ROLE OF NEUROIMMUNE COMMUNICATION IN CUTANEOUS IMMUNOLOGICAL PATHOPHYSIOLOGY

FINAL REPORT

The research program of this grant was split into 6, partly parallel tasks.

Task 1 involved the validation of our previously published RNA-Seq data using RT-qPCR, Western blot, and immunohistofluorescence. While this task was planned to be completed in the first year, parts of it were still underway during the second year. During this time we successfully validated our preliminary results at the RNA level by determining the expression of calcitonin gene related peptide (CGRP; CALCRL and RAMP1), neurotensin (SORT1), and B type natriuretic peptide (BNP; NPR1) receptors with RT-qPCR on human monocyte-derived Langerhans cells (moLCs) and immature and mature dendritic cells (iDCs and mDCs). We found that all the investigated receptors are expressed on all investigated cell populations (monocytes, monocyte-derived LCs and DCs), with the exception of NRPR1, which is not expressed on monocytes, and is most highly expressed on moLCs. Based on this unique expression profile of NPR1 we next determined the expression of NPR1 on the protein level using Western blot and immunohistochemistry. Due to the difficulty of attaining fresh samples because of COVID-19 induced restrictions in place at the time, we decided to use paraffin-embedded sections as the first step, to avoid extended delays in the execution of the project. This minor deviation from our plans did not result in significant changes to the overall timeline, as we still collected and utilized fresh frozen sections as well. As a positive corollary of this adaptation, we were also able to investigate the expression of these receptors on a wider range of clinical samples, as paraffin-embedded blocks were (and still are) more readily available from the Department of Dermatology. This change allowed us to execute Task 6 earlier than planned, as detailed below.

Task 2 focused on investigating the functional effects of neuropeptide ligands of the receptors studied in Task 1 (namely, CGRP, neurotensin and BNP) on both iDCs and moLCs. We found that none of the tested concentrations (which were determined based on data available in the literature) were cytotoxic, nor did they induce apoptotic processes. BNP had the most striking effect on the differentiation of LCs, as it dose-dependently increased the expression of both CD1a and CD207 (Langerin), both markers of differentiated moLCs. Interestingly, other maturation markers were not similarly affected by BNP alone, but cotreatment with TLR agonists (CL-075 and peptidoglycan [PGN]) resulted in increased expression of maturation markers. Task 2 was modified during the second year of the grant to improve the impact and scope of the generated results. This was necessary because the observed effects of some neuropeptides (specifically neurotensin and CGRP) on monocytes, moLCs and iDCs were relatively minor, which would have meant that performing subsequent planned experiments (under Tasks 3 and 4) would likely have wasted resources. To counterbalance the lack of positive findings in this area we decided to expand the investigated cell types into THP-1 derived macrophages, as an additional antigen presenting cell (APC) population. Although not originally part of our planned experiments, we decided to investigate THP-1 derived macrophages since they are an accepted model of macrophage functions, and performing experiments on these immortalized model cells did not increase the cost of our planned experiments in a significant way. In spite of initially promising results most neuropeptides that target the receptors expressed on the cells had no significant effects on macrophage polarization derived from THP-1 cells. Overall in

this task, which was started in the first year of the project and was finished in the first couple of months of the third year, we identified BNP as a novel regulator of moLC differentiation and focused on elucidating the effect NPR1 agonists have on the antigen presentation and T cell polarization induced by moLCs in Task 3.

Task 3 was split into three parallel subtasks, which focused on distinct aspects of APC activation. We investigated the effect of NPR1 agonist neuropeptides on allogenic T cell cocultures in Task 3a, on the secretome of APCs in Task 3b and on the polarization of activated T cells in Task 3c. All three subtasks were initiated in the second year of the project and concluded at the beginning of the final year. Of the investigated APCs and neuropeptides, we found that both A-type natriuretic peptide (ANP) and BNP had similar effects as both decreased the ratio of proliferating T cells in activated moLC-T cell cocultures, and both decreased the release of proinflammatory cytokines. This is not unexpected as both act as ligands of the same receptor, namely NPR1. The activated T cells characterized under Task 3c were found to be neither Th1 nor Th2 cells, and are most likely polarized toward a regulatory phenotype. moLCs differentiated in the presence of NPR1 agonists were found to upregulate pathways and genes associated with tolerance, namely Indoleamine 2,3-dioxygenase 1 (IDO1), as well as ICOSLG, IL10RA, CCR7 and others, which collectively show a migratory and tolerogenic phenotype of moLCs.

We investigated the secondary messenger pathways activated by ANP and BNP in Task 4, which was performed in the third year of the project. We performed extensive calcium homeostasis experiments that proved that intracellular calcium is not measurably affected by BNP and ANP treatment either alone, or in combination with TLR agonists. As NPR1 has been associated with increased intracellular cGMP in previous studies, we also assessed the involvement of this secondary messenger pathway and could prove a dose-dependent increase in cGMP in treated cells.

Task 5 focused on the potential bidirectional crosstalk between nerves and APCs, in which we planned to use indirect and direct cocultures between these cells. Because of changes we had to make in the planned budget of the project, which necessitated a decrease in the amount of funds available for research expenditures to cover the salary of one of the researchers working on the project we were only able to establish indirect cocultures. In these, conditioned media from neuropeptide and TLR-ligand-treated moLCs were applied to dorsal root ganglia (DRG) cultures established from mice, and we performed fluorescent calcium imaging on DRG neurons. As the conditioned media from treated cells showed no marked effect compared to conditioned media from vehicle treated control cells, we decided that with the limited resources available executing the rest of this task would most likely not yield sufficient data to justify the expenditure.

In contrast to the limited success of Task 5, in Task 6 – where we aimed to increase the translational potential of our results by validating them on human samples – we were able to start our experiments sooner than planned, thanks to two major factors. On the one hand the protocols optimized in year 1 and 2 as well as access to paraffin-embedded tissue enabled rapid investigation of LCs and neuropeptide receptors in common inflammatory skin diseases such as atopic dermatitis and psoriasis. On the other hand, restructuring of the grant to cover the salary of one of the researchers involved in executing the research plan necessitated streamlining some of the planned experiments pertaining to other tasks, mainly Task 5 as detailed above. To increase the impact of these *in situ* experiments we also initiated a collaboration with the Department of Biophysics to acquire full-slide scans of skin

combined with high magnification confocal images of LCs and their environment. This allowed us to accurately pinpoint the neuropeptide receptors expressed on these cells. We have successfully identified the presence and distribution of LCs in both healthy donors and common inflammatory skin disorders, as well as the expression of neuropeptide receptors on LCs in these lesions.

The above detailed results will be assembled into two separate manuscripts, one detailing the effects of neuropeptides on LC differentiation, and the second on the effect of NPR1 agonists on LC functions and APC-T cell communication. These manuscripts will be submitted in the year following the close of the project, as both manuscripts are currently being written.

Dissemination of the results obtained during the project was achieved through oral and poster presentations at national and international conferences (a total of 6 poster presentations and one oral presentation), three review articles published regarding the roles of APCs in psoriasis, the role of cutaneous immuno-neuronal crosstalk in the development of itch, the role of opioidergic signaling in atopic dermatitis, as well as four research articles in highly acclaimed journals detailing (i) the effect of anandamide on the inflammatory responses of human corneal epithelial cells, (ii) the function of TRPV4, a non-specific cation channel, on LCs, (ii) the role of cannabidiol, a phytocannabinoid, on monocyte-derived DC differentiation and maturation, and (iii) the effect of anandamide on moLCs. The first article investigated the effect of anandamide, a prototypical endocannabinoid, on the inflammatory response elicited by TLR3 agonism and UV radiation in human corneal epithelial cells. We found that in this context anandamide is more pro- rather than anti-inflammatory, which highlights the need to specifically investigate the effects of putatively anti-inflammatory substances in a cell-specific manner. The second research article was published in the Journal of Investigative Dermatology, a D1 ranked journal in the field of dermatology. In this manuscript we showed that TRPV4, which is implicated in multiple skin pathologies ranging from sunburn to atopic dermatitis, and has thus emerged as a potential therapeutic target in dermatological conditions, is functionally active on LCs. Therapies targeting this channel therefore will also have effects on the immune system, which might be beneficial as activation of the channel resulted in an anti-inflammatory response. The third paper was published in Frontiers in Immunology, which is listed as a Q1 journal in Immunology. As phytocannabinoids are increasingly utilized in various therapies including epileptic disorders, tumor treatments, and inflammatory disorders among others, the characterization of direct effects of phytocannabinoids on immune cells is essential. Many newer therapies targeting the skin utilize topical formulations, which will allow phytocannabinoids to directly access both LCs in the epidermis and dendritic cells in the dermis. In our results we showed that contrary to described effects on other immune cells CBD was only anti-inflammatory on DCs when applied in parallel to other activating stimuli, but not when applied alone. This highlights the fact that care must be taken when using these compounds for extended periods of time, as in differing inflammatory contexts their effects might be drastically different. The fourth article was also published in Frontiers in Immunology, and detailed the effects of one of the prototypical endogenous cannabinoids, anandamide, on the differentiation and activation of moLCs. As mentioned above cannabinoids are increasingly used in both systemic and topical formulations, and these preparations can influence the endogenous cannabinoid system. Since endocannabinoids can be considered neurotransmitters (if not neuropeptides due to their lipid-like structure), investigating the effects of one of the most abundant such transmitters in the skin is essential to understanding LC physiology. In this article we found that anandamide has nuanced effects on the differentiation, maturation, cytokine secretion, metabolism and function of activated moLCs.

Among these changes the decrease in CD1a expression on moLCs holds promise to selectively dampen inflammation induced by CD1a restricted T cells, while the increase in T cell activatory capacity of moLCs and the shift toward a Th1 phenotype provides important immunological context to its varied effects.