

THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

The role of sebaceous lipids and proteins in the differentiation,
polarization and function of dermal macrophages and T cells

by Marianna Lovászi

UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF HEALTH SCIENCES

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2. LIST OF ABBREVIATIONS

α -MSH	α -melanocyte-stimulating hormone
Δ 15-PGJ2	15-deoxy-delta(12,14)-prostaglandin J2
13CRA	13-cis retinoic acid
5 α -DHT	5 α -dihydrotestosterone
9CRA	9-cis retinoic acid
9-PAHSA	9-palmitic-acid-hydroxy-stearic acid
AA	arachidonic acid
ABC transporters	ATP-binding cassette transporters
ACTH	adrenocorticotrophic hormone
AD	atopic dermatitis
Akt	a.k.a Protein kinase B
ANOVA	analysis of variance
ATRA	all trans retinoic acid
BSA	bovine serum albumin
CD	cluster of differentiation
CFU	colony forming unit
CH	cholesterol
CHE	cholesterol ester
COX	cyclooxygenase
CRH	corticotropin-releasing hormone
CTR	control
ctrl. med	control medium
DAPI	40,6-diamidino-2-phenylindole
DBD	DNA- binding domain
DC	dendritic cell
DGAT	diglyceride-acyltransferase
DHA	docosahexaenoic acid
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylene-diamine-tetra-acetic acid
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
FA	fatty acid
FADS2	fatty acid desaturase 2
FATP4	fatty acid transport protein 4
FBS	fetal bovine serum
FFA	free fatty acid
FITC	fluorescein isothiocyanate
FoxO1	forkhead box O 1
FXIII-A	Factor XIII subunit A
GH	growth hormone
hBD2	human β -defensin-2

HRP	horse radish peroxidase
IFN- γ	interferon gamma
IGF	insulin-like growth factor
IGF-R	insulin-like growth factor receptor
IL	interleukin
LA	linoleic acid
LC	Langerhans cell
LCFA	long chain fatty acid
LD	lipid depleted
LDL	low-density lipoprotein
LOX	lipoxygenase
LPS	lipopolysaccharide
LT	leukotriene
LTB4	leukotriene B4
LXR	liver X receptor
MC-R	melanocortin receptor
mTORC1	mammalian target of rapamycin complex 1
MUFA	monounsaturated fatty acid
NF- $\kappa\beta$	nuclear factor kappa-light-chain-enhancer of activated B cells
NHR	nuclear hormone receptor
NLRP3	nucleotide-binding oligomerization domain, leucine rich repeat and pyrin domain containing 3
OA	oleic acid
<i>P. acnes</i>	<i>Propionibacterium acnes</i>
PA	palmitic acid
PASI	Psoriasis Area Severity Index
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffer saline
PFA	paraformaldehyde
PG	prostaglandin
PGD2	prostaglandin D2
PGE2	prostaglandin E2
PI3K	phosphatidylinositol 3-kinase
PPAR	peroxisome proliferator activated receptor
pre-DC	precursor dendritic cell
PUFA	polyunsaturated fatty acid
RAR	retinoic acid receptor
RIPA	radio immunoprecipitation assay
ROS	reactive oxygen species
RT	room temperature
RXR	retinoid X receptor
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
SCD	stearoyl-CoA desaturase
SCFA	short chain fatty acid
SFA	saturated fatty acid

SFPB	serum-free protein block
SG	sebaceous gland
SP	substance P
SQ	squalene
SREBP	sterol regulatory element-binding protein
STA	stearic acid
SZ95 sup	SZ95 sebocyte supernatant
TCR	T cell receptor
TFA	trans fatty acid
TG	triglyceride
TGF- β	transforming growth factor β
Th	T helper
TLR	Toll-like receptor
TNF	tumour necrosis factor
Treg	regulatory T cells
Tris	tris(hydroxymethyl)aminomethane
UV	ultra violet

3. INTRODUCTION

For a long time, it was a widely-accepted concept in dermatology, that sebaceous glands (SGs) are atavistic remnants of the human skin and only play important roles in mammals, which resulted in the overlook of human sebaceous gland biology.

However, in the last 25 years, cell lines (such as SZ95, SEB-1 and Seb-E6E7) (1) and new molecular techniques were introduced also into SG research, leading to the identification of numerous signalling pathways, transcription factors and enzymatic molecules in sebocytes. As a result, although the primary function of the SGs is still considered to be the production of sebum, which contributes to the lipid barrier of the skin, more and more data has revealed that SGs might also have a role in various (patho)physiological functions from skin aging to UV protection and that sebocytes could also have immune properties as well.

The aim our work was therefore, to focus on their involvement in inflammatory processes and identify possible mechanisms behind it, especially addressing their possible interaction with various cells types of the immune system.

4. LITERATURE OVERVIEW

4.1 Sebaceous gland structure and function

Sebaceous glands (SGs) are found in the dermal layer of the skin all over the body, except for the palms and soles (2). Localizing mostly to hair-covered areas, they can also be found in non-hairy regions of the skin, such as the eyelids, nipples, labia minora and the glans penis (3). Attached to the hair follicles, SGs form the pilosebaceous unit together with the arrector pili muscles. (Figure 1.) (4).

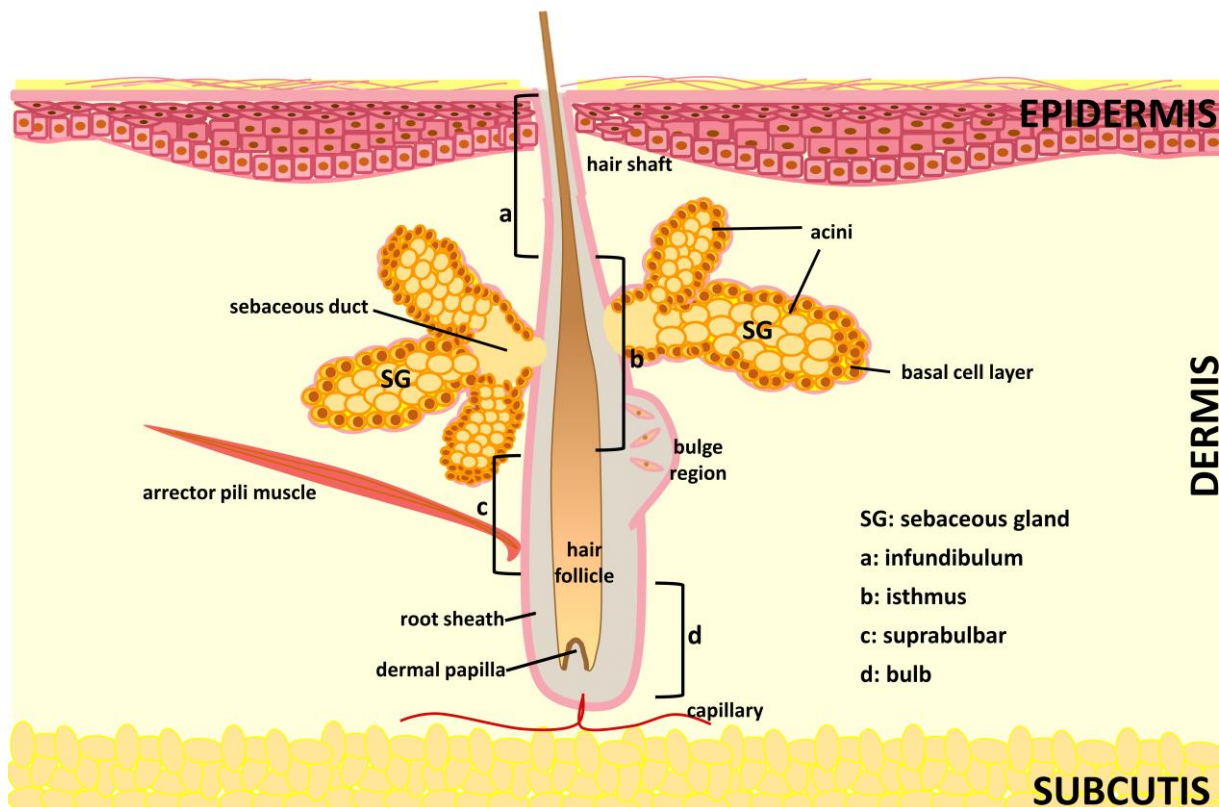


Figure 1. The structure of the pilosebaceous unit

SGs have an acinar structure, where multiple glands branch off a central duct. The glandular structure consists of modified epithelial cells called sebocytes than can be divided into proliferating basal cells, mature sebocytes synthesizing sebum and cells that have already lost their nucleus and are ready for the so called holocrine secretion excreting sebum into the sebaceous duct which is connected to a hair follicle (Figure 2.) (5, 6).

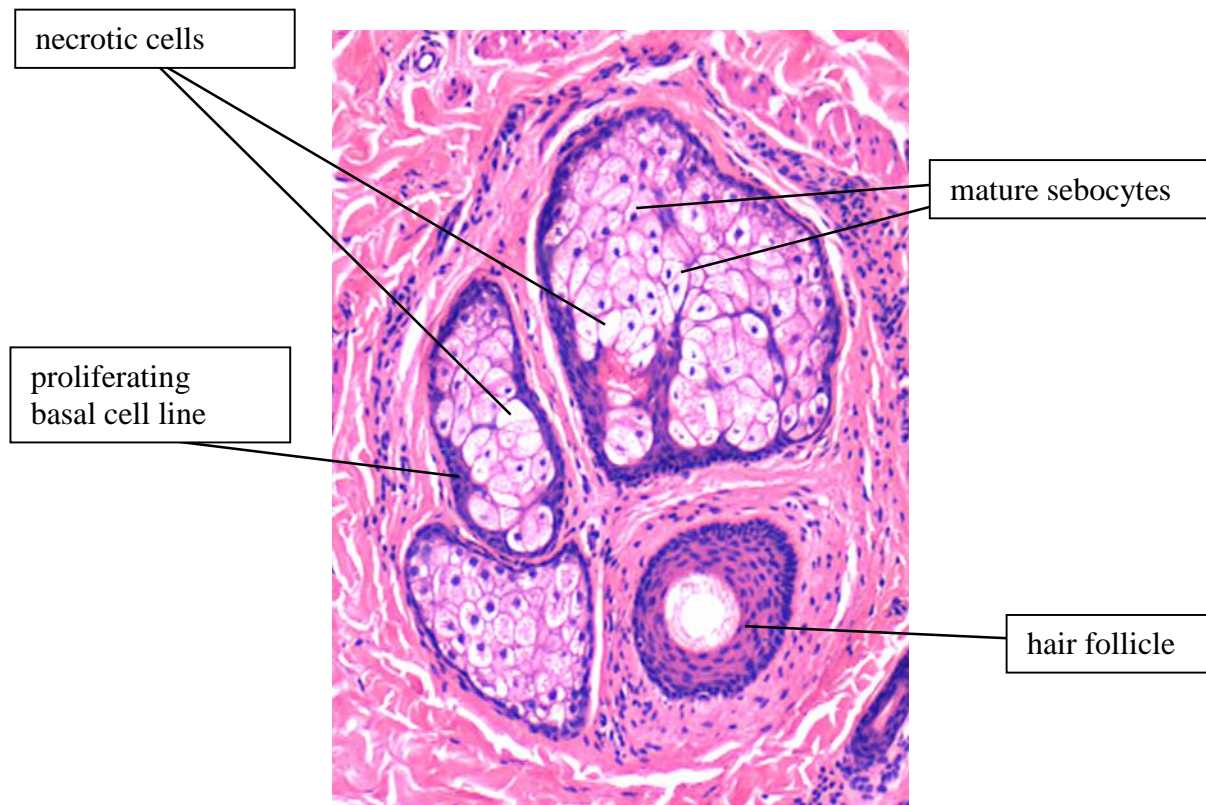


Figure 2. Differentiation of sebocytes

Haematoxylin-eosin staining of a healthy human skin biopsy (shoulder). Magnification 100X.

Sebum reaches the surface of the skin along the hair shaft through the upper most part of the pilosebaceous unit, called the infundibulum. Under the infundibulum lies the isthmus region, which extends until the bulge region followed by the suprabulbar segment, which consists of the outer and inner root sheath and the hair follicle. The pilosebaceous unit ends in the bulb region where the dermal papilla anchors the hair follicle, surrounded by capillaries which nourish the matrix cells (Figure 1.) (5, 6).

The primary function of human sebaceous glands is considered to be the production and secretion of sebum to cover the hair and contribute to the lipid barrier of the skin (4, 7, 8). In mammals sebum provides waterproofing and lubrication for the skin and hair (9), furthermore, together with apocrine glands, SGs also play an important role in thermoregulation. Additionally, sebum offers protection from several natural hazards such as ultra violet (UV) radiation and various microbes with its antimicrobial activity (10, 11). In the next chapter we will be focusing on the various aspects of sebum production.

4.2 The endocrine regulation of sebaceous glands

The proliferation and differentiation as well as sebum production of sebocytes is controlled by complex endocrine processes, of which the most prominent changes take place at puberty (12). However human sebocytes are not only receptive of hormonal changes, but rather are capable of producing androgens, estrogens, corticotrophin-releasing hormone (CRH), all trans retinoic acid (ATRA), eicosanoids, cortisol and cholecalciferol/vitamin D3 themselves (13, 14). A prominent example is the androgen 5α -dihydrotestosterone (5α -DHT), converted by sebocytes from testosterone with the enzyme 5α -reductase type I (15). While androgens are known to facilitate sebaceous gland proliferation and differentiation, furthermore increase lipid production (16, 17), estrogens, cortisol and ATRA have an opposite effect on the proliferation and differentiation of sebocytes moreover the accumulation of lipids is also decreased in their presence (18-22).

Sebocytes are capable of producing CRH and expressing its receptors as well as CRH-binding protein (23). CRH activates sebocyte proliferation and directly induces lipid synthesis via the expression of $\Delta 5$ - 3β -hydroxysteroid dehydrogenase, which is responsible for the conversion of dehydroepiandrosterone into testosterone (23).

Melanocortin receptors 1 and 5 (MC-1R, MC-5R) are also expressed on the surface of sebocytes (8, 24). α -melanocyte stimulating hormone (α -MSH) and adrenocorticotrophic hormone

(ACTH) are both ligands of MC-1R and MC-5R. α -MSH have been shown to downregulate nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) activation thus directly regulate the production of proinflammatory cytokines in sebocytes (25).

Neuropeptides are also present in sebaceous glands and may provide a link between stress and acne pathogenesis (14, 26). Among neuropeptides substance P (SP) has a significant role in modulating sebaceous cell size and the number of sebum vacuoles, suggesting that SP promotes both sebocyte proliferation and differentiation (27). Another interesting feature is the presence of SP in sebaceous glands surrounding inflamed nerve fibers, raising the possibility that neuronal inflammatory processes and sebaceous gland function may be connected (28).

In understanding the role of diet in sebaceous lipogenesis, a widely accepted mechanism is that a diet rich in carbohydrates increases the activity of the insulin-like growth factor 1 (IGF-1) pathways (29-31). The presence of the IGF-1 receptor (IGF-1R) in sebocytes has been described and was shown to activate the phosphatidylinositol 3-kinase/Akt/Forkhead box O1 (PI3K/Akt/FoxO1) pathway thus providing evidence that FoxO1 may be a key factor in the regulation of growth-factor stimulatory effects on sebaceous lipogenesis and inflammation in response to elevated IGF-1 and insulin levels (32). *In vitro* studies have also shown that IGF enhances lipid production in sebocytes via the sterol response element binding proteins (SREBPs) (33). IGF is also capable of activating the growth hormone (GH) (34), thus GH can also upregulate sebocyte differentiation and increase sebum synthesis (34).

Notably, in a previous study we have demonstrated that leptin “the satiety hormone”, besides inducing the expression of inflammatory cytokines such as interleukin (IL)-6 and CXCL-8 in sebocytes also affects lipogenesis by increasing triglyceride (TG) levels, thus increasing the ratio of monounsaturated fatty acids (MUFAs) or polyunsaturated fatty acids (PUFAs) to saturated fatty acids (SFAs) and by decreasing the levels of vitamin E (35, 36).

Peroxisome proliferator-activated receptors (PPARs) belong to the family of nuclear hormone receptors (NHRs), which are transcriptional factors either residently or transiently located in the nucleus. Their main function is to attach to their target genes through their deoxyribonucleic acid (DNA)- binding domain (DBD) thus regulating major cellular signalling pathways at the level of gene expression (37). All PPAR isotypes (PPAR α , PPAR β/δ and PPAR γ) are present in sebocytes and regulate multiple lipid metabolic genes taking part in lipogenesis and differentiation (38, 39). Particularly PPAR γ came to the focus of research with its sebostatic effect via inhibiting apoptosis and thus obstructing holocrine secretion altogether leading to decreased sebum production (40). However recent studies have also shown that the arachidonic acid (AA) induced lipogenesis in SZ95 sebocytes was PPAR γ mediated (41, 42), furthermore PPAR γ agonist not only increased lipid production, but also enhanced the release of inflammatory cytokines (43). On the contrary PPAR α agonists inhibit lipogenesis via intercepting the proinflammatory leukotriene (LT) activity (44, 45). Interestingly, *P. acnes* and its soluble factors may also participate in the augmentation of sebaceous lipogenesis by increasing the production of the 15-deoxy-delta(12,14)-prostaglandin J2 (Δ 15-PGJ2), an endogenous activator of PPAR γ (46).

Retinoids are vitamin A derivatives with well-known beneficial effects in acne patients (47). Target receptors for retinoids are retinoid X (RXR) and retinoic acid (RAR) receptors both of which are expressed in sebocytes. While RXRs have a role in influencing cell differentiation (48), RARs modulate cell proliferation (22). 9-cis retinoic acid (9CRA) and ATRA have been identified as natural ligands of RXR and RAR respectively, while 13-cis retinoic acid (13CRA) an isomer of ATRA acts as an inhibitor of proliferation in SZ95 sebocytes (49-51). Due to the above-mentioned properties 13CRA is used worldwide as an effective anti-acne medication (20).

Also belonging to the nuclear receptor family, liver X receptors (LXRs, α and β isoforms) play a decisive role in cellular cholesterol (CH) homeostasis and lipid metabolism (26, 52, 53). LXRs regulate various ATP-binding cassette transporters (ABC transporters), fat metabolizing enzymes and other transcription factors such as sterol regulatory element-binding protein-1c (SREBP-1c) (53, 54). SREBP-1c not only regulates total TG levels in the sebum, but also increases the amount of MUFAs by regulating the expression of $\Delta 6$ -desaturase and stearoyl-CoA desaturase enzymes (55). Moreover, application of the natural LXR α ligand 22(R)-hydroxycholesterol inhibits proliferation and increases lipogenesis in SZ95 sebocytes indicated by the accumulation of lipid droplets, a hallmark of sebaceous lipogenesis (56).

4.3 Sebum composition and production

Sebocytes are able to uptake lipids from the circulation and *de novo* synthesize polar lipids and TGs elongating the carbon chains by adding acetate groups to it. The maturation of sebocytes is followed by a cell type specific cell death. This procedure results in the holocrine secretion of sebum (4, 8).

Human sebum is a lipid mixture composed of cell debris and nonpolar (neutral) lipids, namely TGs and fatty acids (FAs) (57.5%), wax esters (26%), squalenes [SQ (12%)], cholesterol esters (CHEs) (3%) and CH (1.5%) (57, 58). The composition of human sebum varies greatly from what is secreted by mammalian sebaceous glands, SQ, sapienic acid and sebaleic acid are only synthesized by human sebocytes (59, 60).

While some sebum lipid fractions are unique products of the human sebaceous gland, others are also synthesized elsewhere in the body. Such example for the latter is SQ, an unsaturated hydrocarbon, which is a precursor of CH. While in other tissues SQ is rapidly converted to CH, in human sebocytes SQ is highly accumulated in the produced sebum. Due to its prominent lubricant and penetration efficiency SQ is capable of neutralizing the formation of UV irradiation-induced reactive oxygen species in the skin (10, 11). On the other hand, peroxidated

SQ has been reported to be comedogenic and therefore responsible for pathological skin conditions (61, 62).

As opposed to SQ, wax esters are uniquely produced by sebocytes. FAs are direct precursors of wax esters, moreover fatty acid transport protein 4 (FATP4) along with cluster of differentiation (CD)36 and low-density lipoprotein (LDL) receptors are strongly expressed in sebocytes (58, 63). FATP4 is a trans-membrane protein that increases the uptake of long chain fatty acids (LCFAs) into sebocytes (64). The enhanced uptake of LCFAs results in an enhanced wax ester biosynthesis in human sebaceous gland cells (60).

While diglyceride-acyltransferase (DGAT) is a major enzyme partaking in sebaceous TG synthesis (65, 66), stearoyl-CoA desaturase (SCD) plays a major role in the synthesis of unsaturated FAs. SCD oxidases stearoyl-CoA and palmitoyl-CoA, which serves as substrates for the biosynthesis of phospholipids, TGs and CHEs (58). For example, SCD creates a double bond between the 9. and 10. carbon atom thus creating oleic acid [OA (18:1 Δ 9)] from stearic acid [STA (18:0)] (63, 67). OA (18:1 Δ 9), a major omega-9 MUFA of sebum TGs, exerts the strongest antibacterial and anti-inflammatory properties among sebaceous FAs and strongly enhances the innate antibacterial defence of the skin (68, 69).

Linoleic acid [LA (18:2, Δ 9,12)], an omega-6 polyunsaturated compound, and α -linolenic acid (18:3, Δ 9,12,15), an omega-3 polyunsaturated compound, are essential FAs (70). Ge et al. identified stearoyl-CoA Δ -6 desaturase/fatty acid desaturase 2 (FADS2) as the major FA desaturase in human SGs, which induces rapid oxidation and degradation of LA and its derivatives in sebocytes (67) and converts palmitic acid [PA (C16:0)] into sapienic acid (C16:1 Δ 6) (57). Sapienic acid is a unique sebaceous MUFA with a single double bond at the sixth position (71, 72) exerting strong antibacterial and antifungal activities (73). The elongation and further desaturation of sapienic acid yields another unique sebaceous gland FA, sebaleic

acid (18:2 Δ 5, 8) (57). An excerpt of the complex process of lipid synthesis is presented in Figure 3.

During lifetime sebum production shows significant alterations. At birth due to maternal hormones it is relatively high, however after the diminishing maternal hormonal effects sebum levels decrease in childhood. At the onset of puberty hormones, mainly androgens, radically induce sebum production, which decreases by the end of puberty, and remains low during the rest of adulthood with a further decrease in old age (74, 75). The composition, quality and quantity of sebum may slightly vary depending on race or the use of certain drugs (76, 77), however it can even have bigger variations in case of pathological skin conditions. In the following chapter these alterations in lipid composition will be discussed.

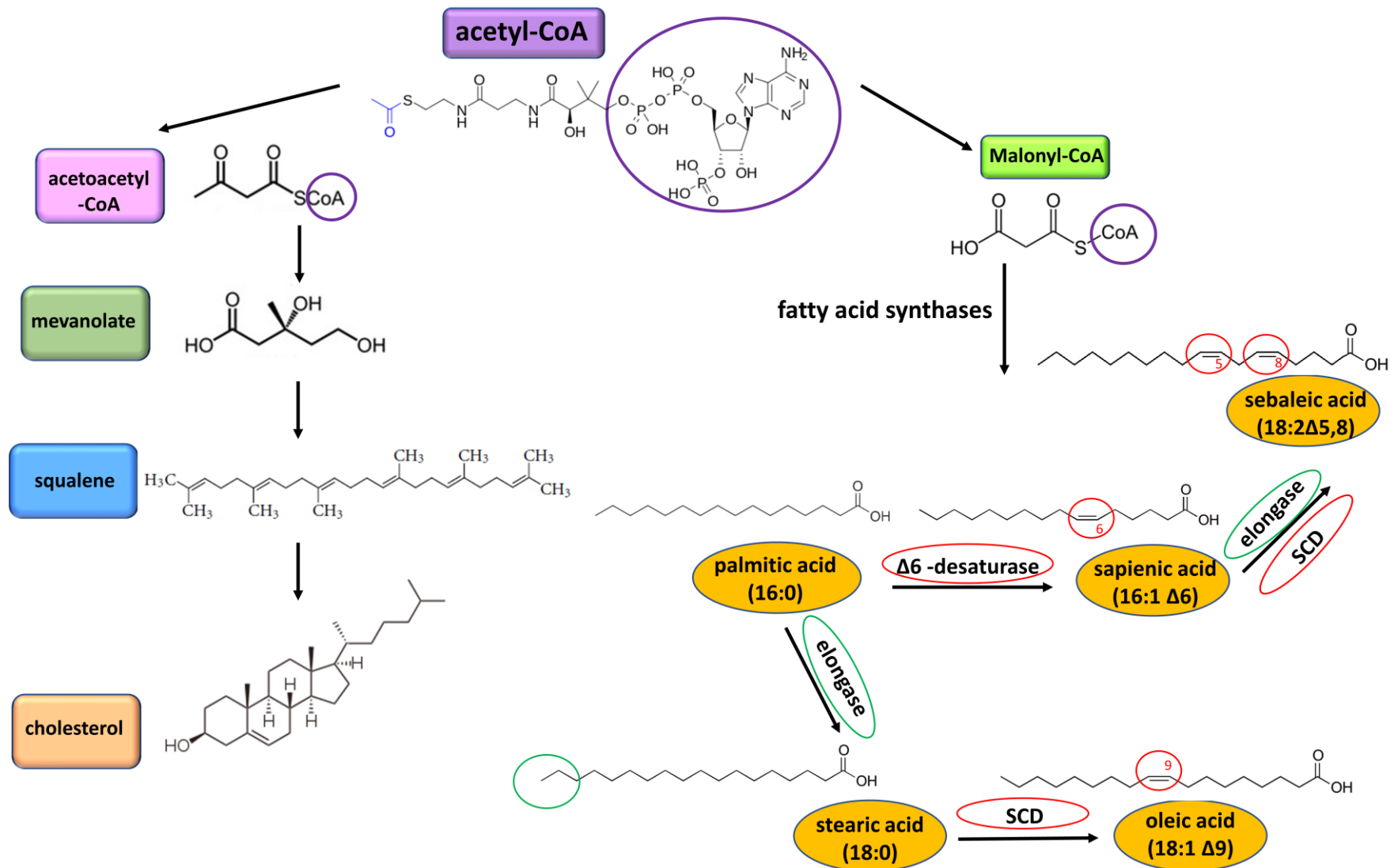


Figure 3. A segment of lipid synthesis [adapted from Horton et al., J Clin Invest. 2002 May;109(9):1125-31. (78)]

4.4 Sebum alterations in various skin diseases

Variations in the composition and amount of sebum in different skin diseases have been detected, which are suggested not to be simply disease markers, but may also play significant roles in disease pathogenesis.

4.4.1 Acne

Acne predominantly affects 60-80% of adolescents (79, 80). Both genetic and environmental factors contribute to its onset, however the exact mechanisms are yet to be identified. In the recent years numerous studies have showed, that in addition to an elevated sebum production, acne patients have a markedly altered sebum composition (35). Due to endogenous desaturases, peroxidases as well as bacterial lipases and phosphatases, an altered ratio between saturated and unsaturated FAs has been identified, particularly, an increase in the C16:1 Δ 6/ C16:0 lipid ratio has been shown in acne, accompanied by diminishing levels of LA content (35, 81). These data imply that desaturation of FAs may drive towards acne development. Remarkably, dietary PA (C16:0) directly activates Toll-like receptor 2 (TLR2) in mouse monocytes (82) and activates both the nucleotide-binding oligomerization domain, leucine rich repeat and pyrin domain containing 3 (NLRP3) inflammasome and the mammalian target of rapamycin complex 1 (mTORC1) pathway (83, 84), however, it also provides direct antibacterial activities and enhances the skins' innate antibacterial defence by inducing the expression of human β -defensin-2 (hBD2) in human sebocytes as well as the expression of murine β -defensin-2 in mouse skin (69). Sapientic acid (C16:1 Δ 6) also has potent antimicrobial properties against various nasal, oral and skin bacteria (85, 86), however interestingly it is not effective against *Propionibacterium acnes* (*P. acnes*) (73, 87). Furthermore, the application of C16:1 to the skin of mice enabled Ca²⁺ influx into keratinocytes, resulting in hyperproliferation and comedogenesis (88).

Another interesting aspect of the detected changes are the accumulation of lipid peroxides, specifically SQ peroxide, which activates the PPARs, stimulate keratinocyte proliferation and lipoxygenase (LOX) activity, overall increasing the expression and secretion of proinflammatory cytokines in acne (89-91).

Furthermore, studies also detected decreased levels of vitamin E in acne subjects, which also leads to inflammation and decreased protection against reactive oxygen species (ROS), which altogether underlines a general importance of lipid peroxidation in the development of acne (57).

The role of P. acnes in the pathogenesis of acne

Several hypotheses attribute a key role for *P. acnes* in the development of acne (92), although it is still not fully understood how *P. acnes*, a part of the healthy skin microbiome, can become pathogenic (93, 94). According to the most accepted theory, *P. acnes* might induce the hyperproliferation of the keratinocytes in the hair follicle duct, which blocks the opening of the pilosebaceous unit forming the so called “follicular plug” thus preventing the flow of sebum. The accumulating sebum then forms a microenvironment in which *P. acnes*, could reach significant levels to induce and augment inflammation (95, 96). Based on *in vitro* experiments, *P. acnes* not only promotes the activity of NLRP3 and thus enhances the production of IL-1 β , but also increases the production of CXCL-8, IL-12 and tumour necrosis factor (TNF)- α in keratinocytes macrophages and sebocytes (97). Although, it is still a question whether *P. acnes* can come into direct contact with sebocytes, its products such as lipases can undoubtedly have an effect on sebaceous glands (98).

4.4.2 Seborrheic dermatitis

Seborrheic dermatitis, also known as seborrhea, is a chronic inflammation of the skin, with symptoms such as redness, itching and scaling localizing mainly to the scalp and face (99). In relation to seborrheic dermatitis, studies suggest that the lipases and phosphatases produced by

Malassezia hydrolyse sebaceous lipids, resulting in decreased TGs and a corresponding increase in free fatty acid (FFA), which may cause the characteristic irritation (100).

4.4.3 *Rosacea*

Rosacea is a chronic inflammatory cutaneous condition that typically affects the cheeks, forehead chin and nose resulting in redness, swelling and dilated blood vessels (101). Despite the occurrence of rosacea in the sebaceous gland-rich areas of the body, the amount of sebum excretion does interestingly not show alterations in rosacea patients compared to healthy controls. However, there are changes in the overall relative composition of sebum (102, 103). Affected individuals exhibit elevated levels of myristic acid (C14:0) and reduced levels of saturated LCFAs (103). These lipids have a role in maintaining the skin barrier integrity, therefore it is reasonable to assume that the altered sebaceous FA profile is an important contributor to skin barrier dysfunction in rosacea (103).

4.4.4 *Psoriasis*

Psoriasis is a chronic inflammatory skin disease characterized by patches of hyperproliferating skin, which severely affects the quality of life (104). Psoriatic lesions are usually characterized by various degrees of sebaceous gland atrophy, however there has been no correlation found between sebaceous gland size and either total Psoriasis Area Severity Index (PASI) or the degree of erythema and inflammatory infiltration (105). Despite having sebaceous gland atrophy, the total amount of sebum production in psoriatic skin is not significantly changed (106), although an increase in the levels of phospholipids, triacylglycerols and CH were found in the epidermis of psoriatic patients, which correlated with the severity of psoriasis (107-109). Moreover, an increased ratio of CH esterification was also observed, especially in severe psoriasis (110).

4.4.5 Atopic Dermatitis (AD)

AD is a chronic inflammatory skin condition that often starts in early childhood and results in dry, red and itchy skin (111). Although AD is mainly characterized by the dysfunctional synthesis of ceramides in keratinocytes (112), AD patients seem to exert a severely reduced sebum production, which contribute to diminished skin hydration. Since the proportion of sebaceous lipids in the casual lipids is decreased and epidermal lipids (e.g. CH) are increased, therefore a link between an altered sebaceous gland function and skin barrier dysfunction seem to contribute to the development of the disease, although the exact mechanisms have not been elucidated (113-115).

4.5 Therapeutic perspectives in modulating sebum composition

The observations of altered sebum composition made so far in various skin diseases puts forward possible therapeutical solutions through the modulation of sebum amount and composition (116).

New compounds might be utilized as topical treatments in acne, such as long-chain PUFAs, which could target infections, e.g. with *P. acnes*, *Staphylococcus aureus* (*S. aureus*), due to their antimicrobial and anti-inflammatory properties, thus providing an alternative for antibiotics in acne treatment (117). PA, OA, and lauric acid have been already proposed as alternatives to antibiotic therapy in acne vulgaris (117, 118). Additionally, clinical studies are currently ongoing to create sebocyte specific PPAR α agonists and PPAR γ antagonist, which may beneficially decrease sebum production and inflammation in acne patients. (119-121).

Controlling lipid production not only can be beneficial for acne patients, where the overproduction of sebum and its compositional imbalance represents the main problem. Severe skin dryness as observed in AD patients might be remedied by manipulation of sebum production towards a normal composition via the stimulation of androgen or PPAR receptors,

which could potentially repair the defective skin barrier (122). Furthermore, the activation of LXRs by synthetic ligands may have therapeutic relevance, which could also augment lipid production along with exerting anti-inflammatory effects (122).

Novel compounds, such as endocannabinoids may also provide solutions to various sebaceous gland-related diseases. Recently Dobrosi et al. provided evidence, that sebocytes express cannabinoid receptor-2, more interestingly arachidonoyl ethanolamide/anandamide, 2-arachidonoyl glycerol are also present in sebocytes and can induce lipid production and apoptosis (123). Endocannabinoids generally up-regulate the expression of genes involved in lipid synthesis, such as PPAR transcription factors, thus cannabinoid receptor-2 antagonists or agonists may be utilized in the management of sebaceous gland disorders (123, 124).

4.6 Inflammatory processes exerted by sebocytes

Although, sebocytes are able to alter their sebum production in response to different inflammatory stimuli (36, 123, 125, 126), to position them in the inflammatory network, research has mostly focused on identifying the role of the co-produced proteins. Recent findings have shown that sebocytes, similarly to immune competent cells, are able to produce a vast array of cytokines and chemokines both *in vivo* and *in vitro*, such as: IL-6, CXCL-8, IL-1 α , IL-1 β and TNF- α (41, 89, 127-129).

Moreover, sebocytes have been shown to exert inflammatory properties with the production of antimicrobial peptides, cytokines and chemokines (35), in which the activation of TLR2 and -4, shown to also be expressed in sebocytes, play a crucial role (41, 92, 130). Furthermore, sebocytes have also been identified as sources of various adipokines, such as adiponectin, leptin, serpin E1, resistin and visfatin, within the skin, highlighting that sebaceous glands could represent a link between inflammation and lipid metabolism similarly to adipocytes (36, 131). However, it is still largely unrevealed how sebum lipids could contribute to the inflammatory milieu of the skin, despite previous findings that FFAs may exert proinflammatory effects (132).

Besides protein production, inflammation is also characterized by lipid mediators, such as prostaglandins (PGs) and LTs, which PGs and LTs are derivatives of AA metabolized by cyclooxygenase (COX) and lipoxygenase (LOX) respectively (41). Sebocytes express both isoforms of COX (COX-1 and COX-2) moreover, COX-2 expression has been shown to be upregulated in acne-involved SGs (41). PGs have major regulatory roles over sebocyte differentiation and lipogenesis, both under physiological and pathological conditions. Henke et al. identified prostaglandin D2 (PGD2) and prostaglandin E2 (PGE2) in mouse sebaceous gland cells (133), while Iwata et al. determined that Δ 15-PGJ2 plays a key role in lipid droplet formation and increased triglycerol synthesis in hamster sebaceous glands (134). 5-LOX is an essential enzyme in sebocytes for the production of leukotriene B4 (LTB4) shown by Alestas et al. LTB4 has an important pro-inflammatory regulatory role in sebocytes, moreover its chemoattractant activity for various immune cells is also known. Additionally, the activation of 5-LOX via AA or calcium ionophores resulted in elevated CXCL-8 production in sebocytes (41).

4.7 Immune functions of sebum lipids

Sebaceous glands secrete acids that form the acid mantle, a fine, slightly acidic (between pH 4.5 and pH 6.0) film on the surface of the skin that provides a barrier against pathogens, such as bacteria and viruses that might penetrate the skin and are primarily of alkaline nature (135). Moreover, sebaceous lipids make an important contribution in maintaining the integrity of the skin barrier along with lipids of epidermal origin (4, 13) thus contributing to the body's first line of defence (119).

The healthy human skin is populated with a characteristic microbiome (136). Interestingly, its diversity was found to be associated with the differences in the quantitative levels of sebum and hydration even between various parts of the face (137). One of the most studied colonizing member is *P. acnes*, an anaerobic bacterium, which is capable of metabolizing sebaceous TGs

into FFA on the surface of the skin via its lipases and peroxidases (54, 98). This bacterial lipase activity not only leads to an increased presence of FFA on the surface of the skin, but also to an altered sebum composition (138). SQ in particular has been proposed as the precursor of highly toxic proinflammatory mediators, produced by bacterial lipoperoxidase activity (139). Ottaniavi et al. showed that SQ peroxide induces the enzymatic activity of 5-LOX in keratinocytes thus increasing the production of IL-6. Moreover, SQ peroxide activates NF- κ B, which also mediates proinflammatory signalling in cells (140). These changes lead to hyperkeratinisation and chronic inflammation, which further increases the pathological microbial colonization of the skin, thus sustaining acne formation.

Sebum may also act as a delivery system for antioxidants and antimicrobial peptides. Such molecules with antimicrobial properties are cathelicidin, psoriasin, dermcidin and human hBD-2 (141-143). As previously described, Nakatsuji et al. found that LA, PA and OA, typically found in human sebum enhanced the hBD-2 expression and antimicrobial activity of human sebocytes against *P. acnes* (69).

Other studies have showed that lauric and sapienic acid as well as long-chain bases (sphingosine, dihydrosphingosine and 6-hydroxysphingosine) have broad-acting antimicrobial properties (85). These antimicrobials act as part of the innate immune system of the skin, and are generated through the action of ceramidases from the stratum corneum (85).

These findings altogether show, that sebocytes are capable of producing inflammatory mediators both of protein and of lipid nature (35) and are capable to alter their sebum production/composition in response to different inflammatory stimuli (36, 123, 125, 126). These data already suggest that sebocytes may not just be a target of inflammation, as previously considered, but could actively contribute to skin homeostasis and the inflammatory environment (116, 119, 144-146).

As depicted in the previous chapters, sebocyte proliferation, differentiation, lipogenesis as well as inflammatory processes are regulated by multiple signalling pathways (Figure 4.).

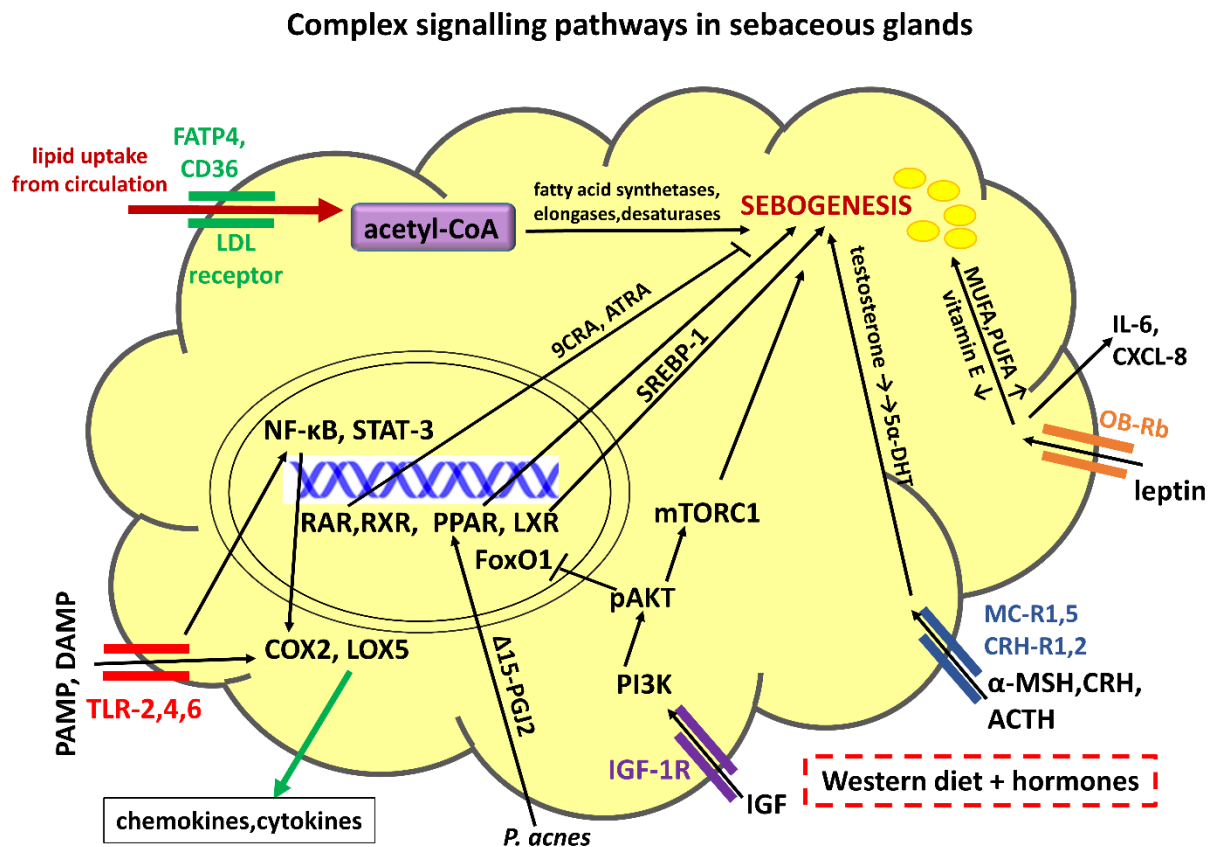


Figure 4. Signalling pathways influencing sebaceous differentiation, proliferation and lipogenesis

4.8 Skin resident immune cells

Resident macrophages and dendritic cells (DCs) of prenatally seeded yolk sac- or fetal liver-derived precursors can be found in the epidermis (Langerhans cells, LCs) and the dermis. These cells are distinct in their phenotype and function (147) from CD14⁺ monocytes and precursor dendritic cells (pre-DCs), which are derived from bone marrow cells and circulate in the blood stream, from where upon attracting signals and stimulation they passage through the endothelium into the surrounding tissues differentiating into macrophages and DCs (148, 149). Interestingly McGovern et al. found that these CD14⁺ cells have a rapid turnover in the dermis in contrast to resident macrophages, spending approximately 6 days in the dermal layer of the skin, followed by cell death and phagocytosis, only to be replaced by freshly arriving CD14⁺ monocytes (150). With the identification of novel monocyte/macrophage and dendritic cell markers, new subsets of myeloid cells are discovered continuously, making the myeloid cell population of the skin highly heterogeneous, however the available tolls to characterize their functions is very limited (151-153).

4.8.1 *Macrophages and macrophage heterogeneity*

Being the key phagocytes of the immune system, macrophages sense and transform signals and stimuli towards the initiation of inflammation and activation of the adaptive immune system. In order to fulfil these complex roles, macrophages exert a prominent morphological and functional heterogeneity in both the healthy and the inflamed skin, outlining the importance of the different stimuli behind their activation (154). Based on these stimuli, a widely accepted classification separates macrophages into classically (M1) and alternatively (M2) activated subsets (Figure 5.) (148).

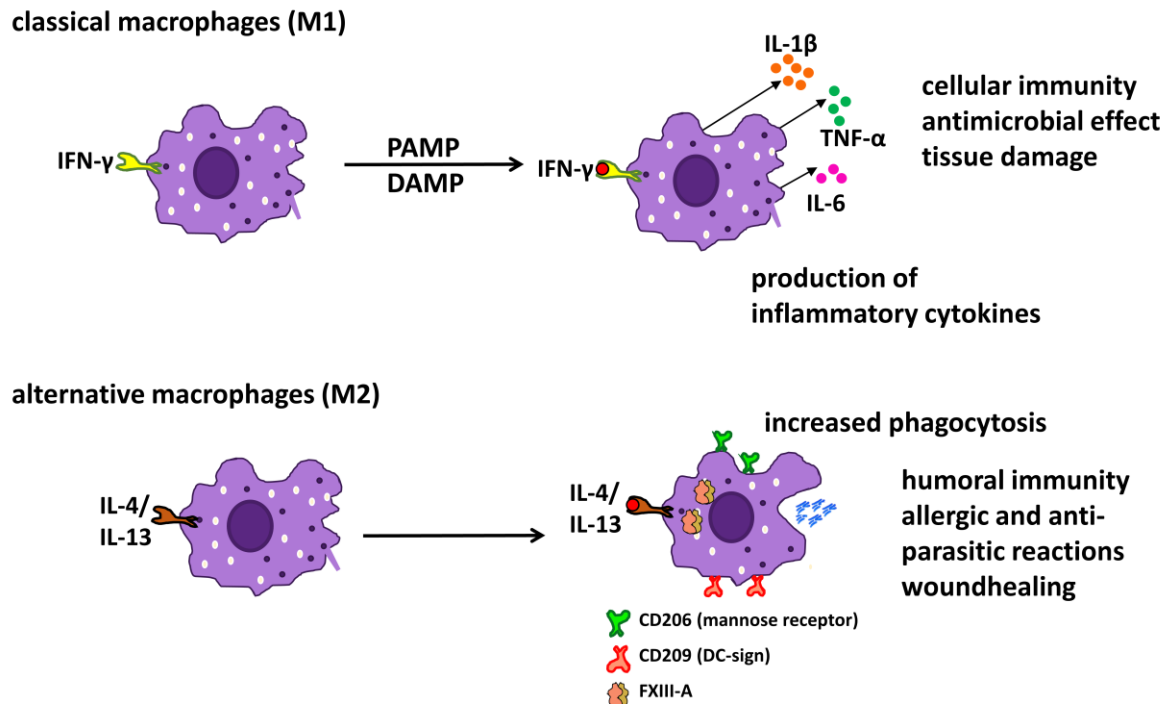


Figure 5. Activation pathways of macrophages

Based on Gordon S, Taylor PR: *Nat. Rev. Immunol.* 2005, 12:953-964. (148).

While alternative macrophages are activated by IL-4/IL-13 and by various lipids that are associated with tissue maintenance and remodelling processes, and are increased in high numbers in pathological conditions, such as wound healing or tumour matrix formation, the classically activated macrophages typically develop in response to interferon gamma (IFN- γ) and/or TNF- α (155, 156). Classic examples of M2 macrophages can be found in skin diseases of granuloma annulare and necrobiosis lipoidica (157), while a hallmark of M1 macrophages are present in tuberculoid granulomas (148). Human dermal macrophages in a healthy conditions typically express MHC-II, CD14, and Factor XIII subunit A (FXIII-A) (158) and are superior at phagocytosis, but inferior at T cell activation compared to DCs (153).

Macrophage markers used in the study

CD163 is a high affinity scavenger receptor for the haemoglobin-haptoglobin complex (159). Notably it has been identified as a marker of cells originating from the monocyte/macrophage lineage (160).

CD206, also known as mannose receptor, is a C-type lectin mainly present on the surface of macrophages and immature dendritic cells (161). The receptor recognises terminal mannose and N-acetylglucosamine residues found on the surface of certain microorganisms (162), thus playing an important role both in the innate and adaptive immune systems.

CD209, also known as DC-SIGN, is a C-type lectin receptor present on the surface of both macrophages and dendritic cells. In case of macrophages CD209 recognises mannose type carbohydrates commonly found on various pathogens, therefore CD209 has an important role in activating phagocytosis (163).

Factor XIII (FXIII) is a transglutaminase and an important member of the blood coagulation system that crosslinks fibrin. While circulating FXIII consists of two catalytic subunits (FXIII-A) and two carrier subunits (FXIII-B), in monocytes/macrophages FXIII-A (but not FXIII-B) can also be found in an intracellular localization where it could contribute to gene expression regulation as well as alter cellular functions such as phagocytosis (164, 165). Recently FXIII-A has been identified as a marker of alternative activation and was successfully applied to detect these macrophages in granuloma annulare and necrobiosis lipoidica (157, 166).

Role of lipid metabolism in macrophage activation

The importance of lipid metabolism in macrophage activation has been recently reviewed by Ménégaut et al. (167). Both external (dietary) and internal FA sources are able to influence and drive macrophage polarization: generally, M2 polarization is associated with FA oxidation, whereas M1 polarization is linked with lipogenic pathways (167). Several studies highlighted

the importance of PPAR γ , which activated by oxidized FAs or IL-4, is capable of driving M2 polarization (168-170). Recent papers suggest that FA synthesis directly contributes to the inflammatory response in M1 macrophages, however it is not clear yet, whether the induction of FA synthesis is necessary for M1 polarization (167, 171-173). Additionally, saturated FFAs, such as PA has been suggested to trigger the metabolic activation of macrophages in adipose tissue (174). In another study Shi et al. showed that *in vivo* FFA released by adipocytes potentially influenced macrophage polarization (175).

Based on these observations it is reasonable to assume, that sebum component lipids may also take part in the polarization of macrophages.

4.8.2 DCs

Besides macrophages, the skin also contains distinct populations of DCs, characterised by unique morphology and the surface expression of CD11c (176). Cutaneous DCs serve as sentinels, which along with producing inflammatory cytokines also process and present antigens to antigen-specific T lymphocytes (177). Depending on their location cutaneous DCs can be divided into subsets: epidermal LCs and bone marrow-derived dermal cDCs, which can be further classified into type 1 (cDC1) and type 2 (cDC2) subsets (178). These three subgroups are well defined by developmental origins and surface marker expression patterns (Figure 6.), however under inflammatory conditions additional subtypes of DCs appear, such as inflammatory myeloid DCs and plasmacytoid DCs (177, 178).

Similarly to macrophages, DCs also respond to changes in FA metabolism. Interestingly, the oxidation of FAs creates a state in which DCs become tolerogenic (167). A recent paper also highlighted that DCs from tumours accumulate oxidized lipids, which inhibit T cell function and facilitate tumour progression (179).

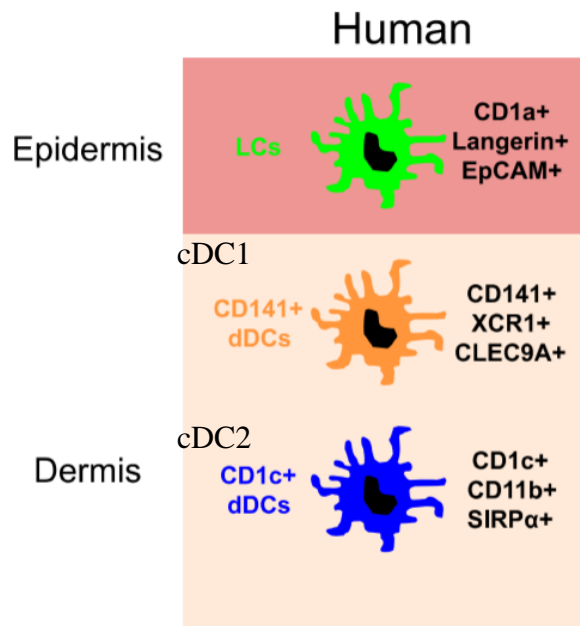


Figure 6. Major DC subsets in normal human skin [Kim et al. *Int. J. Mol. Sci.* 2018, 19, 42; doi:10.3390/ijms19010042 (178)]

Furthermore, Weatherill et al. demonstrated that SFAs activated the TLR4 pathway leading to the up-regulation of MHC class II molecules, whereas PUFAs such as docosahexaenoic acid (DHA) counteracted the ability of SFAs to induce DC maturation (180).

4.8.3 Helper T cell subsets

Cell-mediated immune responses are mainly controlled by T cells. T cells mature in the thymus, and the ones that express CD4 glycoprotein on their surface are classified as T helper (Th) cells (181). Th cells obtain highly specialized effector functions during activation and can be categorized into several subsets. These distinct CD4⁺ subsets differentiate from naïve CD4⁺ T cells as a result of specific cytokine exposure (Figure 7.) (181). The very first classification system was set up by Mosmann et al., who defined the Th1 and Th2 subsets based on their distinct cytokine production of IFN- γ and IL-4, respectively (182). Later on, a new subset of regulatory T cells (Treg) has been also identified, that typically express transforming growth

factor- β (TGF- β) and IL-10 (183-185). Recently the Th family has notably expanded to include Th9, Th17 and Th22 cells, which also have their specific cytokine expression profile (Figure 7.) (186-189).

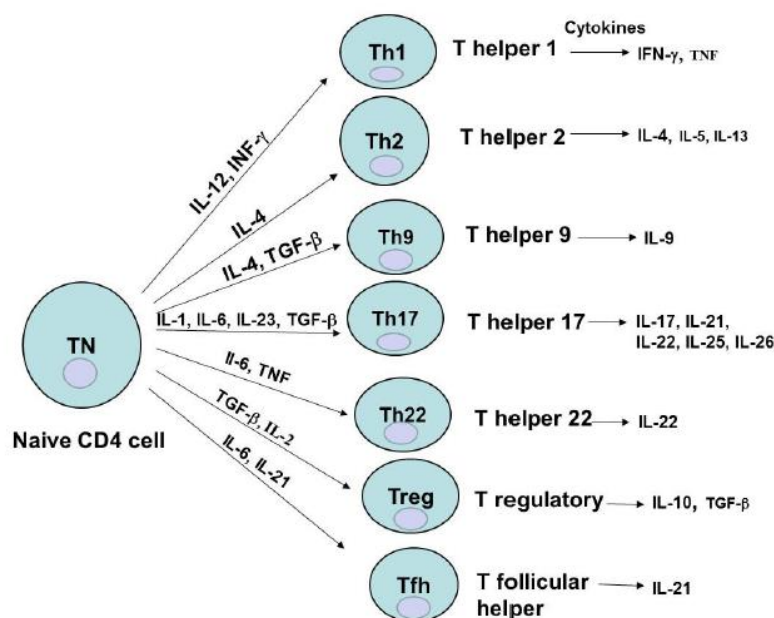


Figure 7. Summary of the different CD4⁺ T cell subsets differentiating from CD4⁺ naïve T cells

Golubovskaya et al. Cancers 2016, 8, 36; doi:10.3390/cancers8030036 (190)

As with the case of macrophages besides specific cytokines that define the different Th differentiation pathways, lipids can also contribute to their polarization. In previous studies, dietary n-3 PUFAs reduced systemic and local Th1 (191, 192), and Th17 abundance and *ex vivo* differentiation of naïve CD4⁺ T cells into a Th17 phenotype in mice (193-195). Furthermore, trans fatty acids (TFAs) (mainly C16:1, C18:1, C18:2, C20:1, C20:2 and C22:1) promoted Th17 polarization and upregulated the expression of proinflammatory cytokines in the inflamed colonic mucosa of mice (196). On the contrary dietary 9-palmitic-acid-hydroxy-stearic acid (9-PAHSA) reduced colonic T cell activation and proinflammatory cytokine and chemokine expression in mice. 9-PAHSA belongs to a newly identified group of branched FA esters that are products of endogenously synthesized in mammals (197).

In addition to the contribution of FAs, the presence of *P. acnes* may amplify immune responses by stimulating the development of subclasses of T cells (90, 198). It has recently been shown that *P. acnes* induced a Th17 response in human peripheral blood mononuclear cells (PBMCs), the expression of key Th17 related genes and IL-17 secretion from CD4⁺ T-cells (199).

5. OBJECTIVES

In recent years sebaceous gland research has come a long way; with the establishment of sebocyte cell culture models and the development of new molecular techniques more and more information became available regarding sebocyte proliferation, differentiation and function. However, their possible role as mediators or even initiators in inflammatory skin diseases has not been defined well yet. Furthermore, their main profile namely sebum production and the potential role of sebum component lipids in influencing various inflammatory pathways and immunological processes has been barely investigated. Therefore, we aimed to:

1. determine whether sebum component lipids could contribute to the dermal lipid content.
2. evaluate whether sebocytes are able to recruit immune cells to sites of skin inflammation.
3. investigate if sebocytes could contribute to the differentiation, polarization and function of macrophages via their secreted lipids and proteins.
4. address a possible interaction between human sebocytes and *P. acnes* in driving inflammatory responses, which may be active in skin homeostasis (symbiosis) and/or in immune reactions such as acne vulgaris.
5. analyse the functional communication between human sebocytes and T cells.

6. MATERIALS AND METHODS

6.1 Oil-Red-O staining

Anonymised frozen skin biopsies were sectioned and fixed in 4% Paraformaldehyde (Sigma-Aldrich, Dorset, UK) for 10 min. Slides were placed in 100% Propylene Glycol (Amresco, Solon, OH, USA) for 1 min, followed by a rinse with distilled water. The sections were stained with 0.7% Oil-Red-O (Sigma-Aldrich) solution for 7 minutes than rinsed with 85% Propylene Glycol solution. A counter staining for nuclei was done with methylene green. Finally, the slides were covered using Mount Quick Aqueous mounting medium (Bio Optica Milano, Italy).

6.2 Raman spectroscopy

Raman spectroscopy measurements were executed and evaluated by our collaborators Dr. Attila Gácsi and Dr. Erzsébet Csányi in the Institute of Pharmaceutical Technology and Regulatory Affairs, Szeged. Skin biopsies were obtained from abdominal reconstruction surgery. Approximately 1 cm² skin samples were treated with either SQ, LA, OA, PA or STA on 4 mL volume Franz diffusion cells using phosphate buffer saline solution (PBS) (Biochrom, Berlin, Germany) to avoid skin drying. In all cases the treatment duration was 24 h and the treated area was 66.5 mm². Tissue samples of the treated areas were frozen and sectioned onto aluminium coated slides. Raman spectra of each section were obtained with a DXR Raman microscope (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a 532nm diode laser. Instrument operation and the evaluation of measurements were done by the OMNIC Dispersive Raman 8.2 software (Thermo Fisher Scientific). In all mapping measurements 24 spectra were collected, the spectral resolution was approximately 2 cm⁻¹, and the spectral window ranged from 200 to 3200 cm⁻¹. The individual spectra of each FA were used as a reference when comparing the treated vs untreated samples.

6.3 Immunohistochemistry

6.3.1 *CD163*

Anonymised formalin-fixed and paraffin embedded sections of human skin from the tissue archive of the Department of Dermatology, University of Debrecen, were used after approval by the Regional and Institutional Ethics Committee, University of Debrecen. Paraffin sections were deparaffinised, rehydrated, and incubated in 3% H₂O₂ in distilled water for 15 min. For antigen retrieval, slides were treated with tris(hydroxymethyl)aminomethane- ethylene-diamine-tetra-acetic acid (Tris-EDTA) (10mM Tris Base, 1mM EDTA solution, 0.05% Tween 20, pH 9.0) buffer in a boiling pressure cooker (120°C) for 20 min. To reduce non-specific binding, slides were incubated in 5% bovine serum albumin (BSA)-PBS for 30 min at room temperature. Tissue sections were incubated with CD163 antibody (LifeSpan BioSciences, Seattle, WA, USA) overnight at 4°C in a humidity chamber. As negative control, samples were incubated in 2.5% BSA-PBS without primary antibody. Appropriate horse radish peroxidase (HRP)-conjugated secondary antibodies were used in accordance with the manufacture's instruction (SuperSensitive One-step Polymer-HRP Detection System, BioGenex, CA, USA). Immunoreaction was visualized by Vector VIP Kit (Vector Laboratories Ltd, Cambridgeshire, UK). Sections were counterstained with methylene green. For antibody specifications see Table 1.

Images were analysed with ImageJ software by marking three equal sized fields representing the three examined area (epidermis, sebaceous gland, neutral area). The built-in cell counter feature of ImageJ was used to count the CD163⁺ cells that are marked with blue (subepidermis), green (neutral area) and red (sebaceous gland). For antibody specifications see Table 1.

6.3.2 *CD206, CD209 and FXIII-A*

Frozen sections were fixed in acetone for 10 min and incubated in 5% normal goat serum diluted in serum-free protein block (SFPB) (Dako, Glostrup, Denmark). FXIII-A was detected by rabbit

affinity purified anti-human FXIII-A antibody (Affinity Biologicals, Ancaster, Ontario, Canada) for 2 h at room temperature. This procedure was followed by visualization using DyLight 488 horse anti-sheep IgG antibody for 45 min (Vector Laboratories Ltd). For co-expression, the detection of FXIII-A was sequentially combined with different reference markers using monoclonal anti-human antibodies against CD antigens [CD206, CD209 (Abcam, Cambridge, UK), CD163 (Enzo Life Sciences, Farmingdale, NY, USA)]. Following a 10 min incubation with normal horse serum containing SFPB, the second primaries' specific binding was visualized by DyLight 594 goat anti-rabbit/mouse IgG antibody (Vector Laboratories Ltd). Slides were washed in PBS and mounted with Vectashield Mounting Medium with DAPI [40,6-diamidino-2-phenylindole (Vector Laboratories Ltd)] to counterstain nuclei. For negative controls, the appropriate non-immune control sera (rabbit IgG from Vector Laboratories Ltd, mouse IgG1 or mouse IgG2b from BD Pharmingen, Heidelberg, Germany) were used in place of primary antibodies followed by the same procedure as above. Images were acquired with an Axioplan microscope (Carl Zeiss, Oberkochen, Germany) equipped with selective filters and connected to a CCD IMAC camera (Sony, Tokyo, Japan) and ISIS fluorescent imaging system (MetaSystems, Altlussheim, Germany). For antibody specifications see Table 1.

6.3.3 *IL-17 and CD4*

Stainings were performed by Dr. Natalie Garzorz-Stark in collaboration with the ZAUM institute in Munich. Paraffin-embedded skin biopsies of acne patients were incubated at 65°C for 25min and rehydrated by incubation with xylol (2x10 min) followed by 100% isopropanol (2x5 min), 96% ethanol, 70% ethanol and deionized distilled H₂O (1x5min each). Antigen retrieval was achieved by boiling in a pressure cooking device with citrate buffer (pH=6) for 7min and blocking with 10% normal goat and 10% normal horse serum (both Life Technologies, Carlsbad, CA, USA) for 1h at room temperature. Slides were incubated with

primary antibody mix or isotype controls (polyclonal goat anti-IL-17, R&D Systems and monoclonal rabbit anti-CD4, Zytomed Systems) for 1h at room temperature and then overnight at 4°C. After a Tris buffer washing step, sections were stained with secondary antibody mix (Alexa Fluor 488 goat anti-rabbit IgG, Life Technologies and NorthernLights 557 donkey anti-goat IgG, R&D Systems) for 1h at room temperature. To quench tissue autofluorescence, sections were incubated in 0.1% Sudan Black B (Sigma-Aldrich). For nuclear staining, slides were incubated for 5min in a 1:1000 DAPI solution (Sigma-Aldrich), washed with distilled water and mounted with VectaShield (Vector Laboratories Ltd). Fluorescence images were obtained using an Olympus IX73 inverted fluorescence microscope equipped with cell Sens Software (Olympus, Tokyo, Japan) and processed with ImageJ software (<https://imagej.nih.gov/ij/>). For antibody specifications see Table 1.

6.4 Migration assay

Migration assays were performed by our collaborators in Munich. Monocytes, neutrophils and T cells were isolated from PBMCs of healthy donors using CD14 and CD3 microbeads respectively (Miltenyi Biotech, Vienna, Austria) following the manufacturer's protocol. Cells were resuspended in complete RPMI 1640 (Sigma-Aldrich) supplemented with 0.5% BSA (Amresco) and their numbers were adjusted to achieve a final concentration of 2×10^6 cells ml⁻¹. The migration assays were performed by using 5 µm pore polycarbonate membranes (ChemoTx Disposable Chemotaxis System, NeuroProbe, Gaithersburg, MD, USA). Supernatants were transferred into the bottom chambers. Different concentrations of recombinant CXCL-8 (10 ng/ml, 50 ng/ml and 100 ng/ml, respectively, PromoKine, Heidelberg, Germany) were used as positive controls. Cells were added on top of the membrane and were incubated for 2 h at 37°C in a humidified atmosphere containing 5% (v/v) CO₂. Each sample was measured in duplicates. Subsequently the cell suspension was removed and cells

that had transmigrated into the lower chamber were collected and counted with LSRFortessa (BD Biosciences New Jersey, NJ, USA) flow cytometer. Migrated T cells were additionally stained for CD4, CD8, CD56, CD45RO and CD45RA. For CXCL-8 neutralization, the supernatant was incubated with the anti-CXCL-8 antibody (R&D Systems, Minneapolis, MN, USA) for 1 h prior to the migration assay.

6.5 SZ95 sebocyte cell culture and treatment

The immortalized human sebaceous gland cell line SZ95 (129) was cultured at 37°C in a humidified atmosphere containing 5% (v/v) CO₂, in Sebomed medium (Biochrom) supplemented with 10% fetal bovine serum (FBS) (Biowest, Rue de la Caille, France), 1 mM CaCl₂ solution, 1% penicillin/streptomycin (Sigma-Aldrich) and 5 µg/ml epidermal growth factor (EGF) (Sigma-Aldrich). Cells were kept in culture until reaching approximately 80% confluence. Prior to supernatant collection the used medium was replaced with Sebomed medium containing 0.5% FBS, 1 mM CaCl₂ solution, with or without 1% penicillin/streptomycin, lacking EGF. 24 h supernatant was collected and filtered using 0.2 µm syringe filters (Sarstedt, Nümbrecht, Germany) and used for experiments. In each *in vitro* experiment Sebomed medium containing 0.5% FBS, 1 mM CaCl₂ solution, with or without 1% penicillin/streptomycin, lacking EGF was used as a control treatment of monocytes or macrophages.

For lipid depletion of SZ95 sebocyte supernatant Cleanascite lipid clarification reagent (Biotech Support Group, Monmouth Junction, NJ, USA) was used according to the manufacturer's instructions. Lipids; SQ, LA, OA, PA and STA (Sigma-Aldrich) dissolved in ethanol:DMSO (1:1); were replaced individually subsequent to lipid depletion in a concentration of 150µM. ethanol:DMSO (1:1) was used as a vehicle control.

For T cell and DC culturing experiments SZ95 sebocytes were stimulated with recombinant cytokines (50ng/ml each) or lipopolysaccharide/lipoteichoic acid (LPS/LTA) (1µg/ml) or the *P. acnes* strain 889 (50:1 ratio) for 6h, extensively washed and cultured for additional 24h in Sebomed medium. SZ95 sebocyte supernatants were collected and filtered using 0.2µm syringe filters (Sarstedt) and frozen until use in experiments.

6.6 *P. acnes* strains

Strain *P. acnes* 889 was cultured at the Department of Microbiology, University of Debrecen, Hungary. *P. acnes* bacteria were cultured on pre-reduced Columbia agar base (Oxoid, Basingstoke, UK) supplemented with 5% cattle blood, vitamin K1, and hemin, incubated at 37°C for 72 h under anaerobic conditions (Ruskinn Concept 400 Anaerobic Workstation, Pencoed, UK). The 72 h culture of the bacteria was dissolved in suspension medium broth and the suspension was adjusted to 0.5 McFarland standard turbidity. This resulted in 10⁵-10⁶ colony forming units (CFU)/ml. Cells were then centrifuged at 3500 rpm for 5 min and the supernatant was discarded. After a washing step in PBS (Lonza, Verviers, Belgium) cells were harvested in 1 ml RPMI1640 medium (Sigma-Aldrich) lacking antibiotic/antimicotic solution and used for experiments.

6.7 Monocyte isolation and differentiation

6.7.1 Macrophage differentiation and culturing

Monocytes were isolated from PBMCs of healthy donors using the CD14 microbeads (Miltenyi Biotech) following the manufacturer's protocol. 1x10⁶ monocytes were plated in a 24-well plate in RPMI 1640 (Sigma-Aldrich) supplemented with 10% of FBS (Biochrom) and with or without 1% antibiotics (Invitrogen, Carlsbad, CA, USA); and were treated with 40% or 80% of SZ95 sebocyte supernatant (collected as previously described). An appropriate concentration of Sebomed medium containing 0.5% FBS, 1 mM CaCl₂ solution, with or without 1%

penicillin/streptomycin, lacking EGF was used as control. Monocytes were differentiated in the presence of SZ95 sebocyte supernatant or Sebomed medium for 5 days at 37°C in a humidified atmosphere containing 5% (v/v) CO₂ and were used for experiments. For further differentiation and activation IL-4 (20ng/ml), IFN- γ (20ng/ml) or *P. acnes* was used (1:50). Results presented are obtained with 80% SZ95 sebocyte supernatant supplementation after confirming dose dependence.

6.7.2 DCs generation and mixed leukocyte reaction

Experiments were a joined effort with Dr. Martina Mattii in collaboration with the ZAUM institute in Munich. 1×10^6 CD14⁺ monocytes were seeded in a 24-well plate containing 500 μ l RPMI 1640 (Invitrogen) supplemented with 1.5% FCS (Biochrom), 0.5% penicillin/streptomycin solution (Invitrogen), 500 μ l of SZ95 sebocyte supernatant as well as IL-4 and GM-CSF (100U/ml of each) (PromoKine) and incubated for 5 days at 37°C, 5% CO₂. At day 5, DCs were stimulated with LPS (1 μ g/ml, Invitrogen) for 24 hours, washed twice with PBS and plated in a 96-well plate in a 1:10 ratio with CD4⁺CD45RA⁺ or CD4⁺CD45RO⁺ T cells for the mixed leukocyte reaction. Supernatant of CD4⁺CD45RO⁺ memory T cells was collected at day 3, CD4⁺CD45RA⁺ naïve T cells were kept in culture for 6 days and re-stimulated for 72 hours with plate-bound human α CD3 and soluble α CD28 (each 0.75 μ g/ml) (BD Biosciences) before supernatant collection. Samples were assayed in duplicates.

6.8 Flow cytometry

For flow cytometric analysis macrophages were collected and washed with PBS (5 min, 1500 rpm) and were resuspended in staining buffer (PBS containing 1% BSA (Amresco). Cells were stained for surface markers CD206, CD209 using phycoerythrin (PE) conjugated mAbs (BD Biosciences) for 30 min at 4°C; PE conjugated IgG1 κ (BD Biosciences) was used as isotype control. The stained cells were subsequently washed in staining buffer (5 min, 1500 rpm) and

were fixed with 4% paraformaldehyde (PFA) for 20 minutes at RT. Data was collected by flow cytometric analysis using FACSCalibur (BD Biosciences) and was analysed with Flowing Software (Cell Imaging Core, Turku, Finland). For antibody specifications see Table 1.

6.9 Phagocytosis assay

For fluorescein isothiocyanate (FITC) staining of *P. acnes*, bacteria were collected and washed in PBS and resuspended in 1 ml 0.1 M sodium bicarbonate buffer pH 9. 1 µl of 10 mg/ml FITC (Sigma-Aldrich) was used for labelling 10^8 bacteria at 4°C for 1 h. Finally, cells were washed in PBS twice and used for phagocytosis. Macrophages were incubated with FITC-labelled *P. acnes* for 2 h at 37°C, with or without lipid depletion and replacement, to allow bacteria uptake. The process of phagocytosis was stopped by adding ice-cold PBS. Cells were then collected and washed twice with cold PBS by centrifugation for 5 min at 1500 rpm. Cells were fixed with 4% PFA for 20 min at RT, data was collected by flow cytometric analysis using FACSCalibur (BD Biosciences) and was analysed with Flowing Software (Cell Imaging Core).

6.10 Western blotting

Cells were collected at 12 h following *P. acnes* treatment and were washed in PBS and lysed in radio immunoprecipitation assay (RIPA) buffer containing a phosphatase-protease inhibitor mix (Sigma-Aldrich). After centrifugation (12000 rpm, 20 min, 4°C) supernatant was collected, protein concentration was determined using BCA Protein Assay Kit (Thermo Fisher Scientific). Proteins were separated by electrophoresis using a 6% or 12% polyacrylamide gel and transferred to nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA). After blocking, membranes were probed with anti-IL-1 β (R&D Systems), anti-FXIII-A (Acris Antibodies, Herford, Germany) and anti- β -actin (Cell Signalling, Danvers, MA, USA). The Ag-Ab complexes were labelled with appropriate HRP-conjugated secondary antibodies (Bio-

Rad Laboratories) and visualized by Immobilon Western HRP Substrate kit (Millipore, Bedford, MA, USA). For antibody specifications see Table 1.

6.11 Chemokine and cytokine detection

6.11.1 Enzyme-linked immunosorbent assay (ELISA)

Supernatants from macrophages cultured in the presence of SZ95 sebocyte supernatant or appropriate controls were collected at 24 h after *P. acnes* 889 treatment and were stored at -20°C until they were analysed for IL-6, TNF- α , IL-1 β , IL-17, IL-22, IFN- γ (R&D Systems) and IL-4 (BD Biosciences) using the appropriate ELISA kits according to the manufacturer's instructions. For the analyses of CXCL-8 and IL-4, supernatants from SZ95 sebocytes were collected at 12 h, 24 h and 36 h at approximately 80% confluence and were stored at -20°C until they were used for measurements with specific ELISA Duosets (R&D Systems) according to the manufacturer's instructions.

6.11.2 Bio-Plex assay

Experiments were performed on our samples by our collaborators in the ZAUM institute in Munich. Bio-Plex analysis was performed using the Bio-Plex Pro Human Cytokine 27-Plex Assay (Bio-Rad Laboratories). Magnetic beads were coated with cytokine specific capture antibodies and added to 96-well plates. Samples were analysed in duplicates and serially diluted standards were used as reference. Plates were incubated for 30 min at room temperature (RT) and subsequently washed three times. In the next step the samples were incubated with specific detection antibodies for 30 min at RT and washed three times afterwards. Detection was achieved by incubation for 10 min with streptavidin-phycoerythrin fluorescent reporter. The wells were washed three times and re-suspended in assay buffer. Quantification of protein content was determined with the Bio-Plex 200 System (Bio-Rad Laboratories).

6.12 Purification and stimulation of naïve and memory CD4⁺ cells

Experiments were a joined effort with Dr. Martina Mattii in collaboration with the ZAUM institute in Munich. CD4⁺ cells were magnetically sorted using the CD4 T cell isolation kit II followed by a positive selection with CD45RO or CD45RA beads (Miltenyi Biotech). 2x10⁵ cells were seeded in a 96-well plate and stimulated with plate-bound human α CD3 and soluble α CD28 (each 0.75 μ g/ml) (BD Biosciences) in presence of 100 μ l SZ95 sebocyte supernatant and 100 μ l RPMI 1640 (Invitrogen) supplemented with 2.5% human serum (Lonza), and 0.5% of penicillin/streptomycin solution (Invitrogen) at 37°C with 5% CO₂. Supernatant of CD4⁺CD45RO⁺ memory T cells was collected at day 3. CD4⁺CD45RA⁺ naïve T cells were kept in culture for 6 days, and re-stimulated for 72 hours with plate-bound human α CD3 and soluble α CD28 (each 0.75 μ g/ml) (BD Biosciences) before supernatant collection. Samples were assayed in duplicates.

6.13 Neutralization experiments and protein removal

Experiments were performed on our samples by our collaborators in the ZAUM institute in Munich. SZ95 sebocyte supernatants were incubated for 1h with the following antibodies: anti-IL-1 β (1ng/ml), anti-TGF β (1ng/ml), anti-IL-6 (0.8ng/ml) or antiCXCL-8 (0.8 μ g/ml) (all R&D Systems). For total protein digestion, SZ95 sebocyte supernatants were treated with Proteinase K (0.5mg/ml) (Qiagen, Hilden, Germany) and incubated for at 56°C for 30 min and 80°C for 10 min for enzyme inactivation.

6.14 Statistical analysis

Statistical analysis for our joint project with the ZAUM institute was carried out by Dr. Alexander Konstantinow. Further analysis was verified by Eszter Anna Janka. Each experiment was performed in technical duplicates. The given n-number displayed at each result represent independent experiments performed as biological replicates. Statistical analysis was performed

using the Graph Pad Prism and Excel softwares. Statistical significance was either determined using the Wilcoxon matched-pairs signed rank test or One-way analysis of variance (ANOVA) for multiple comparisons. Additionally, Tukey post-hoc test was used in the analysis of ELISA data. Asterisks represent statistical significance defined as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Graphically, the mean \pm standard deviation is shown.

Table 1.

Antibodies and their used dilutions in the study.

Antibodies used for immunofluorescence					
Antigen	Manufacturer	Clone	Isotype	Dilution	Detection
FXIII-A	Affinity Biologicals	-	APIgG	1:100	Horse anti-sheep DyLight 488
CD206	Abcam	15-2	IgG	1:100	Goat anti-mouse DyLight 594
CD209	Abcam	5D7	-	1:100	Goat anti-mouse DyLight 594
CD163	Enzo Life Sciences	K20-T	IgG	1:100	Goat anti-rabbit DyLight 594
CD4	Zytomed Systems	SP35	IgG	1:50	Goat anti-rabbit Alexa Fluor 488
IL-17	R&D Systems	-	IgG	1:100	Donkey anti-goat NorthernLights 557
Antibodies used for IHC					
CD163	LSBio	MRQ-26	IgG1	1:50	SuperSensitive One-step Polymer-HRP
Antibodies used for flow cytometry					
Antigen	Manufacturer	Clone	Isotype	Dilution	Detection
CD206	BD Pharmingen	19.2	IgG1, κ	1:100	PE-conjugated
CD209	BD Pharmingen	DCN46	IgG2b, κ	1:100	PE-conjugated
Antibodies used for Western blotting					
Antigen	Manufacturer	Clone	Isotype	Dilution	Detection
FXIII-A	Acris Antibodies	-	-	1:200	Goat anti-rabbit HRP
IL-1 β	R&D Systems	8516	IgG1	1:500	Goat anti-mouse HRP
β -actin	Cell Signalling	-	IgG2b	1:1000	Goat anti-mouse HRP

7. RESULTS

7.1 Sebaceous gland rich skin is enriched in its dermal lipid content

As a starting point for our studies we first aimed to assess whether the lipid content of a given skin sample correlates with the presence of sebaceous glands. The used Oil-Red-O staining clearly demonstrated that in samples from sebaceous gland rich areas the lipid staining had an increased intensity in the dermis compared to samples from regions that were poor in sebaceous glands (Figure 8.).

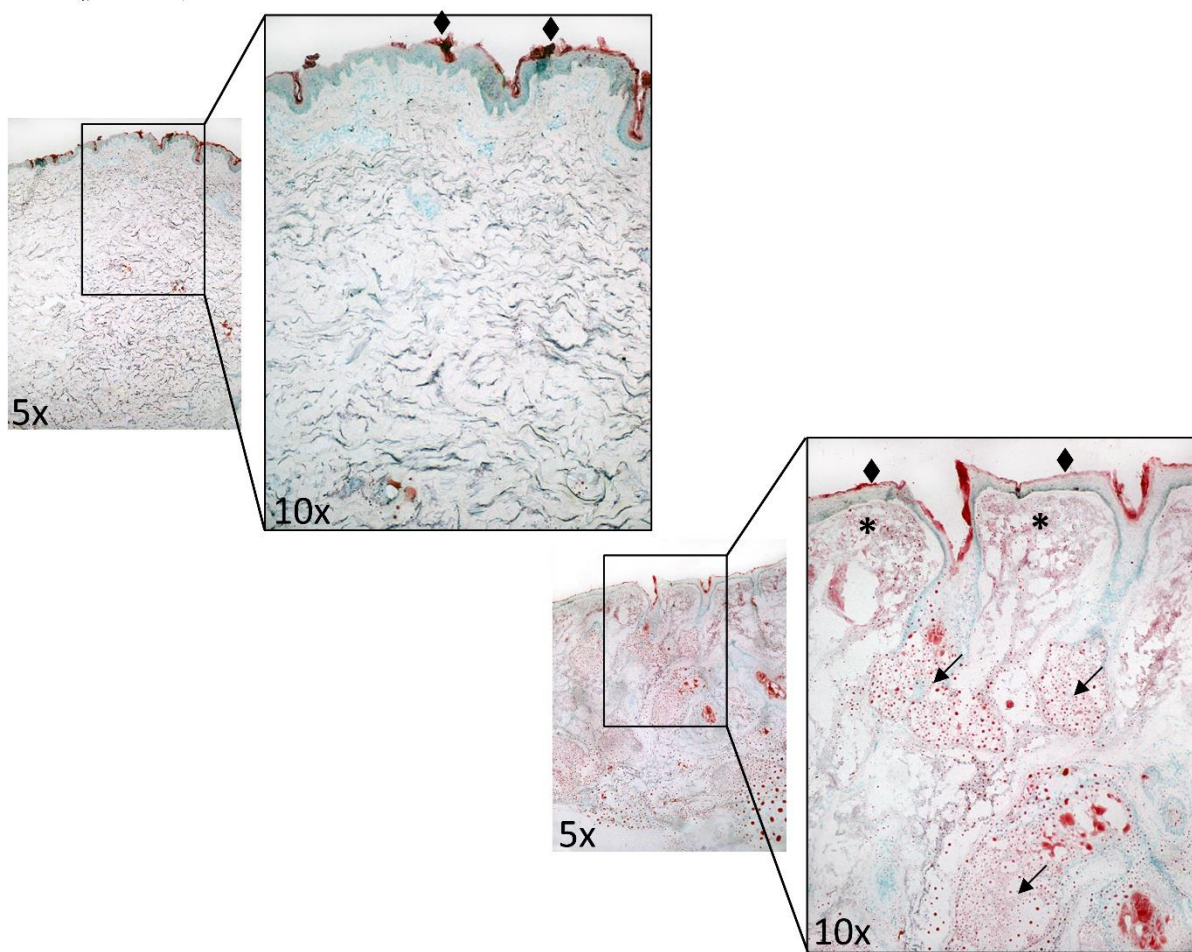


Figure 8. Dermal lipid content is in direct correlation with the presence of sebaceous glands

Oil-Red-O staining of sebaceous gland poor (stomach; left) and sebaceous gland rich (nose; right) skin. Arrows indicate sebaceous glands, asterisks indicate lipid rich areas in the subepidermal region, while diamond arrows point at the epidermal lipid barrier. Note that in the sebaceous gland (SG) rich sample an increased staining was observed not just in the vicinity of the SGs, but also beneath the epidermis. The comparable intensities in the epidermal staining of the SG rich and poor samples supports that keratinocytes are important in the epithelial lipid barrier, showing no correlation with the dermal lipid content.

Interestingly, this increase was most prominent in the subepidermal part and around the sebaceous glands. These results suggest that sebocytes might have a primary role in supplying the dermis with lipids.

7.2 Sebum component lipids are able to penetrate through the epidermis and accumulate in the dermis

Next, we aimed to verify whether sebum lipids are able to penetrate into the dermis through the epidermis. As a starting point, we examined the whole spectrum of lipids in an untreated human skin sample. Figure 9. depicts peaks that are typical of organic skin samples. Since these appear in a low intensity, treatment with specific lipids that yield unique peaks are easily differentiable from native samples. Analysing the Raman spectra of each lipids individually, OA, LA and SQ showed discrete patterns in accordance to the number of C=C bonds (1, 2 and 6 respectively), while PA and STA had nearly identical spectra (Figure 10. A, B). Therefore, simultaneous quantification of the lipids was not possible, however each pattern could be identified if the lipids were applied individually.

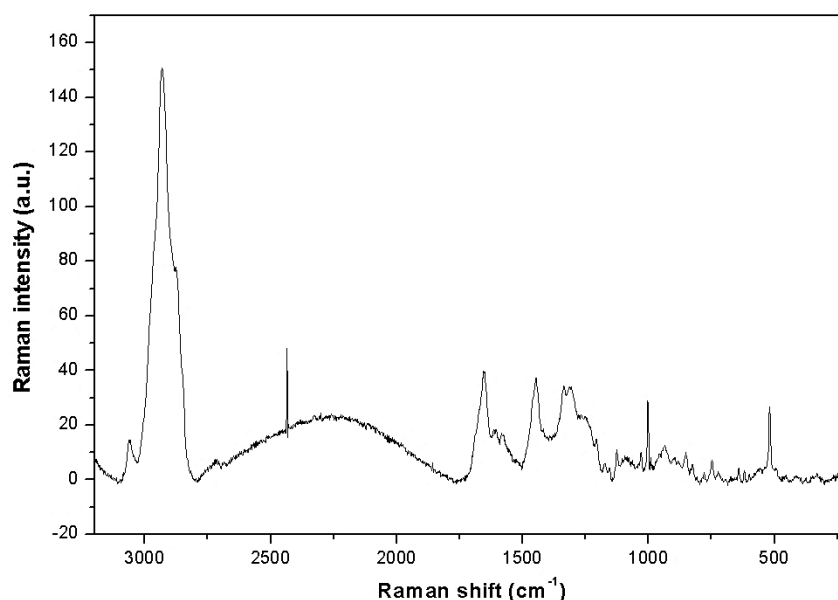


Figure 9. Determination of dermal specific Raman peaks

The normal spectrum of human skin was taken by Raman spectroscopy to provide background information for comparative purposes.

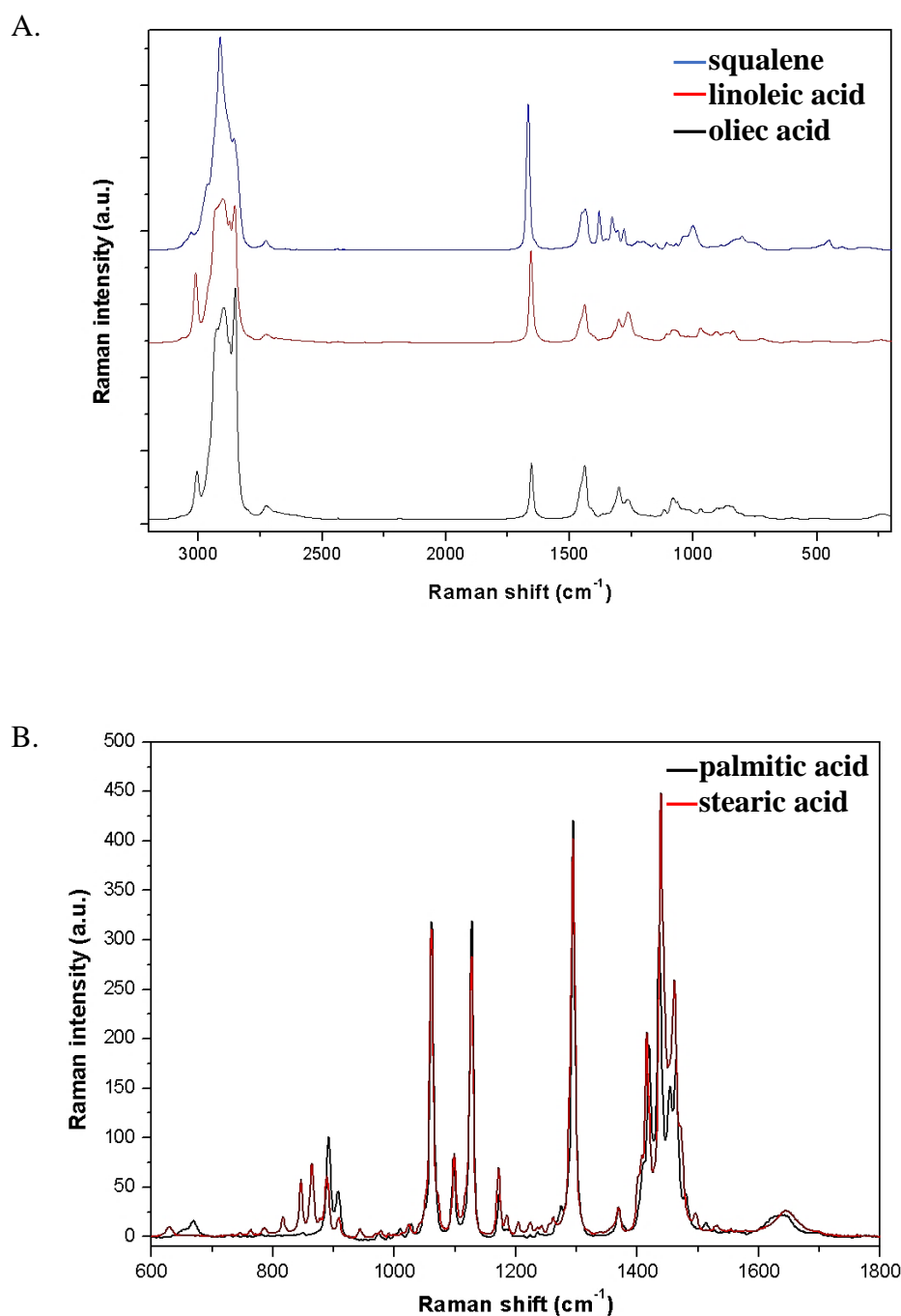


Figure 10. Determination of individual lipid specific Raman peaks

The Raman shift of SQ, LA and OA (A) as well as PA and STA (B) were measured in order to identify uniquely distinguishable peaks for further analysis.

Applying SQ, OA, PA, LA and STA individually onto skin grafts from sebaceous gland poor regions of the body, we used Raman spectroscopy to determine the extent of penetration and accumulation for each of the lipids.

Our results clearly showed that all of the tested lipids could penetrate through the epidermis without disrupting its structure (Figure 10.).

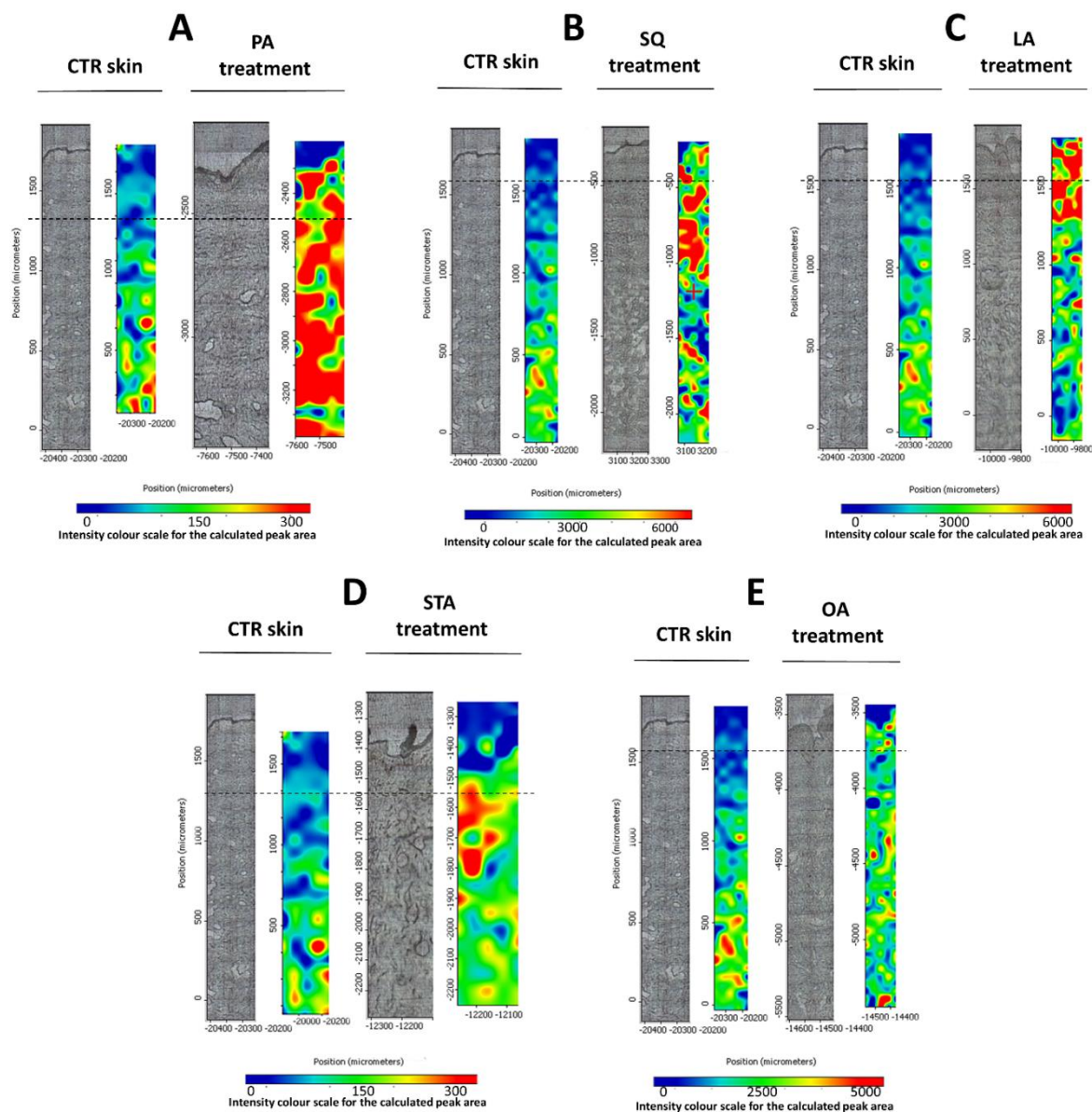


Figure 10. Raman map of control versus squalene (SQ), stearic- (STA), palmitic- (PA), linoleic- (LA) or oleic acid (OA) treated skin samples

From blue to red colouring the map indicates the increasing presence of the various FAs in a semi-quantitative manner. Dotted lines mark the estimated dermoepidermal border.

While PA (A) could be detected in high concentrations in the full depth of the dermis, SQ (B) and LA (C) acid accumulated in the upper region. Interestingly STA (D) displayed a prominent enrichment in the intermediate dermal region, while OA (E) was only present in moderate amounts however it showed a scattered distribution throughout the skin.

These findings provide evidence that sebum lipids could have a far more complex physiological role than being only topical moisturizing agents, and call for further studies to investigate their effects on various cell types of the dermis, such as macrophages.

7.3 Alternatively activated macrophages show characteristic distribution beneath the epidermis and around sebaceous glands

Macrophages have been described in higher numbers beneath the epidermis and in the vicinity of sebaceous glands, but the reason for their accumulation has not been challenged so far (200). In our studies we also confirmed these observations by comparing the number of CD163 positive dermal macrophages around the sebaceous glands, beneath the epidermis and in “neutral areas” in physiological conditions (Figure 11.).

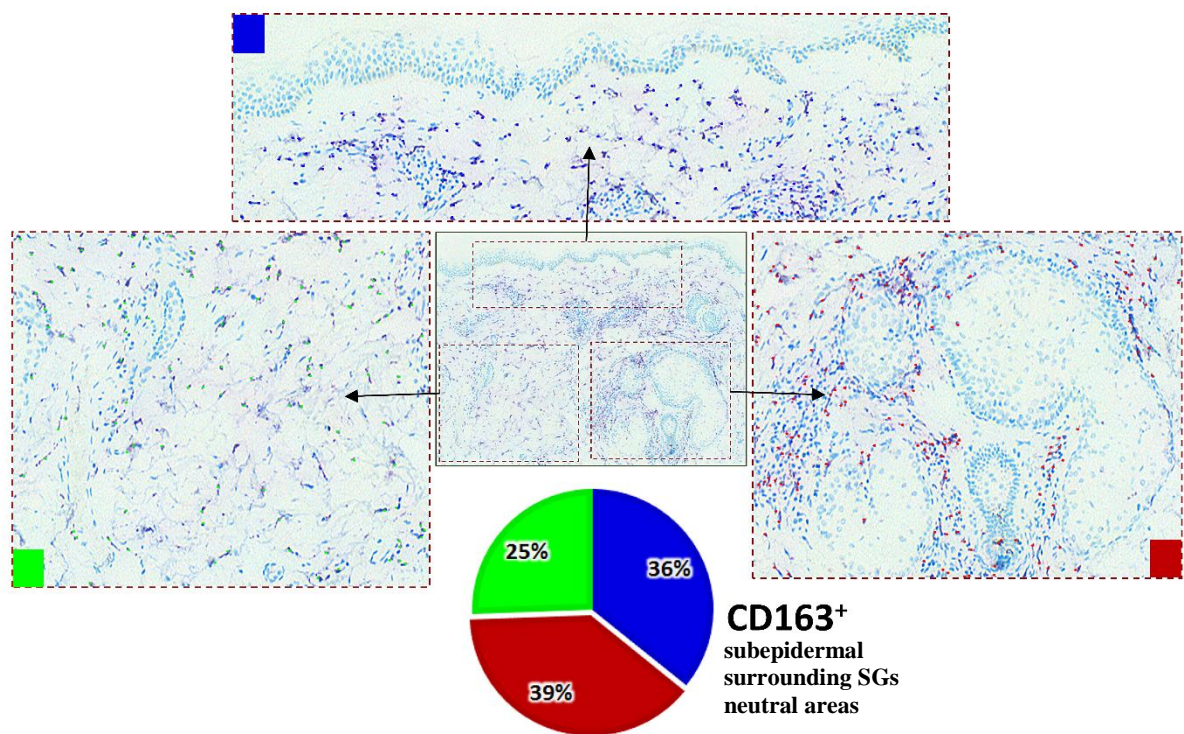


Figure 11. CD163⁺ macrophages surround the sebaceous glands in healthy skin samples. Macrophages reside within the dermis in high numbers beneath the epidermis (marked with blue) and around the sebaceous glands (marked with red). Sections were counterstained with methylene green and digitally magnified. Magnification 100x. Cell numbers were determined and compared with ImageJ software; n=5.

Importantly macrophages besides showing an increased number in the vicinity of the sebaceous glands (comparable to the numbers from beneath the epidermis) also displayed a characteristic distribution, by lining up almost exclusively parallel with the basal cell layers (Figure 12.) of the sebaceous glands, indicating that their presence might not be a random occurrence (200, 201).

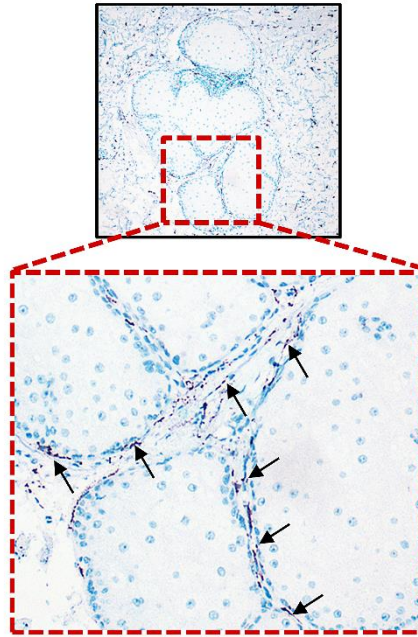


Figure 12. Sebocytes show distinct localization around sebaceous glands

Representative image of CD163⁺ macrophages (black arrows) that line up almost exclusively parallel with the basal cell layers of the sebaceous glands. Magnification 200x.

In order to further characterize the accumulating macrophages, we used double label immunofluorescence staining for the marker combinations FXIII-A and CD206 or CD209 that are reliably used to classify them into the alternative (FXIII-A⁺/CD209⁺/CD206⁺) or the classical subset (CD163⁺/FXIII-A⁻) (157). We found that all detected macrophages in healthy skin were expressing the markers of alternative activation (FXIII-A⁺/CD209⁺/CD206⁺) (Figure 13.).

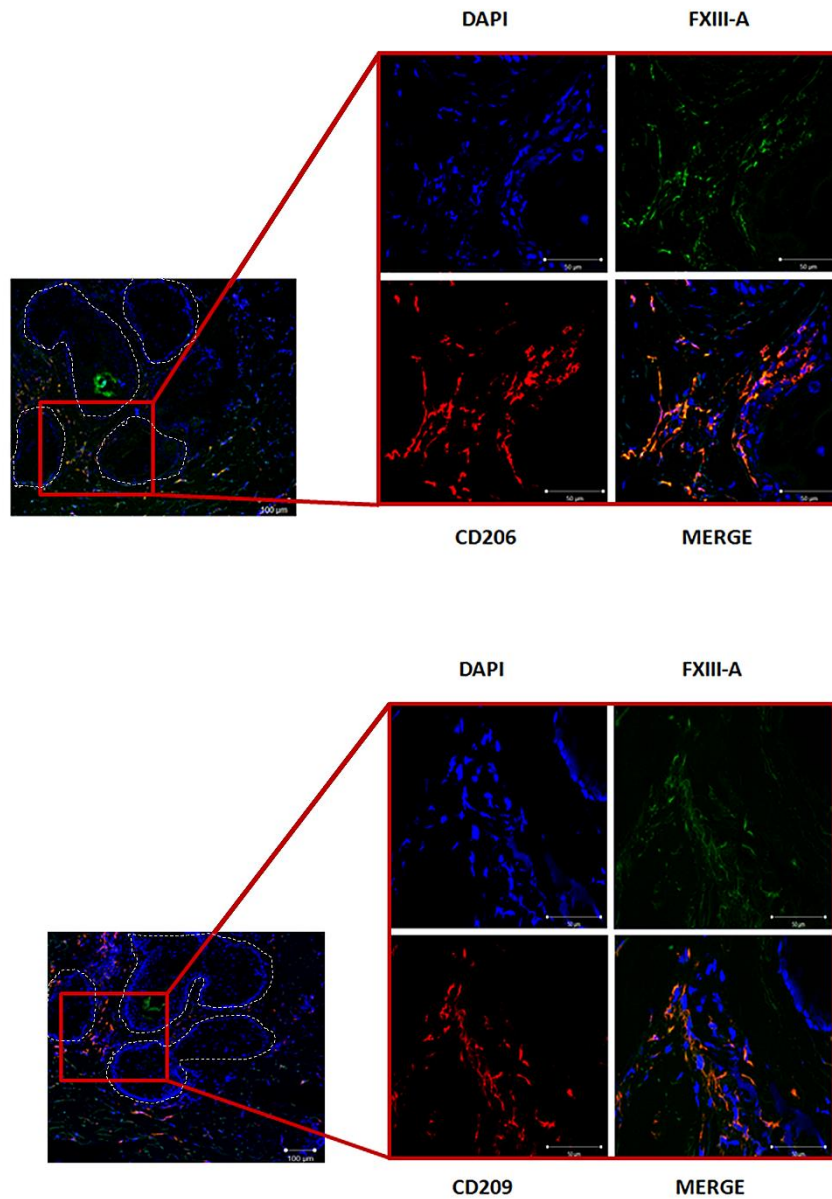


Figure 13. Alternatively activated macrophages surround the pilosebaceous unit in healthy skin

Double immunofluorescent labelling for FXIII-A [green] and CD206 (upper) or CD209 (lower) [red] in healthy skin samples detecting macrophages around the sebaceous glands to be alternatively activated [orange] (FXIII-A⁺/CD206⁺/CD209⁺). Scale bar 100 µm and 50 µm.

Notably, classically activated ones ($CD163^+/FXIII-A^-$) could be detected only under pathological conditions such as acne with a characteristic distribution localizing to the centre of inflammation (Figure 14.).

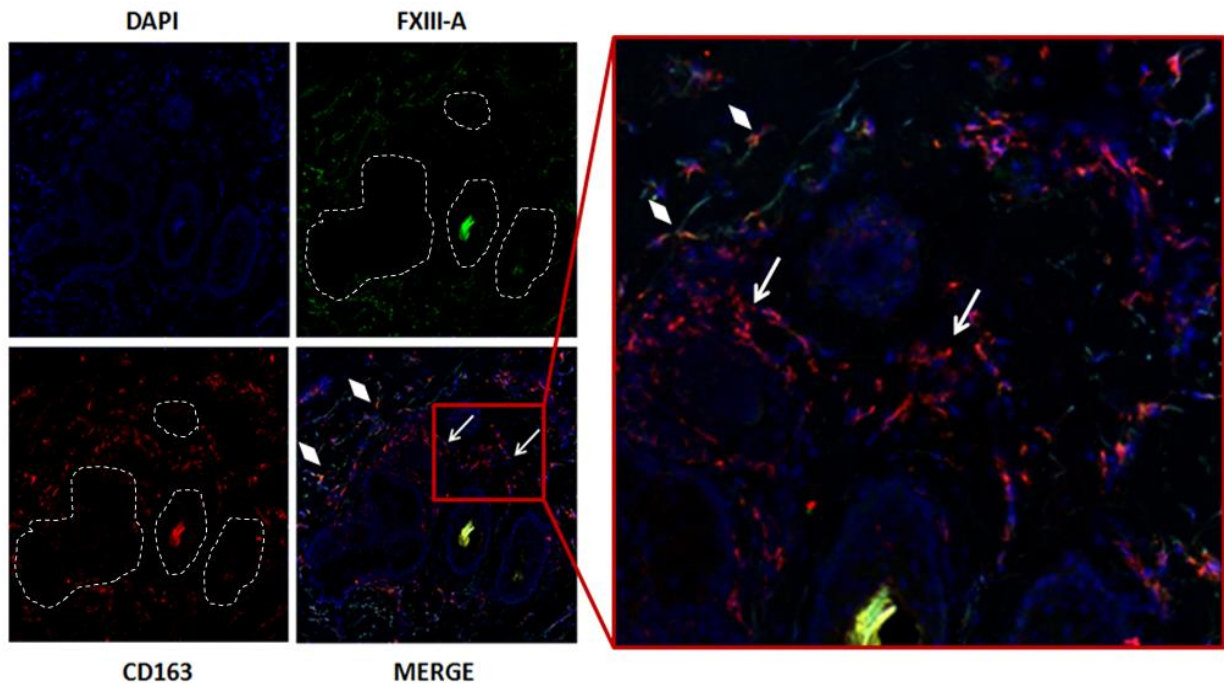


Figure 14. Classically activated macrophages ($CD163^+/FXIII^-$) could only be detected under pathological conditions

Immunohistochemical staining of acne samples revealed that classically activated macrophages accumulate in a well-defined focus of acne lesions (white arrows). Note that dual positive $CD163^+/FXIII-A^+$ alternatively activated macrophages are also detected on the periphery of the sebaceous glands (white rhombuses). Magnification 100x and 200x.

These results suggest that the local microenvironment might have a role in the accumulation and activation of macrophages around the sebaceous glands to which sebocytes could be potential contributors.

7.4 Th17 cells surround the pilosebaceous unit in acne lesions

Staining of paraffin embedded skin sections of acne lesions also revealed a high number of CD4⁺IL-17⁺ double-positive T cells accumulating in close proximity to the pilosebaceous unit (Figure 15. A). Despite the fact that healthy control skin did not show signs of inflammation, CD4⁺IL-17⁺ double-positive T cells were detected next to the sebaceous gland indicating a potential crosstalk of Th17 cells and sebocytes not only during inflammation, but also in physiological conditions (Figure 15. B).

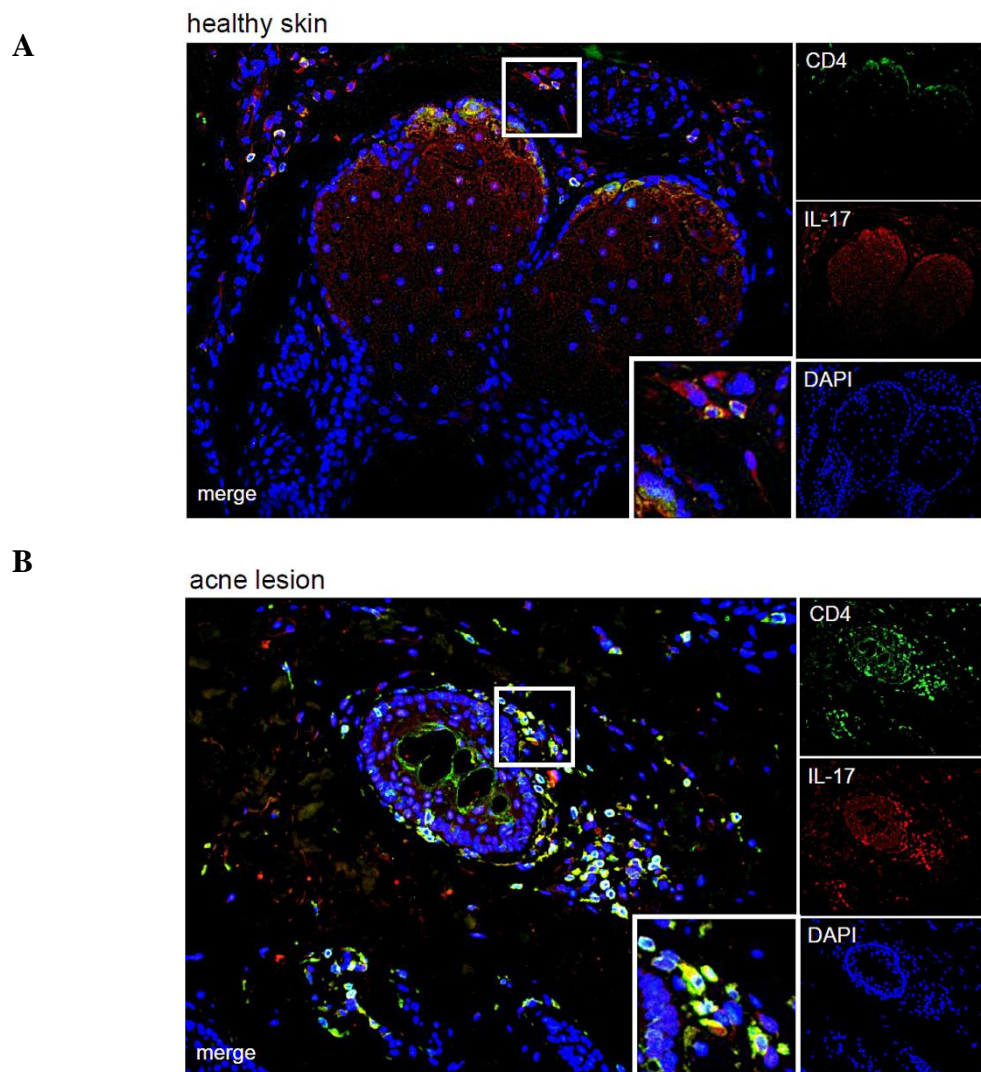


Figure 15. CD4⁺IL-17⁺ T cells surround the pilosebaceous unit

Paraffin-embedded sections of acne vulgaris patients were immunofluorescently stained for CD4 (green) and IL-17 (red). The nucleus was counterstained with DAPI (blue). Fluorescence images were obtained using an Olympus IX73 inverted fluorescence microscope equipped with cell Sens Software (Olympus) and processed with ImageJ software. Shown is one representative staining for healthy (A) and lesional skin (B).

7.5 Sebocytes attract immune cells through CXCL-8 release

The histological findings that macrophages and T cells are lined up around the sebaceous glands in the healthy pilosebaceous unit, called us to assess if sebocytes could actively attract these cells. We analysed supernatants derived from SZ95 sebocytes, the most widely used and accepted sebocyte cell line (129), for the production of cytokines, chemokines and growth factors by Bio-plex technology. Supernatants were collected from both unstimulated SZ95 sebocytes as well as from cultures treated individually with IL-4, IFN γ , TNF α , IL-17, LPS, LTA and *P. acnes*, or the combination of IFN γ and IL-17. The baseline concentrations of the analysed components in the unstimulated SZ95 cells were: CXCL-8: 311,46 pg/ml; CCL-2: 71,41 pg/ml; CCL-5: 14,37 pg/ml; CXCL-10: 32,31 pg/ml; IL-1 β : 2,9 pg/ml; IL-6: 417,29 pg/ml; IL-12^{p70}: 41,47 pg/ml; G-CSF: 39,88 pg/ml; VEGF: 218,61 pg/ml.

Our results revealed a robust secretion of chemokines such as CXCL-8, CCL-2, CCL-5 and CXCL-10, which could be further increased with the applied stimuli presented in fold changes compared to control medium in Table 2.

		CXCL-8	CCL-2	CCL-5	CXCL-10	IL-1 β	IL-6	IL-12 ^{p70}	G-CSF	VEGF
	SZ95	114 \pm 77.87	4.1 \pm 2.81	2.4 \pm 1.40	18.9 \pm 29.38	4.9 \pm 4.91	145.8 \pm 54.57	5.5 \pm 3.85	11.4 \pm 6.78	20 \pm 19.59
SZ95	+IL-4	208.6	1.3	3.7	4.8	2.8	444.1	3.6	2.7	23.3
	+IFNγ	617.5	3.4	30.6	2383.3	4.6	2201.5	5.3	12.2	28.8
	+TNFα	418.1	1.8	14.3	10.4	3.4	747.0	2.9	3.7	18.3
	+IL-17	807.0	2.6	9.3	27.6	5.0	1801.6	4.9	12.3	31.3
	+IFNγ+IL-17	985.5	3.7	8.5	51.7	5.3	1967.5	4.3	15.3	27.6
	+LPS	1048.6	3.8	8.1	53.4	5.5	2122.4	4.6	16.4	30.5
	+LTA	845.4	3.2	7.5	45.0	5.8	2181.9	5.8	15.7	37.1
	+<i>P. acnes</i>	228.7 \pm 2.48	17.8 \pm 11.71	3.7 \pm 3.20	10.6 \pm 7.16	6.7 \pm 4.99	6.6 \pm 4.76	163.0 \pm 8.96	33.1 \pm 22.90	10.8 \pm 6.65

Table 2. Bio-Plex results of chemokines and cytokines analysed in SZ95 supernatant after 24h of culture with various stimuli

The average fold change with SD of each stimulation condition compared to control medium is shown. SZ95 and SZ95 + *P. acnes* (n=3), all others (n=1). Measurements have been performed in duplicates.

As these chemokines are important migration factors, first we analysed the migratory capacity of monocytes towards the SZ95 sebocyte supernatant. By performing migration assays, we found that blood-derived monocytes showed a significant increase in their capacity to migrate towards the sebocyte supernatant in a time dependent manner (Figure 16. A). To confirm the possible role of CXCL-8 in the migration, a potent chemoattractant known to be expressed also *in vivo* in the sebaceous glands (41), CXCL-8 was neutralized in the SZ95 sebocyte supernatant prior to the migration assay resulting in a restored migratory capacity of monocytes (Figure 16. B). These results indicate that sebocytes may be active players in attracting monocytes to the pilosebaceous unit with the regulated expression of CXCL-8.

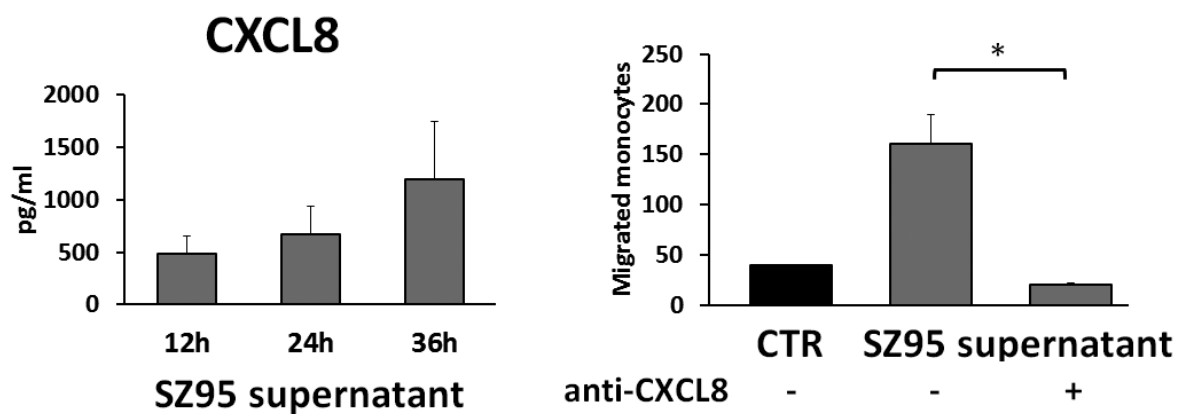


Figure 16. SZ95 sebocytes have a chemoattractant effect towards monocytes

CXCL-8 secretion of SZ95 sebocytes was detected by ELISA. Mean \pm SD of samples assayed in duplicate is depicted (n=4) (left graph). Monocyte migration towards the SZ95 sebocyte supernatant or CXCL-8 depleted supernatant was detected using migration chambers and counting the migrated cells via flow cytometry. Mean \pm SD of samples assayed in duplicate is depicted (n=3), * $p < 0.05$ (right graph).

Next, we analysed the migratory capacity of neutrophils and T cells towards the SZ95 sebocyte supernatant. We have found that neutrophils (A) and T cells (B) also migrated towards the SZ95 sebocyte supernatant in a CXCL-8 dependent manner (Figure 17.).

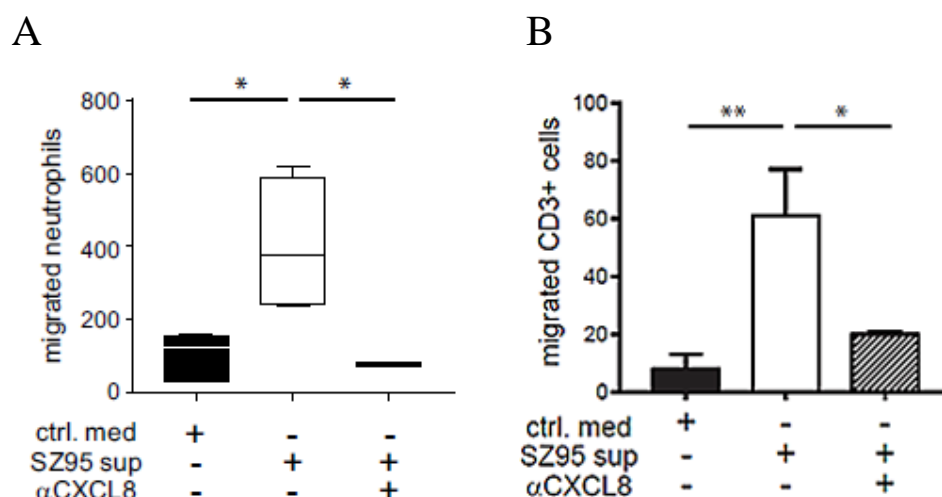


Figure 17. SZ95 sebocytes induce the migration of neutrophils and T cells via secretion of CXCL-8

Neutrophils (A) and CD3⁺ T cells (B) migrated towards the SZ95 sebocyte supernatant after 2 hours of incubation. Graphs show absolute numbers of migrated cells towards control medium (ctrl. med), SZ95 sebocyte.

By further analysis we have determined that amongst T cells CD4⁺ and CD45RO⁺ effector T cells represented the main migratory subsets (Figure 18.).

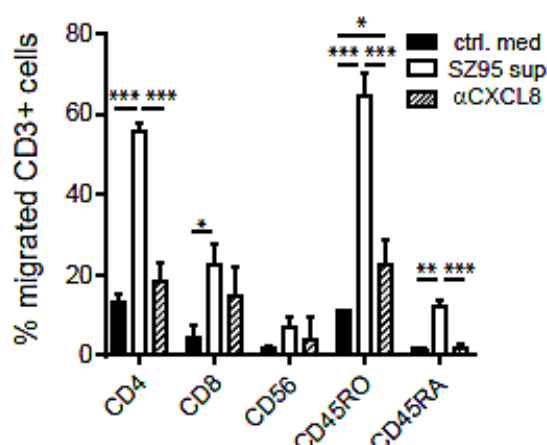


Figure 18. Flow cytometric analysis of CD3⁺ T cells

Migrated CD4⁺, CD8⁺, CD56⁺, CD45RO⁺ and CD45RA⁺ cells are expressed as the percentage of total migrated CD3⁺ T cells (n=3). Statistical significance was determined using the Kruskal-Wallis test and the Dunn's multiple comparisons test to correct for multiple testing and expressed as *p<0.05; **p<0.01; ***p<0.001.

Next, we wanted to understand if cultivation of SZ95 sebocytes in different proinflammatory environments alters the secretion of proteins as well as the subsequent migration of cells.

Therefore, SZ95 sebocytes were pre-stimulated for 6h with IL-4, IFN- γ , TNF- α , IL-17, LPS, LTA and *P. acnes*. Whereas all conditions lead to increased secretion of CXCL-8, CCL-5 and CXCL-10; IFN- γ , IL-17, LPS and LTA were the predominant activators of sebocytes (Table 2.). Also, the migration of neutrophils (A), T cells (B) as well as monocytes (C) followed the course of induced chemokines (Figure 19.).

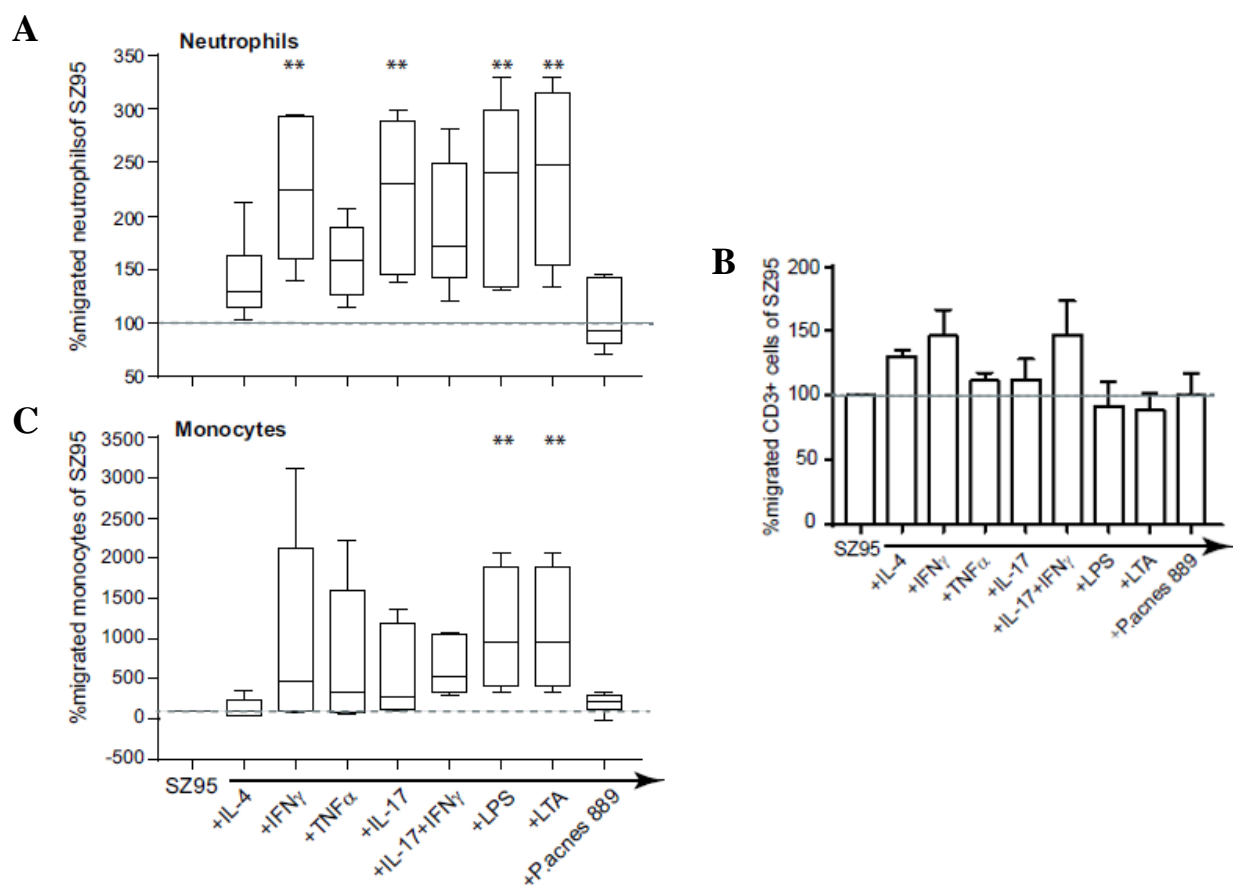


Figure 19. Migration analysis of neutrophils, T cells and monocytes to various stimuli

Flow cytometric analysis of migrated neutrophils, CD3⁺ T cells and monocytes towards pre-stimulated SZ95 sebocyte supernatant expressed as % of SZ95 migration. Statistical significance was determined using the Kruskal-Wallis test and the Dunn's multiple comparisons test to correct for multiple testing and expressed as *p<0.05; **p<0.01; ***p<0.001.

Despite the migration of total T cells towards SZ95 supernatant not being significant, CD3⁺CD45RO⁺ T cells showed a significant migration towards IL-4 and IFN- γ pre-stimulated sebocytes (Figure 20.).

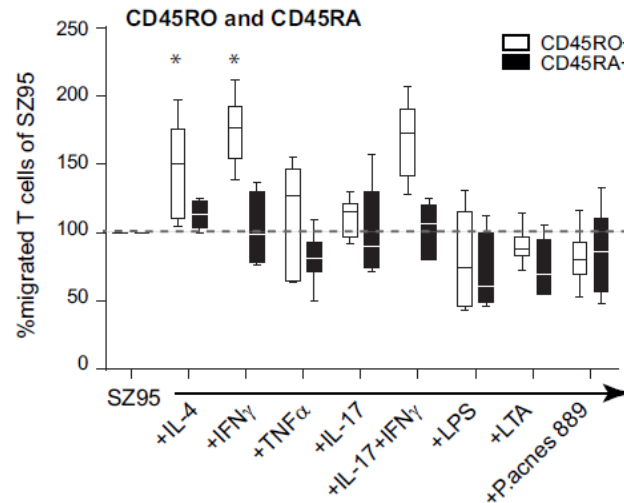


Figure 20. Effector and naïve T cells migrated towards SZ95 supernatant

Migrated CD45RO⁺ and CD45RA⁺ T cells towards pre-stimulated SZ95 supernatant expressed as % of SZ95 migration (n=3). Statistical significance was determined using One-way ANOVA and Tukey post-hoc test and expressed as *p<0.05; **p<0.01; ***p<0.001.

These data provide evidence that resting sebocytes can attract immune cells *in vitro* in a CXCL-8 dependent manner and that this chemoattractant effect is further raised in a proinflammatory environment.

7.6 SZ95 sebocytes promote alternative polarization of monocyte-derived macrophages

Our histological findings and the chemoattractant properties of sebocyte supernatant put forward a probable communication between sebocytes and macrophages. In order to test such interaction between sebocytes and macrophages, we established an *in vitro* model, where peripheral blood monocytes were differentiated into macrophages in the presence of SZ95 sebocyte supernatant.

First, we characterized the effects of SZ95 sebocyte supernatant on the polarization of macrophages and measured the expression of the widely-accepted markers via Western blotting (FXIII-A) and flow cytometry (CD206 and CD209). Since CD206 and CD209 cell surface protein level expressions revealed a dose dependence on the amount of the SZ95 sebocyte supernatant used for supplementation of the culturing medium, for all subsequent experiments an 80% SZ95 sebocyte supernatant supplementation to the culturing medium was adopted (Figure 21.).

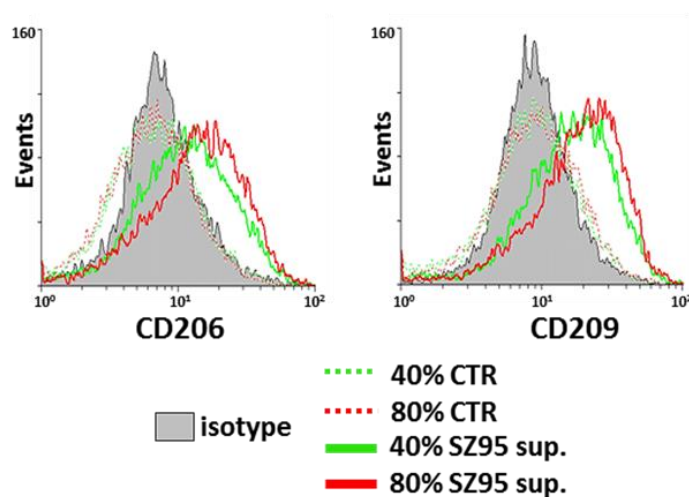


Figure 21. CD206 and CD209 markers reveal a dose dependence on SZ95 supernatant treatment

CD206 and CD209 cell surface protein level expression on *in vitro* differentiated macrophages was assessed by flow cytometry. For the differentiation RPMI medium was supplemented with 40% (green line) or 80% (red line) of SZ95 supernatant (n=5).

An enhancement of the expression of all markers studied was detected when monocytes were differentiated in the presence of the SZ95 sebocyte supernatant. Importantly, when using lipid depleted SZ95 sebocyte supernatant, we found a prominent down-regulation of FXIII-A (Figure 22. A) and an almost complete inhibition in the induced expression of CD206 and CD209 (Figure 22. B) pointing to a possible participation of sebaceous lipids in macrophage differentiation.

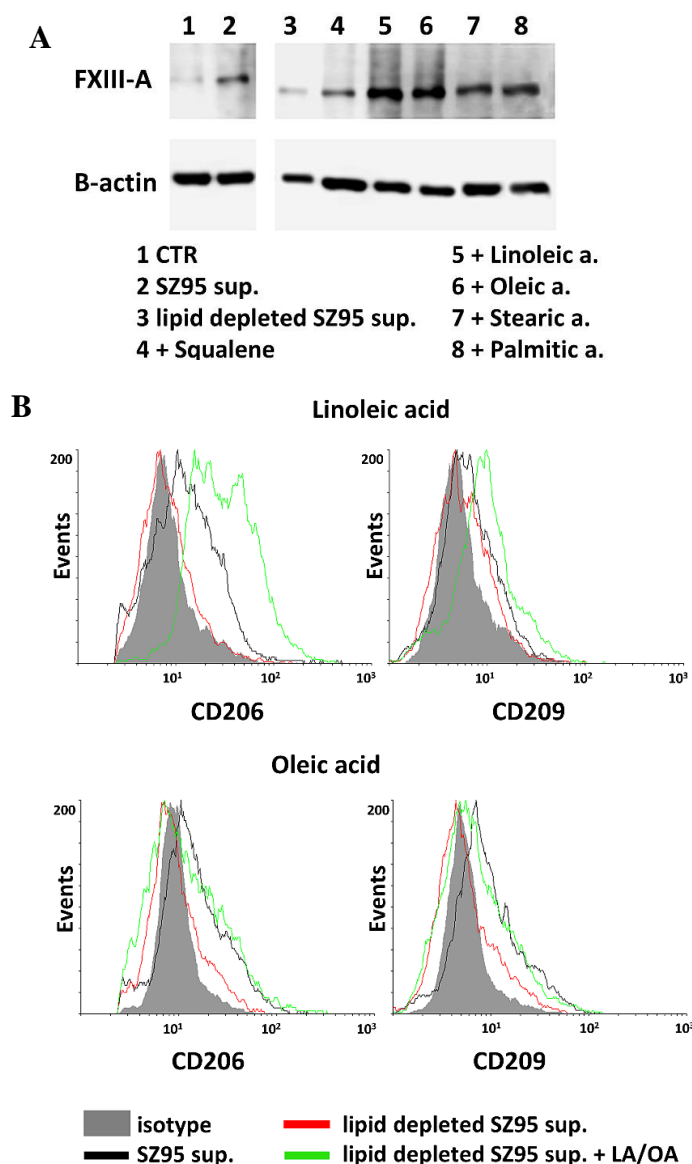


Figure 22. Linoleic- and oleic acids are responsible for the alternative phenotype in macrophages

Western blot analysis of FXIII-A protein levels (A) (n=3) and flow cytometric analysis of CD206, CD209 cell surface markers (B) (n=5) is depicted in *in vitro* differentiated macrophages in the presence SZ95 sebocyte supernatant containing lipids (SZ95 sup.), after lipid depletion (lipid depleted SZ95) or supplementation with various sebum lipids (SQ, LA, OA, STA or PA). Supplementation of lipid-depleted SZ95 sebocyte supernatant with linoleic (LA) and oleic (OA) acids partially restored the induction of CD206 and CD209.

Individually replacing the major components of the sebum such as OA, LA, PA and STA and SQ in the lipid-depleted SZ95 sebocyte supernatant used for macrophage culturing, we found LA and OA to be potent inducers of the measured markers of alternative macrophage activation (Figure 22. B), while SQ, STA and PA had no effect (Figure 23.).

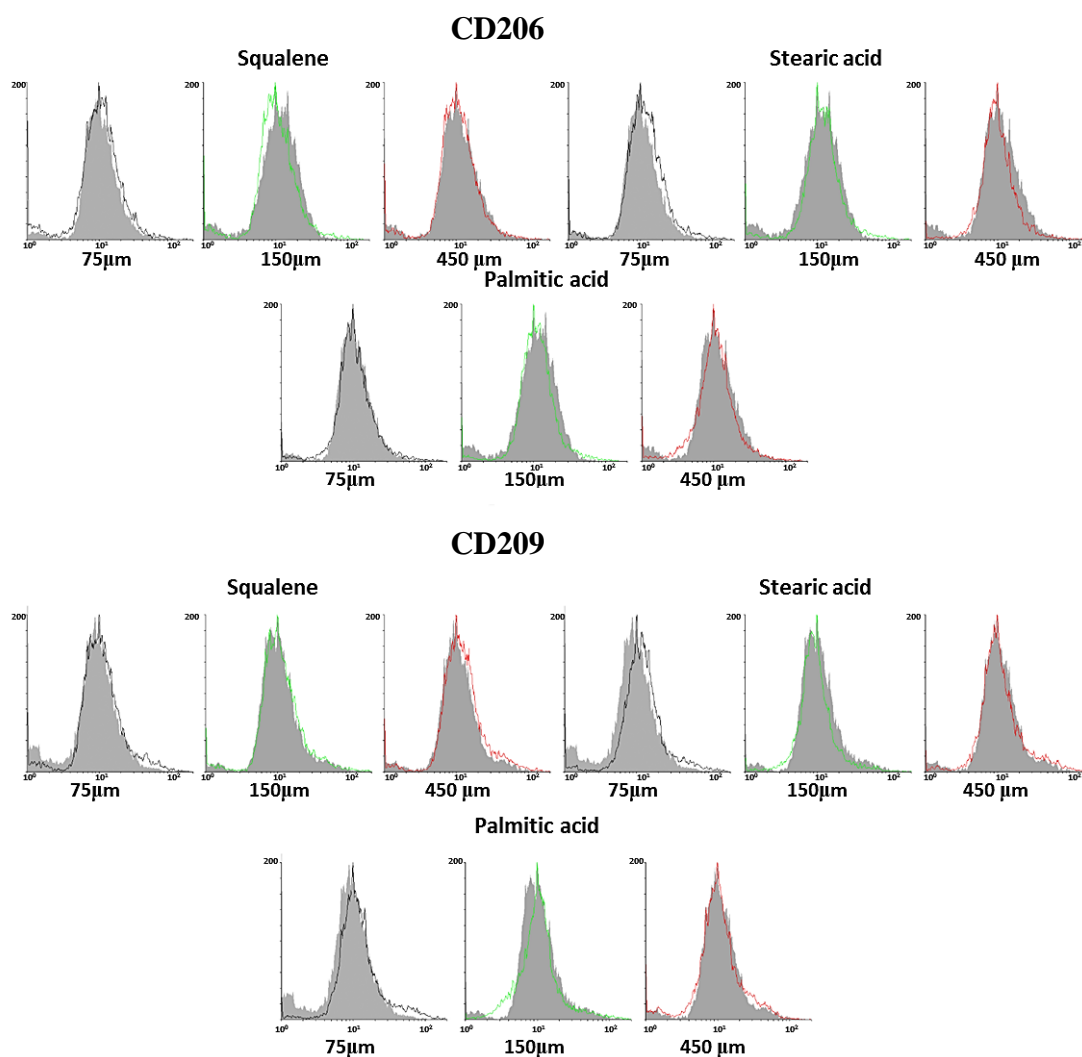


Figure 23. Squalene, stearic- and palmitic acid replacement had no effect on CD206 and CD209 expression

Flow cytometry measurements were carried out to detect CD206 and CD209 cell surface protein expression levels of *in vitro* differentiated macrophages treated with increasing concentrations of SQ, STA and PA. ethanol:DMSO (1:1) was used as a vehicle control (grey histograms). One representative experiment of three performed is shown.

Interestingly, we found that the high levels of CD206 and CD209 in response to IL-4 treatment, the primary stimulus to induce alternative activation, could be further increased when macrophages were cultured in SZ95 sebocyte supernatant (Figure 24.).

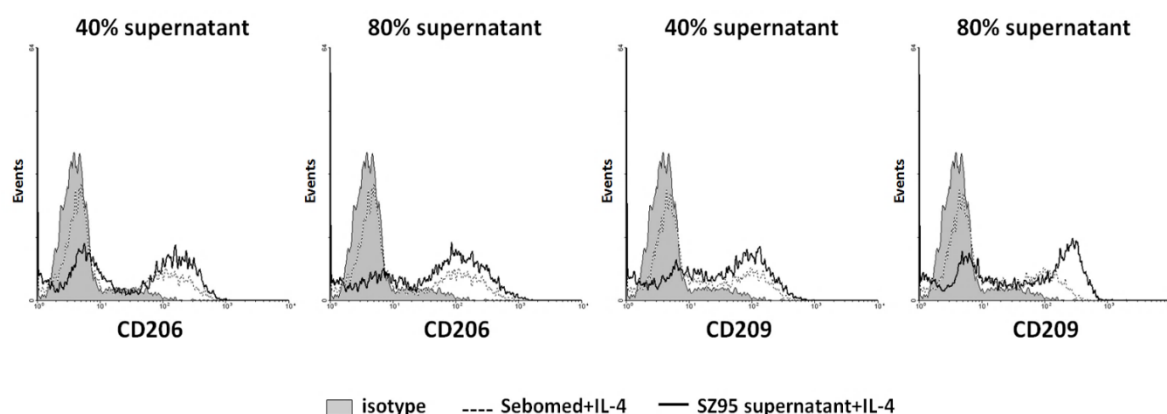
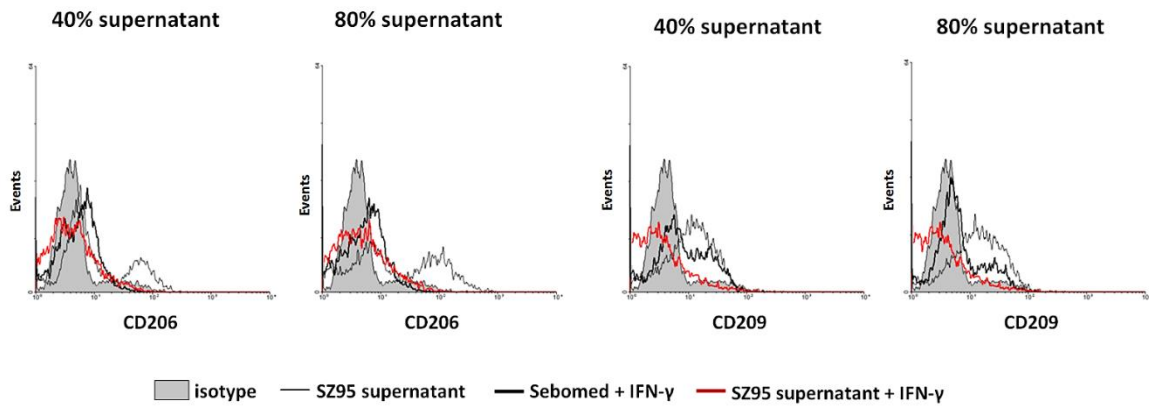


Figure 24. SZ95 sebocyte supernatant further enhances the effect of IL-4 on the markers of alternative macrophage activation

CD206 and CD209 cell surface protein expression were determined by flow cytometry on IL-4 activated macrophages that were differentiated *in vitro* in the presence of SZ95 sebocyte supernatant in different concentrations (40% and 80%) as described in Materials and Methods (solid black line) or culture medium (control, dotted line). One representative experiment of five performed is shown.

While, despite the strong polarizing effect towards the alternative activation, the SZ95 sebocyte supernatant treated macrophages conserved their potential to differentiate into classical activation suggesting that the polarizing effect can be overwritten in case of a danger signal. The co-treatment could neither relieve the down regulation of the measured markers nor the increased production of TNF- α in macrophages treated with IFN- γ , the underlying stimulus behind the classical activation pathway (Figure 25.).

A



B

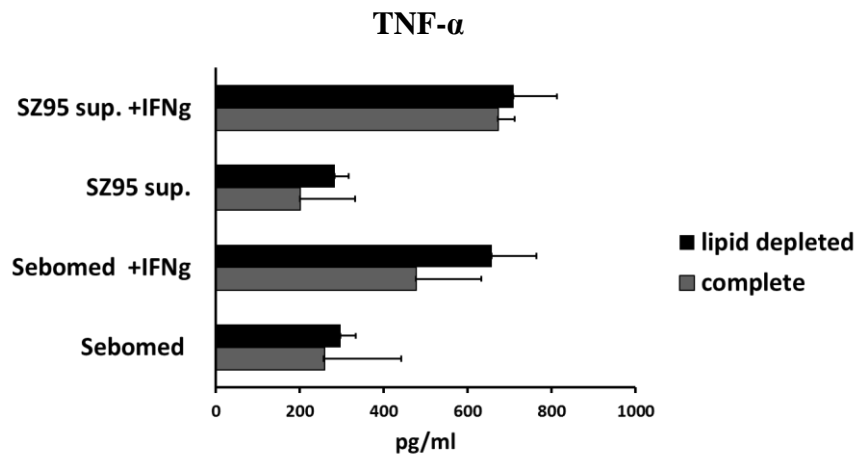


Figure 25. Macrophages conserve their potential to differentiate along the classical pathway

Flow cytometry data displays that SZ95 sebocyte supernatant could not relieve the down regulation of the measured markers due to co-treatment with IFN-γ (red line) in macrophages. One representative experiment of three performed is shown (A). This was further confirmed by TNF-α ELISA measurements, where monocytes were differentiated in the presence of either complete (grey bar) or lipid depleted (black bar) SZ95 sebocyte supernatant administered together with IFN-γ. Mean ± SD of samples assayed in duplicate is depicted (n=3) (B).

7.7 Sebocyte-derived lipids contribute to the potential of macrophages to uptake *P. acnes*

Alternatively activated macrophages have an enhanced potential for phagocytosis as one of their most important functional features. As *P. acnes* has been shown to contribute to the pathogenesis of acne, we next measured the uptake of FITC-labelled *P. acnes* by macrophages cultured with and without the SZ95 sebocyte supernatant. We found that in the presence of sebocyte-produced lipids macrophages gained an increased potential to uptake the bacteria. To address the possible lipids that may contribute to the enhanced phagocytosis, we selectively supplemented single lipids in the lipid-depleted SZ95 sebocyte supernatant and found LA and OA to be potent players in the increased uptake (Figure 26.).

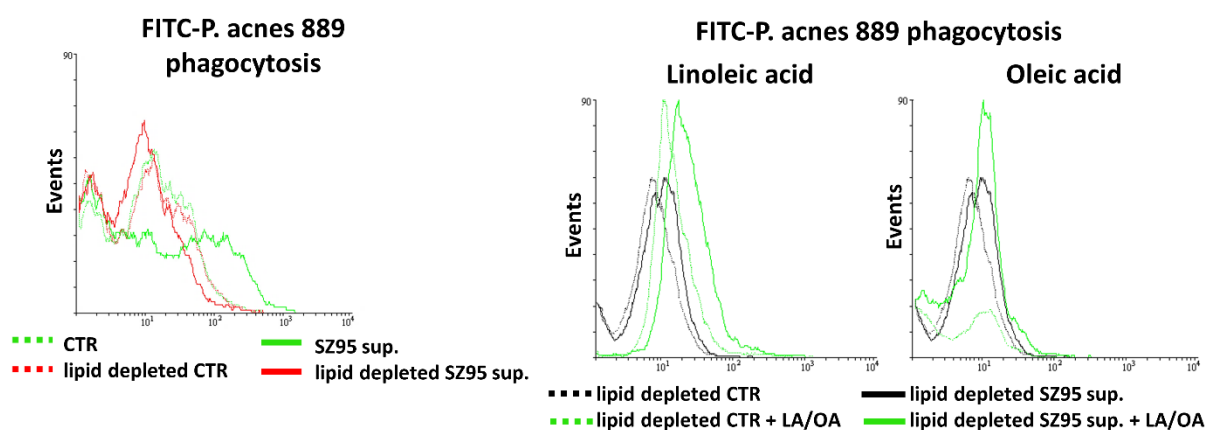


Figure 26. SZ95 supernatant enhances the phagocytic abilities of macrophages

Contribution of sebocyte-derived lipids to the uptake of FITC-labelled *P. acnes* by macrophages was assessed when macrophages were cultured in the presence of SZ95 sebocyte supernatant, lipid-depleted SZ95 sebocyte supernatant or lipid-depleted SZ95 sebocyte supernatant supplemented with linoleic- (LA) or oleic (OA) acid (n=3).

7.8 Sebum component lipids modulate the macrophage activating potential of *P. acnes*

P. acnes is crucial in the pathogenesis of acne by inducing IL-1 β production in macrophages, however, in *in vitro* settings, *P. acnes* itself only induces IL-1 β production and secretion at a very moderate level suggesting that other factors are also required for its pathological role(97, 202). Therefore, we first measured if supplementation with the SZ95 sebocyte supernatant could alter the IL-1 β levels in *in vitro* *P. acnes*-treated macrophages. Supporting an important role for sebocyte-derived products, a prominent induction in the protein levels of IL-1 β was found when *P. acnes* was added in combination with SZ95 sebocyte supernatant to the culturing medium, as revealed by Western blot from cell lysates (Figure 27.). Interestingly, IL-1 β production was further increased in the *P. acnes*-treated macrophages when lipids were depleted from the SZ95 sebocyte supernatant (Figure 27.).

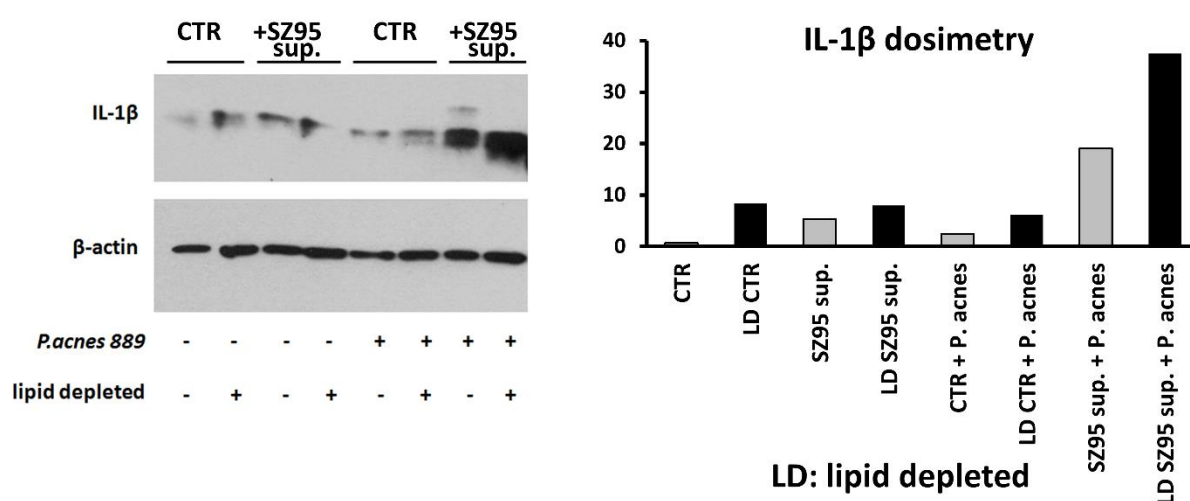


Figure 27. IL-1 β production in *in vitro* cultured *P. acnes* treated macrophages with and without SZ95 sebocyte supernatant.

Note that *P. acnes* induced IL-1 β production only in the presence of SZ95 sebocyte supernatant which could be further increased when lipids were depleted from the SZ95 sebocyte supernatant as revealed by Western blot (n=3). Dosimetry data depicts the relative differences in IL-1 β production of macrophages as a result of *P. acnes* treatment differentiated in the presence of complete or lipid depleted (LD) SZ95 sebocyte supernatant.

To address the contribution of the previously tested lipids to the IL-1 β secretion in *P. acnes* activated macrophages, of which PA is known to induce IL-1 β via TLR2, while SFAs are recognised as TLR4 agonists (82, 203), we extended our studies by selectively replacing the different lipids in the lipid-depleted SZ95 sebocyte supernatant. Our ELISA measurements revealed that the lipids had different effects on the IL-1 β secretion. Besides confirming the potential of PA, STA, OA and SQ to induce IL-1 β , we could also show that PA, OA and STA sensitized and further augmented the macrophage response to *P. acnes*. On the contrary, LA inhibited IL-1 β production even in the presence of *P. acnes* (Figure 28.).

IL-6 and TNF- α are other characteristic inflammatory cytokines produced by infected macrophages. Measurement of these cytokines in the supernatants showed that the pro-inflammatory effect of STA and PA and the anti-inflammatory effect of LA are not limited to IL-1 β secretion, but could also affect TNF- α secretion in a similar manner, while IL-6 was only slightly affected. Interestingly, OA decreased the levels of both IL-6 and TNF- α , suggesting that it may have a special role among sebum lipids by selectively and differentially interacting with the different inflammatory signalling pathways (Figure 28.).

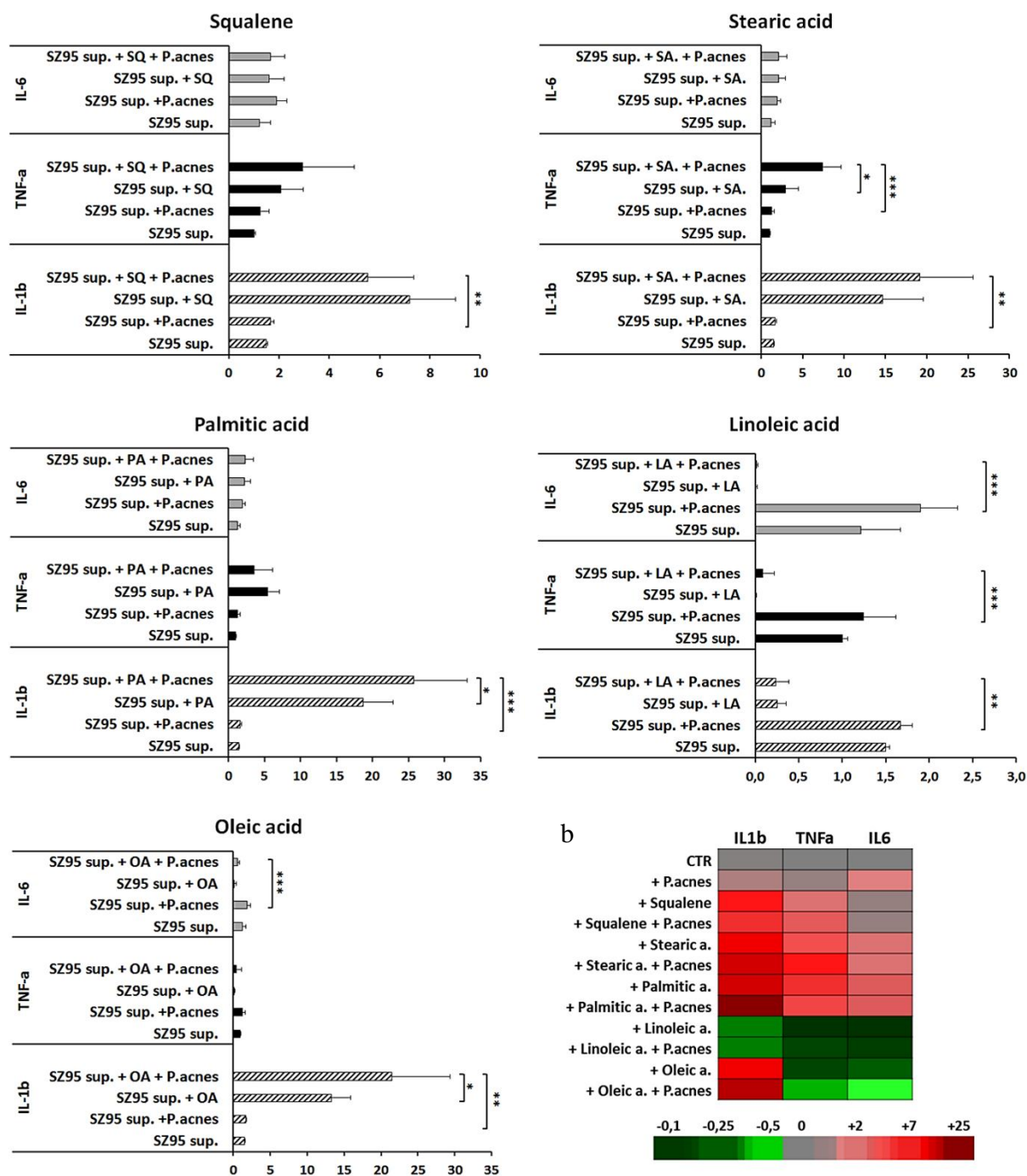


Figure 28. SZ95 lipids induce a specific cytokine profile in macrophages

IL-1 β secretion of macrophages differentiated in the presence of lipid depleted SZ95 sebocyte supernatant supplemented with *P. acnes* and/or the indicated lipids were analysed for IL-6, TNF- α and IL-1 β from their supernatants by ELISA. Mean \pm SD of samples assayed in duplicate is depicted (n=3); * p<0.05, ** p<0.01, *** p<0.001; SQ: Squalene, SA: Stearic acid, PA: Palmitic acid, LA: Linoleic acid, OA: Oleic acid (a). Heat map summarizing the IL-1 β , IL-6 and TNF- α secretion of macrophages (with and without *P. acnes* stimuli) to display the selective inflammatory effects of the various sebum component lipids on differentiated and activated macrophages (b).

These data show that the role of sebocytes in regulating macrophage function is not limited to altering their polarization, but can also be integrated into disease-specific settings, such as acne, where sebocytes may regulate inflammation via the secretion of its biologically active lipids (Figure 29.).

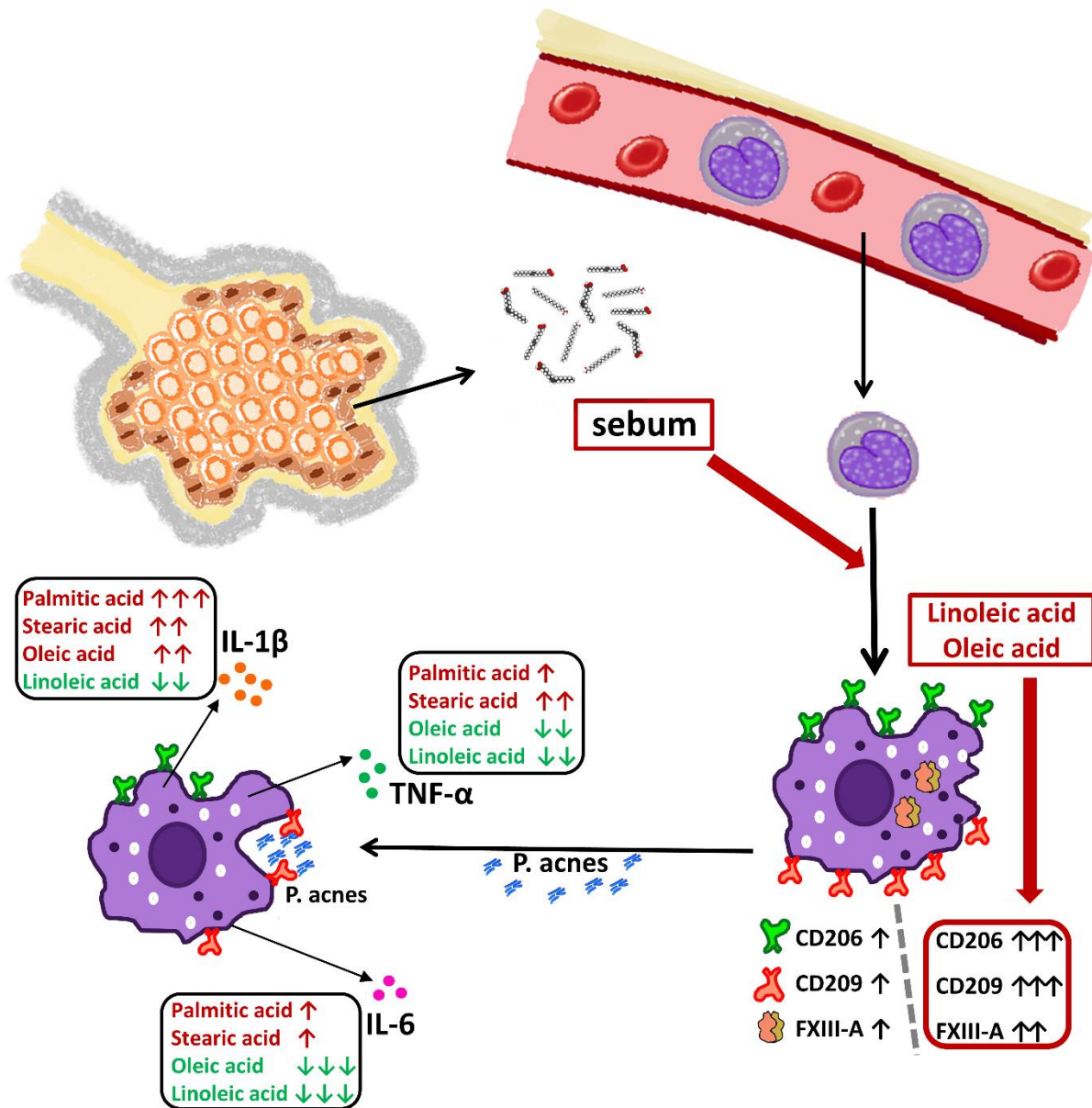


Figure 29. Overview figure on the possible role of sebocytes in modulating macrophage activation, differentiation and function

Sebocytes are able to contribute to the polarization of monocytes towards the alternative activation with their produced lipids, as marked by increased levels of CD206, CD209 and FXIII-A expression, of which LA and OA are essential. Furthermore, LA and OA contribute to an increased potential of macrophages to uptake *P. acnes*, while PA, OA and STA augment the macrophage response to the bacteria.

7.9 Sebocytes do not influence CD4⁺CD45RO⁺ effector T cell cytokine secretion

With CD4⁺CD45RO⁺ being the largest T cell subset that is attracted by sebocytes, we questioned whether their function is actively influenced by sebocytes. Therefore, human, blood-derived CD4⁺CD45RO⁺ cells were stimulated with plate-bound α CD3 and soluble α CD28 (T cell receptor stimulation) in the presence of SZ95 sebocyte supernatant or control medium for 72 hours. Here, no significant alteration in the secretion of IL-17, IFN- γ , TNF- α and IL-4 production compared to control medium could be detected. However, a significant induction for IL-22 secretion was detectable (Figure 30.).

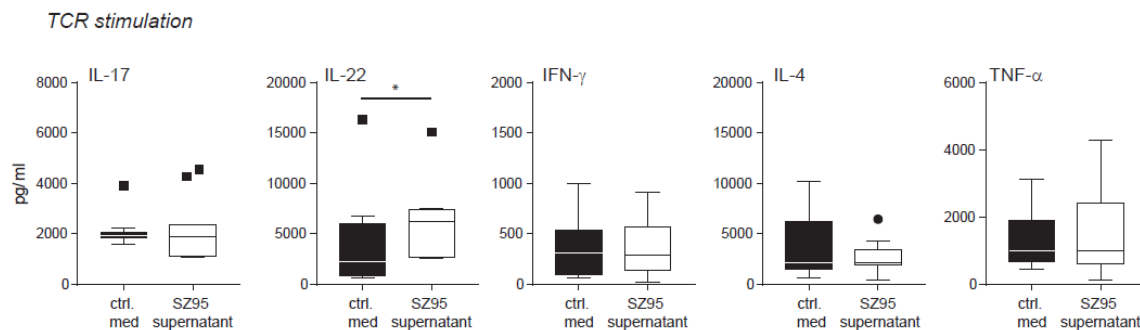


Figure 30. CD4⁺CD45RO⁺ effector T cell function is not altered by SZ95 supernatant

CD4⁺CD45RO⁺ effector T cells were cultured for 72 hours in presence of SZ95 supernatant or control medium and stimulated with α CD3/ α CD28. Levels of IFN- γ , TNF- α , IL-17, IL-22 and IL-4 were measured by ELISA (n=5).

As in this setting, T cell receptor (TCR) stimulation alone might not be sufficient for inducing alterations in cytokine secretion, we next investigated if sebocytes trigger functional changes in T cell activation *via* DCs. Therefore, CD14⁺ monocytes were differentiated into DCs in presence of SZ95 sebocyte supernatant or control medium and stimulated with LPS prior to co-culture with allogenic CD4⁺CD45RO⁺ cells (mixed leukocyte reaction).

SZ95 supernatant did not impact on DC maturation (Figure 31.) and did also not impact on T cell activation, as no significant release of all cytokines analysed was detected (Figure 32.).

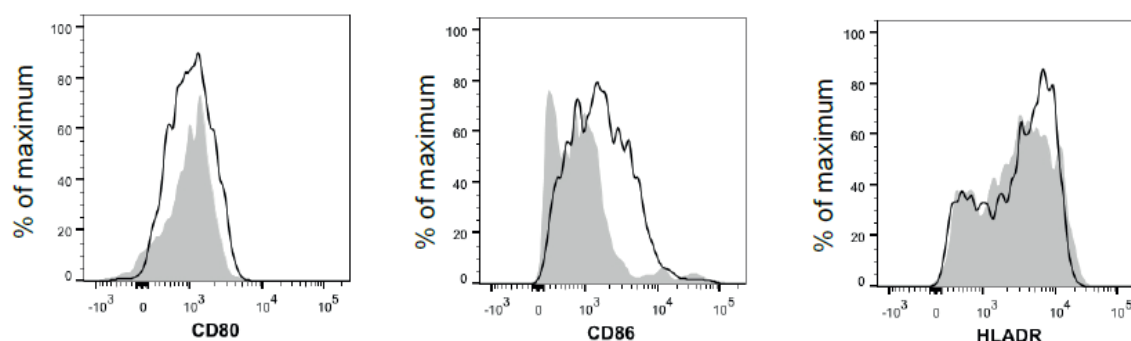


Figure 31. SZ95 supernatant does not affect DC maturation

DCs generated in presence of SZ95 supernatant (black line) or control medium (grey filled line) show an equal increase of CD80, CD86 and HLA-DR expression after exposure to LPS. Show is one representative experiment out of five.

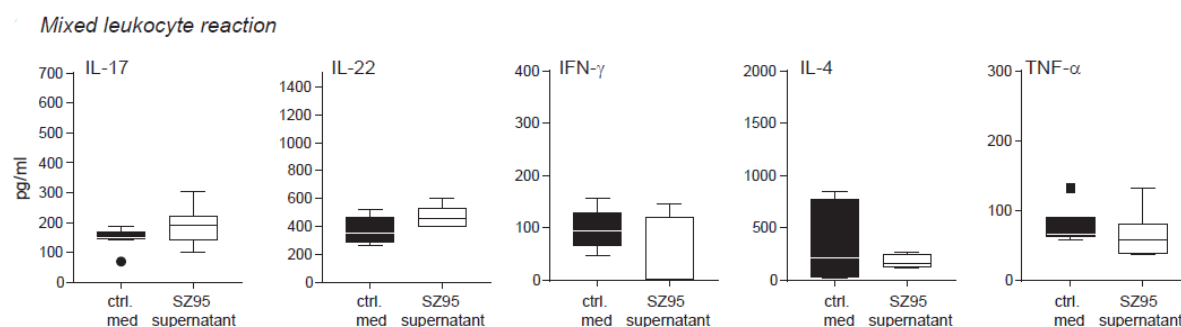


Figure 32. Mixed leukocyte reaction of effector T cells

Mixed leukocyte reaction of DCs that have been differentiated from monocytes in presence of SZ95 supernatant or control medium as well as GM-CSF and IL-4. At day 5, DCs were stimulated with LPS for 24 hours and subsequently co-cultured with allogeneic CD4⁺CD45RO⁺ T cells for 72 hours. Culture supernatants were collected and levels of IFN- γ , TNF- α , IL-17, IL-22 and IL-4 measured by ELISA (n=3). Statistical significance was determined using the Wilcoxon matched-pairs signed rank test and expressed as *p<0.05.

Our data suggest that human sebocytes affect memory T cell cytokine secretion neither directly nor mediated by DCs.

7.10 Sebocytes trigger a Th17 immune response

As also naïve T cells were attracted in small numbers by sebocytes, we investigated the influence of sebocytes on T cell differentiation. For this purpose, CD4⁺CD45RA⁺ T cells were stimulated with α CD3/ α CD28 in presence of the SZ95 sebocyte supernatant or control medium and the secretion of effector cytokines was assessed by ELISA. Whereas SZ95 sebocyte supernatant did not induce a Th1 or Th2 immune response, as no significant IFN- γ , TNF- α and IL-4 release was detected in the supernatant of differentiated T cells, a significantly higher production of IL-22 and IL-17 was detected after 6 days in culture (Figure 33. a). In line with that, also DCs that were generated in presence of SZ95 sebocyte supernatant were able to drive naïve T cell polarization towards the Th17 phenotype with significant increased expression of IL-17 and IL-22 (Figure 33. b).

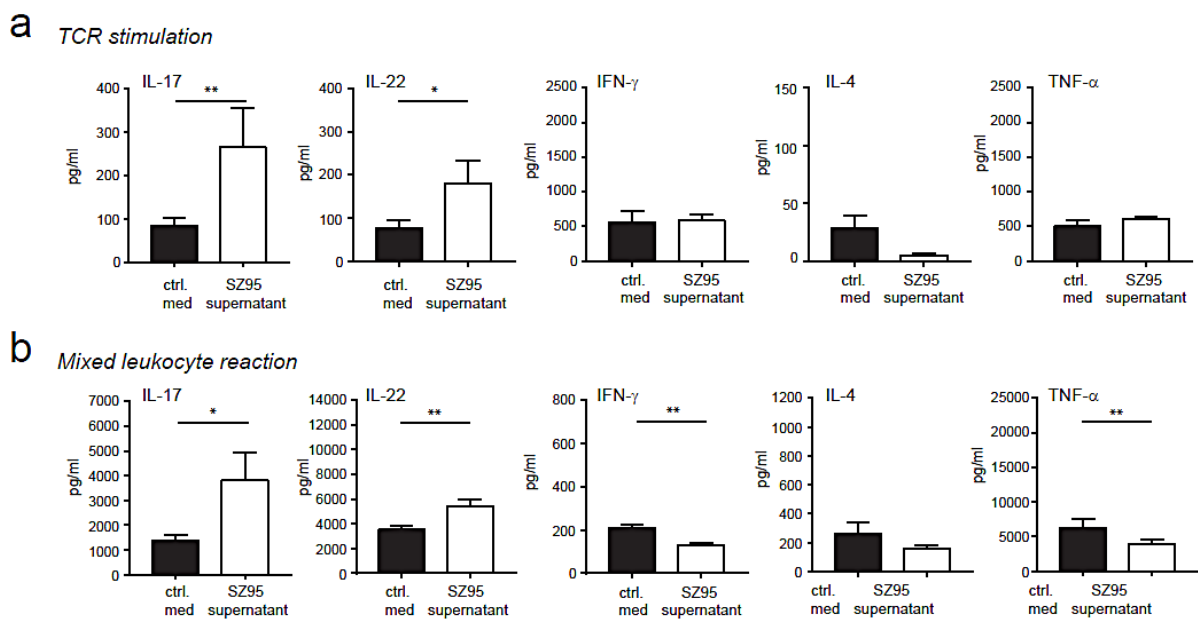


Figure 33. Sebocyte supernatant polarizes naïve T cells into Th17 cells

(a) CD4⁺CD45RA⁺ naïve T cells were cultured with SZ95 sebocyte supernatant or control medium and stimulated with α CD3/ α CD28. After 6 days, cells were re-stimulated for 72 hours and supernatants analysed for levels of IL-17, IL-22, IFN- γ , IL-4 and TNF- α by ELISA (n=5). (b) DCs generated from monocytes in presence of the SZ95 sebocyte supernatant and control medium were stimulated with LPS and co-cultured with allogeneic CD4⁺CD45RA⁺ naïve T cells. After 6 days, T cells were re-stimulated with α CD3/ α CD28 for 72 hours and supernatants analysed by ELISA (n=3). Statistical significance was determined using the Wilcoxon matched-pairs signed rank test and expressed as *p<0.05; **p<0.01.

Interestingly, IL-17 and IL-22 cytokine levels were even higher compared to the solely stimulation with α CD3/ α CD28 whereas levels of IFN- γ and TNF- α were significantly reduced in this set-up.

Thus, our *in vitro* data imply that human sebocytes have the capacity to skew immune responses towards a Th17 profile.

7.11 Sebocytes induce Th17 differentiation via secretion of IL-1 β

To address the contribution of lipids or proteins to the observed effect on Th17 differentiation, we incubated the SZ95 sebocyte supernatant with proteinase K. CD4⁺CD45RA⁺ naïve T cells were stimulated with α CD3/ α CD28 and cultured in presence of the protein-digested SZ95 sebocyte supernatant resulting in a reduction of IL-17 secretion in absence of the whole protein fraction (Figure 34. a). This suggests that the sebocyte effect on naïve T cell polarization is primarily protein-mediated. However, effects of sebocytes produced lipids and interactions of lipids and proteins cannot be ruled out without further experiments.

As in steady-state SZ95 sebocytes secrete cytokines that are known to contribute to Th17 polarization, such as IL-1 β and IL-6 (204) (Table 2.), we next neutralized these cytokines in SZ95 supernatant and performed a subsequent naïve T cell differentiation. Neutralization of IL-1 β , lead to a decrease of IL-17 secretion in differentiated T cells of 35%, whereas neutralization of TGF- β and IL-6 alone only had marginally effects. Conversely, the depletion of all three cytokines simultaneously abrogated IL-17 production by 44% (Figure 34. b).

Therefore, it is likely that sebocytes drive a Th17 immune response via the production of IL-6, TGF- β , and largely IL-1 β .

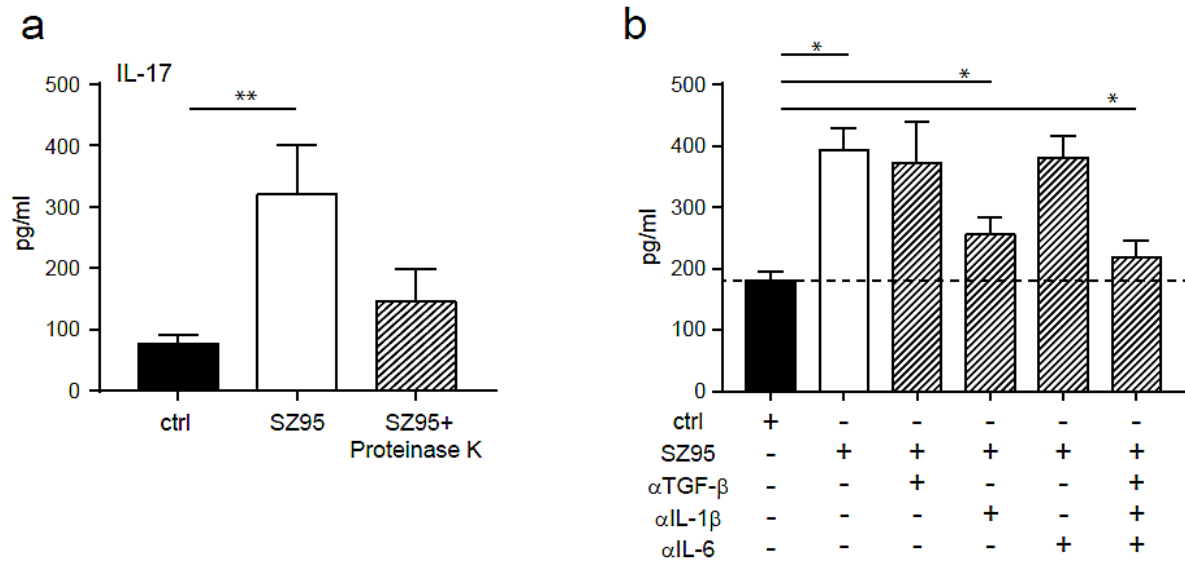


Figure 34. Sebocytes induce Th17 differentiation by release of key polarizing cytokines
 (a) Proteins in SZ95 sebocyte supernatant were removed by digestion with proteinase K. CD4⁺CD45RA⁺ naïve T cells were cultured with the protein-depleted supernatant, the entire SZ95 sebocyte supernatant (SZ95) or control medium (ctrl) in presence of αCD3/αCD28 antibodies for 6 days. Secretion of IL-17 was measured by ELISA after restimulation with αCD3/αCD28 antibodies (n=3). (b) SZ95 sebocyte supernatant was incubated for 1 hour with TGF-β, IL-1β and IL-6 neutralizing antibodies either alone or in combination and used to differentiate CD4⁺CD45RA⁺ naïve T cells for 6 days. After re-stimulated, levels of IL-17 were measured in the supernatants by ELISA (n=2). Statistical significance was determined using One-way ANOVA and Tukey post-hoc test and expressed as *p<0.05; **p<0.01.

7.12 *P. acnes* does not influence immune cell recruitment, but affects priming capacity of DCs

To explore whether *P. acnes* synergistically acts with sebocytes to reinforce the local symbiosis and/or immune response, SZ95 sebocytes were pre-incubated for 24h with *P. acnes* sonicate 889, extensively washed to remove bacteria and further incubated for 24h prior to supernatant collection. In migration assays, *P. acnes* did not alter the chemoattractant potential of sebocytes as compared to SZ95 supernatant, as migration of neutrophils, monocytes or lymphocytes was not altered when sebocytes were pre-incubated with *P. acnes* (see previously at Figure 19. and 20).

However, when DCs were generated in the presence of *P. acnes* pre-stimulated SZ95 supernatant and subsequently used for differentiation of allogeneic CD4⁺CD45RA⁺ naïve T

cells, these T cells showed a slight, however not significant, increase in IL-17 and IL-22 production (Figure 35.). Interestingly, DCs matured with the SZ95 sebocyte supernatant (both unstimulated, and pre-stimulated with *P. acnes*) induced a significant reduction of the Th1 cytokine IFN- γ ($p=0.03$), whereas TNF- α and IL-4 levels were not significantly altered (Figure 35.).

Mixed leukocyte reaction

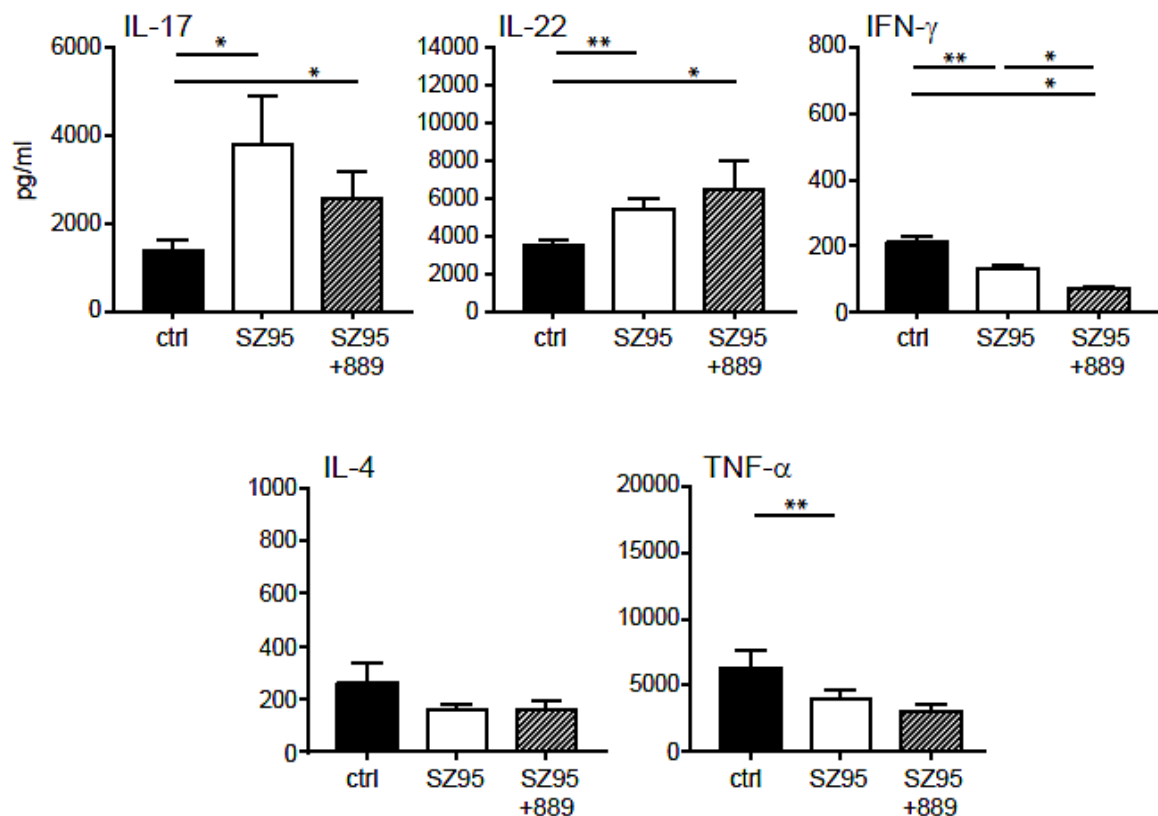


Figure 35. Mixed leukocyte reaction of naïve T cells

DCs were generated in presence of IL-4 and GM-CSF and supernatant derived from SZ95 sebocytes that have been pre-stimulated with the *P. acnes* strain 889. At day 5, DCs were stimulated with LPS and subsequently co-cultured with CD4⁺CD45RA⁺ naïve T cells for 6 days. Differentiated T cells were re-stimulated with α CD3/ α CD28 antibodies prior to supernatant collection and analysis by ELISA for IL-17, IL-22, IFN- γ , IL-4 and TNF- α levels ($n=3$). Statistical significance was determined using One-way ANOVA and Tukey post-hoc test and expressed as * $p<0.05$; ** $p<0.01$.

These data indicate that sebocytes induce Th17 polarization, and *P. acnes* indirectly contributes to this phenomenon by inhibiting Th1 differentiation.

8. DISCUSSION

The presence of macrophages around the sebaceous glands in healthy skin with higher numbers than found in other parts of the dermis has been mentioned in previous publications (130, 205, 206) however these findings have been left at the level of observations with no conclusions or discussion. Moreover, a possible involvement of sebocytes under pathological conditions is also supported by findings in diseases such as acne, which is characterized by the extensive infiltration of the sebaceous glands by immune cells (207), such as Th17 cells, suggesting that sebocytes indeed could have complex roles also in inflammation. A possible explanation for the lack of interpretation of these findings is most likely due to the relative ignorance, which considered SGs to be atavistic remnants of the skin that lost their important role in former human wildlife and, nowadays, only have one remaining function - sebum production that contributes to the triggering of acne (7, 8, 208). The studies of the last decade, however, highlighted that sebaceous lipid metabolism is under a complex regulation that can be altered by different inflammatory stimuli (13, 209). Moreover, upon these stimuli sebocytes are also capable of producing various cytokines and chemokines (36, 41, 92, 126, 145, 210, 211) making sebocytes not only an interesting model to study, but suggesting other potential (patho)physiological roles such as the link between lipid metabolism and inflammation at a cellular level. These features largely resemble adipocytes (212-214), which serve the best example for a cell type with a primary function to metabolize lipids, but also having immunomodulatory effects.

The concept of immunologically competent skin cells with no myeloid or lymphoid origin has been a challenging issue in skin biology. After decades of research keratinocytes, primarily via their cytokine production, are now accepted as immune regulators (215-217) that are important in the pathogenesis of various inflammatory diseases, such as psoriasis and AD (218, 219). In contrast, sebaceous glands are still widely considered to contribute only to the lipid barrier of

the skin by producing the lipid rich sebum (90, 208), however, as we show in this work, there is an impressive difference already in the dermal lipid content between sebaceous gland rich and poor skin.

The key question whether sebum lipids could also contribute to the dermal lipid content (220), was first assessed by Butcher in the 1950s, who demonstrated that the sebum component lipids, STA (radioactive) OA and LA (with an increased fluorescence intensity) absorbed readily through rat skin with a notable accumulation around the sebaceous gland in the case of STA, while LA passaged even to the blood vessels (221, 222). In this study, we extended and completed the characterization on the penetration of sebum component lipids by using Raman spectroscopy and showed that all of the examined lipids (STA, LA, OA, PA and SQ) were able to make their way through the epidermis and accumulated in the dermis to various degrees. While Butcher proposed that lipids are mostly penetrating through the pilosebaceous duct, our results support that secreted sebum lipids from the skin surface could also penetrate and contribute to the dermal lipid milieu.

As lipids, besides cytokines and pathogens, also have a key role in the differentiation and activation of various immune cells, therefore, in our studies we aimed to challenge the interaction of sebocytes with macrophages and lymphocytes, the key immune cells in maintaining the immune milieu of the healthy skin and also in initiating various diseases.

In order to provide explanation for the previously described findings of macrophage accumulation around sebaceous glands, we relied on currently available histological methods and markers to distinguish different subsets of macrophages (166, 200). Famous for their “Janus face”, macrophages exert different functions depending on the tissue environment (148). One extreme is their activation towards the alternative pathway, in which primarily IL-4/IL-13, but also different lipids might act as inducers (148, 154, 155, 223). This pathway, best represented by “skin resident macrophages” which express all the characteristic markers such as CD206,

CD209 or FXIII-A, is central in the maintenance of the tissue environment by producing extracellular matrix components and contributing to tissue remodelling. On the other end of the spectrum is the classical activation, whose prototype is the formation of tuberculoid granulomas, where IFN- γ and TNF- α are the key cytokines (148). In this work, we characterized the macrophages in the vicinity of the sebaceous glands according to this classification and found that under physiological conditions only alternatively activated macrophages could be detected, while classically activated ones appeared under pathological conditions such as acne. Integrating our findings into the current knowledge regarding macrophage activation pathways, we suggest that under normal conditions the exclusive presence of alternatively activated macrophages may be a sign and consequence of a continuous “non-danger signal” contributing to the homeostasis of the skin, of which sebocytes are a potential source (4, 116). However, based only on histological findings, it would have been an over-interpretation of our work to conclude a possible interaction between sebocytes and macrophages.

In steady-state, SZ95 sebocytes release several chemokines and cytokines. This is line with previously published reports highlighting the *in situ* production of e.g. CXCL-8, IL-6 and IL-1 β by sebocytes in the sebaceous gland (41, 116) and underlining the importance of SZ95 sebocytes as *in vitro* model for sebocyte research. Among the steady-state chemokines, CXCL-8 has a key role in recruitment of immune cells such as monocytes and neutrophils to sites of skin inflammation. Although previous reports suggested that neutrophils are the first immune cells in acne lesions (224, 225), some studies revealed that, along with macrophages, also T lymphocytes infiltrate sites of evolving inflammatory lesions (89, 128). Considering the fact that sebocytes undergo holocrine secretion, we used cell free supernatant of SZ95 sebocytes in migration assays and indeed, found that, in a CXCL-8-mediated fashion, sebocytes recruit monocytes, neutrophils and different subsets of T cells such as CD4⁺CD45RO⁺ effector, but also CD4⁺CD45RA⁺ naïve T cells to the skin. However, during inflammatory responses

sebocytes become further activated by proinflammatory cytokines and/or bacterial products leading to enhanced secretion of chemokines and cytokines. In line with that, pre-stimulated SZ95 sebocytes showed an increased chemoattractant potential on immune cells *in vitro* that was mainly following the concentration of CXCL-8 and is reflected *in vivo* by high numbers of immune cells surrounding the sebaceous gland in acne lesions.

These findings strongly suggest that the regulated expression of CXCL-8 by sebocytes could be pivotal in initiating and accelerating the accumulation of immune cells around the sebaceous glands.

To further investigate the probable interaction, we approached the possible effects of sebocytes on macrophages from different aspects such as regulation of the expression of macrophage markers related to the differentiation pathways, phagocytosis and cytokine production of *P. acnes* infected macrophages.

In support of our histological findings we have proved that treatment with SZ95 sebocyte supernatant resulted in the upregulation of all the widely-accepted markers for the alternative macrophage activation pathway (CD206, CD209 and FXIII-A). This suggests that sebocytes can, indeed, affect the polarization of macrophages favouring the alternative pathway and may also provide an answer for the phenomenon of macrophages expressing these detected markers in conditions where IL-4/IL-13 is not involved (226). In addition, the supernatant-treated differentiated macrophages *in vitro* also exerted an increased capacity for phagocytosis of *P. acnes*, a previously defined hallmark of CD209⁺ macrophages (227), suggesting that sebocytes might affect not just the marker profile, but also functions of macrophages. These results support that sebocyte derived lipids could be potent contributors to the homeostasis of the skin which is in line with recent findings, that sebaceous gland rich healthy skin has a distinct non-inflammatory immune surveillance (228).

Based on the findings, that the lipid composition of sebum is not a steady-state condition, but can be changed, as it was observed both in *in vitro* sebocytes in response to various stimuli as well as in the sebum of acne patients where the variations in the quantity and mostly the quality of sebum lipids could significantly affect inflammation by targeting both keratinocytes and sebocytes (35, 228, 229), we extended our experiments also with *P. acnes*-treated macrophages. Our results provided convincing data that macrophages could also be possible targets for the altered sebum production in acne. With dissecting the role of sebocyte-produced lipids in regulating the secretion of IL-1 β and other inflammatory mediators, such as IL-6 and TNF- α by *P. acnes*-infected macrophages (92, 97, 202), we found that each of the major lipid components had a well-defined inflammatory property, that has been overlooked so far perhaps not just in acne, but most likely in the pathogenesis of other dermatological diseases as well. Interestingly, PA and STA, which are both major sebum components, were proven to be potent stimulators of inflammatory cytokine production in macrophages themselves even without the presence of *P. acnes* (82). Moreover, our results that certain lipids, such as LA and OA, had a significant effect on sensitizing macrophage response towards *P. acnes* even when the bacteria only had a minor effect on the secretion of IL-1 β itself, addresses and (partially) explains another intriguing question, namely, how an otherwise commensal bacterium *P. acnes*, could still be a potent macrophage activator in diseases such as acne. Our results shed light on the essential role of the sebum lipids in this process. With ratio alterations, lipid fractions could determine the extent of macrophage' response to *P. acnes* and most likely to other pathogens as well. Keeping the limits of our study in mind, we also provide data supporting the biological relevance for the significant decrease in the levels of LA and thus the increased ratio of OA/LA in the sebum of acne patients (57) and suggest that LA may be a key player, not just in comedo formation, but also in the impaired symbiosis between pathogens and macrophages with a primarily anti-inflammatory effect, which is further affected by the changing levels of the potent

and selective IL-1 β inducer OA. These results altogether fully corroborate that sebaceous lipogenesis does not only contribute to the accumulation of sebum, but is also an instrument for several coordinated actions (20, 39, 116, 230), including the communication with macrophages. Based on these results we can conclude that sebocytes, similarly to keratinocytes, may contribute to the inflammatory milieu and act as a guard at a “locus minoris resistentiae” within the dermis, especially for the pilosebaceous unit, where the lipids, cytokines and resident bacteria (that are all at elevated levels and unique to the pilosebaceous unit) form a unique symbiotic environment that has to be conserved and balanced. Moreover, the analysis of sebum lipid fractions should also be addressed from the scope of their potential immunoregulatory functions, which may give new explanations for the altered lipid profile in sebum of various pathological conditions such as acne and rosacea (103, 116, 231).

In the second part of this study, we provide evidence for a functional communication between sebocytes and T cells as well, resulting in the induction of a Th17-dominated immune response. On the other hand, we demonstrate an indirect contribution of *P. acnes* via sebocytes and DCs towards acne-associated inflammation.

Due to the important role of T cells in the inflammatory tissue response, we investigated whether sebaceous glands influence T cell differentiation similarly to that observed in macrophages. We could demonstrate that factors released by sebocytes do not alter cytokine secretion of CD4⁺CD45RO⁺ effector T cells indicating that sebocytes do not impact on previously determined T cell phenotype. One exception is a slight increase in IL-22 production implying that sebocytes ensure barrier homeostasis by fostering the IL-22/TNF- α axis (232).

Unlike effector cells, we found that sebocytes impact on the differentiation of CD4⁺CD45RA⁺ naïve T cells. As sebocyte secreted various cytokines, and most importantly IL-6 and IL-1 β that represent the key cytokines for *de-novo* differentiation of Th17 cells (189, 233), we could show

that sebocyte supernatants alone are capable to fully induce the Th17 phenotype in naïve T cells and that this interaction is mainly dependent on IL-1 β production. As T cell priming does not take place in peripheral tissues, we assume that sebocytes contribute to the generation of a local micro milieu that skews differentiation of naïve T cells towards the Th17 phenotype in skin draining lymph nodes.

The Th17 population bridges innate and adaptive immunity and has a key role in mediating host defence. Alone or in synergy, the Th17 effector cytokines IL-17 and IL-22 induce an array of antimicrobial peptides to produce a robust antimicrobial response (234-236). However, Th17 cells can also induce pathological inflammation and are associated with several inflammatory skin conditions such as psoriasis, atopic eczema and allergic contact dermatitis (237-239). Moreover, a role for Th17 cells in acne pathogenesis has recently been described. Kelhala *et al.* showed an enhanced expression of Th17 associated cytokines and differentiation factors in lesional skin (240). In line with our data, Agak *et al.* showed that Th17 cells are present in the perifollicular infiltrate of comedones. However, and in contrast to our findings, the authors hypothesized that the Th17 immune response is mainly regulated by *P. acnes* (199). Similarly, a recent study showed that *P. acnes* induces a Th1/Th17 response even though acne pathogenesis has been associated with a Th1 type immunity (241, 242). Our data indicate that sebocytes induce neither a Th1 nor a Th2 cell differentiation, but skew the immune response towards a Th17 profile that is further enhanced by presence of *P. acnes*.

Furthermore, it has been reported that *P. acnes* efficiently induces IL-1 β secretion in sebocytes by activating the NLRP3 inflammasome (116). We also could detect an increase in IL-1 β levels when sebocytes were pre-treated with *P. acnes*, however, we did not find a further increase in Th17 differentiation arguing for a quite high intrinsic production of IL-1 β that is totally sufficient for Th17 priming, even in a resting state. Our data argues for a steady-state induction of Th17 cells by sebocytes to maintain skin homeostasis. However, when the pilosebaceous

unit is colonized with *P. acnes* under pathological conditions the Th17 response is further enhanced *in vivo*. In line with this, we could detect CD4⁺IL-17⁺ cells only sparsely around sebaceous glands in healthy individuals, whereas these cells were frequently co-localized with sebocytes in acne lesions.

Several *in vitro* studies show that *P. acnes* whole cells or cell fractions stimulate cytokine release from immune cells, keratinocytes and sebocytes through binding to TLR2 (92, 243-245). However, the mechanism by which *P. acnes* exerts its activity *in vivo* is still unknown. *P. acnes* can reside in the deeper portions of sebaceous follicles (243), but rarely in the sebaceous gland (246). When this commensal bacterium proliferates, it can come into contact with DCs and activate their maturation with the consequent immune response crucially depending on presence of local commensals or pathogens, biofilm production and additional signals from tissue cells (247). It has been reported that DCs stimulated with *P. acnes* show an increased expression of adhesive molecules and cytokines, which is similar to DCs activated by LPS (248, 249). In presence of naïve T cells, *P. acnes*-matured DCs induced a strong secretion of IFN- γ that is comparable to LPS-matured DCs confirming the capacity of *P. acnes* in eliciting a powerful Th1-type immune response (250). However, presence of sebocyte supernatant reduced the capability to induce Th1 responses and instead drove the symbiotic and/or immune response to *P. acnes* specifically towards a Th17 commitment. Taken together, we assume that sebocytes intend to maintain the epithelial barrier by i) by homeostatic priming of Th17 cells, ii) initiation of effective inflammatory responses and iii) reduction of pathogenic IFN- γ production to reach homeostasis after inflammation.

Our study provides evidence that sebocytes actively participate in inflammatory processes in the skin via recruitment of immune cells and a functional cross-talk with T cells leading to a pronounced Th17 differentiation. This interaction might be of importance for the pathogenesis of acne vulgaris; however, further studies have to clarify whether the sebocyte-Th17 axis

contributes to a beneficial host defence or the perpetuation of a vicious circle of inflammation.

In order to elucidate the conditions and the stimuli that could drive sebocytes to harmonize and balance their production of inflammatory proteins and lipids as well as the identification and characterization of additional lipids that have an immune modulatory role further studies are required. Our findings therefore set the basis and open several new avenues for research of scientific and of therapeutic interest, which can lead to the identification of new players of protein and lipid nature in the inflammatory stimuli/sebocyte/immune cell axis that could be involved in various skin diseases.

9. SUMMARY

Sebaceous glands and hair follicles together form the pilosebaceous unit, which primary function is the production of sebum. Sebaceous glands have already been attributed to have a role in inflammatory processes via pattern recognition receptors such as TLR2, TLR4, and TLR6. Furthermore, an altered lipid composition and increased sebum production is a hallmark in the development of acne.

Despite these findings, up until recent years, sebaceous glands have been only considered as passive players in inflammation. In this work we challenged this dogma and addressed sebocytes as potential immunomodulators.

Based on our Raman spectroscopy findings we have showed that sebum can penetrate through the epidermis and accumulate in the dermal region of the skin, suggesting a more complex role for sebocytes than only contributing to the lipid barrier of the skin. Utilizing various immunohistochemical techniques we displayed that both macrophages and Th17 cells surround the pilosebaceous unit both in physiological and pathological conditions.

On the basis of these observations we have set up an *in vitro* model, where we used SZ95 sebocyte supernatant to culture either monocytes, DCs or T cells. Using migration assays, we have demonstrated that by CXCL-8 production sebocytes attracted all of the above-mentioned cell types via chemotaxis. Furthermore, we proved that sebum component lipids, primarily LA and OA promoted the alternative activation of monocyte derived macrophages and contributed to the potential of macrophages to uptake *P. acnes*. Moreover, sebum component lipids selectively and differentially altered the macrophage activating potential of *P. acnes*.

When investigating a potential sebocyte – T cell interaction, we have determined that sebocytes trigger a Th17 immune response via the secretion of IL-1 β .

Finally, we have demonstrated that *P. acnes* does not influence immune cell recruitment, but affects the priming capacity of DCs.

These results therefore suggest that the proteins (chemokines, cytokines) and even more importantly the sebum component lipids produced by sebocytes, both in amount and in their ratios, should be integrated into our thinking. Further research is required, both in understanding sebocyte function and lipidomics, to provide useful data for therapeutic advances in sebaceous gland-related skin diseases. Therefore, placing (sebum) lipids in the centre of research provides a vast and intriguing field for further studies to come.

10. ÖSSZEFOGLALÁS

A faggyúmirigy, mely a szőrtüszővel és szőrszállal együtt alkotja a piloszebáceus egységet, elsődleges funkciója a bőr zsírosítása a faggyútermelésen keresztül. A faggyúmirigynek ugyanakkor a gyulladásos folyamatokban is lehet szerepe, elsősorban a mintázatfelismerő receptorokon keresztül, mint például a TLR2, a TLR4 és a TLR6, való aktiválódásuk eredményeként. Bár számos betegségnek, így az akne kialakulásának jellegzetessége a mennyiségében és minőségében megváltozott faggyútermelés, felvetve, hogy a termelt faggyú megváltozott lipid-összetétele és a gyulladás között kapcsolat lehet, a közelmúltig a faggyúmirigyekre csak, mint gyulladásos folyamatok passzív résztvevőire tekintettek.

Munkánk során célul tűztük ki annak megvizsgálását, hogy a faggyúsejtek, milyen formában képesek részt venni a gyulladásban, középpontba állítva az immunesejtekkel való esetleges interakciójukat, és ezáltal lehetnek-e akár aktív kezdeményezői is az immun-folyamatoknak.

Raman-spektroszkópiai eredményeink alapján bemutattuk, hogy a faggyú nem csupán a bőr felszíni zsírosításához képes hozzájárulni, de áthatolva az epidermiszen felhalmozódik a bőr dermális régiójában is, felvetve, hogy a faggyút alkotó zsírok hatással lehetnek az immunsejtekre is. Ennek további vizsgálatához kísérleteinket makrofágokon és T sejteken végeztük, melyeket jelentős számban azonosítottunk a piloszebáceus egység környezetében.

Az *in vitro* modellünkben, ahol SZ95 szebocita felülűszó jelenlétében differenciáltattunk monocitákat, illetve T-sejteket, migrációs mérések segítségével meghatároztuk, hogy CXCL-8 termeléssel a szebociták kemotaxison keresztül képesek magukhoz vonzani az összes fent említett sejtípust.

További munkánkkal bebizonyítottuk, hogy a faggyúban jelenlevő lipidek, elsősorban a linol- és olajsav elősegítették a monocita eredetű makrofágok alternatív aktiválását, és hozzájárultak a makrofágok fokozott *P. acnes* felvételéhez. Ezenkívül bemutattuk, hogy a faggyú komponens

lipidek szelektív és differenciált módon járulnak hozzá a *P. acnes* makrofág aktiváló képességéhez is.

Vizsgálva egy lehetséges faggyúsejt - T-sejt interakciót, megállapítottuk, hogy abban elsősorban a faggyúsejtek által termelt fehérjék vehetnek részt, mely közül az IL-1 β -t találtuk kulcsfontosságúnak, mely termelésével a Th17 immunválaszt indukálhatják a faggyúsejtek.

Végül bemutatjuk, hogy a *P. acnes* nem befolyásolja az immunsejtek toborzását, de befolyásolja a DC-k priming kapacitását.

Eredményeink rámutatnak arra, hogy faggyúsejtekkel, az általuk termelt zsírokkal és fehérjékkel, egyaránt számolnunk kell a bőr (pato)fiziológiás működésének megértésében.

Ahogy ma egy-egy bőrgyógyászati betegség esetében kulcskérdés a résztvevő citokinek azonosítása, úgy vizsgálataink felvetik annak is a szükségességét, hogy megismerjük a faggyúzsír „mintázatot” is, melynek egyaránt lehet diagnosztikai és terápiás hasznosíthatósága is, ezáltal érdekes távlatokat megnyitva mind az akadémiai mind pedig az ipari kutatás/hasznosíthatóság terén.

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12. KEY WORDS

sebaceous gland
sebaceous-immunobiology
bioactive sebum lipids
dermal immunity
Propionibacterium acnes
Th17 polarization
M2 macrophage polarization

KULCSSZAVAK

faggyúmirigy
faggyú-immunbiológia
bioaktív szébum lipidek
dermális immunitás
Propionibacterium acnes
Th17 polarizáció
M2 makrofág polarizáció

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14. PUBLICATIONS



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List of publications related to the dissertation

1. *Mattii, M., ***Lovászi, M.**, Garzorz, N., Atenhan, A., Quaranta, M., Lauffer, F., Konstantinow, A., Küpper, M., Zouboulis, C. C., Kemény, L., Eyerich, K., Schmidt-Weber, C. B., Törőcsik, D., Eyerich, S.: Sebocytes contribute to skin inflammation by promoting the differentiation of Th17 cells.
Br. J. Dermatol. [Epub ahead of print], 2017.
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2. **Lovászi, M.**, Mattii, M., Eyerich, K., Gácsi, A., Csányi, E., Kovács, D., Rühl, R., Szegedi, A., Kemény, L. V., Stähle, M., Zouboulis, C. C., Eyerich, S., Törőcsik, D.: Sebum lipids influence macrophage polarization and activation.
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*These authors contributed equally to this work.



List of other publications

3. Szentkereszty-Kovács, Z., **Lovászi, M.**, Zatik, Z., Duró, E., Kovács, D., Törőcsik, D.: Perspektívák a faggyúmirigy kutatásban.
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Br. J. Dermatol. 171 (6), 1326-35, 2014.
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