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Sebocytes contribute to skin inflammation by promoting the differentiation of Th17 cells

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What is already known about this topic?

Sebocytes are part of the pilosebaceous unit and produce lipids for moisturizing the skin. They long were regarded as bystander cells during skin inflammation with no impact on the immune response

What does this study add?

We show that sebocytes actively contribute to inflammatory processes by recruitment of immune cells into the skin and by skewing T cell differentiation towards Th17

Abbreviations:

P.acnes: *Propionibacterium acnes*

Th: T helper

PBMCs: peripheral blood mononuclear cells

IL: interleukin

TLR: toll like receptor

DCs: Dendritic cells

LPS: Lipopolysaccharides

TGF-β: transforming growth factor β

Abstract

Background: The main function of sebocytes is considered to be the lipid production for moisturizing the skin. However, it became recently apparent that sebocytes release chemokines and cytokines and respond to pro-inflammatory stimuli as well as presence of bacteria.

Objectives: To analyze the functional communication between human sebocytes and T cells.

Methods: Immunofluorescence stainings for CD4 and IL-17 were performed on acne sections and healthy skin. Migration assays and T cell stimulation cultures were performed with supernatants derived from unstimulated or pre-stimulated SZ95 sebocytes. DCs were generated in presence of SZ95 supernatant and subsequently used in mixed leukocyte reactions.

Results: We could show that CD4+IL-17+ T cells accumulate around the pilosebaceous unit and are in close contact with sebocytes in acne lesions. By using SZ95 sebocyte supernatant, we demonstrate a chemotactic effect of sebocytes on neutrophils, monocytes and T cells in a CXCL-8 dependent manner. Furthermore, sebocyte supernatant induces the differentiation of $CD4^+CD45RA^+$ naïve T cells into Th17 cells via the secretion of IL-6, TGF-β and, most importantly, IL-1β. No direct effects of sebocytes on the function of $CD4^+CD45RO^+$ memory T cells were detected. Moreover, sebocytes functionally interact with *Propionibacterium acnes* in the maturation of dendritic cells leading to antigen presenting cells that preferentially prime Th17 cells.

Conclusions: Our study provides evidence that human sebocytes actively participate in inflammatory processes in the skin by recruiting and communicating with immune cells. This interaction leads to the generation of Th17 cells that might contribute not only to the pathogenesis of acne vulgaris, but to several inflammatory skin diseases.

Introduction

Sebocytes are specialized epithelial cells that construct the sebaceous gland acini and form together with hair follicles the pilosebaceous unit. The main function of sebocytes is considered to be the synthesis and accumulation of lipid droplets that are released along the hair shaft by cellular disintegration to moisturize the skin surface[1]. More recently, it became

apparent that sebocytes may also act as immune competent cells regulating immunological and inflammatory processes in the skin by production of cytokines and inflammatory mediators[2,3]. It was shown that sebocytes constitutively produce CXCL-8[4] and after stimulation with proinflammatory agents also secrete IL-6[5]. Furthermore, sebaceous triglycerides serve as nutrient for *Propionibacterium acnes (P.acnes* [6] and its presence stimulates the production of the inflammasome protein IL-1β[7]. In addition, the presence of *P.acnes* may amplify immune responses by stimulating the development of subclasses of T cells[8,9]. It has recently been shown that *P.acnes* induces a T helper (Th) 17 response in human peripheral blood mononuclear cells (PBMCs), the expression of key Th17 related genes and interleukin (IL)-17 secretion from $CD4^+$ T-cells[10].

Both sebocytes and *P.acnes* are key players in the pathogenesis of acne vulgaris that represents a chronic inflammatory disorder of the pilosebaceous unit[8,11]. Various immune factors, including both adaptive and innate immune responses, have been implicated in acne pathogenesis and the pilosebaceous gland itself also plays an active role in skin inflammation as it releases certain immunologic factors such as lipid mediators and proinflammatory cytokines[12,13]. Several studies mapping the progression of inflammatory lesions at different time points revealed that, apart from neutrophils and macrophages, $CD4^+$ cells infiltrate sites of early acne lesions[14,15]. Although the role of sebocytes in acne inflammation and innate immunity has been widely studied, data regarding the implication of these cells in T cell recruitment and activation are still missing. Herein, we evaluated whether sebocytes are able to recruit immune cells to sites of skin inflammation and to modulate their function. Moreover, we evaluated a possible synergism between human sebocytes and *P.acnes* in driving an inflammatory response, which may be active in skin homeostasis (symbiosis) and/or in immune reactions such as acne vulgaris.

Materials and methods

SZ95 sebaceous gland cell culture

Immortalized human SZ95 sebocytes[16] were cultured at 37°C in a humidified atmosphere containing 5% (v/v) CO_2, in Sebomed® medium (Biochrom) supplemented with 10% fetal bovine serum (Biowest), 1mM $CaCl_2$ solution, 1% penicillin/streptomycin (Sigma-Aldrich) and 5µg/ml epidermal growth factor (EGF) (Sigma Aldrich). At approximately 80% confluency, SZ95 sebocytes were stimulated with recombinant cytokines (50ng/ml each) or 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, filtered using 0.2µm syringe filters (Sarstedt) and frozen until use in subsequent experiments. Sebomed medium lacking EGF served also as control in stimulation experiments.

Migration assay

Neutrophils, monocytes and T cells were isolated from heparinized whole blood of healthy donors by density centrifugation, CD14 and CD3 microbeads (Miltenyi Biotech), respectively, resuspended in complete RPMI 1640 (Invitrogen) plus 0.5% BSA (Sigma Aldrich) with a final concentration of $2x10^6$ cells/ml and added to the top of a 5µm pore polycarbonate membrane (ChemoTx Disposable Chemotaxis System) (NeuroProbe). After 2h at 37°C with 5% CO_2 migrated cells to further analyzed with a LSRFortessa (BD Biosciences) flow cytometer. Migrated T cells were additionally stained for CD4, CD8, CD56, CD45RO and CD45RA. Migration was performed in duplicates.

Purification and stimulation of naïve and memory $CD4^+$ cells

$CD4^+$ cells were magnetically sorted using the CD4 T cell isolation kit II followed by a positive selection with CD45RO or CD45RA beads (Miltenyi Biotech). $2x10^5$ cells were seeded in a 96-well plate and stimulated with plate-bound human αCD3 and soluble αCD28 (each 0.75µg/ml) (BD Biosciences) in presence of 100µl SZ95 sebocyte supernatant and 100µl RPMI 1640 (Invitrogen) supplemented with 2.5% human serum (Lonza), and 0.5% of penicillin/streptomycin solution (Invitrogen) at 37°C with 5% CO_2. 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 αCD3 and soluble αCD28 (each 0.75µg/ml) (BD Biosciences) before supernatant collection. Samples were assayed in duplicates.

DCs generation and mixed leukocyte reaction

$1x10^6$ $CD14^+$ monocytes were seeded in a 24-well plate containing 500µl RPMI 1640 (Invitrogen) supplemented with 1.5% FCS (Biochrom), 0.5% penicillin/streptomycin solution (Invitrogen), 500µl 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% CO_2. At day 5, DCs were stimulated with LPS (1 µg/ml, Invivogen) 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 αCD3 and soluble αCD28 (each 0.75µg/ml) (BD Biosciences) before supernatant collection. Samples were assayed in duplicates.

Statistical analysis

Each experiment was performed in technical duplicates. The given n-number represent independent experiments performed with different donors. Statistical analysis was performed using the Graph Pad Prism software. Statistical significance between two stimulation conditions was determined using the Wilcoxon matched-pairs signed rank test. Statistical analysis with more than two stimulation conditions was performed with the Kruskal-Wallis test and Dunn's multiple comparison test to correct for multiple testing. Asterisks represent statistical significance defined as *$p<0.05$; **$p<0.01$; ***$p<0.001$. If no asterisk is given, no statistical differences could be detected. Graphically, boxplots with Turkey whisker plots are shown.

Further information on culture of Immune fluorescence staining, P.acnes, cytokine neutralization, protein digestion and ELISA/Bioplex can be found in the supplementary information.

Results

Th17 cells surround the pilosebaceous unit in acne lesions

Staining of paraffin embedded skin sections of acne lesions revealed a high number of $CD4^+IL\text{-}17^+$ double-positive T cells accumulating in close proximity to the pilosebaceous unit (Fig.1). Isotype control stainings are given in Fig.S1. Despite the fact that healthy control skin did not show signs of inflammation, $CD4^+IL\text{-}17^+$ double-positive T cells were detected next to the sebaceous gland indicating a potential crosstalk of Th17 cells and sebocytes during inflammation, but also homeostasis.

Sebocytes attract immune cells through CXCL-8 release

We next analyzed if sebocytes actively contribute to the recruitment of immune cells and especially T cells into the skin. Here, we used cell-free supernatants from the human sebaceous gland cell line SZ95 that represents an accepted and widely used model in sebocyte research[16]. In a first step, these supernatants were analyzed for production of 27 cytokines, chemokines and growth factors by Luminex technology revealing a robust secretion of chemokines such as CXCL-8, CCL-2, CCL-5 and CXCL-10 (Table S1). As these chemokines are important for granulocyte and leukocyte migration into tissues, we next analyzed the migratory capacity of neutrophils, monocytes and T cells towards the SZ95 sebocyte supernatant. Neutrophils, monocytes and T cells migrated towards the SZ95 sebocyte supernatant in a CXCL-8 dependent manner (Fig.S2a, Fig.2a). Amongst T cells, $CD4^+$ and $CD45RO^+$ effector T cells represented the main migratory subsets (Fig.2b).

Next, we wanted to understand if cultivation of SZ95 sebocytes in different pro-inflammatory environments alters the secretion of proteins as well as the subsequent migration of cells. Therefore, SZ95 sebocytes were pre-stimulated 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 S1). Whereas IFN-γ stimulation of sebocytes seems to foster, hence not significantly, the migration of CD3+ T cells (Fig.2c) with a significant migration of the CD45RO+ T cell subset (Fig.S2c), LPS or LTA stimulation significantly induced migration of neutrophils and monocytes (Fig.S2b).

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 pro-inflammatory environment.

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 αCD3 and soluble αCD28 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. Only for IL-22 secretion a significant induction was detectable (Fig.S3a).

As in this setting, T cell receptor 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* dendritic cells (DCs). Therefore, CD14$^+$ monocytes were differentiated into DCs in presence of SZ95 sebocyte supernatant or control medium and stimulated with lipopolysaccharides (LPS) prior to co-culture with allogenic CD4+CD45RO+ cells (mixed leukocyte reaction). SZ95 supernatant did not impact on DC maturation (Fig.S4) and did also not impact on T cell activation, as no significant release of all cytokines analyzed was detected (Fig.S3b).

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

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 αCD3/αCD28 in presence of the SZ95 sebocyte supernatant or control medium. As also in our hands intracellular cytokine stainings of differentiated T cells did not work[17], 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 (Fig.3a). 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 (Fig.3b). Interestingly, IL-17 and IL-22 cytokine levels were even higher compared to the solely stimulation with αCD3/αCD28 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.

Sebocytes induce Th17 differentiation via secretion of IL-1β

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 αCD3/αCD28 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 (Fig.4a). 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-1β and IL-6[18] (Table S1), we next neutralized these cytokines in SZ95 supernatant and performed a subsequent naïve T cell differentiation. Neutralization of IL-1β, 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% (Fig.4b). Therefore, it is likely that sebocytes drive a Th17 immune response via the production of IL-6, TGF-β, and largely IL-1β.

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 SZ9Z supernatant as migration of neutrophils, monocytes or lymphocytes was not altered when sebocytes were pre-incubated with *P.acnes* (Fig.S2b, c; Fig.2c).

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 (Fig.5). Interestingly, DCs matured with the SZ95 sebocyte supernatant (both unstimulated or 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 (Fig.5).

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

Discussion

Current research on human sebocytes has indicated that these cells are not only bystanders during skin inflammation, but actively modulate immune responses via secretion of chemokines and cytokines[2,3,19]. In this study, we provide evidence of a functional communication between sebocytes and T cells 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.

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-1ß by sebocytes in the sebaceous gland[5,7](Li Alestas) 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 neutrophils and monocytes to sites of skin inflammation. Although previous reports suggested that neutrophils are the first immune cells in acne lesions [20,21], some studies revealed that, along with macrophages, also T lymphocytes infiltrate sites of evolving inflammatory lesions[14,15]. Indeed, we found that, in a CXCL-8-mediated fashion, sebocytes recruit 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 pro-inflammatory 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.

Owing to the important role of T cells in the inflammatory tissue response, we investigated whether do not only attract T cells next to the sebaceous gland, but also influence their function. 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[22].

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-1ß, that represent the key cytokines for *de-novo* differentiation of Th17 cells[23,24], 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-1ß production. As T cell priming does not take place in peripheral tissues, we assume that sebocytes contribute to the generation of a local micromilieu that skews differentiation of naïve T cells towards the Th17 phenotype in skin draining lymphnodes.

The Th17 population bridges innate and adaptive immunity and has a key role in mediating host defense. 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[25-27]. 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[28-30]. 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[31]. 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[10]. 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[32,33]. 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-1ß secretion in sebocytes by activating the NLRP3 inflammasome[7]. We also could detect an increase in IL-1ß 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-1ß 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 $CD4^+IL\text{-}17^+$ 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[2,34-36]. 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[34], but rarely in the sebaceous gland[37]. 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[38]. 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[39,40]. 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[41]. 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 barrier integrity by i) homeostatic priming of Th17 cells, ii) initiation of effective inflammatory responses and iii) reduction of pathogenic IFN-γ production to reach homeostasis after inflammation.

The limitations of our study lie clearly in the *in vitro* nature of the performed experiments and the use of a cell line. However, the SZ95 sebaceous gland cell line is a widely accepted and used human sebocyte model that shows comparable behavior to other sebocyte lines and mostly to primary sebocytes that only deliver limited amounts of material[42]. Furthermore, additional *in vivo* mouse experiments are needed to fully characterize the contribution of sebocyte-induced Th17 cells to acne pathogenesis, and, probably, also other inflammatory skin diseases.

Despite these limitation, 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 defense or the perpetuation of a vicious circle of inflammation.

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Figure legends

Figure 1. 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 (upper part) and lesional skin (lower part).

Figure 2. SZ95 sebocytes induce the migration of T cells via secretion of CXCL-8.

(a) CD3+ T cells 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 supernatant (SZ95 sup) and CXCL8 depleted SZ95 sebocyte supernatant (n=3). (b) Flow cytometric analysis of CD3+ cells. Migrated CD4+, CD8+, CD56+, CD45RO+ and CD45RA+ cells are expressed as the percentage of total migrated CD3+ cells (n=3). (c) Flow cytometric analysis of migrated CD3+ cells 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 comparions test to correct for multiple testing and expressed as *$p<0.05$; **$p<0.01$; ***$p<0.001$.

Figure 3. Sebocyte supernatant polarizes naive T cells into Th17 cells

(a) $CD4^+CD45RA^+$ naive T cells were cultured with SZ95 sebocyte supernatant or control medium and stimulated with αCD3/αCD28. After 6 days, cells were re-stimulated for 72 hours and supernatants analyzed 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 $CD4^+CD45RA^+$ naive T cells. After 6 days, T cells were re-stimulated with αCD3/αCD28 for 72 hours and supernatants analyzed by ELISA (n=3). Statistical significance was determined using the Wilcoxon matched-pairs signed rank test and expressed as *$p<0.05$; **$p<0.01$.

Figure 4. Sebocytes induce Th17 differentiation by release of key polarizing cytokines.

(a) Proteins in SZ95 sebocyte supernatant were removed by digestion with proteinase K. $CD4^+CD45RA^+$ naive 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 $CD4^+CD45RA^+$ naive 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 the Kruskal-Wallis test and the Dunn's multiple comparions test to correct for multiple testing and expressed as *$p<0.05$; **$p<0.01$.

Figure 5.

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 $CD4^+CD45RA^+$ naive T cells for 6 days. Differentiated T cells were re-stimulated with αCD3/αCD28 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 the Kruskal-Wallis test and the Dunn's multiple comparions test to correct for multiple testing and expressed as *p<0.05; **p<0.01.

Figure 1

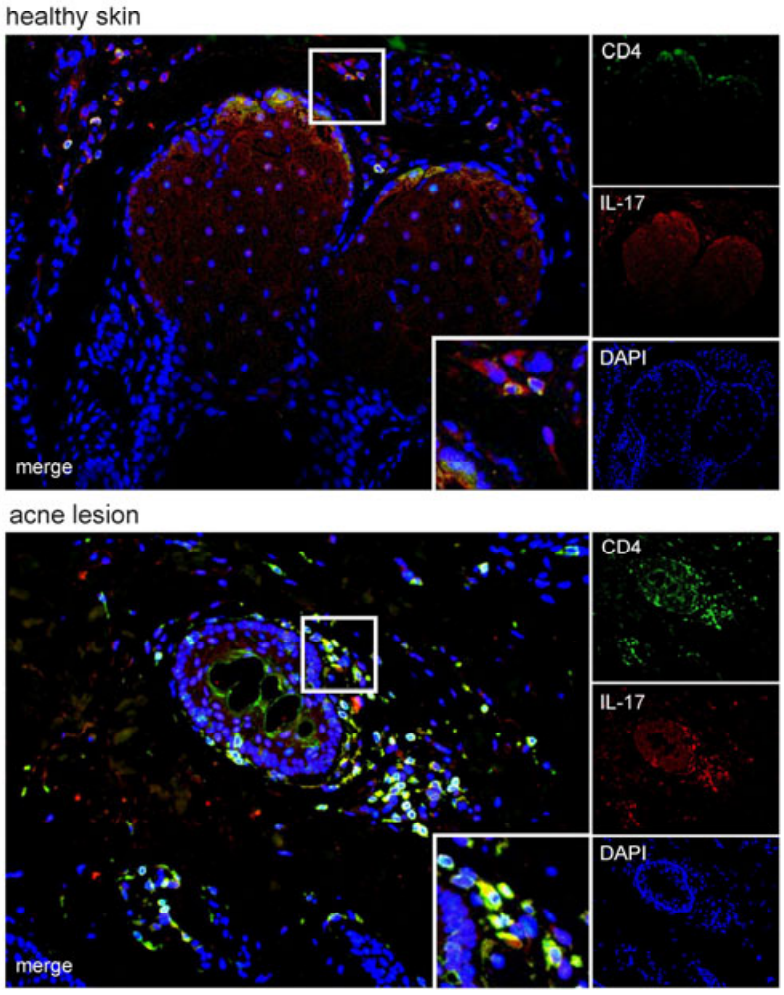

Figure 2

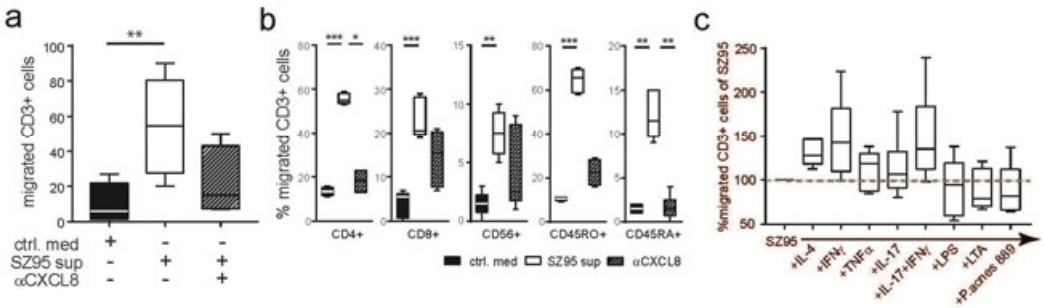

Figure 3

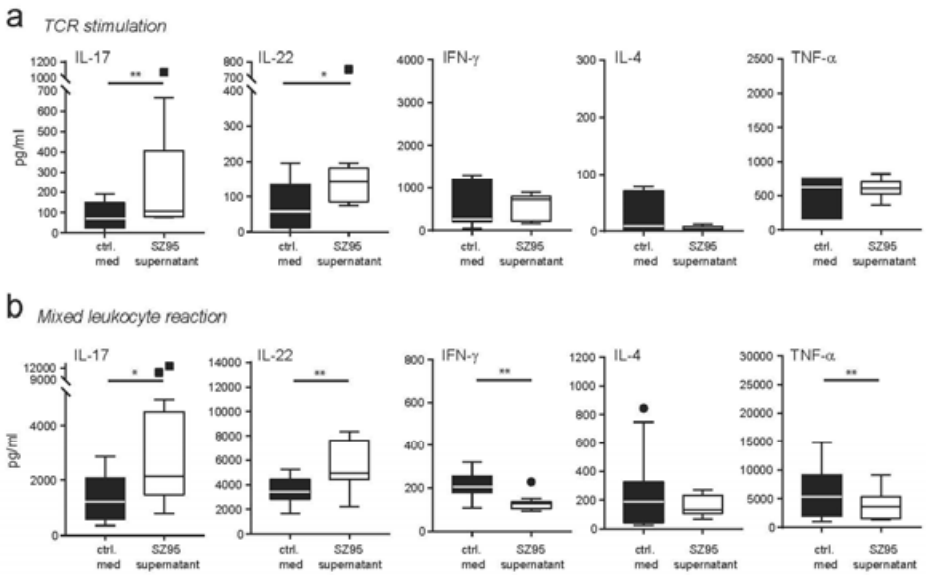

Figure 4

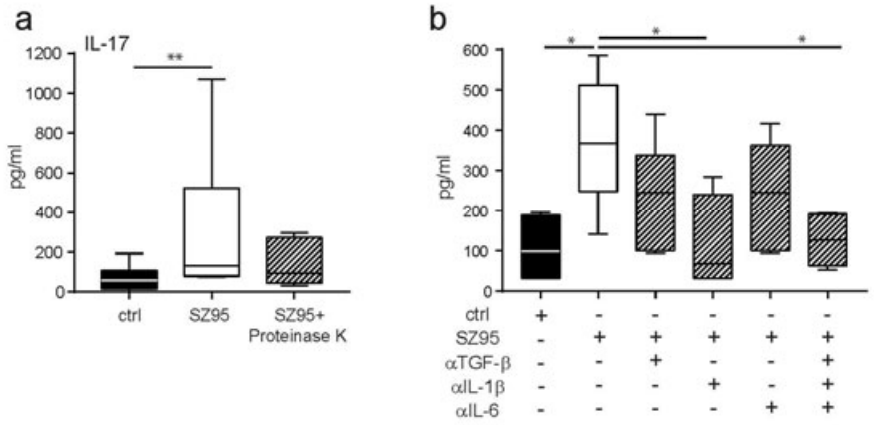

Figure 5