Results of the NKFIH project K 124813 (Signal transduction therapy in cancer and inflammation)

1.The results of the investigations on macrophage polarization

Macrophages differentiated from HL-60 cell line and isolated from human blood monocytes (HBM) were identified by the expression of CD68 and CD11b markers. The localization of these markers was different, the former is intracellular, the latter is rather membrane localized. Macrophages were polarized with LPS+IFNγ (M1) and IL-4 (M2) treatment, respectively. Nitric oxide synthase (NOS) 2, a marker of M1 polarization has been expressed in M1 polarized cells, but its level was much lower in HL-60 cells compared to HBM cells. Inflammatory cytokines, as IP-10 and TNFα were detected in both cell types, but the cytokine pattern was different. IL-8 was found higher in M2 polarized cells. The relationship between polarization markers and signalling pathways was also studied, by using specific protein kinase inhibitors. Since the phosphorylation of Erk1/2 and p38 increased and their inhibition by kinase inhibitors caused the decrease of NOS2 and inflammatory cytokines, the role of MAPK pathway has been certified, in addition to the canonical JAK/STAT pathway. In M2 polarized HL-60 cells, the role of HSP27 phosphorylation has been proved: kbNB 142, a protein kinase D (PKD) inhibitor decreased the phosphorylation of HSP27 and the expression of IL-8 cytokine. In addition, dorsomorphine, an AMPK-inhibitor also reduced IL-8, but in a PKD-independent way. On the other side, MAPK and JAK inhibitors caused an increase of IL-8 in M1 polarized cells, indicating a partial repolarization form M1 to M2 macrophages, while M2 inhibitors caused the increase of IP-10 in IL-4 treated cells, suggesting an M2 to M1 repolarization. Nevertheless, these effects were restricted only to certain markers, therefore general repolarization could not be achieved by the specific protein kinase inhibitors. Publication: a paper recently published in Heliyon (https://doi.org/10.1016/j.heliyon.2021.e08670)

In rat peritoneal macrophages, CD68 markers were used to identify their macrophage character. Interestingly, both polarization markers (CD80 and CD206) were found in resident and inflammatory macrophages as well, i.e., it could not be determined whether these macrophages are M1 or M2 polarized cells. The tested kinase inhibitors caused only a slight or no reduction in the expression of CD80 and CD206 markers. The Erk1/2 phosphorylation increased in casein-elicited cells, but the p38 phosphorylation was lower. Most of STAT proteins were phosphorylated higher in inflammatory cells, except STAT3, and IL-4 treatment did not reduce it. NOS 2 expression was higher in inflammatory cells, which also produced high amount of nitrite, the end product of NO synthesis. Inhibitors of MAPK, p38 and JAK/STAT pathways decreased the expression of NOS2. Arginase expression was lower and not different in resident and inflammatory cells. IP-10 was higher in inflammatory cells, and its production decreased by certain kinase inhibitors. RANTES expression was higher in resident cells (RANTES), but the inhibitors of MAPK and JAK/STAT pathways did not increase but decreased further the level of this cytokine. Phagocytic capacity was significantly higher in inflammatory macrophages and could be inhibited by MAPK and JAK/STAT inhibitors. Summarizing our results, the polarization of the resident and inflammatory macrophages could not be identified unambiguously, and the effects of kinase inhibitors cannot be explained exclusively by their specificity on the signalling pathways. Publication*: „The effects of selected protein kinase inhibitors on the inflammatory markers of rat peritoneal and human blood-derived macrophages – relation to polarization"* submitted to Immunology Letters, during review.

2. Investigation of Warburg effect and its relationship to macrophage polarization

Numerous s described the occurrence of the Warburg effect in inflammatory macrophages. The purpose of our experiments was to study the possible reversion of Warburg effect by kinase inhibitors targeted to the pathways involved (mTOR-Akt-PI-3K axis). The level of IP-10 and TNFα was only slightly increased in rat inflammatory macrophages. RANTES, an M2 cytokine was lower in inflammatory cells, and kinase inhibitors caused further reduction of this cytokine. NOS2, a marker of inflammatory state was expressed both in M1 polarized HL-60 and rat inflammatory macrophages

and attenuated by kinase inhibitors. The high phagocytic capacity of M1 polarized HL-60 and rat inflammatory cells was not reduced significantly by the tested kinase inhibitors.
We could detect a slight increase of the expression of HIF-1α both in HL-60 derived and rat peritoneal inflammatory macrophages, which was attenuated in HL-60 derived cells by the tested kinase inhibitors. Pyruvate kinase M2, another Warburg-marker, increased only in rat inflammatory macrophages and reduced by the kinase inhibitors.
The metabolic outcomes of Warburg effect were studied with a Seahorse instrument capable to measure the oxidative (OCR) and glycolytic metabolism (ECAR) in the same samples. In HL-60 derived cells, OCR was very low and oligomycin insensitive, despite of a high total respiratory capacity. ECAR was normal, significantly higher in M1 polarized cells. In rat macrophages, OCR was very low, ECAR was detected, but at lower level than in HL-60 cells, without the effect of kinase inhibitors. In both cell types, deoxyglucose and succinate significantly decreased ECAR, without an increase of OCR, suggesting, that this „Warburg-effect" is not reversible. The inhibition of NOS2 enzyme did not restore OCR even in rat peritoneal cells, denying the damaging role of NO on mitochondria. Dichloroacetate (DCA), an inhibitor of PDH kinase did not cause an oxidative shift in metabolism. Oligomycin insensitivity raised the issue of an impairment of mitochondrial membrane potential, but it could not be proved by fluorescent microscopic experiments.
The NADPH oxidase (NOX2), another oxygen consuming enzyme is also expressed both in HL-60 derived and rat peritoneal macrophages. The specific activity of NOX2 was higher in rat macrophages than in HL-60 derived macrophages, but no significant difference was found between M1-M2 polarized or resident and inflammatory macrophages and the inhibitors could not reduce it.
Summarizing these results, the tested macrophages markedly prefer the anaerobic metabolism, and it could not be transformed into oxidative metabolism by any attempted method (glycolysis inhibition, NOS2 inhibition, addition of DCA), despite of a high respiratory capacity (at least in HL-60 derived macrophages), and in the intact state of mitochondrial membrane potential. Therefore, this phenomenon does not seem to be a „Warburg-effect", but an inherent anaerobic preference of these macrophages, without a close relationship to the macrophage polarization.
Publication: a paper is under editing, submission planned before 31 March 2022

3. Studies on the effect of NO and cytokines produced by macrophages on cancer cells

Two rapidly proliferating cancer cell lines (FaDu and Panc-1) were used for this study. NO-donors, IP-10, PDTC, deoxyglucose and DCA were added to their cultures for 72 h and then ^{3}H-thymidine was used to monitor their DNA synthesizing capacity. Simultaneously cell viability was tested by MTT. Significant decrease in thymidine incorporation and cell viability was observed in the presence of sodium nitroprusside, PDTC and deoxyglucose, while only slight effect was found with other NO-donors. Rat peritoneal macrophages adhered to cover slips were added to the cancer cell lines in the presence and absence of NOS inhibitors and arginase. Rat macrophages decreased the thymidine incorporation of cancer cells and N-iminoethyl-lysine antagonized its effect, suggesting the role of NO in the antiproliferative effect. On the other hand, arginase did not inhibit NOS activity, but caused a further decrease of DNA synthesis and cell viability. The Warburg effect was also studied in both cell lines by Seahorse experiment. The OCR of Panc-1 cells was low and inhibited by succinate and SIN-1 (NO donor). The total oxidative capacity was high and decreased by succinate. ECAR was much higher in Panc-1-cells, reduced by succinate. FaDu cells had higher OCR, inhibited by SIN-1, while ECAR was lower than in Panc-1 cells. The effects of the inhibitors of mTOR-Akt-PI3K axis on OCR, ECAR, cell viability, proliferation and the possible apoptosis are under investigation.
Based on these experiments, both cell lines are sensitive for NO. FaDu cells have a more oxidative metabolism than Panc-1 cells and its glycolytic metabolism is more sensitive for various effectors. Interestingly, FaDu cells have only a low total respiratory capacity (released by DNP) compared to

Panc-1 cells. These results suggests that the faster proliferation rate of Panc-1 cell line compared to FaDu may be related to its higher glycolytic capacity, but it requires further investigations.
A manuscript is planned for submission during the first part of 2022, but further results are required.

4. The characterization of the Hsp90-PKD interaction

Hsp90-PKD connection

In our pilot experiments to evaluate the potential interaction of Hsp90 and the PKD isoforms, we treated the PKD1 expressing endothelial derived EA.hy926, pancreatic tumor Panc1, mouse fibroblast NiH3T3 and the PKD3 expressing androgen-independent prostate cancer DU145 and PC3 cell lines with the Hsp90 inhibitor geldanamycin. We monitored PKD1 and 3 protein level using western blot analysis and the dose dependent decrease of PKD1 and 3 level was observed, indicating the Hsp90 dependence of the stabilization of the two isoforms. In the following experiments we focused specifically on prostate cancer, hence instead of DU145 and PC3 cell lines, we employed the PKD1 expressing LNCaP cell as well. We treated these cell lines with the clinically investigated ganetespib and followed the PKD isoform protein level by western blot. The experiments showed that ganetespib was also able to decrease PKD1 and PKD3 protein level in a dose-dependent manner. We investigated the level of the previously identified Hsp90 client PKD2 as well, which, in accordance with the literature, also diminished. The level of the other reference protein Akt kinase was also reduced after by ganetespib treatment.

After Hsp90 inhibition, its client proteins are degraded by the proteasome. To address this possibility for PKD1 and 3, we treated the prostate cancer cells simultaneously with ganetespib and the proteasome inhibitor bortezomib. After separation of the soluble and insoluble fraction by sedimentation, we solubilized the insoluble proteins using SDS and urea. Due to the high toxicity of bortezomib to PC3 cells, this experiment was performed only on LNCaP and DU145 cells. Akt and Raf kinases were also used as a reference. In DU145, bortezomib in combination with ganetespib, caused the appearance of both PKD3 and Akt proteins in the insoluble fraction, indicating that misfolded PKD3 is degraded by the proteasome, indicating the requirement for Hsp90 in the conformational stabilization of PKD3. However, the treatment of LNCaP cells with ganetespib and bortezomib using the same conditions as in the case of DU145 cells did not cause the appearance of PKD1 in the aggregate fraction. In our opinion, this means that Hsp90-PKD1 interaction has different dynamics than Hsp90-PKD3 connection. Next, we investigated the direct physical interaction between Hsp90 and the PKD isoforms. Performing co-immunoprecipitation, we observed that PKD1 and PKD3 are in complex with Hsp90 in our cellular models.

In order to confirm the interaction between Hsp90 and PKD isoforms we employed Proximity Ligation Assay (PLA). PLA is a sensitive method which allows to detect direct protein-protein interactions at 40 nm distance between endogenous proteins *in situ*. These results also identified a direct interaction between the three PKD isoforms and Hsp90. The reference protein Akt1 also showed strong signal, but Lamin B, used as negative control, showed no or negligible signal compared to the other kinases.

Investigation of the role of PKD isoforms in cell viability and migration in prostate cancer cells

The exact role of the different PKD isoforms is still elusive, hence in this project we made effort to clarify their function in cell viability and migration. We performed the specific gene silencing of the three different PKD isoforms in LNCaP, DU145 and PC3 prostate cancer cells. First, we optimized the siRNA and transfection reagent concentration and incubation time to minimize toxicity and maximize protein depletion. The isoform specific siRNA-s were used alone and in combination as well. After gene silencing, both cell viability and cell migration experiments were performed. The efficiency of gene silencing was confirmed by western blotting. For cell viability, we performed trypan blue exclusion. In these experiments, we observed almost similar results in all three cell lines. The silencing of PKD1 and 2 (but not PKD3) considerably reduced cell viability, however the combined silencing of PKD1 and 2 did not caused any additive effect on cell viability reduction. Interestingly, when PKD1 or 2 siRNA was combined with PKD3 siRNA, the cell viability reducing effect was slightly attenuated. The simultaneous silencing of the three PKD isoforms was also performed, however, interestingly the rate of cell death was not as high as in the case of PKD1 or 2 silencing alone.

Cell migration experiments were performed using Boyden chamber method. In contrast to the literature our results showed that the depletion of PKD1 diminished cell migration at the highest extent, at the same time the effect of PKD2 silencing was no significant. However, the depletion of PKD3 also caused significant decrease in cell migration. The combination of PKD1/2, PKD1/3 or PKD1/2/3 siRNA-s showed almost the same effect as the PKD1 siRNA alone. The most effective way to reduce cell migration was the combination of PKD1 and 3 silencing. Surprisingly, the simultaneous silencing of PKD2 and 3 decreased cell migration significantly only in PC3 cells. According to our results, it seems like the silencing of PKD2 is weakening the anti-migratory effect PKD1 or PKD3 depletion.
On the other hand, we performed PKD (PKD1wt, PKD2wt, EGFP-PKD3wt, EGFP-PKD3CA, EGFP-PKD3DN) overexpression as well. First, the DNA plasmids were transformed into bacteria (XL10-Gold), multiplied and isolated. Then, we determined the optimal parameters of gene transfection with the GFP expressing control plasmid. Due to the toxicity, we probed more transfection reagents and unfortunately the optimization took more time than we expected. Moreover, we realized that DU145 cells were not suitable for plasmid transfection, hence we performed these experiments only with LNCaP and PC3 cell lines. The efficacy of the gene transfection was verified by Western blot. Using the transfected cell lines, we analyzed the changes in signal transduction focusing on PKD implicated pathways like MEK/Erk and Akt. Unfortunately, the results of our parallel experiments did not show correlation whit each other. We also performed experiments to evaluate the effect of PKD overexpression on cell viability, nevertheless there was no difference among the samples within 48 hours and after 48 hours the transfected cells – even the control – perished. Thus, we could not conclude the valid effect of PKD isoform overexpression on prostate cancer viability.
On the other hand, we performed cell migration experiments applying the transfected cells using the Boyden-chamber method. Our results showed that all wild-type PKD isoforms enhanced cell migration in LNCaP cells, but only PKD1 and 2 increased it considerably in PC3 cells. These results were partially unexpected, because - according to the literature - PKD1 inhibits cell migration. We transfected LNCaP cells with the constitutive active (CA) or dominant negative (DN) PKD3 mutants as well. Both the PKD3DN, and the PKD3CA caused the decrease of cell migration, in correlation with a previous publication.

The investigation of the function of the Hsp90-PKD3 complex

Based on our previous results, established cellular models and the literature, we examined the functional role of the Hsp90-PKD3 complex in prostate cancer cell migration. We found that the overexpression of PKD3wt caused more than 30% increase in cell migration in the low metastatic and low PKD3 expressing prostate cancer LNCaP cell line. Treatment of the cells with ganetespib depleted the overexpressed PKD3 and the cell migration was also decreased, indicating that Hsp90 stabilization is essential for PKD3 to exert its cell migratory effect. On the other hand, we performed the ganetespib treatment of DU145 and PC3 cells after the genetic depletion of PKD3. The results showed that PKD3 silencing did not augmented further the effect of ganetespib, showing that the function of PKD3 entirely depends on Hsp90 stabilization.
A manuscript about the Hsp90-PKD3 interaction, titled: "*PKD3 requires Hsp90 for stability and promotion of prostate cancer cell migration"* is before submitting to Cancers (IF:6.6) journal. We also planning to further clarify the role of PKD1 and to characterize its interaction with Hsp90, but more experiments are needed.

5. Identifying novel prognostic and predictive biomarkers in head and neck cancers

Due to the unfortunate personal changes during the project, we were obliged to change the originally proposed plan. Thus, in cooperation with the Department of Oto-Rhino-Laryngology, Head and Neck Surgery in Semmelweis University, performed investigations in order to identify novel prognostic and predictive biomarkers, and to investigate the role of PKDs in head and neck cancers. First, employing three head and neck cancer cell lines (Detroit 562, FaDu, SCC25) we observed the inverse relationship between the levels of the tumor suppressor connexin 43 (Cx43) and the anti-apoptotic protein Bcl-2, furthermore we found that the cells having high Cx43 expression are more sensitive to paclitaxel treatment. We published these results in Cancers (IF: 6.162), doi: 10.3390/cancers11121848

The next step was to identify new, potential therapeutic targets in head and neck cancer cell lines, thereby modeling the possibilities of personalized tumor therapy. Among the analyzed cell lines SCC25 proved to be the most sensitive to MEK inhibitors. A possible explanation of this observations is the lack of feedback activation through EGFR/AKT in the SCC25 cell line upon using MEK inhibitors. In contrast to SCC25 cells, in the FaDu cell line this feedback activation has an important role. The insensitivity of Detroit562 cells to MEK inhibitors might be caused by its PIK3CA mutation. Our main achievement is the notion that the feedback activation of the EGFR/AKT pathway caused by MEK inhibitors is not only related to the increased amount of EGFR but also to the activity of pY1068-EGFR. A manuscript on the effects of MEK inhibition, titled: "MEK is a potential indirect target in head and neck cancers" will be submitted to the International Journal of Molecular Sciences (IJMS; IF: 5.924).
In cooperation with the Department of Oto-Rhino-Laryngology, Head and Neck Surgery (Semmelweis University), and the 1st Department of Pathology and Experimental Cancer Research (Semmelweis University) we immunohistochemically (IHC) stained the different PKD isoforms in tumor samples gained from 97 therapy naive patients having squamous cell carcinoma of the oropharynx, hypopharynx and larynx. We found that the high PKD1 expression level correlated with the tumor localization, namely tumor samples from larynx showed low level of PKD1 expression, but samples from oropharynx had high PKD1 protein level. Furthermore, high PKD1 expression level associated with high PKD2 expression. In the Western world, high percentage of oropharyngeal tumors are associated with Human Papillomavirus (HPV). Our results showed that HPV infection was associated with high PKD2 protein level. Moreover, high PKD2 level indicated aggressive prognosis (high grade and stage). The tumor samples originated from hypopharynx or oropharynx also has high PKD2 expression level. The results of PKD3 showed that its lower expression level associated with poorer prognosis.
We purchased the Targeted Inhibitor Library from MedChemExpress, which contains 105 chemical compounds used in the clinics. In viability assay, we combined them with the PKD inhibitor CRT0066101 and we identified novel potential inhibitor combinations. We found that, EGFR and Alk seems to be a good therapeutic target in combination with PKDs.
The studies with PKD isoforms in head and neck cancers are promising, but more experiments are needed for a publication.

6. Novel signal transduction therapy approaches

We made effort to develop novel signal transduction approaches as well. We synthetized crizotinib–GnRH (gonadotropin-releasing hormone) conjugates to target specifically GnRH expressing tumors thereby selectively introduce crizotinib. According to our results, crizotinib–GnRH conjugates are transported directly into lysosomes, bypassing the ATP-binding sites of tyrosine kinases. This finding may explain the lower effect of crizotinib-GnRH conjugates than predicted based on their binding on GnRH-receptors and indicates the importance of the lysosomal drug escape in kinase inhibitor therapies. We published these results in International Journal of Molecular Sciences (IF: 4.556), doi: 10.3390/ijms20225590
In cooperation with ELTE Institute of Chemistry we investigated novel ferrocene-imipridone molecular hybrids. Compounds were synthesized at ELTE. Our initial biological screening was performed on four cancer cell lines (Fadu, EBC-1, A2058, PANC-1) based on MTT cell viability assay. Initial screening revealed that four newly synthesized molecular hybrids were significantly more effective than the reference imipridone ONC201 on all tested cancer cell lines. Colony formation assay was performed on PANC-1 cells to demonstrate the benefit of ferrocene conjugation to overcome the resistance against imipridon treatment. Colony formation assay proved successfully that two of the emergent hybrids eliminated the resistance and no viable PANC-1 cells were detected after treatments at 10 µM concentration.
Based on these promising results an article will be submitted until the end of 2022. January.

Troubles hampering the realization of the original project:

1. Personal problems (mainly the death of the PI and an important senior researcher)

2. Institutional problems – some researchers left the group and the department because of the incertitude of the research network of MTA; in the last year the MTA group has been removed by the University authorities from their original site in the EOK building, causing several weeks delay in working, and worsening the conditions of their experimental work.
3. Covid pandemic – during the “first wave”, the availability of EOK building was restricted, later the purchase of consumables was much slower, prices were higher, conferences could not be visited, publication became more difficult (slow reviewing, preferences for Covid papers vs others by the journals). Our liquid scintillation instrument could not be repaired for more than a year, causing delays in experiments requiring radioactive measurements. For this reason, we asked 3 additional months, which was approved by NKFIH. Nevertheless, these troubles caused delays mainly in publications. 3-4 publications, indicating the support of NKFIH are now under review or planned for submission after the deadline of the project. In the case of acceptation, we will inform the NKFIH Office immediately.