 2005; Dobrosi et al., 2008). Our research group has previously shown that classical eCB ligands, such as AEA and 2-AG, are locally produced by human sebocytes. In addition to this, we found that the maintenance of homeostatic SLP is also regulated via a CB₂-dependent manner, and that the lipogenesis of sebocytes can dramatically be increased by direct AEA or 2-AG treatment (Dobrosi et al., 2008). Certain non-psychotropic phytocannabinoids, namely CBD and THCV, were shown to be able to normalize AA- and other mediator-induced excessive lipid synthesis, while others (such as CBG and CBGV) could significantly induce SLP, although only in a moderate way. Furthermore, CBD and THCV could even exert multifaceted anti-acne effects (Oláh et al., 2014; Oláh et al., 2016b). Moreover, a CBD containing topical formulation has already successfully passed a phase Ib clinical trial, and is currently being investigated in phase II (Spleman et al., 2018). Having explored the effects of multiple phytocannabinoids, in this thesis, we aimed to further investigate the role of ECS in SG biology.

To the best of our knowledge, our results show for the first time that major enzymes of ECS (i.e., NAPE-PLD, DAGL α and β , MAGL and FAAH) are expressed not only in human sebocytes *in vitro*, but also in SGs of the human skin *in situ* (**Fig. 21A-D**), with the sole exception of DAGL α , which exhibited dubious immunostaining pattern (**Fig. 22**). These findings support well the already available murine data on the expression of MAGL (Ma et al., 2011) and FAAH (Wohlman et al., 2016). Moreover, we also demonstrated that eCB transport is functionally active in human sebocytes and it can be inhibited pharmacologically (**Fig. 24**). Using VDM11, we showed that this AEA uptake inhibitor significantly enhanced the samples'

AEA level and it even tended to elevate the concentration of 2-AG (**Fig. 25A-B**). Based on these intriguing findings, we came to the conclusion that these increases might rather be connected to the (re-)uptake/degradation of eCBs than their synthesis/release. Even though VDM11 was found to be able to alter the activity of FAAH in sebocytes in a minor way (**Table 1**), we showed that it did significantly elevate OEA levels and tended to enhance PEA concentrations (**Fig. 25C-D**). These findings might be highly relevant, since these eCB-related substances are both very effective in alleviating inflammation (Facci et al., 1995; Impellizzeri et al., 2015; Pontis et al., 2016).

Elevated eCB tone-induced anti-inflammatory effects have already been reported both in human and mouse skin. In these studies, it has been shown that skin immune responses that have key roles in the development of local allergy and inflammation are tightly regulated by ECS (e.g. Karsak et al., 2007; Oláh et al., 2016a). As we have already mentioned, the concentration of OEA and PEA was increased after VDM11 treatment, although to varying degrees. This could mean that SGs might not only be able to regulate the metabolism of these “eCB-related” substances, but that they can maintain their levels in the adjacent tissue microenvironment (Facci et al., 1995; Impellizzeri et al., 2015; Pontis et al., 2016; Yang et al., 2016).

What if the incomplete development of SGs, that have been described in AD (Shi et al. 2015), would also be associated with lower magnitude of PEA (and/or OEA), next to their reduced sebum production. This might be an important factor in the development and aggravation of atopic inflammation. Knowing that PEA has effectively been used as an adjuvant therapy for AD (Eberlein et al., 2008), it would be good to know whether a SG underdevelopment-related putative PEA/OEA-deficiency could also be considerable in the emergence of this disease. Moreover, since besides PPARs, these molecules can activate certain recently de-orphanized GPCRs (GPR55 and GPR119, respectively), our data highlight the

possibility that these novel receptors may also be expressed in human SGs (Godlewski et al., 2009; Maccarrone et al., 2015).

We subsequently investigated the effects of EMT inhibitors on the viability, lipid synthesis, and immune responses of human sebocytes. We determined that non-cytotoxic concentrations of VDM11 and AM404 significantly increased the SLP of the cells, although only in a moderate way (**Fig. 27, 28B**). In addition, VDM11 also suppressed the LPS-induced pro-inflammatory cytokine expression of the cells (**Fig. 32A-B**). The differentiation of CD4⁺/CD45RA⁺ naïve T cells into T helper (Th) 17 cells can be evoked by certain sebocyte-derived cytokines, such as IL-6 (Mattii et al. 2017), which finding further strengthens the concept that in the development of such skin diseases that are associated with Th17-driven inflammation (e.g. AD or psoriasis), the abnormal sebocyte functions can also play an important role. Consequently, such reduction of IL-6 level, which has been shown above, could very well be advantageous in the treatment of such disorders (**Fig. 32B**).

These results collectively show that the subsequent elevation of “eCB-tone” promotes SLP in human sebocytes via the inhibition of eCB degradation (**Fig. 34**). Even though the FAAH inhibitor URB597 could efficiently inhibit the FAAH activity in sebocytes (**Table 1**), surprisingly it did not cause any significant change in their SLP (**Fig. 29B**). This can be explained by the low level of FAAH activity that was determined by measuring hydrolysis of radiolabeled AEA.

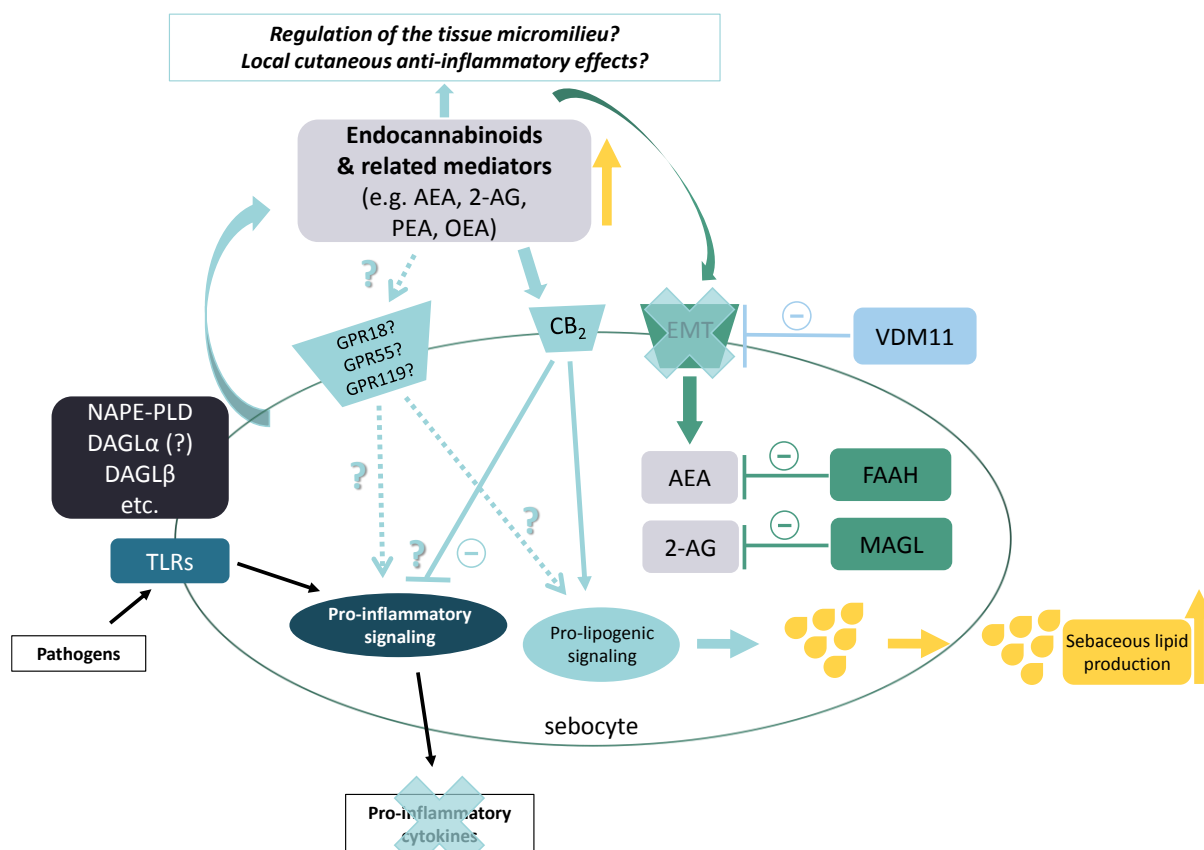


Fig. 34: Overview of the sebaceous gland's endocannabinoid system

Major enzymes of the eCB metabolism (i.e. NAPE-PLD, DAGL β , MAGL, FAAH, and maybe DAGL α) are expressed on human sebocytes. Thus, the cells are able to locally metabolize several “classical” eCBs, e.g., AEA and 2-AG, as well as related acylethanolamides, such as PEA and OEA. Using VDM11, the concentration of these ligands increase leading to a moderate elevation in SLP and potent anti-inflammatory actions.

It should also be noted that, based on our data, an alternative hypothesis can also be formulated regarding the mechanism of lipogenic actions. As mentioned above, EMT mediates a bidirectional transport, therefore, EMT-inhibitors might also suppress the *release* of eCBs (Chicca et al., 2012). What if locally produced eCBs are transported outside of the cells by EMT rather than being up-taken? This would mean that by using specific inhibitors, such export across the cell membrane could be reduced, and therefore, eCBs would be more processed within the cells. By increasing their intracellular concentrations, the level of cytoplasmic AA (an important precursor of several pro-inflammatory lipid mediators) should also be elevated in a FAAH-mediated manner (Bennett and Gilroy 2016). Considering the substantial lipogenic effect of AA (Géczy et al., 2012), it is possible that the elevated intracellular AA levels could

be responsible for the lipogenic effect of VDM11. The absence of such AA-elevation would also elucidate why URB597 was unable to sufficiently affect the SLP of sebocytes.

Even though it seems very interesting, the former hypothesis may not explain all aspects of this complex regulatory process. We have previously found that the lipogenic property of AEA was CB₂-dependent (Dobrosi et al., 2008), while AA was shown to have no activity on this receptor (Di Marzo, 2008). Moreover, cyclooxygenase and lipoxygenase metabolites of AA are more likely to exert pro-inflammatory effects (Bennett and Gilroy 2016), we have pointed out that VDM11 suppressed LPS-induced pro-inflammatory response (**Fig. 32A-B**). Last but not least, if the formation of AA from externally administrated AEA was really necessary to facilitate SLP, why VMD11 did not impact on the lipogenic effect of the former?

It has already been mentioned that the treatment of diseases associated with skin dryness could theoretically well be complemented with the controlled (i.e. moderate, not acnegenic) elevation of physiological SLP (Pappas 2009; Shi et al., 2015). As we have shown on **Fig. 27**, the lipogenic effect of VDM11 was much lower compared to responses seen after direct AEA treatment or after the application of other compounds that can also facilitate lipogenesis (e.g., 2-AG, AA, and linoleic acid plus testosterone) (Dobrosi et al., 2008; Géczy et al., 2012; Oláh et al., 2014; Oláh et al., 2016b). Although exact effects of VDM11 on the sebaceous lipidome is to be assessed in future, targeted studies, all aforementioned effects of VDM11, including its anti-inflammatory efficiency, suggest that this AEA uptake inhibitor, maybe together with other eCB transport-blocking-agents, might be promising for the prevention and/or treatment of dryness- and inflammation-accompanied skin diseases. Thus, our data should encourage one to test next in appropriate clinical studies the putative therapeutic potential lying in SG-targeted ECS-modulators in the clinical management of such skin diseases that are associated with skin dryness and inflammation (e.g., AD).

7. SUMMARY

Endocannabinoid system has been shown to be important in the regulation of numerous physiological and pathological processes in human skin. In this thesis we have investigated the role of endocannabinoid (eCB) tone in the biology of selected non-neuronal skin cells, more specifically in human skin mast cells (MCs) and human sebocytes.

We found that human MCs express functionally active cannabinoid receptor type 1 (CB₁). Via abrogating the homeostatic CB₁ signaling *in situ*, the inverse agonist AM251 could enhance maturation and activation of human skin MCs. These effects were most probably mediated by direct actions on MCs, as well as by modulation of the expression and release of stem cell factor in human hair follicle epithelium. Since CB₁ agonists prevented secretagogues-induced degranulation of the MCs, our data suggest that CB₁ stimulation might be a promising strategy in the future management of allergy and other MC-dependent inflammatory diseases by limiting skin MC activation and maturation.

On human sebocytes our data has shown that important eCB metabolic enzymes (NAPE-PLD, DAGL β , FAAH, and MAGL) are present both in SZ95 cells and in sebaceous glands, except for DAGL α , whose expression was not conclusive based on the histological examinations. We have also shown that the AEA-uptake inhibitor VDM11 moderately elevated the sebaceous lipid production of SZ95 cells and also the concentration of different eCB ligands. In addition, VDM11 could inhibit some of the Toll-like receptor 4 activator lipopolysaccharide-induced pro-inflammatory actions.

Collectively, our results indicate that eCB transport inhibitors might be advantageous in treating cutaneous inflammatory conditions associated with skin dryness.

8. ÖSSZEFOGLALÁS

Az endokannabinoid rendszer bizonyítottan nagy jelentőséggel bír a bőr számos fiziológiás és patológiai folyamatának szabályozásában. Jelen munkánkban célul tűztük ki az endokannabinoid (eCB) tónus bőrre kifejtett hatásainak behatóbb vizsgálatát egyes ott megtalálható nem neuronális sejtcsoportokon, névszerint humán hízósejteken, valamint humán szebocitákon.

Eredményeink szerint a humán hízósejtek kifejezik az 1-es típusú kannabinoid receptor (CB₁) funkcionálisan aktív formáját. A homeosztatisz CB₁ jelátvitel felfüggesztése, a CB₁-inverz agonista AM251-gyel képes volt nemcsak a hízósejtek aktivációját, de azok rezidens progenitor sejtekből történő érési folyamatait is fokozni. A hatások feltehetőleg részben közvetlenül a hízósejteken, részben pedig a szőrtüsző epitéliumában termelődő őssejtfaktor szintjét befolyásolva alakultak ki. Minthogy a CB₁ agonisták emellett kivédtek a szekretagóg-indukálta degranulációt is, eredményeink arra utalnak, hogy a CB₁ receptor stimulációja ígéretes lehetőség lehet olyan allergiás- és gyulladásos bőrbetegségek jövőbeni kezelésében, melyek okai között szerepel a fokozott hízósejt szám és aktiváció.

Humán szebocitákat és faggyúmirigyeket vizsgálva megállapítottuk, hogy azokon az ECS enzimapparátusának számos tagja (NAPE-PLD, DAGL β , FAAH és MAGL) megtalálható. Ezek alól kivételt képzett a DAGL α , melynek kifejeződése a szövettani festések során nem volt meggyőző. Megállapítottuk, hogy az anandamid felvételt gátló VDM11 mérsékelten növelte a faggyúlipid-termelést, valamint több eCB koncentrációját, és képes volt lipopoliszacharid által kiváltott gyulladásos válasz egyes elemeinek kivédésére is.

Összefoglalva, eredményeink felvetik az eCB transzportot gátló szerek alkalmazásának lehetőségét a bőrszárazsággal járó gyulladásos bőrbetegségek kezelésében.

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10. KEYWORDS/TÁRGYSZAVAK

Endocannabinoid system, endocannabinoid tone, non-neuronal human skin cells, mast cell, CB₁ receptor, inflammation, sebocyte, inhibition of endocannabinoid transport, sebaceous lipid production, skin dryness.

Endokannabinoid rendszer, endokannabinoid tónus, a bőr nem-neuronális sejtjei, hízósejt, CB₁ receptor, gyulladás, szebocita, endokannabinoid transzport gátlás, faggyúlipid-termelés, bőrszárazság.

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12. LIST OF PUBLICATIONS



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List of publications related to the dissertation

1. **Zákány, N.**, Oláh, A., Markovics, A., Takács, E., Aranyász, A., Nicolussi, S., Piscitelli, F., Allarà, M., Pór, Á., Kovács, I., Zouboulis, C. C., Gertsch, J., Di Marzo, V., Bíró, T., Szabó, T.:
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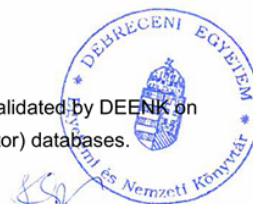
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Total IF of journals (all publications): 33,7

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The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.



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13. APPENDIX: PUBLICATIONS RELATED TO THE DISSERTATION