The goal of this project was to investigate the role of antigen presenting cells, such as dendritic cells (DCs) and Langerhans cells (LCs), in the complex intercellular communication network of the skin. We planned to determine the receptor-arsenal of *in vitro* differentiated DCs and LCs with Next-Generation Sequencing, and to use these generated data to find possible targets of keratinocyte- and sebocyte-derived factors. The putative communication between these cells is bidirectional, so we also planned on finding LC and DC-derived factors that influence the major biological functions of keratinocytes and sebocytes.

As a first step we mapped the receptor repertoire of antigen presenting cells, which we performed using both RNA sequencing and RT-qPCR. The total exome of these cells yielded a large amount of data that holds promise to serve as the basis for future experiments. In the current project we decided to focus on receptors that have already been implicated in the common inflammatory skin diseases that we aimed to target to exploit the translational potential of our results. Of these receptors (histamine receptors H1, H3 and H4; proteinase-activated receptors PAR2 and PAR4; endothelin receptors ETAR and ETBR; Toll like receptor 3 [TLR3]; kappa opioid receptor; niacin receptor; transient receptor potential vannilloid 4 [TRPV4]) we identified the presence of H1 and 4, TLR3, HCA2, TRPV4. With the exception of histamine, which has been characterized on LC before, we tested the effect of agonists to these receptors (Niacin, GSK1016790A and polyI:C) on the expression of markers of antigen presenting cell activation (CD83, CCR7), pattern recognition receptors (e.g.: TLR3), and secreted factors that activate neighboring immune cells (IL-6, CXCL-8, CCL20). Based on the results of these pilot experiments we identified TRPV4 as an interesting target for our further investigations.

TRPV4 is part of a group of thermosensitive transient receptor potential ion channels that, while nonselective cation channels, are mostly calcium permeable. Activation of TRPV4 led to upregulation of multiple markers listed previously (e. g. IL-6, CD83, TLR3), which hinted that it is functionally expressed on these cells. TRPV4 is an especially good candidate to influence LCs, since two of its major activating factors (hyperosmolarity and UV radiation) are easily found in the epidermis, where LCs reside *in vivo*. The channel has also been shown to be expressed on keratinocytes where its activation can contribute both to pain and itch sensation. These results were presented at two international conferences in 2018.

To test whether TRPV4 expression on LCs is functional, we performed fluorometric calcium measurements, which showed that the specific agonist of TRPV4 (GSK1016790A) dose-dependently increased the calcium concentration in the nanomolar range, which effect could be completely abrogated by the addition of a TRPV4-specific antagonist (HC 067047). Since the calcium homeostasis of immune cells is tightly regulated and is an important factor in their differentiation and maturation (as evidenced in our previous work on the role of TRPV1 and 2 on DCs, (Toth et al., FEBS Lett. 2009; Szollosi AG et al., FEBS Lett. 2013), we next investigated the effect of long-term agonist treatment on the differentiation and maturation of the cells. We found that applying GSK from day 0 or day 2 of the differentiation protocol doubled the amount of CD207 and CD1a positive cells in our culture, which effect was once again abrogated by the application of the specific antagonist. Interestingly, the TRPV4 antagonist HC applied alone decreased the amount of differentiated cells when applied from day 0, but had no effect from day 2, which hints that TRPV4 might be important in the dedication of monocytes into the differentiation program. We also investigated the acute effect of GSK on differentiated LCs, where we found that TRPV4 activation decreased the endocytotic activity of the cells.

To gain a broader understanding regarding the secretome of LCs we next performed cytokine arrays, and then validated these results with specific ELISAs (proving that the cells produce IL-6, CXCL-8, MIF, MMP-9, CCL-17). We have also investigated the efficacy of these LCs in stimulating T cell proliferation. LCs from multiple donors were capable of inducing significant T cell expansion, and TRPV4 activation decreased this function of LCs. Overall we found that TRPV4 agonist treated cells tend more toward anti-inflammatory, rather than a classical inflammatory phenotype. Due to the COVID-19-induced restrictions we were not able to finish the manuscript based on these results, but we hope to submit the manuscript soon, since the experiments required for the publication have been completed. Although the manuscript based on these results is still not completed, the results outlined above were presented at two conferences, a national conference in 2018 and an international one in 2019.

The second major branch of the project was to investigate the effect of DC and LC-derived factors on the major functions of keratinocytes and sebocytes. Supernatants from both types of antigen presenting cells were collected from multiple donors, and we first determined the proper dilution of conditioned media to use without influencing the viability of skin cells. We next

investigated the effect of the conditioned media on the barrier function, the neutral lipid production and the expression of immunomodulatory proteins of skin derived cells (keratinocytes and sebocytes). In spite of the promising preliminary results presented in the grant application, the effects of these supernatants on skin cells was not consistent from donor to donor. This forced us to shift our focus into more consistently reproducible stimuli that would mimic at least some aspects of the intercellular network we wished to investigate. Building on results from previous work performed by the PI we investigated the role of TLR3 on human keratinocytes. We found that the expression of pattern recognition receptor TLR3 increases in keratinocytes in inflammatory dermatoses characterized by chronic itch, and that the activation of these receptors results in the production of inflammatory cytokines such as IL-6 and CXCL-8, which can influence the functions of neighboring immune cells. We have also shown that the endothelin produced by keratinocytes activates nerve endings in their close proximity, and that this may form the basis of a positive feedback loop that underlies chronic pruritus. The manuscript based on these results was submitted and accepted to the Journal of Investigative Dermatology, one of the most prominent dermatological journals (currently third in Scimago rankings for dermatology).

We also investigated the role of the abovementioned TRPV4 ion channel on the regulation of hair growth. In this manuscript we detailed not only the expression of TRPV4 on various compartments of the hair follicle, but also the effect of TRPV4 agonism on the hair follicle. The TRPV4 agonist GSK1016790A caused a significant decrease in hair shaft elongation in a concentration-dependent manner, suggesting that TRPV4, dose dependently inhibits hair shaft elongation *in vitro*. GSK1016790A treatment also decreased the ratio of anagen HFs and increased the number of catagen HFs, showing that TRPV4 activation induces premature catagen regression. Quantitative analysis of Ki67/TUNEL-positive cells (which mark proliferating and apoptotic cells, respectively) in the area of matrix keratinocytes showed that TRPV4 activation significantly decreased the ratio of proliferating cells and increased the number of apoptotic cells. All of the observed effects—on elongation, catagen induction, and ratio of apoptotic/proliferating cells—could be blocked by HC067047. These effects highlight the important role that the TRPV4 ion channel plays in the complex paracrine-autocrine communication network of the hair follicle, which most probably effects other neighboring cells such as LCs and DCs. These results were also published in the Journal of Investigative Dermatology.

We also investigated the effect of plant derived cannabinoids on the maturation and differentiation of DCs, which we presented at two national conferences. Although this latter result is not directly outlined in the research plan, the results of these investigations are especially interesting in the context of skin-resident antigen presenting cells, since the phytocannabinoids tested (cannabidiol, cannabigerol, cannabinol and tetrahydrocannabinol) are increasingly common in topical treatments for dermatitises. Further information about their effect on the orchestrators of the immune response in skin, i.e. DCs and LCs, is of paramount importance to understand their possible therapeutic applications. The signaling networks uncovered in these experiments can then be investigated in the context of endogenous ligands of the cannabinoid system (endocannabinoids), many of which can be produced by sebocytes and keratinocytes. We have found that cannabinoids differentially modulate the differentiation and maturation of DCs, as determined by their surface marker expression.

During the final year of the project we continued our research into the complex intercellular communication network between antigen presenting cells of the skin and their closest "neighbors". In the course of this period, we modified the planned experiments based on our RNASeq results from the previous year. According to our results monocyte-derived DCs and LCs express several distinct receptors that are capable of responding to sensory nerve ending-derived peptides (neuropeptides). Since LCs are commonly associated to free nerve endings in the epidermis in human skin, we expanded the focus of our research to neuropeptides to supplement our work with keratinocytes and sebocytes. We validated the expression of neuropeptide receptors found in our RNASeq results (RAMP1, CALCRL, SORT1, NPR1) on LCs with RT-qQPCR. To assess the functionality of these receptors we treated DCs and LCs with agonists of these receptors (calcitonin gene related peptide, neurotensin, B-type natriuretic peptide [BNP]) and observed their effects on the differentiation and maturation of the cells. Of the tested agonists BNP had the most striking effect, as it was capable of significantly increasing both the differentiation and maturation of LCs, and based on these results we plan on examining this effect in more detail in the future. To support these further experiments an FK2020 grant proposal was submitted and approved, which will allow us to continue our research into this exciting field, and shows that the current project was successful in leading into further research areas. These results were also presented at a national conference.

We have also published a review article detailing the role of heme oxygenase in healthy and diseased human skin. In this review we highlighted the role of heme oxygenase as an important cytoprotective enzyme with anti-inflammatory and anti-apoptotic properties.

In addition to the direct work of the group detailed above we have also contributed to work done by our collaborational partners. A full research article regarding the effect of volatile anesthetics on transient receptor potential channel melastatin 3 was accepted in the current year, on which two members of the research team were coauthors.