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1. Abbreviations

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<tr>
<td>Ab</td>
<td>Antibody</td>
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<tr>
<td>APC</td>
<td>Antigen-presenting cell</td>
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<td>CCR</td>
<td>Chemokine receptor</td>
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<td>CD</td>
<td>Cluster of differentiation</td>
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<td>CLA</td>
<td>Cutaneous lymphocyte antigen</td>
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<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
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<td>DC</td>
<td>Dendritic cell</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<td>Fb</td>
<td>Fibroblast</td>
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<tr>
<td>Gro</td>
<td>Growth-related oncogene</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>ILC</td>
<td>Innate lymphoid cell</td>
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<tr>
<td>LC</td>
<td>Langerhans cell</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MIP</td>
<td>Macrophage inflammatory protein</td>
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<tr>
<td>NTS</td>
<td>Non-tape-stripped</td>
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<td>OCT</td>
<td>Octenidine dihydrochloride</td>
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<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
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<tr>
<td>PRR</td>
<td>Pattern recognition receptors</td>
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<tr>
<td>SP</td>
<td>Standard procedure</td>
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<tr>
<td>TARC</td>
<td>Thymus- and activation-regulated chemokine</td>
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<tr>
<td>TCR</td>
<td>T cell receptor</td>
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<td>TE cell</td>
<td>T effector cell</td>
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<td>TH cell</td>
<td>T helper cell</td>
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<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
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<td>TM</td>
<td>Memory T cell</td>
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<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
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<td>Treg</td>
<td>Regulatory T cell</td>
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<td>TS</td>
<td>Tape-stripped</td>
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<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<td>µm</td>
<td>Micrometer</td>
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2. Introduction

2.1 Human skin architecture and immune system

Human skin is responsible for the protection of the host against harmful toxins and infections and serves as the front line defense of the immune system. In addition, the skin protects the body from dehydration and functions as a thermoregulator and sense organ. It is organized in three compartments, epidermis and dermis and hypodermis all of which contain a variety of different cell types.  

The epidermis is a stratified, squamous and non-vascularized epithelium that is separated by a basement membrane from the extracellular matrix (ECM) and its appendages (e.g. sweat glands, hair follicles) of the underlying dermis. It consists of 4 different layers: stratum basale, containing basal keratinocytes (KCs), stem cells and dividing transit-amplifying cells which give rise to differentiated cell layers of the stratum spinosum, stratum granulosum and stratum corneum. The KC is the most abundant cell type of the epidermis. Melanocytes are located in between basal KCs and produce melanin, the pigment responsible for skin color, which is transferred and stored in KCs to protect skin from ultraviolet radiation. Merkel cells represent a rare population of epithelial cells and are unique due their close contact formation with Aβ sensory neurons at the epidermal-dermal junction, and representing a unique touch receptor. Langerhans cells (LCs) and T cells are major immune sentinels in the epidermis (Figure 1).

Beneath the epidermis, the dermis forms the second compartment of the skin. It is much thicker compared to the epidermis with a higher diversity of cell populations. It harbors...
different subsets of dendritic cells (DCs), macrophages, mast cells, CD4+ T helper (TH) cells, CD8+ T cells, ψδ T cells, αβ T cells, regulatory T cells (Treg), natural killer T (NKT) cells and innate lymphoid cells7-9, 10-12 (Figure 1). Even though there is a wide range of cells in the dermis, they are not the principal cell population. The dermis is mainly populated with collagen and elastin fibers, largely produced by fibroblasts (FBs), which are the predominant dermal cell type. Further, capillary beds, nerves, blood vessels and draining lymphatics can be found in the dermis and serve as a connection to deeper lying lymph nodes6.

The hypodermis represents a subcutaneous tissue consisting of 50% of body fat with the function to i) bind the dermis to the underlying bones or muscles, ii) regulate the body temperature, iii) store lipids and cushions, and iv) insulate the body. It also contains macrophages, adipocytes and FBs13.

2.1.1 Keratinocytes
KC s form an effective barrier against different pathogens and minimize moisture loss. They are dividing as they arise as basal cells at the innermost layer of the epidermis14. While differentiating, KCs move to the next layer, the stratum spinosum, where they change their columnar shape to polygonal and start producing keratins. On their way to the stratum granulosum KCs commence to synthesize additionally other proteins and lipids. The migrating cells terminally differentiate and finally form the cell surface layer called stratum corneum, which contains dead KC-derived cells with a leakage of nuclei2, 14. This layer and the tight junctions in the stratum granulosum represent the physical and chemical barrier of the skin2, 7.

In addition, KCs can act as immune sentinels due to the expression of multiple pattern recognition receptors (PRRs) such as nucleotide binding oligomerization domain (NOD)-like receptors (NLRs), Toll-like receptors (TLRs), and retinoic acid inducible gene I (RIG-I)-like receptors (RLRs). Additionally, they take part in the defense against pathogens by producing antimicrobial peptides (AMPs) and therefore are important mediators of epithelial homeostasis15. KCs also produce different cytokines and chemokines either upon induction by various stimuli or constitutively (e.g. CCL20, CXCL9, CXCL10, interferons (IFN) α, β, γ, tumor necrosis factor (TNF), interleukin (IL)-1, -6, -7, -8, -10 etc.)16-21. After skin injury, KCs release IL-1, which represents the primary event in skin defense. IL-1 stimulates the release and production of IL-6, IL-8 and GM-CSF from neighboring KCs. These cytokines represent the activators for pro-inflammatory cells. IL-8 is responsible for the recruitment of macrophages, neutrophils and T cells to the injured site22. KCs also produce specialized inflammatory factors, such as IL-33, a cytokine secreted mainly from stromal cells and its expression is upregulated by following pro-inflammatory stimulation23. Vascular endothelial growth factor (VEGF) produced by KCs24, but also macrophages25, tumor cells26, 27, platelets28 and renal mesangial cells29, plays an important role in physiological processes like hematopoiesis30, wound healing31 and development32.

2.1.2 Langerhans cells
LCs were initially described as skin nerve cells by Paul Langerhans in 186833. After years of further research, it was discovered that these cells have immunological functions and are a part of the DC family34. LCs derive from the bone marrow and represent the first immunological
defense line in the skin to the environment. In the epidermis they are forming a network with external dendrites and account 3-5% of the total epidermal cells in humans (Figure 2).

![Figure 2. LCs form a network in the epidermis. Immunohistochemistry of a human epidermal sheet stained with a mAb directed against CD207. Scale bar = 100 μm.](image)

Birbeck granules, an organelle only found in LCs, were for a long time the only possibility to discriminate LCs from other cells. Today, LCs can be identified by the expression of Langerin (CD207), a type II transmembrane mannose-specific C-type lectin receptor localized in Birbeck granules and on the cell surface. Besides CD207, resident immature LCs express various markers such as CD45, CD1a, CD1c, CD39, CD45, CCR6, MHC I and II/HLA-DR molecules, common leukocyte antigen (CLA), EpCAM and E-cadherin. During inflammation, LCs mature in that they upregulate the expression of several markers (CCR7, CD40, CD80, CD83, CD86, intercellular adhesion molecule (ICAM)-1, very late antigen (VLA)-9-4). When the inflammation process in the skin occurs, LC precursors from the dermis and blood are recruited. In order to enter the epidermis, LC precursors are depending on the release of transforming growth factor beta (TGFβ) and the chemokine CCL20, also known as macrophage inflammatory protein-3 alpha (MIP-3α). The role of this chemokine is to attract LC precursors, whereas TGFβ is necessary for the formation of Birbeck granules. LCs interact with naive T cells once they arrive from epidermis in the skin draining lymph node through lymphatic vessels and mount an immune response. Recent studies have shown that LCs induce the proliferation of pathogen-specific skin-resident memory Treg cells and skin resident effector T cells (TE), where Treg cells show a potent suppressive activity. LCs and Treg cells colocalize in epidermis and follicular epithelium. The primary function of LCs in the skin is most likely that they are responsible for immune tolerance and to mediate protective immunity when activated by infection.

2.1.3 Fibroblasts

FBs produce the ECM that forms the connective tissue of the skin consisting of collagens, reticular and elastic fibers. FBs play a crucial role in wound healing, where tissue damage induces their mitosis. Besides their role as structural components, FBs play an important
role in an immune response following injury. Upon chemokine synthesis through the presentation of receptors on their surface, immune cells respond and initiate different events to remove the invasive pathogens. After infection, FBs regulate the hematopoietic cells which infiltrate a damaged tissue.

### 2.1.4 T cells

The innate immune system represents the first line of defense against various pathogens using its phagocytes and DCs, while the adaptive immunity is carried out by T cells and B cells. Further, adaptive immunity can be divided in the humoral and cellular type. Antibodies (Abs) and B cells are part of the humoral immunity, while the cellular type is being carried out by T cells. T cell production takes place in the bone marrow and their maturation in the thymus. Upon antigen contact they can differentiate into TH, cytotoxic T lymphocytes (CTL), Tregs, memory (TM) or TE cells. The T cell receptor (TCR) is responsible for antigen recognition. T cells can be divided into TCR αβ and γδ T cells. During maturation in the thymus T cells express a unique TCR on their surface as a result of developmental and clonal selection, where the TCR is associated with nonpolymorphic CD3 proteins. The skin of a healthy adult human contains about 10-20 billion T cells predominantly expressing TCR αβ rather than TCR γδ and consisting of CD4+ and CD8+ TM cells. Four human skin T cell subsets (two populations of resident memory T cells (TRM) and two distinct populations of recirculating T cells) have been reported and are known to contribute to human diseases such as psoriasis and cutaneous T cell lymphoma. Over 90% of skin T cells in healthy adult human skin can be found in the dermis, whereas a small fraction resides in the suprabasal and basal KC layer of the epidermis. Remaining naive T cells (>5%) mostly express the skin homing cutaneous lymphocyte antigen (CLA). CLA is described as a skin homing T cell receptor that binds to E-selectin, expressed by inflamed endothelium. Naive T cells can be found primarily in the blood and lymph nodes and can migrate under the influence of the homing addressins L-selectin and CCR7. These cells encounter DCs carrying antigens from peripheral tissues. These cells also express CD62L, responsible for immune surveillance.

CD4+ T cells recognize antigens presented via MHC class II molecules on B cells, macrophages and professional antigen-presenting cells (APC). As a result, they produce cytokines as TH cells and mount the immune response. Professional APCs such as DCs and LCs recognize pathogen-associated molecular patterns (PAMPs), such as microbial nucleic acid, lipoproteins, peptidoglycans, lipopolysaccharides (LPS), other unique bacterial structures and damage-associated molecular patterns (DAMPs) released from injured cells via PRRs. TH cells were first subdivided into TH1 and TH2 cells according to the cytokine production and therefore classified as type 1 and 2 immune responses, which produce IFNγ and IL-4/-13, respectively. Meanwhile, more TH subsets have been described: TH9, TH17, TH22, as well as Treg and follicular T cells. Antigens presented to CD8+ T cells via MHC class I molecules leading to the differentiation of CD8+ T cells into CTLs. CD8+ CTLs are able to detect and destroy the infected cells and pathogens. CD4+ and CD8+ T cell subsets can be further distinguished by their secreted cytokines upon activation. The functional role of γδ T cells is not yet understood, but research
studies show their ability to secrete insulin-like growth factor 1 upon activation and influence wound healing.\textsuperscript{71}

2.2 Octenidine dihydrochloride

2.2.1 Chemical characteristics
Octenidine dihydrochloride \([\text{N,N-\{1,10-decanediylid-1[4H]-pyridinyl-4-ylidene\)-bis-(1-octanamine) dihydrochloride, OCT}]\) is a cationic surface active antimicrobial substance, which consists of 2 pyridine rings within its molecules that are separated by a long aliphatic hydrocarbon chain (Figure 3). OCT differs clearly from bisguanidines such as chlorhexidine and quaternary ammonium compounds like benzalkonium chloride. As OCT has no amide or ester structures, it is stable under various physical and chemical conditions and cannot hydrolyze. Due to this structure, the molecule contains two positive charges that are responsible for the action of the binding to negatively charged surfaces, such as microbial cell walls and eukaryotic cell membranes. OCT is an amphiphilic molecule and has a strong adherence to lipid bacterial cell membrane components which explains the high antimicrobial effect without affecting epithelial or wound tissue. It is stable at the pH range from 1.6-12.2 under the influence of light, which is crucial in wound care due to pH change in the wounding process\textsuperscript{72, 73, 74}.

Figure 3. Structure of OCT. A long carbohydrate chain separates the 2 pyridine rings. It has 2 positive charges that are responsible for binding to the negatively charged surfaces (adapted from Hubner et al.\textsuperscript{75}).

2.2.2 Antimicrobial efficacy
OCT has antibacterial and antifungal efficacy already at low concentrations (0.1% and less)\textsuperscript{75}. Due to its unspecific and strong interaction with the cell wall and the cell membrane, OCT has a remarkable widespread antimicrobial effectiveness against gram-positive and gram-negative bacteria, chlamydia\textsuperscript{76}, mycoplasma\textsuperscript{77}, plaque-forming bacteria (e.g. streptococcus, actinomyces) and fungi\textsuperscript{78}. OCT induces changes in the lipid and sterol components of candida albicans and binds to human buccal epithelial cells \textit{in vitro}\textsuperscript{79}. It was also shown that OCT is highly effective against biofilms that were induced by species isolated from orthopedic implant infections as well as by laboratory strains of \textit{Pseudomonas aeruginosa} and \textit{Staphylococcus epidermidis}\textsuperscript{74, 80, 81}. OCT has a limited anti-viral efficiency against f2- and MS2-coliphages and low efficiency against PhiX174-phages. Previous investigations suggest a virucidal effect of OCT against enveloped viruses (e.g. hepatitis B, herpes simplex viruses) but no effect on hydrophilic and non-enveloped viruses (e.g. adenoviruses)\textsuperscript{82, 83}.  

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2.2.3 Residual effects and resistance

OCT has a strong residual (remanence) effect on skin that is observable for 48 hours (h) after application\textsuperscript{84-90}. The remanence effect is particularly robust against a broad range of pathogens present on the skin after disinfection\textsuperscript{75}. It binds readily to negatively charged surfaces and is not locally absorbed, in which at least one part of the applied substance remains on the application site and thus provides a constant antimicrobial effect. As an effectively working antiseptic, OCT does not develop a resistance in its multiple applications\textsuperscript{74, 91-94}.

2.2.4 Toxicology

OCT is not locally absorbed by the skin or mucous membranes. Experiments performed in mice using \textsuperscript{14}C-OCT found no radioactivity in the serum. Similar results have been achieved with rats 24 and 48 h after oral dosing of \textsuperscript{14}C-OCT. No systemic effects are expected as a result of OCT being only used topically and not virtually absorbed\textsuperscript{74}.

Previous investigations have shown that OCT binds to primary KCs, murine FBs and human epithelial cells \textit{in vitro}. It is very stable after binding to the cell surfaces and once bound, it cannot be removed easily\textsuperscript{73, 95}. \textit{In vitro} cell culture studies have shown that an OCT-based solution has a cytotoxic effect on cultured cells such as human KC cell line HaCaT, primary human KCs and FBs\textsuperscript{96}. Favorable clinical results are in contrast to these results showing that OCT has good tissue tolerability in wound tissue samples\textsuperscript{97, 98}. These contradicting results can be explained by the fact that dermal cells are embedded in the ECM, while cultured cells are missing this potentially protective feature\textsuperscript{99, 100}. When applied, OCT forms stable complexes with cells which might lead to a favorable effect on tolerability due its exposure only on the top cell layer where it works as a high effective antibacterial shield to the deeper regenerating tissue. The preserved release of OCT from these complexes has non-cytotoxic, low and antimicrobial concentrations in the wound\textsuperscript{73}. The use of OCT has been shown also very suitable for a sensitive group of patients, such as newborns\textsuperscript{101} and pregnant women\textsuperscript{102, 103}.  

3. Objective of the thesis

Topical antiseptics are less potent to induce resistance using their unspecific mode of action and the high concentrations in which they can be used comparing to the use of antibiotics. Contrasting with other antiseptic substances (e.g. polihexanide, chlorhexidine, PVP-Iodine), OCT shows high efficiency already within a short time of application at low concentrations. In addition, the remanence effect lasts for more than 48 h\textsuperscript{84-90}. Recent studies in humans demonstrated that OCT does not delay the wound epithelization and significantly lowers bacterial colonization on skin wounds leading to a greater reduction in wound size and higher healing rates of skin graft wounds in burn patients\textsuperscript{104, 105}. Results from animal\textsuperscript{89, 94, 106} and clinical\textsuperscript{93, 99, 105, 107, 108} observations imply that OCT may have a positive influence on wound healing. Yet, the exact influence of OCT on human skin cells has not been fully investigated. Therefore, the main objective of my thesis was to uncover the mechanism of action of OCT on human skin cells. To address this we used different experimental set-ups:

\textit{i)} \textit{in vitro} cell culture of different skin cell types and
\textit{ii)} \textit{ex vivo} skin explant cultures

\textit{In vitro} experiments included the influence of different concentrations of OCT on primary human KCs and FBs. Furthermore, we applied a human skin explant model to study OCT effects on injuries caused by tape-stripping that were localized to the \textit{stratum corneum}, the most upper skin layer. The primary purpose was to study the influence of OCT on LCs and T cells and the quantification of multiple inflammatory mediators associated with wound healing in culture supernatants, such as cytokines, chemokines and growth factors.
4. Results

4.1 Standard OCT concentrations induce toxicity in human KCs and FBs in vitro

The effect of different OCT concentrations (ranging from 0.1-0.001%) on cell migration was assessed by using the in vitro scratch assay. For this purpose, cell monolayers consisting of primary human KCs and FBs, which were always isolated from the same donor, were scratched and cell migration into cell-free areas was followed after 48 h.

We found that 0.1% OCT, in contrast to the control medium, induce toxicity in both KCs and FBs as evaluated by trypan blue staining of the cells (data not shown) and consequently, failure of migration (Figure 4a, b), thus confirming and extending previously reported results obtained with a KC line\textsuperscript{100}. Migration of FBs with the lowest concentration of OCT (0.001%) was comparable with FB culture behavior without OCT (control) (Figure 4b), while KCs failed to migrate even at this low concentration (Figure 4a).

Figure 4. Scratch assay with human KCs and FBs. The standard and highest OCT concentration (0.1%) used induced toxicity in both KCs (a) and FBs (b). The lowest OCT concentration (0.001%) was not toxic for FBs thus allowing a migration behavior that was comparable with the untreated control (b), while in KC cultures many detached, dead cells, but no migratory cells were observed (b). Data with KCs and FBs from one representative donor are shown (n=6). Scale bars = 100 µm.
4.2 OCT does not alter skin anatomy upon culture

An *ex vivo* human skin organ culture model was employed to examine morphological and behavioral changes of skin cells in response to the topically applied OCT gel (OCT) as this culture system is currently the closest laboratory model akin to *in vivo* conditions. In a first step, we investigated the influence of OCT on skin morphology. To address this, we comparatively assessed non-tape-stripped (NTS) and tape-stripped (TS) human skin explants after 24, 48 and 72 h of culture without or with topical application of OCT and Normigel (control). TS was performed to study OCT effects on wounded skin. Paraffin-embedded skin samples were stained with haematoxylin and eosion (H&E) and analyzed.

No obvious morphological changes were identified in skin cells upon OCT treatment compared to the control-treated as well as untreated groups at all time points investigated. Although the *stratum corneum* was removed in one group (= comparable to a mild injured skin) to allow a better penetration of OCT through the skin, we observed no changes on skin morphology with OCT (Figure 5). Of note, while no OCT was observed on the top of the skin biopsy after the incubation period of 48 h, Normigel was still present and even persisted for 72 h (data not shown). These observations lead us to the conclusion that OCT does not seem to alter the human skin architecture upon culture and preserves its morphology similar to controls.
Figure 5. OCT does not change the skin morphology during culture. Shown are histological analyses of H&E stained NTS and TS skin paraffin sections after topical application of either Normigel (control) or OCT gel (OCT) or skin left untreated at the indicated time points of culture. OCT did not alter skin architecture when compared with control and untreated skin samples. Also the removal of the stratum corneum using TS failed to change the skin morphology. One representative donor is shown (n=7). Representative images were acquired with the same magnification. Scale bars = 200 µm.
4.3 OCT does not enhance apoptosis in human skin cells upon TS and culture

To examine whether OCT may influence the viability of human skin cells, we comparatively assessed TS skin explants before and after 48 h of culture without and with topical application of OCT and Normigel (control). Cryosections were stained with an Ab directed against active caspase 3.

We found no caspase 3+ cells in normal skin before culture. In contrast, caspase 3 activation was detected in some epidermal cells of all three groups, suggesting increased apoptosis possibly due to TS and culture rather than OCT treatment (Figure 6).

Figure 6. OCT does not augment apoptosis in human skin cells. Immunofluorescence staining of cryosections with an anti-rabbit caspase 3 Ab (green) and the nuclear counterstain with DAPI (blue) are shown. Occasional caspase 3 active cells (arrowheads and inserts) were observed in untreated, Normigel (control) and OCT-treated TS skin explant cultures but not in normal skin biopsies. The dotted white line indicates the dermal-epidermal junction. One representative donor is shown (n=7). Scale bars = 200 µm.
4.4 OCT apparently does not change LC morphology upon topical application compared with controls

Once human skin is excised and subsequently cultured, epidermal LCs get activated as characterized by the upregulation of markers like CCR7\textsuperscript{42}, CD40\textsuperscript{43}, CD80, CD83, as well as CD86, become mobilized and start to emigrate from the epidermis because of changes in the tissue resulting in the dissemination of migratory signals\textsuperscript{44}. This non-antigen mediated reduction of the LC density in the epidermis is visible in NTS untreated human skin specimens upon culture and was used as a baseline against which LC changes (frequency and mobilization) in skin samples in response to topical application of OCT and the Normlgel control were measured.

Analysis of immunohistochemical stained epidermal sheets, that were isolated from normal skin before any treatment and culture, with an Ab directed against CD207, a marker that is typically expressed on LCs, revealed a network of highly dendritic LCs (Figure 2), essentially as previously reported\textsuperscript{37, 38}. LCs also exhibited many dendrites from their cell surfaces after 24 and 48 h of culture in NTS untreated skin as well as in OCT and Normlgel treated NTS skin. Again, regardless whether NTS skin explants have been left untreated, or were treated with OCT or Normlgel, LCs underwent distinct changes after 72 h of culture in all groups. Many cells appeared round, some cells had only short surface protrusions or formed one or two single dendrites. The dendritic emergence of LCs in NTS untreated ex vivo human epidermis changed during the culture period of 72 h because LCs obviously responded to the culture environment. Of note, we found a general trend toward slightly less LCs in general and fewer dendrites/LCs in particular. No remarkable changes in LC morphology and density became apparent microscopically when OCT or Normlgel were applied onto the top of NTS skin at the beginning of the culture compared to the untreated NTS skin. However, when the skin was TS, a comparable picture to the 72 h culture period of NTS untreated skin was observed at 48 h already, regardless of the group tested (Figure 7). Unexpectedly, however, more LCs appeared to be present with a slightly better preservation of the dendritic morphology in the OCT group compared to controls. In conclusion, we found no major differences with regard to the “natural occurring” morphological changes of LCs in untreated skin during a culture period of 72 h when compared with cultures with OCT or the Normlgel control.
Figure 7. Representative images of immunostained LCs in human epidermal sheets at the indicated time points and treatments. The en face images of epidermal sheets from NTS cultured human skin showed an extensive network of CD207+ LCs (brown) with no observable morphologic changes in these cells between indicated treatments. In contrast, upon TS and 48 h of culture, LCs have changed their dendritic morphology and became round in all groups. After 72 h of NTS explant culture we observed LCs with a similar morphology as observed upon TS after 48 h. One representative donor is depicted (n=7). Scale bars = 200 µm.
4.5 OCT completely inhibits LC cell emigration in TS skin

Enumeration based on CD207 staining of non-cultured epidermal sheets revealed a baseline frequency of 1403±455 CD207+ LCs/mm². A slight depletion of LCs was apparent within 24 h post-culture in all NTS groups (untreated: 1090±157; Normlgel (control): 1125±153; OCT: 1230±162). LCs numbers decreased, though not significantly, during the observation period of 48 h (untreated: 1162±453; Normlgel: 847±97; OCT: 1089±233) and remained numerically quite similar at 72 h (untreated: 896±386; Normlgel: 1045±392; OCT: 997±400) irrespective of the treatment (Figure 8). Surprisingly, we found significantly higher LCs numbers in epidermal sheets prepared from TS skin that were treated with OCT (1545±373) compared to the control group (Normlgel: 945±272). In summary, we found that OCT prevents the emigration of LCs in TS but not in NTS skin explants.

![Figure 8. OCT precludes LC cell emigration.](image)

**Figure 8. OCT precludes LC cell emigration.** Epidermal sheets from indicated groups and time points were stained for CD207, an established LC marker, and LC numbers were counted. The LC density in TS OCT-treated skin biopsies at 48 h was higher than in normal skin (NS) before culture and significantly higher compared with Normlgel (Cont)-treated skin explants. LC numbers in NTS skin biopsies of the Normlgel (Cont) and untreated (UT) group decreased during the indicated observation period. Data presented are mean±SD (n=6). Unpaired t-test, *P≤0.05.

4.6 OCT inhibits the upregulation of the maturation marker CD83 on LCs

As we found that OCT prevents LC emigration in TS skin explants, we wondered whether this may correlate with an inhibition of CD83 upregulation. In normal healthy skin before culture, the vast majority of CD207+ LCs, with exception of a few cells, did not express CD83 in epidermal sheets (Figure 9a). However, after TS and culture for 48 h, many CD207+CD83+ LCs were found in epidermal sheets from untreated and Normlgel-treated skin, while only few were found in OCT-treated epidermis. As expected, enumeration revealed lower numbers of CD83+ LCs in epidermal sheets of OCT-treated TS skin compared to control groups (Figure 9b). Our data clearly indicate that OCT prevents the upregulation of CD83 on LCs in TS skin compared to control groups, implying that OCT may have anti-inflammatory properties.
Figure 9. OCT precludes upregulation of CD83 on LCs. (a) Epidermal sheets were stained with an anti-CD83 mAb (red) and an anti-rabbit CD207 Ab before culture or after TS and 48 h of culture using the indicated treatments. While in TS epidermis without treatment or treatment with Normlgel (Cont) we found an upregulation of CD83 on some LCs, indicating LC maturation, only a few LCs expressed CD83 in OCT-treated TS epidermis. Scale bars: 200 µm. (b) The numbers of CD83+ LCs were obtained from eight donors showing the lowest numbers of activated cells in the OCT-treated group compared to control groups. Unpaired t-test. P=0.0512.

4.7 OCT exhibits anti-inflammatory properties in TS human skin explants
Cytokines and chemokines are signaling proteins with activation, growth and differentiation functions which control immune cell trafficking and the cellular arrangement of immune organs, but also determine and regulate the nature of immune responses. To unravel whether the failure of CD83 upregulation on LCs in TS skin is associated with a different cytokine pattern compared to controls, supernatants from untreated, Normlgel- and OCT-
treated explant cultures of three different donors were collected. Pooled fractions were analyzed using a cytokine array, which represents a sensitive, rapid and economic tool to simultaneously detect a broad range of cytokines within one assay. Cytokines and factors that are expressed during inflammation and wound healing processes and were observed to be up- or downregulated in our experiments will be described as they appear on the array (from top to bottom) (Figure 10a).

Growth-regulated oncogene-alpha (Gro-α/CXCL1) is a member of the CXC family, which plays a major role in the enrollment and activation of neutrophils upon microbial infection or tissue injury. It is expressed in epithelial cells, melanocytes, and FBs and is closely related to IL-8/CXCL8. Gro-α is essential in wound healing processes\textsuperscript{110}, angiogenesis\textsuperscript{111} and tumorigenesis\textsuperscript{112}. Interestingly, we found an upregulation of Gro-α upon OCT but also Normlgel treatment compared to untreated skin.

Thymus- and activation-regulated chemokine (TARC/CCL17) is part of the CC chemokines and stimulates CC chemokine receptor 4 (CCR4). This receptor is expressed by human dermal cells and in KCs of lesional atopic dermatitis skin\textsuperscript{113-115} and recruits CCR4\textsuperscript{+} Th2-polarized T cells to the sites of local inflammation, leading to a Th2-type immune response\textsuperscript{116}. No TARC expression was found upon OCT treatment compared to low level expression in Normlgel-treated and untreated groups.

IL-8 belongs to the family of structurally related cytokines produced by phagocytes, epithelial cells, and mesenchymal cells that are exposed to inflammatory stimuli\textsuperscript{117}. We found marginal higher expression of IL-8 in OCT and Normlgel-treated groups compared to the untreated group.

IL-33 is a cytokine expressed mainly by stromal cells and its expression is upregulated following pro-inflammatory stimulation. It is expressed upon necrosis and alerts the immune system for tissue damage or stress\textsuperscript{23}. While low IL-33 levels were observed upon OCT treatment, supernatants of Normlgel-treated and untreated groups contained slightly upregulated IL-33 expression levels.

Macrophage inflammatory protein-3 alpha (MIP-3α/CCL20) is a CC chemokine, predominantly expressed in extralymphoid tissue and is responsible for directing the migration of DC precursors and memory lymphocytes to sites of antigen invasion\textsuperscript{118}. The expression of this chemokine seemed to be remarkably downregulated in OCT-treated skin explants compared to control groups.

Angiopoietin-2 (Ang-2), and vascular endothelial growth factor (VEGF) are believed to be key regulators of angiogenesis. In our array, Ang-2 was downregulated in Normlgel- and OCT-treated groups compared to untreated controls, while VEGF expression appeared relatively unaltered in the different groups.

The quantitative analysis of the different expression levels of the above mentioned cytokines and chemokines was further analyzed using two different methods: i) LEGENDplex (Figure 10b) and ii) ELISA (Figure 10c). Of note, for the analysis by both methods, supernatants were not pooled but analyzed separately from each of the three donors which may explain the partially different results obtained compared to array data. Data obtained with LEGENDplex more or less mirrored microarray data showing slightly, though not significantly, enhanced Gro-α levels.
upon OCT treatment compared to control groups (Figure 10b). In contrast, when the supernatants from the same experiments were analyzed with ELISA we found exactly opposite results: lower Gro-α levels upon OCT treatment when compared to control groups (Figure 10c). In total contrast to the array data we observed a downregulation of IL-8 expression upon OCT treatment using either method (Figure 10b, c). Of note, lower IL-8 concentrations were observed when using the ELISA method for analysis.

When TARC expression levels were analyzed by LEGENDplex we found a similar expression pattern as identified using array analysis: a tendency towards lower TARC expression levels upon OCT treatment as compared to both controls (Figure 10b).

The analysis of IL-33 expression with ELISA has shown a downregulation of this cytokine upon OCT treatment compared to control groups (Figure 10c).

While array data with regard to MIP-3α revealed a dramatic downregulation of this chemokine by OCT treatment compared to controls, this was less obvious when analyzed by LEGENDplex (Figure 10b).
Figure 10. Anti-inflammatory properties of OCT in TS skin explants. Human skin was TS and left untreated (UT) or was treated with OCT and Normigel (Control gel) for 48 h of culture. Pooled supernatants from three donors were analyzed. (a) OCT leads to either lower (blue boxes) or higher (red boxes) cytokine and chemokine levels when compared to the Normigel (Cont) and untreated (UT) group (black boxes). (b, c) The quantitative analysis of specific cytokines was determined using LEGENDplex (b) or ELISA (c). Data presented are mean±SD (n=3) performed in duplicates (LEGENDplex) or triplicates (ELISA). Unpaired t-test showed no significant differences.
4.8 OCT significantly blocks IL-8 secretion in NTS and TS human skin explants

We have identified in Figure 10 lower IL-8 levels in supernatants of TS OCT-treated skin explant cultures compared to control groups when analyzed by both, LEGENDplex and ELISA. To include more donors and to test whether this effect can also be observed in NTS skin, biopsies obtained from six additional donors were left untreated or were treated with either OCT or Normlgel for a culture period of 24, 48, and 72 h. From the same donors, biopsies were TS and similarly treated and cultured for 48 h. Throughout the observation period of 72 h, we found significantly lower IL-8 concentrations in supernatants of NTS OCT-treated skin explant cultures compared to controls even though a steady increase of IL-8 levels in supernatants of all groups was noticed (Figure 11). One explanation for the notable increase of IL-8 concentrations in supernatants of NTS OCT-treated skin explants upon 72 h of culture may be that OCT have been used up during this long culture period. Even though TS is not a harsh treatment for the skin, higher IL-8 levels in all groups including the OCT group were observed when compared to NTS skin at 48 h of culture (Figure 11). Combining all results (six donors for 24, 48 and 72 h and nine donors for TS 48 h treatment) it can be concluded that OCT has an anti-inflammatory capacity inhibiting IL-8 secretion in human skin biopsies at all time points and treatments investigated.

![Graph showing IL-8 secretion in NTS and TS skin explants](image)

**Figure 11.** OCT significantly inhibits IL-8 secretion in both NTS as well as TS human skin explants. IL-8 levels in supernatants of cultures with skin biopsies treated with OCT were significantly lower compared to controls at all time points and treatments investigated. Albeit IL-8 levels increased from 24 to 72 h upon culture, they remained significantly lower in supernatants of OCT-treated skin cultures compared to controls. Collected supernatants were tested for IL-8 content with ELISA. Data presented are mean±SD (n=6) performed in triplicates. Unpaired t-test, *P≤0.05.

4.9 Mixing of OCT with the culture medium does not significantly change IL-8 levels in the supernatants of explant cultures compared to its topical application

To investigate whether OCT during a potential contact with the culture medium can change IL-8 concentrations, we performed the following experiments. In group one, OCT was applied on the top of the skin biopsy representing our standard procedure (SP)]. In a second group, OCT was mixed with the cell culture medium (Med) before the skin biopsy was added. In a third
group, half of the OCT concentration used in the SP was applied onto the top of the biopsy and the other half of OCT was mixed with cell culture medium (SP/Med). The same experimental setup was performed with the Normlgel. All skin biopsies were incubated for 48 h.

We found significantly lower IL-8 levels in supernatants of all groups containing OCT, irrespective of the application route compared to control groups (Figure 12). Furthermore, our results showed no tremendous changes in the IL-8 levels between the three groups of OCT applications when compared to the groups with Normlgel and untreated controls (Figure 12). Consistently slightly higher IL-8 levels were measured when OCT was directly added to the culture medium, implying that a direct contact of OCT with skin cells is necessary for its strong inhibitory effect on IL-8 secretion. Interestingly, we found almost no IL-8 levels in supernatants when OCT was added onto the top and admixed with the supernatant. No comparable changes in IL-8 levels were observed between the different applications of Normlgel.

![Graph showing IL-8 levels in supernatants](image)

**Figure 12. OCT does not considerably change the IL-8 secretion pattern when mixed with the culture medium.**

OCT (50 µl) was i) applied on the top of the biopsy using the standard procedure (SP), ii) mixed with cell culture medium (Med), as well as iii) applied on the top and mixed with the culture medium (SP/Med; 25 µl OCT each). The same procedure was performed with Normlgel (control). Biopsies from all groups were incubated for 48 h and subsequently the supernatants were collected and analyzed for IL-8 content with ELISA. Data presented are mean±SD (n=3) performed in triplicates. Unpaired t-test, *P≤0.05, **P≤0.01.

We showed that all application routes of OCT significantly blocked IL-8 secretion from skin biopsies during the culture period of 48 h. As it is unlikely that these huge volumes may have dropped from the top of the biopsy into the culture medium during our SP, particularly as each well was manually inspected every day, also smaller and more realistic OCT volumes that may have dropped into the culture medium were tested.

Confirming our results shown in Figure 12, high OCT volumes (25, 50 µl) in the culture medium significantly inhibited IL-8 secretion (Figure 13). Low OCT volumes (10, 5, 1 µl) showed a concentration-dependent, though not significant, trend of IL-8 inhibition.
To assess a potential interference of OCT with the IL-8 ELISA, supernatants with high IL-8 concentrations from three donors, as determined before, were added with selected OCT volumes to the previously prepared ELISA dilution. Subsequently, IL-8 concentrations were determined with ELISA. Our results revealed no significant changes in the IL-8 levels between OCT-treated groups and the untreated group (UT) (Figure 14).

4.10 OCT significantly hinders IL-33 expression in human skin explants
In the cytokine array we found low IL-33 levels in culture supernatants of untreated and Normigel-treated TS skin biopsies and no detectable IL-33 expression upon OCT treatment (Figure 10a). These data were reflected by ELISA results (Figure 10c). To extend these initial
analyses, we have involved skin from other six donors and have included NTS skin in addition to TS skin.

Our results confirmed that IL-33 levels are significantly lower in 48 h culture supernatants of OCT-treated TS skin compared to control groups. Lower IL-33 levels, though only significant at 72 h, were also found upon topical OCT treatment of NTS skin compared to control groups (Figure 15). In contrast to IL-8, the highest IL-33 levels in NTS untreated skin samples and those treated with Normgel were observed at 24 and 48 h and decreased at 72 h of culture. Our results clearly show that OCT inhibits IL-33 expression in human skin explants and has anti-inflammatory properties.

![IL-33 secretion in human skin explants](image)

**Figure 15. OCT significantly inhibits IL-33 secretion in human skin explants.** NTS skin and TS skin was cultured without (UT) and with OCT and Normgel (Cont) at the indicated time points. Supernatants were analyzed with ELISA for IL-33 content. Data presented are mean±SD (n=6). Unpaired t-test, *P≤0.05.

### 4.11 OCT blocks Gro-α expression in human skin explants

Cytokine array and LEGENDplex analyses revealed that OCT leads to a marginal upregulation of Gro-α levels in supernatants of human TS skin explant cultures (Figure 10a, b). However, when supernatants from the same donors were analyzed with ELISA, we received contradictory results showing that Gro-α is rather down- than upregulated (Figure 10c). Subsequently, ELISA data were performed with supernatants of more donors and inclusion of NTS skin which was cultured for 24, 48 and 72 h and treated with either OCT or Normgel or left untreated and compared with TS skin from the same donors which were similarly treated and cultured for 48 h. Over the observation period of 72 h, we found significantly lower Gro-α levels in supernatants from OCT treated NTS skin samples when compared with supernatants from control samples (Figure 16). Gro-α levels in TS OCT-treated skin explant supernatants where comparable with those of NTS treated skin samples (Figure 16), thus confirming previously obtained ELISA results.
4.12 OCT does not alter VEGF levels in human skin explants

Cytokine array data with pooled supernatants from three donors with TS skin have shown that VEGF, one of the key regulators of angiogenesis, appear to be unaltered in control groups and slightly downregulated upon OCT treatment (Figure 10a). We involved additional six donors and included NTS skin at different time points of culture in addition to TS skin to test the validity of these data. Our ELISA results confirmed a trend towards marginal downregulation, though not significant, of VEGF concentrations upon OCT treatment in supernatants of both, NTS and TS skin samples compared to control groups (Figure 17). We found very low VEGF levels in supernatants of all three groups after 24 h of culture, which increased at 48 h and peaked at 72 h.

Figure 16. OCT significantly inhibits Gro-α expression in human skin explants. NTS and TS skin biopsies were cultured without (UT) and with OCT and Normigel (Cont) at the indicated time points. Supernatants were analyzed with ELISA for Gro-α content. Data presented are mean±SD (n=6). Unpaired t-test, *P≤0.05; **P≤0.01; ***P≤0.001.

Figure 17. OCT marginally influences VEGF production in human skin explants. NTS and TS skin explants were cultured without (UT) and with OCT and Normigel (Cont) at the indicated time points. Supernatants were analyzed with ELISA for VEGF content. Data presented are mean±SD (n=6). Unpaired t-test showed no significant difference.
4.13 Determination of selected cytokine levels with LEGENDplex

In order to assess contradictory results (e.g., Gro-\(\alpha\), IL-8) results and to further investigate two relevant cytokines detected by the cytokine array that are important for immune cells in the epidermis (e.g., MIP-3\(\alpha\) and TARC), we analyzed supernatants from six additional donors of TS treated skin samples that were either left untreated or were incubated with OCT or Normigel, with LEGENDplex. Confirming ELISA data described in Figures 10c, 11 and 16, we found significantly lower Gro-\(\alpha\) (Figure 18a) and IL-8 (Figure 18b) levels in supernatants of OCT-treated TS skin cultures compared to controls. Our cytokine array and LEGENDplex results showed to some extent, though not significantly, reduced MIP-3\(\alpha\) levels in supernatants of OCT-treated TS skin samples compared to controls (Figure 10a, b), which we confirmed using more donors (Figure 18c). Similarly, a trend though not significantly, we observed lower TARC levels in supernatants of OCT-treated TS skin biopsies compared to control groups thus validating the cytokine array results (Figure 18d).

Figure 18. Differential effects of OCT on Gro-\(\alpha\), IL-8, MIP-3\(\alpha\) and TARC secretion in TS skin biopsies. (a-d) TS skin was cultured without (UT) and with OCT and Normigel (Cont) for 48 h (n=6). Supernatants were analyzed with LEGENDplex for indicated cytokine/chemokine contents. Data presented are mean±SD (n=6). Unpaired t-test *\(P\leq0.05\), **\(P\leq0.01\).
4.14 OCT treatment remarkably though not significantly reduces T cell numbers in human skin

The influence of OCT on human skin T cells was evaluated by using the SP as described above. Interestingly, we observed in repeated experiments a trend, though not significant, that OCT reduced lymphocyte numbers in NTS as well as TS skin compared to controls upon 48 h of culture (Figure 19). OCT particularly reduced the numbers of CD3\(^+\), CD4\(^+\) and CD8\(^+\) T cells in TS but not NTS skin after 48 h of culture compared to controls. However, more donors need to be included to further investigate these initial observations.

![Lymphocytes](image1)

![CD3\(^+\) T cells](image2)

![CD4\(^+\) T cells](image3)

![CD8\(^+\) T cells](image4)

Figure 19. OCT diminishes lymphocyte and T cell numbers in skin explants. NTS and TS skin was cultured without (UT) and either with Normigel (Cont) or OCT for 48 h (n=4). Unpaired t-test showed no significant differences.
5. Materials and Methods

5.1 Isolation of different cell types from human skin

5.1.1 Primary epidermal KCs
Skin from anonymous healthy female and male donors (aged 20-55 years) was obtained during plastic surgery procedures (abdomen and back). The study was approved by the local ethics committee and conducted in accordance with the principles of the Declaration of Helsinki. A written consent was obtained from the patients. After surgery, the skin was disinfected using Betaisodona solution (Mundi Pharma, Wien, Austria) and Isozid (Gebro Pharma GmbH, Fieberbrunn, AUT). The skin was cut into small pieces (~5 mm²) and transferred into a 15 ml tube (Falcon, Corning, Schiphol-Rijk, NL) with 5 ml 1x Dispase II (100x; Roche, Penzberg, GER) diluted in KBM-2 medium (Lonza, Basel, CHE) and incubated horizontal (overnight (ON), 4°C). On the next day, epidermis and dermis were separated with two plastic forceps (Ärztebedarf Scherer GmbH, Höchst, AUT).

Epidermal sheets were incubated in a 5 ml trypsin/EDTA solution (PAN, 0.25 mg/ml; Gibco/Life Technologies, Paisley, UK) in a 15 ml tube and manually inverted and mixed (7 min, 37°C, water bath). Subsequently, the tube was vortexed (Vortex Genie 2, Bender & Hobein, Zurich, CHE) for approximately 10 seconds and DNase I (1 µl per 1 ml; Roche, Penzberg, GER) was added together with 5 ml medium (DMEM complete, Gibco/Life Technologies). The suspension was filtered through a 70 micron mesh (Miltenyi Biotech, Bergisch Gladbach, GER) into a 50 ml tube (Falcon). The previous tube was washed with 1x PBS (Gibco/Life Technologies) and filtered again. The whole suspension was afterwards filtered through a 40 micron mesh (Miltenyi Biotech) into a new 50 ml tube and the washing process was repeated. The cell suspension was spun down (1500 rpm, 2 min, room temperature (RT); Hermle, Wehingen, GER), the supernatant discarded, cells were resuspended in 35 ml medium (DMEM basal; Gibco/Life Technology) and plated in a 225 cm² cell culture flask. The medium was changed every second day until confluency was observed.

5.1.2 Dermal FBs
Dermal specimens were incubated in FB isolation enzyme working solution (200 µl collagenase (Thermo Fisher Scientific), 350 µl hyaluronidase (Sigma-Aldrich, St. Louis, MO, USA), 7 µl DNase I (Roche), and 7 ml of 5 mM MgSO₄ (Sigma-Aldrich) diluted in distilled water (Aqua bidest, Fresenius Kabi, Graz, AUT) for approximately 4 h (37°C, on a shaker). Afterwards, the suspension was filtered through a 70 micron mesh into a new 50 ml tube. The previous tube was washed with 1x PBS and filtered again. The suspension was then filtered through a 40 micron mesh into a new 50 ml tube and the washing process was repeated as described. The cell suspension was spun down (1500 rpm, 5 min, RT) and cells were washed with 10 ml medium (DMEM basal; Gibco/Life Technology) and spun down again (1500 rpm, 5 min, RT). Subsequently, the supernatant was removed and the cells were resuspended in medium (DMEM complete) and plated in a 225 cm² cell culture flask. The medium was changed every second day until confluency was observed.
5.1.3 Skin T cells
Human full-thickness skin biopsies (Ø=8 mm) were cut into small pieces and mixed with 435 µl L-buffer, 50 µl enzyme D and 2.5 µl enzyme A (whole skin dissociation kit, #130-101-540, Miltenyi Biotech), put into gentleMACS C tubes (#130-093-237, Miltenyi Biotech) and incubated (ON, 37°C). On the next day, the gentleMACS tubes were transferred to the gentleMACS OctoMACS dissociator (#130-095-937, Miltenyi Biotech) where the skin-specific program was run (h_skin_01). Next, the tubes were shortly centrifuged. DMEM complete medium (500 µl) was added to each gentleMACS tube and the suspension was filtered through a 70 micron mesh. The previous tube was washed with 4 ml of the DMEM complete medium and filtered again, after which the tubes were centrifuged (1500 rpm, 7 min). Upon removal of the supernatant, cells were resuspended with 300 µl MACS buffer (1x PBS, 5 mM EDTA (Thermo Fisher Scientific), 0.5 % BSA (Sigma‐Aldrich)). Subsequently, 100 µl of the cell suspension was transferred to FACS tubes (#352052, Thermo Fisher Scientific) and stained with mAbs directed against CD3, CD4, CD8 and CD45 as described in Table I. The suspension was vortexed and incubated (30 min, 4°C). After the washing step (1 ml, MACS buffer), DAPI (#D9542, Sigma‐Aldrich) was added for nucleus staining (1:500) and samples analyzed with flow cytometry (BD FACSVerse, Becton Dickinson, East Rutherford, NJ, USA).

5.2 Scratch assay
Primary KCs and FBs were seeded into a 6-well microtiter plate (TPP, Trasadingen, CHE) and grown in 2 ml complemented medium (DermaLife for KCs, DMEM for FBs) until they reached 80-90% confluency. The complete culture medium was removed before cells were scratched with a yellow pipette tip (Biozym Scientific GmbH, Hessisch Oldendorf, GER) in the middle of the well and washed (twice with 1x PBS) to remove cell debris. Thereafter, 1 ml cell culture medium was added. This was followed by taking a picture with a microscope (EVOS XL, Thermo Fisher Scientific) marking the time point “0 h”. Later, the cells were either left untreated or were incubated with either OCT (stock solution: 1.4% in water, Schülke & Mayr GmbH, Norderstedt, GER) at different concentrations (ranging from 0.1 - 0.001 %) that were diluted in the cell culture medium for 2 min and subsequently washed (2x with corresponding cell culture media) essentially as described previously100. The plates were further incubated at 37°C to assess cell migration for up to 48 h.

5.3 TS, human skin organ culture and epidermal sheet preparation
D102-square standard self-adhesive discs (CuDerm Corporation, Dallas, TX, USA) were applied onto the excised, disinfected full-thickness human skin with a constant pressure for 10 seconds. Fifty consecutive TSs were accomplished on the identical spot by the same performer to reduce variability and is a well-established method in our lab. The efficient removal of the stratum corneum was tested by immunohistochemical staining of punch biopsies (Ø = 8 mm) taken from NTS and TS skin. In parallel, NTS and TS skin biopsies were cultured in triplicates or quadruplicates per each group in medium (DMEM complete) for 24, 48 and 72 h without treatment or application of a control gel (50 µl; Normlgel®, 0.9% w/w sodium chloride solution in gel form, Mölnlycke Health Care AB, Göteborg, SWE) or OCTgel (50 µl; Octenilin® Wundgel,
0.05% OCT in gel form, Schülke & Mayr GmbH) onto the top of each biopsy (=epidermal surface), which was our SP (Figure 20). Supernatants were collected at selected time points as indicated in the respective Figures and frozen at -80°C for further analysis. From both, freshly isolated as well as cultured skin biopsies, one part was embedded in optimum cutting tissue compound (Tissue-plus, Scigen Scientific Inc., Gardena, CA, USA), snap frozen in liquid nitrogen and stored at -80°C until further processing, while the second part was fixed in 7.5% formaldehyde ON and afterwards embedded with paraffin wax in embedding cassettes (#M493-11, Simport, Beloeil, CA). Paraffin- and cryosections were cut (5 µm) and stained with H&E for histological, histochemical and immunofluorescence examinations. From another part of the biopsies, epidermal sheets were prepared by incubation (1 h, 37°C) on 3.8% ammonium thiocyanate solution (Carl Roth GmbH + Co. KG, Karlsruhe, GER) with the epidermal side up. Subsequently, the epidermis was carefully separated from the underlying dermis using forceps, washed twice with 1x PBS (5 min each), fixed (10 min, RT) with acetone (Merck, Kenilworth, NJ, USA) and stored at -80°C until further processing.

Figure 20. A schematic diagram and photograph of the human skin organ culture setup. OCT and Normlgel (=control gel) were pipetted onto the epidermal surface of human NTS or TS skin biopsies (Ø = 8 mm). All specimens were cultured at defined time points (24, 48 and 72 h) as indicated in the respective figure legends.

5.4 Immunofluorescence and enumeration of LCs
Freshly prepared or frozen epidermal sheets as well as cryosections were washed once with 1x PBS (5 min) and incubated (ON, 4°C) with the appropriate primary Abs. After washing steps (twice with 1x PBS, 5 min each), the samples were incubated with the appropriate secondary Ab (1 h, RT). Afterwards, the samples were washed again (twice with 1x PBS, 5 min each) and nucleus staining and mounting was performed with DAPI (20 µl Vectashield with DAPI; Vector Laboratories Inc., Burlingame, CA, USA). LC numbers at defined time points and treatments, were counted using ImageJ (1.51j, Wayne Rasband, National Institutes of Health, Bethesda, MD, USA). The total cell number per treatment group was obtained by counting cells from 6 images with 20x magnification.

5.5 Immunohistochemistry
Epidermal sheets were washed (5 min) with wash buffer (#S300685, Dako) and then incubated (5 min) in peroxidase solution (#S202386, Dako). Next, the samples were incubated (1 h, RT) with an Ab directed against CD207 (#NCL-L-LANGERIN, Leica Biosystems, Wetzlar, GER) diluted in Ab diluent (#S202230, Dako). The samples were washed for 5 min with wash buffer and then incubated (20 min) with a secondary mouse/rabbit Ab (#K5003, Dako). After a washing step with wash buffer (5 min), the samples were incubated (20 min) with a streptavidin peroxidase
solution (HRP, #K5003, Dako). The samples were then incubated (15 min) with AEC+ substrate solution (#K346430, Dako) and afterwards washed twice (wash buffer, 5 min), mounted with Aquatex (#108562, Merck Millipore, MA, USA) and viewed under the microscope (Olympus AX70, Hamburg, GER).

5.6 ELISA
Flat bottomed, 96-well plates were coated with the appropriate capture human Abs such as IL-8 (#M801 M801, Thermo Fisher Scientific) (ON, 4°C), IL-33 (#YYZ0914121, R&D), VEGF (#LD1716033, R&D), and Gro-α (#I2016051, R&D) (ON, RT). On the next day, the plate was washed with the wash buffer (0.05% Tween in PBS) and blocked with the blocking buffer (4% BSA in PBS-Tween 0.05% for IL-8) or reagent diluent (1% BSA in PBS for IL-33, VEGF and Gro-α) (1 h, RT). After blocking, plates were washed again and standards and samples were applied to the plate and incubated for either 1 h (IL-8) or 2 h (IL-33, VEGF, Gro-α). Next, the plates were aspirated and the detection human Ab was applied for IL-8 (#M802B, Thermo Fisher Scientific), IL-33 (#ZCX0714121, R&D), VEGF (#XQ1716032, R&D), and Gro-α (#BEQ1016052, R&D) and incubated for either 1 h (IL-8) or 2 h (IL-33, VEGF, Gro-α). Subsequently, the plate was washed and incubated with a streptavidin-HRP IL-8 (#34028, Thermo Fisher Scientific) and IL-33, VEGF and Gro-α (#338391, R&D) (20-30 min), respectively. The following standards were used: IL-8 (#SIL8), IL-33 (#1381993), VEGF (#1369580), and Gro-α (#1360317).

5.7 Human cytokine array
The human cytokine array for the detection of 36 different cytokines was purchased (#ARY005, R&D) and performed according to the manufacturer’s instruction. Briefly, array buffer 6 (2 ml) was pipetted into each well of a 4-well multi-dish. Each membrane was placed in a separate well. The number on the membrane should be facing upwards. The membranes were incubated (1 h, rocking platform shaker). Array buffer 6 was aspirated from the wells and the prepared supernatants (1.2 ml pooled supernatants from three donors) were added and incubated (ON, 4°C, rocking platform shaker). Each membrane was carefully removed and placed into individual plastic containers with 20 ml of 1x wash buffer. The 4-well multi-dish was washed with deionized or distilled water and dried thoroughly. Each membrane was washed 3x with 1x wash buffer (10 min, rocking platform shaker). For each array 30 μl of the detection Ab cocktail was added to 1.5 ml of 1x array buffer 4/6. Each array was carefully removed from its wash container. Wash buffer was allowed to drain from the array. The array was returned to the 4-well multi-dish containing the diluted detection Ab cocktail and incubated (1 h, rocking platform shaker). The washing was repeated and 2 ml of 1x streptavidin-HRP was added into each well of the 4-well multi-dish. Each membrane was removed from its wash container. The membrane was returned to the 4-well multi-dish containing the 1x Streptavidin-HRP, covered with the lid and incubated (RT, 30 min, rocking platform shaker). Each array was washed and each membrane was removed from its wash container and wash buffer was drained from the membrane by blotting the lower edge onto paper towels. Each membrane was placed on the bottom sheet of the plastic sheet protector with the identification number facing up. Chemiluminescent reagent mix (1 ml) was pipetted evenly onto each membrane. The membranes were covered
carefully with the top sheet of the plastic sheet protector and incubated (1 min). Chemi reagent mix was carefully squeezed out of the plastic sheet. Leaving membranes on the bottom plastic sheet protector, the membranes were covered with plastic wrap taking care to gently smooth out any air bubbles. The membranes were placed with the identification numbers facing up in an autoradiography film cassette and exposed to X-ray film (1-10 min).

5.8 LEGENDplex
The human proinflammatory chemokine panel is a multiplex bead-based assay panel, using fluorescence-encoded beads for the use in flow cytometry and allows simultaneous quantification of 13 human chemokines and was purchased (Biolegend, San Diego, CA, USA). This assay was performed in a V-bottom 96-well plate (#651101, Greier Bio-One, Kremsmünster, AUT). The reagents were warmed up before use. Standards were pipetted in the first two rows (1:4 ratio). Assay buffer 10 µl (#740075) (10 µl) and 10 µl of each supernatant were added to the sample wells (diluted 1:30 with assay buffer). Beads (Gro-α, TARC, IL-8, MIP-3α) were previously sonicated (1 min) in a sonicator bath, vortexed (30 sec) and diluted 1:13 with assay buffer and mixed beads (10 µl) were added to each well. The plate was sealed with a plate sealer, covered with aluminum foil and placed on a plate shaker (500 rpm, 2 h, RT). The fluids were removed inverting the plate and 200 µl of 1x wash buffer was added to each well and was removed by inverting the plate (2x). Detection Ab (#740072) (10 µl) was added to each well. The plate was sealed with a fresh plate sealer, wrapped with aluminum foil and placed on a plate shaker (500 rpm, 1 h, RT). SA-PE 25 µl (#740075) was added to each well. The plate was sealed with a fresh plate sealer, wrapped with aluminum foil and placed on a plate shaker (500 rpm, 30 min, RT). The fluids were removed inverting the plate and 200 µl of 1x wash buffer was added to each well and was removed by inverting the plate (2x). Wash buffer (100 µl) was added to each well and samples were analyzed on a flow cytometer (BD FACSVerse, Becton Dickinson).
5.9 Antibodies

<table>
<thead>
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<th>Ab specificity</th>
<th>Clone</th>
<th>Ig Class</th>
<th>Conjugate</th>
<th>Company*</th>
<th>Product #/Lot #</th>
<th>Working dilution IF/IHC/FACS</th>
</tr>
</thead>
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<tr>
<td>Caspase 3</td>
<td>D3E9</td>
<td>Rabbit IgG</td>
<td>Unconjugated</td>
<td>Cell Signaling Technology</td>
<td>9579</td>
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<tr>
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<td>Unconjugated</td>
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<tr>
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<td>Mouse IgG2b</td>
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<td>Leica Biosystems</td>
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<td>Mouse IgG1</td>
<td>Unconjugated</td>
<td>BD Pharmingen</td>
<td>556854</td>
<td>IF: 1:1000</td>
</tr>
</tbody>
</table>

2nd step reagents

| Goat anti-mouse | (H+L) | Alexa Fluor 546 | Life Technologies | A11018/1719601 | 1:500 |
| Goat anti-rabbit | (H+L) | Alexa Fluor 488 | Life Technologies | A11008/1515529 | 1:500 |

*Cell Signaling Technologies, Danvers, MA, USA; Sigma Aldrich, St. Louis, MO, USA; Leica Biosystems, Wetzlar, GER; BD Pharmingen, San Jose, CA, USA; Life Technologies, Paisley, UK; BD Biosciences, San Jose, CA, USA.

5.10 Cell culture media and solutions

5.10.1 MACS buffer

1x PBS
2 mM EDTA (autoclaved, sterile)
0.5% BSA

5.10.2 Fibroblast isolation enzyme solution

200 μl Collagenase
350 μl Hyaluronidase
7 μl DNase

5.10.3 DermaLife medium (KCs)

0.5 ml rh Insulin LifeFactor
15 ml L-Glutamine LifeFactor
0.5 ml Epinephrine LifeFactor
0.5 ml Apo-Transferrin LifeFactor
0.5 ml rh TGF-α LifeFactor
2 ml Extract P™ LifeFactor
0.5 ml Hydrocortisone Hemisuccinate LifeFactor
1% PenStrep

5.10.4 DMEM medium complete (FBs)
500 ml DMEM
10% FBS
1% PenStrep

5.11 Statistical analysis
Data was analyzed using GraphPad Prism 5. Unpaired t-test was used for comparing means, respectively. The results were considered significant with $P$-values smaller than 0.05. ImageJ (1.51j) was used for the analysis of immunofluorescence staining photos, as well as for scale bars.
6. Discussion

The application of topical antiseptics and antibiotics represents the first strategy of preventing and treating existing wound infection. The unrestrictive use of antibiotics leads to the development of multidrug-resistant germs. Antiseptics, on the other hand, are less likely to cause resistance because of their unspecific mode of action and their wide therapeutic window. Recent clinical observations demonstrated that besides high antimicrobial effects, OCT may also positively influence wound healing processes as it has a better biocompatibility and microbial efficiency than other investigated antiseptics. As the effects of OCT on human skin cells in their natural tissue environment were not well investigated yet, the main goal of the thesis was to study potential impacts of OCT on isolated human skin cells on one hand and on the architecture and phenotype of particular immune and nonimmune cells in excised viable human skin and wound healing on the other hand. In particular, the present study aimed to determine the effect of OCT on living, functional LCs in the biologically relevant environment of viable human skin.

Cell migration of primary skin cells (e.g. KCs and FBs) in vitro after treatment with different concentrations of OCT was assessed with the scratch assay. However, our experiments showed that 0.1% OCT was toxic for both cell populations. Interestingly, lower OCT concentrations were less toxic for FBs than KCs. Thus, our data are in line with and extend previous in vitro cell culture studies showing that an OCT-based solution has a cytotoxic effect on cultured cells such as human amnion cells, primary human KCs and FBs. Favorable clinical observations are in contrast to these results showing that OCT has good tissue tolerability in wound tissue samples. These contradicting results can be explained by the fact that dermal cells are embedded in the ECM, while cultured cells are missing this potentially protective feature as reported previously.

Skin organ cultures have been used previously to investigate the function of the skin immune system, toxicology and many others. We provide further work in this area and used this ex vivo model for the topical application of OCT focusing not only on skin viability and morphology, but also to follow LC behavioral changes over a long culture term. During the observation period of 72 h, we found no remarkable morphological changes of the skin architecture upon OCT treatment compared to control groups. Although the most upper layer of the skin, the stratum corneum, was removed by the TS technique in certain experiments to mimic a mild wound process and allowing a better penetration and thus a superior effect of OCT compared to NTS skin, there were still no obvious changes in skin morphology. Furthermore, no increased induction of cell apoptosis in skin cells after OCT treatment compared to controls was observed. These observations may explain the paradox why OCT has a toxic influence in vitro, but not in vivo. It was hypothesized that the formation of OCT and ECM components could decrease the OCT cytotoxicity while maintaining its antimicrobial activity in vitro. Previous studies reported that OCT reduced its cytotoxicity in the presence of chondroitin sulfate, which represents an ECM component. This may be one explanation why
OCT has no toxic impact onto skin cells and morphology in whole tissue samples and most perhaps also in vivo in patients.

LCs are very potent at uptaking, processing, and presenting antigens from the surrounding epidermis. Upon antigen uptake, a fraction of resident LCs, some of which bear antigen, functionally mature and migrate across the dermal-epidermal junction to draining lymph nodes to present the antigen to naive T cells to elicit an immune response. Several reports have described the properties of LCs in skin organ culture as they conceivably represent the closest laboratory model attainable to the in vivo environment with regard of fidelity to physiology as well as biological complexity, even though tissue viability in general vanishes from the time of excision. When healthy human skin is excised and cultured, like in our experiments, LCs in principal start to migrate from the epidermis due to mechanical trauma, triggering a degree of inflammation, orchestrated by skin cytokines and chemokines that are induced or constitutively produced by KCs or LCs. When following LC behavior, particularly their responses to OCT, over the culture period of 72 h, we found similarly decreased LC densities in NTS skin in all experimental groups. In contrast, LC numbers in OCT-treated TS skin were higher compared with normal skin before culture and significantly higher compared with Normlgel-treated skin. No obvious differences of LC morphology were observed in OCT treated NTS skin throughout the whole observation period compared to changes in the control groups. In all groups LCs partially retracted their dendrites over the whole observation period and was most obvious when viewed en face in epidermal sheets at the end of the culture period. In contrast, when epidermal sheets from TS skin were inspected, LCs in all groups displayed a “rounded” morphology. Previous studies demonstrated comparable changes in LCs after intradermal vaccination, showing a “rounded” morphology and lower LC numbers after 72 h of culture. A slightly more pronounced reduction in dendrites per LC was observed in control groups when compared with OCT treatment at 48 h. Our observations about morphologic changes of LCs in TS skin in all experimental groups extend those in a previous publication. Intriguingly, higher LC numbers in the TS OCT-treated group also correlated with the failure of an upregulation of the maturation marker CD83 on LC when compared with the control groups implying that LCs do not undergo a maturation process. Other receptors need to be investigated to come to a definite conclusion. These results showed that OCT prevents LC migration to the dermis, inhibits the upregulation of CD83 on LCs and thus implies that OCT may have some influence on signals usually favoring their emigration.

To unravel whether migratory signals for LCs may be altered upon TS and OCT treatment, potential changes in the production of cytokines and chemokines were analyzed in skin explant culture supernatants with two different methods (i.e. ELISA and LEGENDplex). Evaluation of the proinflammatory cytokine IL-8 indeed revealed higher IL-8 levels in TS skin compared with NTS skin at 48 h of culture, indicating that IL-8 secretion is upregulated in wounded skin. In contrast, significantly lower IL-8 concentrations were identified in OCT-treated skin cultures compared to controls at all time points and treatments. IL-8 represents a chemoattractant and potent activator of neutrophils during skin injury, can stimulate the endothelial permeability by facilitating leukocyte migration into injured tissue areas and shows increased expression...
levels in wounds. Previous research studies showed that the inhibition of mast cell activation and degranulation led to the downregulation of IL-1β and IL-8 in wounds, which influenced the healing response, characterized by the reduction in wound scar width and improved collagen fiber organization without negative effects. These date demonstrate an IL-8 impact and role in wound repair and its significance in inflammation. Previous experiments also revealed increased IL-8 levels in FBs from keloid scars compared with normal human FBs. This may highlight a possible role of IL-8 in activation in keloid scars and leukocyte recruitment. It was additionally stated that the equilibrium in inflammation regulated by low expression of proinflammatory cytokines like IL-8 and IL-6 is crucial in preventing the scar formation in the fetus. Therefore, downregulation of IL-8 secretion by skin cells, but also of other inflammatory-related cytokines, represents a key point in wound care.

When analyzing other inflammatory-related cytokines we identified that OCT also largely inhibited IL-33 secretion at all time points and treatments. Our findings that OCT has anti-inflammatory capacities are in the line with results from previous research studies, showing that OCT prevented TNF-α secretion, a cytokine involved in inflammation, apoptosis and immune response. Additionally, OCT led to a faster decay of wound inflammation in vivo without occlusive cover in pigs, where redness and swelling of the wound was absent after 4 days of treatment. OCT was also highly effective in the treatment of facial acne lesions. These data suggest that OCT indeed influences the secretion of inflammatory cytokines inhibiting their secretion in skin.

To examine why LCs remained in the epidermis upon TS and OCT treatment, we investigated whether cytokines that normally induce their migration (e.g. IL-1β, TNF-α, IL-18) are altered and have employed a multiple cytokine array system with pooled supernatants from three donors. Unexpectedly, all of the aforementioned cytokines were similarly expressed in the experimental groups tested (data not shown). Unexpectedly, higher IL-8 expression levels in supernatants of OCT treated skin samples were found, which was in contrary to our previous results obtained with other six donors and quantitative analysis by ELISA and may represent inter-individual variation as reported previously. When focusing on the expression of Gro-α, whose expression seemed higher in TS OCT treated skin, quantitative analyses with ELISA and LEGENDplex demonstrated again opposite data, where Gro-α expression was lower in TS OCT treated samples compared to controls. Again, these contradictory results between the cytokine array data and quantitative analyses of cytokines presumably reflect inter-individual distinction. Previous research showed that Gro-α stimulates KC proliferation and angiogenesis in cutaneous wound healing. Its role in our model needs to be evaluated in future experiments. The human cytokine array revealed lower expression of MIP-3α in supernatants of TS OCT-treated skin samples compared to control groups, which was confirmed using quantitative analysis with LEGENDplex. MIP-3α is predominantly expressed in extralymphoid tissue and is responsible for directing the migration of DC precursors and memory lymphocytes to sites of antigen invasion. Our results show markedly, though not significantly reduced MIP-3α levels upon TS and OCT treatment. It remains to be clarified whether both KCs and venular endothelial cells produce less MIP-3α or only KCs which are in close contact with OCT in our skin model. Consequently, it will be interesting to investigate whether LCs still express the exclusive MIP-3α...
receptor CC chemokine receptor 6 (CCR6) upon OCT treatment. Other receptors which are involved in LC migration need to be explored yet.

TARC stimulates the CC chemokine receptor 4 (CCR4). This receptor is expressed on TH2 lymphocytes and recruits CCR4+ TH2-polarized T lymphocytes to the sites of local inflammation, leading to a TH2-type immune response. Human cytokine array from three donors as well as LEGENDplex data including additional six donors showed lower TARC expression in TS and OCT-treated skin compared to control groups. In line with this observation we found lower numbers of lymphocytes, CD3+, CD4+ and CD8+ T cells in OCT-treated skin. As no T cells can be recruited from the periphery into the wounded skin it is unlikely that TARC influences T cell recruitment in our model. Even though not more apoptotic cells were found in TS OCT-treated skin, we cannot exclude that during preparation of the single cell suspension for flow cytometry analysis T cells in this group are more prone to apoptosis than T cells in control groups and remains to be investigated.

A previous study investigating the correlation between IL-8 and VEGF secretion reported a possible mechanism by which IL-8 and other inflammatory mediators may promote the expression of VEGF in endothelial cells. Using both human cytokine array as well as ELISA analyses we identified a minor, but not significant inhibition of VEGF production but significant inhibition of IL-8 secretion in OCT-treated skin. These data imply that OCT does not affect VEGF secretion in normal skin.

In conclusion, our data not only provide novel insights into the host response to OCT within the viable human skin, but also suggest, in addition to its known antimicrobial activity, an anti-inflammatory action that might positively contribute to its wound healing influence.
7. References


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9. Abstract

Prevention of infections by using antiseptics is a key element in professional wound management. Ideal agents for the topical treatment of skin wounds should have anti-microbial efficacy without negative influence on wound healing. Octenidine dihydrochloride (OCT) has become a widely used antiseptic in „modern” wound care, yet its effects on skin physiology are mostly unknown. We have tested its impact on skin cells upon topical application on ex vivo non-tape-stripped and tape-stripped (simulation of wound situation) human skin explants. Hematoxylin and eosin as well as immunofluorescence staining revealed that standard OCT concentrations alter neither human skin architecture nor the viability of skin cells upon a culture period of 72 hours. Immunohistochemistry of epidermal sheets revealed that not only the epidermis of explants but also CD207⁺ Langerhans cells in OCT-treated skin remained morphologically intact and comparable to controls throughout the culture duration. Unexpectedly, OCT inhibited the upregulation of the maturation marker CD83 on Langerhans cells and prevented their emigration in tape-stripped skin that was accompanied by low MIP-3α levels. Additionally, OCT revealed strong anti-inflammatory capacity as shown by the inhibition of IL-8 and IL-33 upregulation in skin explant cultures. OCT largely blocked the upregulation of Gro-α, which is involved in the processes of wound healing and angiogenesis without significantly influencing the production of VEGF involved in vasculogenesis and angiogenesis. In conclusion, our data provide novel insights into the host response to OCT within the biologically relevant environment of viable human skin, suggesting, in addition to its known antimicrobial activity, an anti-inflammatory action that might positively contribute to the wound healing influence of OCT.
10. Zusammenfassung