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CELL BIOLOGY

MULTIOMIC CYTOMETRY - CYTOMETRY AT TRUE RESOLUTION: RESOLVING COMPLEX BIOLOGY WITH SINGLE CELL MULTIOMICS

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B CELL DEVELOPMENTAL TRAJECTORY MODELING – USING COMPLEX IMMUNOPHENOTYPE TO IDENTIFY PRE-MALIGNANT CLL B CELL SUBSETS IN PERIPHERAL BLOOD OF HEALTHY DONORS

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Chronic lymphocytic leukemia (CLL) is a common lymphoproliferative malignancy, often preceded by the pre-malignant state of monoclonal B-lymphocytosis (MBL). The origin of the disease – the cell of origin and the key initiating event - is currently unknown. Epigenetic, transcriptomic and immunophenotyping studies point to the fact that the disease originates in mature, antigen-experienced B-cells, most probably co-expressing CD27 and CD5. On the other hand, it was shown that the malignant clones share features of a whole continuum of B cell developmental trajectory, from naïve to memory B cells. We have therefore applied mass cytometry (cytometry by time-of-flight – CyTOF) and spectral flow cytometry approach to search for B-cell subsets within MBL and CLL-like phenotype in peripheral blood (PB) of heatlhy individuals.

CyTOF methodology enabled us to perform immunophenotyping of complex B-cell samples from PB and bone marrow (BM) using a high number of markers, combining cell surface, intracellular and phosphorylated proteins and multiplexing of several samples in one run. The panel of markers was designed to distinguish normal and leukemic phenotype of B-cells and at the same time describe the B-cell maturation states. Multiplexing of B-cells isolated from BM and PB of healthy donors and PB of MBL and CLL patients was a key step for modeling of the developmental trajectories of normal B cells towards the malignant phenotype. This analysis successfully reconstructed the most probable trajectories of CLL development from normal mature B-cell counterparts via MBL and we were able to distinguish rare B-cell subsets with MBL and CLL-like phenotype in healthy individuals. Subsequently, we screened these cell populations in a larger cohort of healthy donor samples using spectral cytometry.

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SINGLE-CELL RNA SEQUENCING ANALYSIS OF T CELL DIFFERENTIATION AND TRANSDIFFERENTIATION MECHANISMS

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As one of the most crucial components of adaptive immunity, T helper (Th) cells were found to largely affect and orchestrate the immune responses of the host organism against distinct pathogens. They comprise a wide range of effector functions that include stimulation of specific immune cell populations (e. g. macrophages or B cells) via cytokine production and maintenance of immunological memory. All aforementioned functions are based on Th cell differentiation, defined as the process that can trigger changes in immunophenotype of naïve Th cells that lead towards distinct lineage commitment and polarize the Th cells into specialized subsets. The Th cell subsets that display the highest abundance levels in the human organism are pro-inflammatory Th1, Th2 and Th17 cells and immunosuppressive regulatory T (Treg) cells.

In our study, we applied single-cell RNA sequencing (scRNAseq) analysis on naïve Th cells that were stimulated towards Th1, Th2 and Th17 lineage commitment under optimized conditions (using cytokine cocktails) and cultivated for 5 days *in vitro.* Examining a highly heterogeneous population of differentiating Th1, Th2 and Th17 cells, we put emphasis on the key changes in expression profiles (leading us to elucidation the most important signaling pathways regulating the differentiation of each Th cell subset). Using RNA velocity assay, we focused on analyzing rare transient differentiation stages that bring better insight into the continuation of the Th cell differentiation process. Finally, we confirmed the hypothesis of Th17/Treg and Th1/Treg cell transdifferentiation in our dataset, along with elucidating the most crucial protagonists in the Th17/Treg and Th1/Treg transdifferentiation processes, using differential expression analysis and gene enrichment by Gene Ontology and KEGG Pathways database.

Overall, scRNAseq analysis of Th cell differentiation revealed to be a highly effective approach to describe alterations in Th cell differentiation and transdifferentiation that can also lead to identification of druggable targets for treatment of various pathologies of the immune system.

ANALYSIS OF TISSUE INFILTRATING T CELLS BY SINGLE CELL TRANSCRIPTOMICS

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Relatively recently emerging single cell RNA sequencing techniques (scRNAseq) enable studying gene expression at individual cell level. Specifically in T lymphocytes, additional immune receptor mapping enables us to characterize their T cell receptors (TCR) and analyze clonal diversity. However, since there are currently no standardized frameworks for the processing of scRNAseq data, extracting the complete information from scRNAseq is not trivial.

Here, we present our pipeline for scRNAseq data analysis using an example of our mouse model of Addison's disease. The model is based on the immunization of C57BL/6 mice with peptides derived from adrenal-specific autoantigens. We subsequently analyzed potential pathogenic $CD4^+$ T cells infiltrating adrenals by scRNAseq using 10x Genomics' Chromium platform. Specifically, we discuss our approaches to multiplexing cells from multiple samples, to identify potential cell doublets, to map cell clones and to identify T-cell subsets using OPTICS clustering method. Overall, our data analysis revealed heterogeneity of $CD4^+$ T cells within the inflamed adrenals including pro-inflammatory and regulatory T-cell subsets. Importantly, TCR repertoire analyses showed robust clonal expansion of activated $CD4^+$ T cells. These findings provide insights into the pathogenesis of this rare autoimmune disorder.

DATA MINING OF PUBLICLY AVAILABLE SCRNA-SEQ DATASETS: APPLICATION OF MACHINE LEARNING TO INTERROGATE NORMAL CELLULAR COUNTERPARTS OF CHRONIC LYMPHOCYTIC LEUKEMIA

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Chronic lymphocytic leukemia (CLL) is a lymphoproliferative disease of mature CD5+ B cells. There has been no consensus on the origin of CLL. It is also unclear whether the disease is derived from single or multiple precursors and at what stage the transformation occurs. The Extreme gradient boosting (XGBoost) algorithm is a machine learning approach that combines a large number of weak learners based on decision trees into a single strong classifier. This classifier can then be applied to a single sample to calculate a class probability that reflects its similarity to a given class. The aim of this study was to collect publicly available datasets of B cells, unify their annotation, and identify a subpopulation of healthy B cells most similar to CLL cells.

Several single-cell RNA-sequencing (scRNA-seq) datasets of B cells have been published, however, differences in their quality and annotation represent obstacles for their straightforward exploration and generalization of the observations. Here, we reanalyzed the datasets of B cells from multiple tissues. We identified five shared subtypes in peripheral blood (PB) that we denoted as transitional, naïve, IgM+ alternative memory (AMB), CD1C high, and classical memory. To identify the normal counterpart of CLL, we built the XGBoost-based classification model for predicting B cell populations in PB. Then, we multiplexed nine well-characterized CLL patient samples using antibody-based hashtag oligos and performed scRNA-seq. We applied the classifier to predict the similarity of CLL cells to the five B cell populations in healthy PB. Most CLL cells were classified as either naïve or AMB. We, then, subclustered naïve and memory B cells and built two classifiers to predict the similarity of CLL cells predicted as either naïve or memory. Strikingly, within the subclusters most similar to CLL we detected small percentage of cells harboring some CLL markers.

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RESEARCHER ORIENTED TOOL FOR COMPUTER ASSISTED DISSECTION OF DEVELOPMENTAL PATHWAYS IN LARGE SINGLE-CELL DATASETS

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With the onset of highly complex single-cell technologies such as mass cytometry or single-cell RNA sequencing (scRNA-seq) the investigation of cell-to-cell relationships going beyond the simple notion of population came into prominence. The most apparent manifestation of the effort in this line of research is the large number of pathway inference techniques developed in the last couple of years. However these powerful tools seem to fail to establish themself in standard workflows of biological research let alone the clinical diagnostics. One reason for this lack of application is the fact that these tools do not offer enough interaction with the process of finding candidate pathways which eliminates the feedback-loop between computer-suggested solutions and the expert knowledge.
In this study we have developed a novel methodology for pathway inference together with an interactive interface which allows the researcher to interact with the mathematical representation of various topological and geometrical features of the high-dimensional data in an intuitive way not unlike standard manual gating.
We have applied our methodology on 3 human thymi and peripheral blood samples analysed by the means of mass cytometry showing that our computer assisted analysis allows for exhaustive interrogation of the data. We were able to discover all textbook developmental pathways of human T-cells development (including beta-, positive-, negative-selection) and to put several under-described T-cells' population into proper context. We also showed that this approach could be efficiently applied on scRNA-seq data and scales well both in the number of events and dimensions respectively.

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MASS CYTOMETRY-BASED PHOSPHO-KINASE SIGNATURE IN PEDIATRIC T-CELL ACUTE LYMPHOBLASTIC LEUKEMIA (T-ALL) DISSECTS A DOMINANCE OF EITHER JAK-STAT OR PI3K-AKT-mTOR PATHWAYS

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T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive malignant disease arising from lymphoblasts of T-cell origin. While T-ALL accounts for only 15% of childhood and 25% of adult ALL and its initial prognosis is favorable, 30% of patients relapse with a poor outcome. Targeted therapy of resistant and high-risk pediatric TALLs is therefore urgently needed, together with precision medicine tools allowing the testing of efficacy in patient samples. Furthermore, leukemic cell heterogeneity requires drug response assessment at the single-cell level. Here, we used single-cell mass cytometry panel of 15 surface and 15 intracellular antibodies to study signal transduction pathways such as the JAK-STAT, PI3K-AKT-mTOR and MEK-ERK pathways in 16 diagnostic and 5 relapsed T-ALL primary samples and investigated the in vitro response of cells to IL-7 (main activator of JAK-STAT pathways) and BEZ-235 (inhibitor of PI3K pathway). Generally, T-ALL cells showed upregulated activity of the PI3K-AKT-mTOR and MEK-ERK pathways and increased proliferation and translation markers compared to residual non-malignant T-cells. An active JAK-STAT pathway response to IL-7 stimulation was found in 6 out of 16 patients while 11 out of 16 patients showed decreased aktivity of the PI3K pathway upon BEZ-235 ex vivo inhibtion. Interestingly, 13 out of 16 patients showed response to either IL-7 or BEZ-235. Notably, these response signatures were maintained from diagnosis to relapse in individual patients. In conclusion, we demonstrated the power of mass cytometry single-cell profiling of signal transduction pathways in T-ALL. We identified distinct clusters of IL-7- and BEZ-235- responsive T-ALL patients, supporting the hypothesis that alterations in the JAK-STAT and PI3K-AKT pathways have mutually exclusive effects.

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FLOW CYTOMETRY ASSAY FOR THE ANALYSIS OF INTERACTION BETWEEN RECOMBINANT PROTEINS AND SYNTHETIC MEMBRANES: DISHEVELLED BINDING TO SYNTHETIC PHOSPHOLIPID VESICLES

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Protein Dishevelled (DVL) is a key intracellular signal transducer of Wnt signaling pathway. This pathway is essential in regulation of embryonic development as well as in maintenance of homeostasis in adult organisms. Its deregulation is associated with many types of cancer and with various developmental disorders.
DVL has no enzymatic activity and its function is mediated by switching between numerous interacting partners and trafficking between subcellular compartments. Apart from protein interacting partners, it was shown that DVL interacts with cytoplasmic membrane phospholipids via its DEP domain in a pH- and charge-dependent manner[1].
In order to analyse the direct DVL-membrane interaction in the quantitative manner, we have developed an assay that uses fluorescently labelled purified DVL and synthetic phospholipid vesicles of different compositions. We have quantified the interaction of recombinant DVL with phospholipid vesicles using spectral flow cytometry. We have confirmed strong DVL binding to phosphatidic acid (PA) and phosphatidylserine (PS) vesicles and describe the role of pH in DVL-membrane interaction. Moreover, we have shown the contribution of the other DVL conserved domains to DVL-membrane interaction. Our work shows the potential of flow cytometry in addressing the scientific questions studied typically by methods of structural biology and biochemistry.

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RELATION BETWEEN TROP2 AND TETRASPANINS CD9 AND CD63 IN BREAST CANCER

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Members of the tetraspanin family are usually located in Tetraspanin-enriched microdomains (TEMs). Their role includes supporting the trafficking of other membrane proteins, such as EpCAM. The stem cell marker and EpCAM homolog Trop-2 (TACSTD2), is known to be overexpressed in carcinomas and also often associated with cancer progression and poor prognosis. Internalization of Trop-2 induced by antibody conjugated with chemotherapeutic represents a promising approach for cancer therapy. Because a detailed description of Trop-2 internalization is still missing, revealing mechanisms determining Trop-2 membrane dynamics may be beneficial. Our aim was to investigate the possible link between Trop-2 and tetraspanins in breast cancer, focusing on the process of Trop-2 internalization.

As model tetraspanins, we decided to study CD9 and CD63 on breast cancer cell lines. Using confocal microscopy, we analyzed the pattern of CD9 and CD63 in established Trop-2 KO clones. We also introduced deletion of CD9 and CD63 in the MCF7 cell line to describe the impact on the phenotype, Trop-2 expression and its internalization. CD9 and CD63 deleted cells showed impaired ability to form compact viable spheroids. Association of observed phenotype with involvement of Trop-2 has not been evaluated, although Trop-2 is reported to play a role in cell-to-cell contacts. We also optimized internalization assay with flow cytometry readout for monitoring antibody induced Trop-2 internalization. This assay showed decreased internalization of Trop-2 in case of CD63 KO clone.

We can conclude that Trop-2 expression affects the level of tetraspanins CD9 and CD63 in tested breast cancer models. Deletion of tetraspanins CD9 and CD63 affected compactness and viability of spheroids, however the relation of such change with Trop-2 needs to be elucidated. Our results show the effect of CD63 on the internalization of Trop-2, and we would like to elucidate the exact mechanism of this observation in future research.

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PRE-EXISTING CELL SUBPOPULATIONS IN PRIMARY PROSTATE CANCERS DISPLAY SURFACE FINGERPRINT OF DOCETAXEL-RESISTANT CELLS

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Docetaxel resistance represents a leading obstacle in the therapy of prostate cancer (PCa), resulting in lethal disease. Intratumoral heterogeneity, which is frequently driven by epithelial-mesenchymal plasticity significantly contributes to the limited treatment response, chemoresistance, and subsequent poor prognosis of patients with lethal PCa. We employed a high-throughput flow cytometry screening to identify cell surface fingerprint that associates with docetaxel resistance in PCa cells. Using patient-derived xenografts, we validated protein expression of the most robustly changed antigens in vivo and further assessed this 6-molecule surface fingerprint in primary PCa tumors. We revealed the overexpression of SSEA-4 antigen in both in vitro and in vivo docetaxel-resistant models and confirmed the SSEA-4 enrichment in a subpopulation of freshly isolated primary PCa tumors. The level of ST3GAL2, an enzyme that is critically involved in the SSEA-4 synthesis, correlated with increased expression of CD44, CD59, and CD95 and reduced expression of EpCAM and CD9. SSEA-4 was further directly linked to the antimicrotubule agent resistance and poor prognosis in PCa patients. We propose that the 6-molecule surface fingerprint associates with docetaxel resistance and pre-exists in a cell subpopulation of primary PCa tumors even before docetaxel treatment.

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FLOW CYTOMETRY ASSESSMENT OF CERVICAL PRECANCEROUS LESIONS

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Cervical cancer is one of the most common malignancies among female population despite existing prevention options. The development of cervical cancer is preceded by precancerous changes called squamous intraepithelial lesions (SIL) that usually develop over several years. SIL is divided into two stages: low-grade squamous intraepithelial lesions (LSIL) and high-grade squamous intraepithelial lesions (HSIL) depending on severity. Cervical cancer and SILs are associated with persistent high-risk human papillomavirus (HR HPV) infection that is sexually transmitted and responsible for 90-100% of cervical cancer cases among women. Although cervical cancer can be prevented by vaccination, cytological and molecular screening of sexually active women, lack of organized screening programs and/or vaccine hesitancy limit primary preventive measures in some populations. Secondary prevention measures including treatment of precancer come with associated risks and socioeconomic costs despite being highly successful. Due to the above, cervical cancer will remain a significant public health issue in the near future. In this study, gynecological clinical samples were collected with cytobrush and stored in NovaPrep liquid-based cytology (LBC) medium. Samples were classified as normal, LSIL and HSIL by an experienced cytologist. HPV genotyping by Multiplex PCR was performed on the isolated DNA. NovaPrep fixed HeLa cell line and clinical samples were assessed for the presence of several markers including p16, MCM2 and Ck18 by flow cytometry on FACSCalibur instrument. Preliminary data suggest that NovaPrep fixed HeLa cells can be reliably stained with the analyzed markers but protocols need to be further optimized for clinical samples. Further plans are to assess more biomarker candidates and include the information collected through a 2-year follow up period to find those associated with disease or virus persistence or progression.

FLOW CYTOMETRIC ANALYSES OF TNFα INFLUENCE ON BIOLOGY OF MELANOMA AND COLORECTAL CARCINOMA CELLS

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Pleiotropic cytokine tumor necrosis factor alpha (TNFα) has both anti-tumorigenic and pro-tumorigenic activity. Overexpression of TNFα in tumor cells results in reduced tumorigenic potential *in vivo*. In order to study the direct effects of TNFα on tumor cell biology and on cancer stem cell- like subpopulation (CSCs) *in vitro*, we engineered cells of two different origins continually overexpressing human TNFα gene: epithelial colorectal adenocarcinoma line HT29 and malignant melanoma line A375 of neural crest origin.
Using flow cytometry, quantitative RT-PCR and spheroid formation assay, we evaluated CSCs subpopulation and activity of aldehyde dehydrogenase (ALDH). Mitochondrial status and autophagy were also determined by flow cytometry.
Under the TNFα overexpression, we documented significantly lowered mitochondrial mass, lowered ATP production, and changes in mitochondrial morphology in both cell lines. The expression of main CSCs surface markers and stemness related genes *in vitro* remained unchanged. However, we could demonstrate a decrease of ALDH activity (up to 50%), which is linked to the stemness phenotype. The 3D cultivation of spheroids, which are believed to be enriched by CSCs, revealed no significant changes. But the TNFα overexpression resulted in increased autophagy on day 3, followed by senescence induction on day 6 in melanoma cells A375hTNFα.
Our *in vitro* study provides concise report of cellular processes initiated in malignant cells upon the TNFα overexpression. Despite its high overexpression in engineered cells, the subpopulation of CSCs responsible for tumor growth initiation remained unaffected and probably is not directly linked with the loss of tumorigenic potential. Our results point out the pleiotropic nature of TNFα and its diverse effect on cancer cells of different origin.

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INTRATUMOR HETEROGENEITY REFLECTED IN CIRCULATING TUMOR CELLS ASSOCIATES WITH METASTATIC PHENOTYPE OF SINGLE CIRCULATING TUMOR CELL-DERIVED CLONES

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It is presumed that the main mediators of cancer dissemination are the circulating tumor cells (CTCs) released from primary tumors into blood as a consequence of epithelial to mesenchymal transition (EMT). The EMT process supports the plasticity of CTCs, their adaptation to the new microenvironment and successful colonization of the target organ. In the breast and prostate cancer liquid biopsies were found populations of CTCs with different EMT phenotypes, predicting their metastatic potential and development of drug-resistance. Therefore, CTCs are used as prognostic markers in these types of malignancies in clinics.

Based on that we hypothesised that the EMT heterogeneity of primary tumor cells is reflected in circulating tumor cells and the surface EMT-signature in single CTC-derived clones correlates with their metastatic capacity. To test our hypothesis we prepared a syngeneic model of breast cancer, by injecting murine cancer cell line 4T1 12B into the mammary fat pad. This model is recapitulating whole metastatic cascade including the release of CTCs from the primary tumor into systemic circulation. The single CTC-derived clones were isolated, expanded in vitro and re-injected into mammary fat pads for second and third rounds of *in vivo* selection of the most aggressive CTCs-clones. The characterization of EMT-signatures of CTC-clones and their corresponding primary tumors was done simultaneously on a spectral flow cytometer with EMT markers: EpCAM, CD24, CD44, CD49b, CD73 and Sca1. The final analysis of the surface-EMT markers' plasticity was done with a high-dimensional reduction and clustering algorithm (FlowSOME). With this approach we performed complex characterization of surface EMT phenotypes in several single CTCs-clones and their corresponding primary tumors. We identified specific EMT-related signatures of aggressive CTCs-clones enhancing their metastatic ability. This finding can be translated into clinical samples of liquid biopsies and serve as another prognostic indicator of the disease progression towards metastatic spread.

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This work was supported by The Czech Science Foundation, grant nr. 20-22984S and by Czech Health Research Council, grant nr. NV18-08-00245 and NU20-03-00201.

FORMATION OF NOVEL CYTOSKELETAL STRUCTURES IN MEF 3T3 CELLS DURING INTERACTION WITH MICROSPHERES

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The cytoskeleton is an extremely important cell organelle. Any impairment of its functions may cause disturbance in cell homeostasis or even its death. Generally, the cytoskeleton is involved in many cellular process but in the context of mechanotransduction processes in cells, the most important functions are to provide the cell elasticity and its mechanical properties, and to participate in process of endocytosis.

The cytoskeleton is composed of three types of filaments - actin, intermediate and microtubules, which differ in structure, size, and location. While actin filaments are well described, the role and importance of intermediate filaments and microtubules requires more studies.

In our work, we studied the interaction of latex beads, functionalized with a specific protein, with proteins located on the surface of the cellular membrane. Such interaction can significantly affect the cell's behavior. We demonstrated that beads (alone and functionalized with fibronectin protein), placed on the cell surface, significantly change the architecture of actin, microtubules, and intermediate filaments. We also investigated how the cell response depends on the duration of the cell-bead interaction, application of cytoskeletal drugs and substrate stiffness. Elastic substrates can mimic the mechanical properties of physiological cellular environment. Our studies showed the formation of novel "cup-like" structures by the microtubule cytoskeleton upon interaction with latex beads. So far, the participation of microtubules in the endocytosis has not been reported, but our results indicate that they can actively participate in this process. This provides new insights into the function of the microtubule cytoskeleton.

FUCCI CELL CYCLE REPORTER AS A TOOL FOR VISUALIZATION OF CELL CYCLE PROGRESSION AND MALFUNCTIONS

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FUCCI (= Fluorescent Ubiquitination-based Cell Cycle Indicator) is a cell-based system containing fluorescent probes that are reciprocally alternating during cell cycle progression. This technology has found a wide range of applications in biological sciences. I. a., it allows for studying the function of key proteins involved in cell cycle regulation machinery, especially kinases specifically inhibited by selective small-molecule inhibitors. We have constructed cell lines stably expressing the FUCCI4 system that employs four fluorescent sensors to separate all cell cycle phases. Currently, we are developing pipeline that uses continuous fluorescent signal in H1 channel to automatically track individual cells in time lapse images obtained by confocal microscopy. This machine learning-based approach will be able to distinguish different cell fates induced by the currently studied mitotic inhibitors. Particular fates of cells can then be linked to the cell cycle phase in which the treatment was applied. In our work, we describe different phenotypes elicited by treatment with mitotic inhibitors of asynchronous population, such as nuclear mis-segregation, endoreplication or apoptosis. The rate of their appearance can be affected not only by different inhibitory concentration or different types of inhibitors, but also by the cell cycle phase in which the cells were present during the treatment. The FUCCI4 cell cycle reporter is a highly useful tool for tracking individual cells, and it can also indicate their cell cycle phase and differentiate the cell fates caused by the treatment with inhibitors possessing diverse modes of action.

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CLL AND CELL POLARITY: IN VITRO ANALYSIS OF MORPHOLOGY AND MIGRATION OF LEUKEMIC LYMPHOCYTES

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Chronic lymphocytic leukaemia (CLL) is the most common leukaemia in adults (Siegel *et al*., 2019). Even though various molecular markers of faster disease progression in CLL have been linked to cell migration and chemotaxis (Eagle *et al*., 2015; Vaisitti *et al*., 2010), these properties are rarely studied directly (Kaucká *et al*., 2015; Malet-Engra *et al*., 2015). Our research team has previously identified some of these markers as components of a conserved signalling pathway known to establish cell polarity in other tissue contexts (Janovská *et al*., 2016; Kaucká *et al*., 2013).

In our work, we set out to describe the effects of these molecules on CLL cell line morphology and migratory properties using genetic tools (CRISPR/Cas9) and targeted inhibitors. To assess these properties we optimized and tested a directly observable migration assay and developed a standardized image analysis pipelines enabling unbiased quantification. Furthermore, we have employed spectral flow cytometry to describe the phenotypes of these cells in enhanced detail.

Our results suggest that planar cell polarity molecules can influence migratory properties of CLL cells in ways that cannot be observed using indirect migration assays such as transwell assay. We therefore suggest new approach to study CLL cell migration and highlight its advantages for testing of new therapeutic agents.

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DIRECT MEASUREMENTS OF CELLULAR ATP LEVELS USING AUTOMATED, QUANTITATIVE LIVE-CELL ANALYSIS FOR MITOCHONDRIAL TOXICITY SCREENING

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Mitochondrial toxicity has been implicated in several clinical trial failures and withdrawals. Standard approaches to monitoring drug induced metabolic perturbations are limited to endpoint assays that provide population-based measurements and limited kinetic information. We have developed a genetically encoded ATP sensor that enables direct,automated live cell analysis of cellular ATP levels using the Incucyte®Metabolism Optical Module. Our live cel limaging approach to categorize compounds as non toxic, cytotoxic, ormitotoxic using the glucose/galactoses witch model was evaluated. Substitut in g-galactose for glucose in growth media blocks the ability of cells to generate ATP via glycolysis, conferring reliance on mitochondrial oxidative phosphorylation to generate ATP and enhancing sensitivity to mitochondrial-driven toxicity. Reductions in ATP could be observed in minutes, and transient reductions followed by recovery highlight the sensitivity and value of kinetic data using our live cell imaging approach.

96-WELL LIVE-CELL ASSAYS FOR IMMUNE CELL KILLING OF 3D TUMOUR SPHEROIDS

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Immunotherapies such as checkpoint inhibitors, CAR-Ts and immune-targeting Abs have great promise for cancer treatment. Tumour spheroid models potentially offer more translational biological insight than 2D cell models and more relevant analysis of cancer immunotherapy agents in vitro. Here we describe novel image-based, immune cell-killing assays of 3D tumour spheroids based on IncuCyte live-cell analysis. Single spheroids were formed from human tumour cell lines expressing RFP in 96-well ULA plates. Immune cells were then added and spheroid viability was assessed over time (up to 10 days) by measuring the loss of RFP fluorescence. This method is exemplified with a range of immune cell activators, stimulating T cell (anti-CD3 and IL-2) or natural killer cell (IL-12 & IL-2) PBMC sub-populations. As expected the magnitude and rate of cytotoxicity was effector-to-target cell ratio dependent. In an antibody-dependent cell-mediated cytotoxicity (ADCC) format, Herceptin induced a concentration-dependent specific killing of Her-2 expressing tumour cells (SKOV-3). Higher concentrations of Herceptin were required in 3D vs 2D ADCC assays. These data demonstrate the capability to kinetically visualise and quantify 3D immune cell killing and ADCC assays, and illustrates how these assays can be extended from traditional 2D cultures to 3D. These assays will be highly valuable in the search for novel immuno-modulators

EXTRACELLULAR VESICLES REIMAGINED WITH MULTIPLEX COLOR DETECTION IN IMAGING FLOW CYTOMETRY

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Extracellular vesicles (EVs) have caught scientists' attention in recent years. These sub-micron particles are part of a vast cell-to-cell communication network within the body, delivering important biochemical signals from one cell to others. To accomplish this task, EVs carry specific molecular cargo—RNA, DNA, and proteins —which scientists can use to gain insight into diseases and overall health. For instance, EVs derived from cancer cells are promising markers for distinguishing the type and stage of cancer, and EVs themselves may play a critical role in oncogenic signaling pathways. For all of their potential, accessing the vital information EVs encapsulate is no easy task. Because EVs are so small—just 20 nm to 500 nm—they have been difficult to detect, isolate, and study with accuracy. Further, identifying the molecular cargo EVs are carrying requires the simultaneous detection of multiple analytes, which is typically performed with fluorochrome-conjugated antibodies and dyes targeted to specific components of the EV, as well as the proteins or nucleic acids it may be carrying. To help solve these challenges, the Amnis® ImageStream®X Mk II Imaging Flow Cytometer empowers scientists to detect and characterize EVs with exceptional sensitivity. Our newly released High Gain mode for the ImageStreamX enables the accurate detection and enumeration of many types of EVs and viruses. With High Gain mode, the time delay integration (TDI) CCD camera in the ImageStreamX uses a higher gain setting to increase the signal from small particles. A 400 mW 488 nm laser and increased photonic sensitivity supports the simultaneous measurement of up to 10 fluorophores—providing the opportunity to combine EV detection antibodies with dyes for multiple cargo elements.

THE USE OF AN AURORA SPECTRAL CYTOMETER FOR SMALL PARTICLE ANALYSIS

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There is an exploding interest in the use of flow cytometry to assess a variety of small particles such as Extracellular Vesicles (EVs), liposomes, and viruses. Many flow cytometry instruments have been shown to struggle with particles smaller than 300nm. Recent advances in flow cytometry instrumentation design has led to increased opportunities to measure particles less than 300nm. The Aurora spectral cytometer has multiple design attributes that should make this instrument sensitive enough to detect small particles in this range. In this study, we sought to evaluate whether this instrument has the ability to detect small particles in a size range that would be useful for typical biological studies. Various fluorescent small particles (<300nm) with a variety of sizes and refractive indices were run on a Cytek™ 3 laser Aurora. These included apogee beads, viruses (90-250nm), liposomes (100nm), silica (180nm) and EVs (100-300nm). Instrument settings were optimized for either violet/blue SSC or fluorescence triggering so that the buffer only control contained 2-4 events per second (fluorescence triggering) or <40 events per second (scatter triggering). For fluorescence triggering, the detector wavelength closest to the maximum fluorescence emission was used. Parallel samples were run on an ImagestreamX® (ISX) imaging flow cytometer for comparison. All samples were collected for 2 minutes. Data was analyzed using either FCSExpress 6.06 or 7.0 software.

- The Aurora has the sensitivity to detect biological particles as small as 100nm
- Both unmixed and raw (single channel) data demonstrate excellent sensitivity
- Both scatter and fluorescence thresholding provide comparable results
- Aurora can detect small particles even in the absence of fluorescence

DETECTION AND QUANTIFICATION OF CIRCULATING TUMOR CELLS IN PBMCS OR BLOOD USING FLOW CYTOMETRY

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Circulating tumor cells (CTCs) are cells that detach from theprimary solid tumor or metastases and circulates through the peripheral blood. Accumulating evidence shows the importance of CTCs detection also referred to as a “liquid biopsy” for cancer prognosis, as well as a biomarker for metastatic cancer and therapeutic response monitoring. However, detection of CTC in peripheral blood is challenging because they are present at extremely low concentrations; in some patients as little as a few CTC per million blood cells. Previous studies have demonstrated that flow cytometry can be useful for CTCs studies[1-4]. A tumor cell spiking assay is frequently used to evaluate thesensitivity, specificity, accuracy, and repeatability of the methods or systems for enumeration of rare CTCs. In these experiments, the NovoCyte® QuanteonTM was employed to measure and quantify spiked human tumor cells of colon carcinoma (SW620) in PBMCs or peripheral blood. First, EpCAM APC labeled SW620 cells were spiked into PBMCs and analyzed on the NovoCyte QuanteonTM as well as a competitor flow cytometer to determine the rare event detection sensitivity. The results show an excellent linear relationship between the number of cells detected and the predicted number added, with a sensitivity of 0.0001% or 1 CTC per million cells. In the next experiments, SW620 cells were spiked into blood samples to simulate CTC in cancer patients. The spiked tumor cells were isolated together with PBMCs by Ficoll density gradient centrifugation, followed by EpCAM based magnetic bead enrichment. The recovery rate of the spiked tumor cells was then detected by flow cytometry to determine the accuracy and specificity of the enrichment process. A high precision syringe pump allowed the direct enumeration of CTC without the need of reference beads. The results show that a greater number of CTC present in the spiked blood sample increased the recovery rate of CTC through the enrichment process.

IDENTIFICATION OF DUBS REGULATING RIBOSOME STALLING ON ENDOGENOUS mRNA

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Protein synthesis is a critical determinant of every living cell. During translation ribosomes decode genetic information from mRNA. Upon encountering an unreadable or senseless mRNA sequence, ribosomes stall and collide. This leads to translation arrest and subsequent activation of ribosome quality control (RQC) pathway displacing ribosomes from mRNA and inducing mRNA decay. Previous transcriptome-wide studies indicated presence of ribosome collision/pause sites in many endogenous, error free mRNA. Particularly, ribosomes pause and crash on mRNA encoding proteins destined to endoplasmic reticulum. Mechanisms allowing continuous translation after ribosome crashes are yet fully elusive.

Processivity of translation can be studied by various techniques. In addition to laborious and expensive proteomic approaches, recently introduced fluorescent reporters allow fast and simple read out of ribosome activity. We employed modified system based on two fluorescent proteins separated by amino acid/protein sequence of interest in a single reading frame. If a ribosome pause/stall on the inserted sequences, the fluorophore ratio changes. Up to now, these reporters were used only to study the pathological features of ribosome stalling. We have employed the technology monitor ribosome stalling in two error-free mRNAs encoding 1) cytosolic and 2) ER-localised proteins: 1) Ig-like domains of Filamin A and 2) Ig domains from immunoglobulin λ light chain. These molecules are of similar size and identical fold. Additionally, we have performed a flow cytometry-based CRISPR screen to identify deubiquitinating enzymes regulating ribosome processivity during mRNA translation.

We will present new methodology using fluorescence-based reporter system for fast analysis of ribosome activity in human cells. Our results suggest the ribosome stalling occurs during translation of ER-localised proteins but not in cytosol localised ones. Observed stalling was negatively correlated with the size of the protein. To validate dependency of ribosome stalling on the protein size and localisation, novel reporters will be developed.

DISRUPTION OF THE ARYL HYDROCARBON RECEPTOR (AHR) SIGNALING ALTERS FUNCTIONS AND PRODUCTION OF SURFACTANT IN A HUMAN MODEL OF ALVEOLAR TYPE II CELLS

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The aryl hydrocarbon receptor (AhR) is a transcription factor activated by xenobiotics with planar aromatic structures. Its main function is to initiate the expression of detoxifying enzymes, such as cytochromes P450 from family 1. However, it became evident that the AhR plays multiple endogenous roles in the control of cell proliferation, differentiation, metabolism and cell-to-cell communication. Importantly, the AhR seems to contribute to the control of immune cells of the barrier organs, such as skin, gut, or lung. Nevertheless, its functions in epithelial cells of barrier organs are far less explored. Alveolar epithelial type II cells (ATII), also known as type II pneumocytes, are important regulators of functions of alveolar epithelium, which contribute to its regeneration, control of immune responses and production of surfactant. Surfactant lipids and proteins, which cover alveolar epithelium, both reduce surface tension and provide protection to pneumocytes. Here, we studied potential role of the AhR in the differentiation and production of surfactant in a human model of ATII cells, A549 cell line, as well as in ELEP cells (early lung progenitors). We have prepared and used AhR wild type and AhR knock-out cells derived from A549 and ELEP cells, in order to compare their capacity to differentiate, when cultivated at air-liquid interface (ALI). We then evaluated the expression of surfactant proteins and production of surfactant lipids (such as dipalmitoylphosphatidylcholine), as functional markers of differentiated ATII cells. Our results show that cells lacking AhR have an altered differentiation pattern, as compared with wild-type cells. These results suggest that toxicants activating AhR might potentially contribute to disruption of ATII cell functions, which play important role in the maintenance of lung homeostasis. [Supported by the project no. 18-00145S of the Czech Science Foundation].

ACUTE MYELOID LEUKEMIA REGRESSION INDUCED BY PALBOCICLIB AND PONATINIB IN PATIENT-DERIVED XENOGRAFT MODEL

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Aims: We aim to confirm the in vitro effect of palbociclib, a breast cancer approved CDK4/6 inhibitor, and ponatinib, a BCR/ABL1 inhibitor with multikinase activity approved for chronic myeloid and acute lymphoid leukemia, previously shown to be effective against acute myeloid leukemia, in vivo using primary AML cells.
Methods: Two newly diagnosed AMLs were xenotransplanted into NOD SCID gamma mice. The treatment was initiated at the level of approximately 5% hCD45+ cells in mouse peripheral blood. Palbociclib, ponatinib, vehicle, and venetoclax as a comparative treatment, were administered orally for 3 weeks, 5 days per week. Chemotherapy (cytarabine+doxorubicine, AraC/Dox) 5+3 regimen served as a positive control. Azacitidine, served as another comparative drug, was administered subcutaneously, five days per week in 3 cycles – 1 week on, 1 week off.
Results: Palbociclib significantly decreased AML burden and prolonged the overall survival (OS) in both AMLs (Fig. 1). Venetoclax was equally effective. Ponatinib prolonged OS only in AML#2 (Fig. 1). The AraC/Dox regimen effectively suppresed AML but was the only associated with toxicity demonstrated by weight decrease (AML#1, 24 ± 2% [mean ± SD], $p = 0.0001$; AML#2, 19 ± 11%, $p = 0.04$), and possibly with early mouse mortality in the AML#2 (3/4 mice). Azacitidine failed to suppress the disease except for 2/4 mice in AML#2 where it induced a long-term remission. All tested treatments produced only a transient AML decline, followed by a relapse confirmed by high hCD45+ levels in PB, BM and spleens.
Summary: Palbociclib, and partially ponatinib, demonstrated AML suppression *in vivo*, encouraging further investigation. Based on this study, we intend to investigate combinatorial drug effects and modified administration regimens.
Funding: Supported by MUNI/A/1595/2020.

CHK1 INHIBITOR SCH900776 ENHANCES ANTICANCER ACTION OF CISPLATIN AND TRAIL VIA STIMULATION OF BAK-DEPENDENT MITOCHONDRIAL PATHWAY

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Previously, we showed that the selective Chk1 inhibitor SCH900776 (currently profiled in phase II clinical trials) significantly enhances apoptosis and DNA damage response in human cancer cells treated with platinum-based drugs (1). Here we demonstrate that inhibition of Chk1 also increased cooperative cytotoxic effects of cisplatin and TRAIL (tumor necrosis factor-related apoptosis inducing ligand) in human prostate cancer cells. The SCH900776-mediated enhancement of the drug combination-induced apoptosis was associated with stimulation of Bak-dependent mitochondrial pathway and caspase activation. The favorable cytotoxic potential of the triple-combination of the drugs in prostate cancer cells was significantly affected by targeted manipulations (overexpression, siRNA, selective synthetic inhibitor) of the Mcl-1 protein level/activity, suggesting potential impact of this antiapoptotic Bcl-2 family member. The mechanisms involved will be presented in greater detail within our contribution.

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DEREGULATION OF CYTOCHROME P450 EXPRESSION IN HUMAN HEPATOCELLULAR CARCINOMA – POSSIBLE ROLE OF MICRORNAS

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Malignant transformation of hepatocytes leads to significant alterations of their metabolic functions and biotransformation of both xenobiotic and endogenous substances, which may significantly affect the tumour development and the outcome of anticancer therapy. Previously, we demonstrated a significant down-regulation of expression of cytochromes P450 (CYPs), major xenobiotic-metabolizing enzymes, in human hepatocellular carcinoma (HCC) tumour samples as compared to their surrounding non-cancerous tissue. We also showed remarkable differences in the expression of numerous lncRNAs between HCC tumours with severe CYP downregulation and those with weakly altered/unaltered CYP profiles, suggesting a possible involvement of non-coding mRNAs in CYPs regulation (Nekvindova et al., Biochemical Pharmacology, 177, 2020, 113912).
Here, we compared miRNA profiles (TaqMan Array Human MicroRNA v3.0 TLDA qPCR) between human HCC tumours with strong (CYP-) and weak/no (CYP+) downregulation of CYP levels, and identified significant differences in expression of several miRNAs. We further performed *in vitro* functional analyses of their role in regulation of expression of selected CYPs and related xenobiotic receptors, using human hepatic HepaRG cells transfected with specific miRNA mimics or inhibitors. We showed that the two significantly most-upregulated miRNAs found in CYP- tumour samples may act as efficient suppressors of CYP1A2 and AhR, as observed at mRNA or protein levels. The detailed results obtained will be presented in our contribution. Deeper elucidation of the regulation of CYP expression mediated by non-coding mRNAs may help to identify novel regulatory

mechanisms involved, as well as to encourage the discovery of novel biomarkers and therapeutic targets for HCC.

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DEREGULATION OF SIGNALING PATHWAYS CONTROLLING CELL SURVIVAL AND PROLIFERATION IN CANCER CELLS REDUCES INDUCIBILITY OF CYP1A1/1B1 CYTOCHROMES

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Cell proliferation, deregulation of signaling pathways in cancer cells, cell density, or presence of growth factors have been suggested to alter the inducibility of CYP1A1/1B1, major cytochrome P450 enzymes. This has implications both for their impact on the metabolism of xenobiotics and for their use as specific biomarkers of activation of the aryl hydrocarbon receptor (AhR). Here, we evaluated the functional role of p300 histone acetyl transferase (a transcriptional co-activator) in the control of CYP1A1/1B1 expression. We particularly focused on proliferative/survival pathways signaling deregulated in tumor cells. We used a combination of siRNA- or pharmacological inhibition-based approaches in two cell models derived from human liver (HepaRG cells) and colon carcinoma (HCT-116 cells) tumors. We show that the 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-induced CYP1A1 mRNA/protein levels are decreased in exponentially growing cells compared to their non-dividing counterparts. Similarly, siRNA-mediated inhibition of proliferation pathways (e. g. β-catenin and/or Hippo pathway effectors YAP1/TAZ) resulted in an increase in TCDD-induced CYP1A1 mRNA levels in HepaRG and/or HCT-116 cells. These effects seem to be linked to p300 being preferentially utilized for regulation of the expression of genes contributing to cell proliferation. We demonstrate that both inducibility of CYP1A1/1B1 and p300 binding to *CYP1A1* or *CYP1B1* gene regulatory regions, are reduced in cells with activated proliferative Wnt/β-catenin signaling. Collectively, these results indicate that the proliferative status of cells, as well as the activities of signaling pathways contributing to cell proliferation/survival, may substantially alter CYP1A1/1B1 inducibility, via modulation of the activity of histone acetyltransferases, such as p300.

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PROS AND CONS OF IN VITRO CULTURE OF PROSTATE ORGANOIDS AND TISSUE FRAGMENTS

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Current *in vitro* modeling systems do not fully reflect the biologic and clinical diversity of prostate cancer (PCa). Organoids are 3D in vitro cell cultures that may better recapitulate disease heterogeneity and retain parental tumor characteristics. Short term *ex vivo* culture of prostate cancer tissues may also facilitate drug testing in personalized medicine. We aimed to establish both organoid and tissue culture for future drug testing for patients with advanced castration-resistant prostate cancer.

First, we processed both cancer and normal tissue from 50 patients who underwent radical prostatectomy (hormone naïve). In total, we were able to cultivate organoids from 58% tumor (29/50) and 69% of normal tissues (20/29). Whole organoid immunohistochemical staining of two representative cases revealed positive pan-cytokeratin and vimentin, confirming the presence of epithelial and mesenchymal cells, respectively. However, overexpression of AMACR and ERG proteins was not recapitulated in tumor organoids. Another disadvantage of this culture approach in our hands was the propagation of organoids only up to three weeks till the first passage.

Second, the short-term drug test was performed for ten patients using *ex vivo* tissue culture. Samples from hormone naïve prostatectomies presented a low level of proliferation as assessed by Ki-67 staining. Another drawback of this approach is inconsistent tissue morphology between separate tissue fragments and treatments. Only one case showed a high proliferation rate for toxicity testing and tumor tissue was present in all tested tissue pieces.

In conclusion, we have established culture of both organoids and tissue fragments from patients with hormone naïve prostate cancer. However, the organoids did not fully recapitulate primary tissue characteristics and heterogeneity between tissue fragment hampered interpretation of the drug testing. Still, these approaches may be promising using tissues from metastatic castration-resistant prostate cancer.

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THE ROLE OF ALDEHYDE DEHYDROGENASE IN COLORECTAL CANCER CHEMORESISTANCE AND METASTASIS

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Enzymes belonging to the aldehyde dehydrogenase (ALDH) family are involved in the detoxification processes, which puts them in an important position for the proper function of the organism. On the other hand, ALDHs are involved in tumour initiation and progression. In several types of tumours, high ALDH expression is associated with increased chemoresistance and poor prognosis. The aim of the study was to evaluate the impact of ALDH1A1 and ALDH1A3 knockout in chemoresistant colorectal cancer (CRC)-derived cells and the role of c-Met and CXCR4 signalling pathways in CRC chemoresistance and metastasis.

Expression of ALDH isoforms 1A1 1A3 was inhibited by CRISPR/Cas-9 method. Expression of c-Met and CXCL12-CXCR4 was evaluated by flow cytometry. ALDH activity was quantified by ALDEFLUOR™. Signalling pathways were pharmacologically inhibited by AMD3100 and SU11274. The impact on gene expression was evaluated by qPCR and CellTiter-Glo® Luminescent Cell Viability Assay was used to assess the response to chemotherapy.

Inhibition of CXCL12-CXCR4 and c-Met pathways led to significant decrease of ALDH1A1 expression and reduction of overall ALDH activity. ALDH1A3 knockout in chemoresistant cells led to increased sensitivity to 5-fluorouracil and decreased migratory potential in vitro.

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TLR3 AFFECTS CELL MIGRATORY FEATURES IN PROSTATE CANCER

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Prostate cancer (PCa) is one of the most frequent cancer types affecting men in developed countries. Common curative strategies aim to maintain disease under control by androgen-deprivation therapy using anti-androgen molecules, such as enzalutamide and abiraterone. Despite initial effectiveness, patients eventually develop resistance and progress into a condition of castration-resistant PCa (CRPC) associated with further development of the tumor. Curative strategy shifts to the use of chemotherapeutic agents, like docetaxel, which ultimately may also lead to chemoresistance, with disease progression into metastatic condition. Lately, numerous studies showed that several Toll-like receptors (TLRs) are expressed in PCa and their expression and activation status affect prognosis in patients with PCa. We evaluated the levels of TLRs in therapy-responsive and therapy-resistant PCa models and, regardless of the heterogeneous expression of the receptors in our models, we observed up-regulated TLR3 in all resistant models. Given that chemoresistant PCa is associated with metastasis, our aim was to investigate the effect that TLR3 expression and activation has on cell migration and invasion. For this purpose, we created TLR3 OE and TLR3 CRISPR-KO PCa cells. We observed by different methods that TLR3 overexpression leads to increased migration and invasion, which was associated with higher cell motility. The effect of the receptor on these biological processes was confirmed in TLR KO cells, which presented lower cell motility. Taken together, our results show that chemoresistant PCa presents increased TLR3 expression; high TLR3 leads to increased cell motility and pro-migratory ability, which underlines the potential role of TLR3 in PCa aggressiveness related to chemoresistance.

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DIRECT CO-CULTURE MODEL FOR THE ANALYSIS OF CHRONIC LYMPHOCYTIC LEUKAEMIA RESPONSE TO BONE MARROW STROMAL CELLS

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Chronic lymphocytic leukaemia (CLL) cells are largely dependent on stimuli from their microenvironment. Communication with other cells (e. g. stromal cells, nurse-like cells, T cells) is essential for their survival and proliferation. We have previously suggested that casein kinase 1 (CK1), a key component of Wnt signalling pathways, is one of the regulators of CLL-microenvironmental interactions. Our previous work showed that CK1 inhibition by small molecule inhibitor PF-670462 can improve overall survival in a typical CLL mouse model (Eμ-TCL1). The mechanism of action however remained poorly understood.
Here, we describe how CLL cells respond to microenvironmental stimuli provided by stromal cells and how CK1 inhibition modulates their response. To mimic their natural microenvironment, we co-cultured CLL cells of 9 CLL patients with murine bone marrow stromal cells (cell line M210B4). CLL cells and CLL cells co-cultured with stromal cells were treated with CK1 inhibitor (PF-670462) and submitted to RNA sequencing. CLL cells´ reads were mapped to human genome and separated from murine stromal cells´ reads. We identified differentially expressed genes using the limma-voom method and further searched them for enriched signalling pathways and gene ontology terms.
Enriched pathways were largely connected to communication with microenvironment and various proteins binding. Up- and down-regulated genes in CLL cells co-cultured with stromal cells were involved in axon guidance, focal adhesion, ligand-receptor interaction, and cell cycle (PI3K-Akt pathway). Signalling pathways connected to ligand-receptor interaction were also enriched in cells treated with CK1 inhibitor (both CLL cells and CLL cells co-cultured with stromal cells).
Consequent analysis offers enormous potential to in detail describe CK1-dependent and supportive-cells-dependent processes which could uncover new approaches how to target CLL and explain the mechanism of action of CK1 inhibitors.
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BREAST CANCER HETEROGENEITY MEDIATED BY EPITHELIAL-MESENCHYMAL PLASTICITY

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Breast tumor tissues are dynamic structures characterized by profound intratumoral heterogeneity that contributes to disease progression and therapeutic resistance, both representing a major obstacle in treatment. Besides genetic, epigenetic, and microenvironmental stimuli, one of the most prominent processes contributing to the intratumoral heterogeneity is an epithelial-mesenchymal transition (EMT), an evolutionarily conserved developmental program frequently hijacked by tumor cells, strengthening their motile and invasive features. EMT is critical for the spread of cancer cells from the primary tumor to circulation, followed by extravasation and dissemination to distant organs. On the other hand, reverse mesenchymal-epithelial transition (MET) is believed to play a key role in the establishment of macrometastasis at secondary sites. In addition, cancer cells rarely undergo complete EMT and rather exist in a continuum of hybrid E/M intermediate states preserving high levels of plasticity. Despite rich in vitro experimental evidence, reliable studies of both processes in clinical samples are scarce. Therefore, it was in our interest to develop a tool for the investigation of EMT/MET surfaceome and analyze complex EMT status in clinical samples. We introduced a single-tube multicolor flow cytometry protocol for the analysis of EMT states utilizing a previously described set of novel surface antigens referred to as 10-molecule signature that associate with cancer plasticity [1]. Employing multiparametric data analysis, we identified distinct populations of epithelial-like and mesenchymal-like cells in the primary patient tumors and in-house generated patient-derived xenograft models. As a next step,

tumor cells from populations of interest were sorted for bulk RNA-sequencing. Transcriptomic data uncovered distinct gene fingerprint of mesenchymal and epithelial populations that were associated with specific functions and disease progression. Taken together, we described phenotypically defined EMT/MET populations present in treatment-naïve breast cancer samples that exhibit a unique transcriptomic pattern.

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EMPLOYING EXTRACELLULAR VESICLES FOR EARLY DIAGNOSIS OF OVARIAN CANCER

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High grade serous carcinoma of the ovaries, fallopian tube and peritoneum (HGSC) has the highest mortality of all gynecological malignancies and is the most common form of ovarian cancer. The early stage disease is asymptomatic and quickly progressing and thus, efforts for its detection still often fail. 75% patients are diagnosed in advanced stages which results in the five-year survival of 5-25%. However, if HGSC is diagnosed in stage I, the survival rate is 85%. Hence, it is highly important to devise strategies for early HGSC detection.

Extracellular vesicles (EVs), a collective term covering more popular terms such as microvesicles and exosomes, are small membranous particles produced by virtually all cells. EVs function as important conveyors of information between cells, represent a fingerprint of the originating cell and thus can be employed as disease biomarkers. EVs hold great promise in identifying early stage tumors as they deliver enriched biomarkers in blood in considerably higher concentrations and also earlier during cancerogenesis than well-studied circulating tumor cells or tumor DNA.

Ascites, a pathologically accumulated fluid in the peritoneum, is a hallmark of HGSC. We isolated EVs from malignant ascites (the tumor microenvironment of HGSC) and by mass spectrometry identified several candidate proteins present in malignant ascites EVs but absent from EVs of control fluids. We found that the most promising candidate protein, further referred to as 'marker X', was present not only in malignant ascites EVs, but also in plasma EVs of HGSC patients. On contrary, 'marker X' was not found in plasma EVs of healthy individuals nor in non-EV (liquid) fractions of ascites.

We believe our results justify further investigation of this promising candidate (and/or other candidates) as an EV-based biomarker for early detection of HGSC, a goal that would greatly reduce the high mortality rates seen in HGSC patients.

OPTIMALIZATION OF CLICK-IT® EDU ALEXA FLUOR® 488 FLOW CYTOMETRY ANALYSIS AND CORRELATION WITH PROLIFERATION ASSAY

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Oncology research is dependent on variety of research techniques, among them flow cytometry is one of the most important one. Proliferation as an important part of cancer development and progression could be also detected by this method. Cell proliferation is characterized by *de novo* DNA synthesis during the S-phase of cell cycle. Well-established method for monitoring DNA synthesis and cell proliferation uses bromodeoxyuridine (BrdU), a thymidine analog. BrdU is incorporated into newly synthesized DNA and identified using an antibody. Disadvantage is the fact that BrdU antibodies react only with single stranded DNA, therefore denaturation is needed. To overcome disadvantages, 5-ethynyl-2-deoxyuridine (EdU) proliferation assay have been created. EdU proliferation assay is not antibody based and therefore do not require DNA denaturation for detection of the incorporated nucleoside. EdU is a nucleoside analog containing alkyne. In copper-catalyzed reaction, alkyne reacts with dye-labeled azide and form stable covalent bond.
In our team we aimed to introduce Click-iT EdU proliferation kit with Alexa Fluor 488 dye in our laboratory, to measure simultaneously proliferation and a cell cycle. For our experiments we have chosen colorectal carcinoma cell line DLD1.The Click-iT® EdU Alexa Fluor® 488 Flow Cytometry Assay Kit provides a simplified, more robust assay for analyzing DNA replication in proliferating cells. Newly synthesized DNA is analyzed using the 488 nm laser of the flow cytometer. At first, we needed to adjust this protocol for our needs. We followed protocol according to recommendations of the manufacturer, but histogram of cell cycle was not optimal. We tried several modifications (permeabilization of cells with Triton instead of saponin, or use more DNA stain – propidium iodide). Finally, we found out that concentration of EdU was too high, even though we used recommended concentration for short period of time. After all, we have used 10 times lower concentration and finally we obtained a nice histogram of cell cycle and at the same time plot with EdU – Alexa Fluor. Our final observation is that too high concentrations of EdU resulted in weak signal of propidium iodide.

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SINGLE CELL RNA-SEQUENCING OF SORTED LINEAGE TRACED STEM CELL POPULATION SHOWS A GRADUAL DIFFERENTIATION PATTERN IN CONTINUOUSLY GROWING TEETH

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In rodents and several other species, a continuously growing teeth have evolved. This adaptation on specialized style of living ensures permanent replenishing of dental tissues worn by constant gnawing and provides an attractive system for studying of stem cell niche, cell differentiation or injury-induced regeneration. Recent advances in single cells RNA sequencing and lineage tracing methods enabled to perform an unbiased and reliable analysis of this organ and to study different stem cell populations responsible for permanent growth. Using this approach, we found a novel, quiescent and long-lasting population of mesenchymal stem cells contributing to the permanent tooth growth. This, up to now unknown, stem cell population is spatially restricted and gives rise to all mesenchymal parts of dental pulp, including different types of dental pulp cells and dentin-producing odontoblasts. Further analyses showed a multipotent characteristic of this unique population and uncovered molecular background responsible for differentiation (bifurcations) into distinct terminally differentiated cell states. Based on our detailed study of this exemplary model system on single cell level we uncovered the role of the same type of stem cell population during embryonic development of several organ systems. Taken together we discovered a novel, highly specific mesenchymal stem cells which plays role during permanent adult tissue growth and contribute to formation of different organs during development.

ATYPICAL HUMAN KINASE HASPIN – CELL CYCLE FUNCTIONS AND ROLE IN CANCER CELL PLASTICITY

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Mitosis as a crucial step of the cell cycle is essential to genomic stability and mistakes in this process can cause various developmental diseases and cancer. New mitotic kinases have been recently identified as relevant candidates for pharmacological intervention, i.e. attractive targets for drug discovery. Moreover, sufficiently selective small-molecule inhibitors represent not only promising starting points in the development of new drugs, but also frequently serve as invaluable chemical biology tools to explore the cellular functions of kinases. Haspin is an atypical mitotic kinase and as one of the players of the chromosomal passenger complex has specific function in regulation of chromosome alignment, centromeric cohesion, and spindle stability in mammalian cells. The best characterized substrate of Haspin is the threonine 3 residue of histone H3 (H3T3)[1]. It remains unclear whether Haspin is also functional at telophase and interphase. In our work, we compared new proprietary Haspin inhibitors with outstanding selectivity to the state-of-the-art inhibitor CHR6494. The response of breast cancer cell lines to specific Haspin inhibition included decrease in phosphorylation of the only Haspin-specific substrate H3T3 and shift in the cell cycle distribution toward G2/M phase. Moreover, time-lapse imaging using the Fucci2 reporter system in HeLa cells revealed other cell cycle malfunctions. Thus, we aim to link malfunctions and cell fates to interphase- or mitosis-related regulation of the kinase. Furthermore, considering the potential of Haspin as an emerging target for modern anticancer therapy and its involvement in the genome and chromosomal instability, we have investigated its role in the cancer plasticity and EMT using complex flow cytometric analysis. Our data strongly suggests the role and functional link of Haspin and its main interacting partner AuroraB in cancer cell plasticity and EMT phenotype.

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HIGHLY MULTIPLEXED, SINGLE-CELL FUNCTIONAL PROFILING OF CAR-T CELLS ENABLES MORE PREDICTIVE PRODUCT CHARACTERIZATION, CELL MANUFACTURING ANALYSIS,AND CELLULAR BIOMARKERS ACROSS PRODUCT TYPE

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Chimeric antigen receptor (CAR) T cell therapy has already paved the way for successful immunotherapies to fight against liquid tumors and is quickly expanding to solid tumors. Nevertheless, the biggest challenges are how to evaluate the quality of CAR-T cells and how to predict their in vivo behaviors once reinfused into a patient. In this report, we review single-cell polyfunctional profiling results obtained from several different sets of pre-infusion CAR-T samples, including CD19 CAR-T products from Novartis and Kite Pharma (Gilead), GoCAR-T cell products targeting Prostate Stem Cell Antigen from Bellicum, bispecific CD19/22 CAR-T cells from the NIH, trimeric APRIL-based CAR-T cells targeting both BCMA and TACI from MGH and CAR-T cells targeting glypican 3 in hepatocellular carcinoma from NIH. In each case, CD4+ and CD8+ CAR-T cells were stimulated and subsequently analyzed at a single-cell level using IsoPlexis' IsoCode proteomic chips. Our single-cell data revealed highly polyfunctional and heterogeneous responses across each cohorts. The polyfunctional strength index (PSI) of the pre-infused CAR-T products is significantly associated with the clinical outcome of the patients after receiving the treatment, as well as post-infusion grade 3+ CRS. The CAR-T cells secreted a wide range of cytokines/chemokines in response to antigen specific stimulation and a significant portion of the CAR-T cells were polyfunctional (2+cytokines/cell). These results highlight the potential benefits of single-cell proteomics to comprehensively understand how CAR-T products behave in response to antigen-specific stimulation. Analyzing the single-cell polyfunctionality of CAR-T profiles also provides a valuable quality check for optimizing the manufacturing process and a powerful tool for next generation biomarker developments

INTACT NUCLEI SAMPLE PREPARATION FROM FROZEN BRAIN TISSUE WITH THE WOLF CELL SORTER

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The isolation of nuclei for single cell RNA-sequencing has been extremely helpful for cell types that are challenging to isolate such as neurons. However, working with nuclei also comes with its own challenges, nuclei are very fragile and some isolation methods can be too harsh and leave low quality or lysed nuclei. In addition, some isolation methods can leave a significant number of debris that can cause poor sequencing results. Proper sample preparation is a direct reflection of the quality of your sequencing results. The 'Frankenstein' protocol developed by Luciano G. Martelotto has been shown to result in successful isolation of intact nuclei for downstream applications such as 10x Genomics single nuclei RNA-seq (sn-RNA-seq) assays.1 Successful snRNA-seq experiments using the Frankenstein protocol have been demonstrated in several published papers.2-5 This protocol has been shown to be successful with several sample types, including fresh and frozen tissues. Along with the proper nuclei isolation method, sample clean-up using a cell sorter is also beneficial for removing unwanted debris. Moreover, given the fragility of nuclei, gentle sort speeds are essential. The WOLF Cell Sorter is a gentle, microfluidic cell sorter that efficiently removes unwanted particles from samples with very low shear stress.

DEVELOPMENT OF A TRIPLE UV/VIOLET EXCITATION ANALYZER FOR LABEL-FREE FLOW CYTOMETRY

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Background. There is unmet demand for flow cytometry systems that are customized and modular; and in particular, for analyzers tailored to operate in the UV region from customers interested in high sensitive label-free analysis in enviromental research. Using Kinetic River Potomac™ flexible architecture, we have designed and built a Potomac analyzer customized for label-free analysis for the IREA-CNR, Naples, Italy. Methods. It uses three interchangeable excitation sources: two built-in (405 and 375 nm) , both from Pavilion Integration Corp), and one external add-on (266 nm, from CryLas). It has six PMT detectors, FSC, SSC, and four fluorescence channels (Hamamatsu Corp.), with three extra channels built-in. Detection channels (UV to IR) use high performance removable Semrock dichroics and filters (IDEX Health & Science). Ultrastable sheath flow is established with Kinetic River Shasta™ fluidic control system. It incorporates our always-on flowcell monitoring Cavour™ system for simple troubleshooting. Data is collected with proprietary electronics and a PXI National Instruments high speed data acquisition system. Proprietary Panama software™ operates the system, performs a full suite of sample processing and data visualization, and is intuitive and user-friendly. Results. The system was tested for performance using a variety of dyes, microspheres, and cell-based assays, including Coefficients of variation (CVs) for the scatter channels and each fluorescence channel, 8-peak beads dynamic range, Megamix-Plus SSC nanoparticles beads and cell cycle using DAPI. Conclusions. We have designed, built, and delivered a completely customized commercial flow cytometry solution that enables researchers at IREA-CNR to perform label- free excitation of algae and bacteria for water quality analysis. The system includes only those features required to meet the end-user's unique needs, with built-in room to grow.

BUILDING ARNO, TIME-RESOLVED FLOW CYTOMETER FOR COMPENSATION FREE ASSAYS

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Background. In polychromatic flow cytometry, compensation is required to account for and remove the contribution of signal spillover between spectral channels. In order to address to need of high complexity assays avoiding spectral spillover, a novel Lifetime Fluorescence platform was designed. Methods. The new 15-parameter "Arno" platform has two spatially separated, pulsed excitation sources (Toptica iBeam 405 and 488); one FSC and two SSC channels; and 12 fluorescence detection channels (6 per laser), from violet to infrared. The 12 fiber-coupled fluorescence signals are sensed by only 6 physical detectors (Hamamatsu PMTs). Each detector collects light from two spectrally overlapping fluors, and the contribution from each fluor is resolved using Kinetic River proprietary time-domain multiplexing technology . Ultrastable sheath flow is established with our custom-built Shasta fluidic control system. Data acquisition is performed using 8-channel 1.25-GHz sampling on a National Instrument PXI platform and custom-written LabVIEW code. Signal processing is performed on a dedicated computing platform running custom algorithms. Results. Arno system has been fully characterized with respect to sensitivity, dynamic range, CVs, and time resolution in each of the 12 fluorescence 'channels' (six spectral bands, three from each laser, each with both a short- and a long-lifetime component). By having only three detectors per laser, each wavelength band can be spectrally well-separated, leading to less than 5% spillover in all channels, with most below 1%. For the proof-of-concept CD3 and CD4 are detected simultaneously in the same spectral band and lympho populations can be easily distinguished. Conclusion. Our Arno platform leverages Kinetic River's proprietary time-resolved flow cytometry technology to achieve compensation-free 12-color (plus one forward and two side scattering channels) using only two lasers and six spectral detectors.

PRODUCTION OF A MICROFLUIDIC CHIP TO STUDY THE CD44-HIPPO PATHWAY CROSS-TALK

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During the last decade, demand for physiologically relevant 3D in vitro models has arisen since these models are capable to better recapitulate complex cell behaviour than 2D cell cultures as well as reduce the need for animal testing. However, commercially available chips usually have a rectangular cross-section of the channels, which may cause non-physiological behaviour of the cells due to disturbances in shear stress. It is now well established that laminar flow generating constant shear stress poses a protective effect on vasculature. On contrary, disturbed flow is responsible for the pathologies. Among many mechanosensitive pathways, the Hippo pathway was shown to sense the shear stress in endothelial cells. CD44 is a promiscuous mechanoreceptor that senses the ECM components in addition to other functions. Literature has indicated that it may interact with the Hippo pathway.
To overcome problematic rectangular cross-section, we designed a microfluidic chip with a circular cross-section of the channels that generates uniform shear stress. The chip was made of polydimethylsiloxane (PDMS). The surface of native PDMS was plasma oxidized and further coated by collagen IV to overcome its limiting hydrophobicity that strongly limits cell adhesion. Channels with modified surface were then seeded with endothelial cells that were cultivated under flow. Using this chip, we were able to repeatably get a uniform layer of highly viable endothelial cells having physiological behaviour.
The monitoring of the Hippo pathway was optimised in a static HUVEC cell culture. First, a fluorescence reporter of Hippo pathway-related transcription was introduced. It, however, worked well with confocal microscopy only. The expression of Hippo pathway downstream genes was determined by RT-qPCR, reference gene for HUVECs was optimised. Hippo pathway activation was further examined using western blotting. Next, the Hippo pathway activity will be studied under flow using our model with unique surface chemistry.

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PHENOTYPIC VARIABILITY IN LYMPHOID CELL LINES: FACS-BASED ANALYSIS OF SINGLE CELL CLONES AND DESIGN OF QUALITY CONTROL FLOW CYTOMETRIC PANELS

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Cellular heterogeneity in tumor cells is a well-established phenomenon. Individual cells vary in many aspects, for instance ploidy, ability to survive, proliferate or migrate. These complex characteristics can be reflected by distinct expression profiles and patterns of surface molecules. In this study we have asked what is the cellular heterogeneity within established cancer cell lines.
We created several clones of lymphoid cell lines engineered by CRISPR-Cas9 technology or stably expressing doxycycline-inducible vectors. We sought to establish easy-to-use method for characterization of subpopulations within original cell lines and comparison with the generated clones. Flow cytometry-based phenotyping technique seemed to be promising method for assessment of populations in original cell lines and similarity and representation of these populations in resulting clones originated from a single cell. We assembled panel of surface molecules that are non-uniformly expressed in original cell lines and their expression was meaningful for our studies. Dimensionality reduction and clustering methods were applied and representation of subpopulations of cells with similar expression patterns was assessed. Our study raises a quality control issue in cell lines and especially in the individual single cell clones – a procedure routinely used as part of almost any genetic manipulation in cell lines.

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MEF 3T3 CELLS REACTION TO PHO- BIOPOLYMER OF BACTERIAL ORIGIN

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In the era of growing ecological awareness, interest in polymers of natural origin increases due to their exceptional properties such as biocompatibility, biodegradability and biointegrity. Bioplastics are considered as valuable materials that benefit the environment and have a growing potential for medical applications, such as: bone scaffolds, dentures or wound patches production. Biopolymers such as polylactide (PLA) and poly (3-hydroxyoctanate) (PHO) favor cells growth, which can play an important role in medical products. The biopolymers, due to their chemical properties, can be modified with antibiotics or other anti-inflammatory drugs supporting physiological processes such as wound healing and tissue regeneration. However, the path to the final medical product must begin with basic cellular research. Their aim is to determine the cells' response to physicochemical changes in their environment, and to check whether given material or substance has a toxic effect on them. The interaction between cells and the environment (extracellular matrix or other cells) is one of the factors which strongly modulate the mechanisms governing the wellbeing of the entire organism. Morphology of the cells is an important indicator showing the reaction of cells to the variable surrounding conditions, including the substrate properties.

Our study shows that cells grown on our biopolymers show a much more extensive, denser network of both actin filaments and microtubules, which however are much thinner than those observed in cells grown on glass. The analysis of cellular morphology changes, including cytoskeleton dynamics, was performed using confocal microscopy technique which also allowed to visualize the biomaterial structure in three dimensions. Data obtained gave detailed information about the sample spatial organization, its porosity and internal structure. Application of advanced research methods to study the properties of new biomaterials can contribute to significant advances in research and open up new horizons for the medicine of the future.

ANALYSIS OF PLANT GENOME SIZE IN THE FIELD USING GUAVA MUSE

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Analysis of plant genome size often involves collection of samples at remote locations. Therefore, it would be convenient to be able to perform the flow cytometric analysis in the field. The aim of this study was to assess the usability of Guava Muse (Luminex) for the analysis of plant genome size in the field.
Leaves of plants commonly used as size standards were collected and cell nuclei were isolated and stained using propidium iodide (PI) or other DNA stains and DNA content was analyzed using Guava Muse.
Guava Muse was found to be truly portable thanks to its size (20x22x28cm), weight (5.9kg) and no need for sheath fluid. In locations with no access to power grid, Guava Muse was powered from car battery using an AC/DC converter. Its optics (532nm laser and two fluorescence detectors) allowed detection and quantitation of DNA stained with PI, 7-AAD, DRAQ5, LDS-751 and DyeCycle Orange or Ruby. Plant standards with various genome sizes were successfully analyzed and CVs were assessed.

ARYL HYDROCARBON RECEPTOR (AHR) DOWNREGULATESTHE INFLAMMATORY RESPONSE IN HUMAN LUNG ALVEOLAR TYPE II CELLS MODEL

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The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor involved in the metabolism of carcinogens. In addition, there is evidence suggesting that the AhR could actively contribute to the control of inflammation. However, there is limited information regarding this particular role within lung alveolar type II (ATII) cells. Using a human model of ATII cells, A549 cell line, we studied the participation of the AhR in the modulation of inflammatory responses and the relevant mechanisms. We activated wild type (WT) and AhR knockout (AhR KO) A549 variants with a pro-inflammatory cytokine, IL-1β. We found that the inflammatory response in AhR KO cells led to a significantly higher induction of cyclooxygenase-2 (COX-2), production of prostaglandins, and the release of pro-inflammatory cytokines (IL-6 and IL-8). This corresponded with increased induction of PTGS2, mPTGES, CXCL8 and IL6 mRNAs in AhR KO cells. The increased production of inflammatory mediators in AhR KO cells was linked with a significantly higher activity of transcription factor NF-κB, which regulators IKKα/β and their target IKBα, were more efficiently phosphorylated in AhR KO cells. Finally we mimicked the exposure to airborne mixture of pollutants activating inflammatory responses in ATII cells, using organic extract of a standard mixture of diesel exhaust particles (SRM1650b). We found that both crude extract and polar fraction of SRM 1650 induced an exacerbated inflammatory response in AhR KO cells (increased induction of *COX-2, TNF-α, CCXCL8* and *IL6* genes), and that AhR countered this response as we found only a slight increase in induction of the pro-inflammatory genes in WT cells. Overall, these results suggest that AhR may act as a negative regulator of the inflammatory process in human ATII cells, which impacts NF-κB pathway as the major mechanism being responsible for this modulation. [Supported by the project no. 18-00145S of the Czech Science Foundation].

ANOTHER ONE BEATS THE DUSP - DUAL SPECIFICITY PHOSPHATASES IN MES DIFFERENATAION

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The mitogen activated protein kinase (MAPK) pathway is one of the best-studied signaling axes important for numerous cellular processes, which has been described for example in context of cancer biology, immune response or animal development. Members of the MAPK protein family are active when phosphorylated and can be inactivated by number of phosphatases, including dual specificity phosphatases (DUSP). This family includes more than twenty different enzymes and in our study we focus on three cytosolic DUSPs – DUSP6, DUSP7 and DUSP9 and their role during the *in vitro* differentiation of mouse embryonic stem (ES) cells. Using the CRISPR/Cas9 method we have created cell lines depleted of these phosphatases and we show, that even though these cells do retain some of their basic characteristics, their absence affects the cell differentiation. We have observed that in the case of depletion of any of the studied DUSPs, embryonic stem cells preferentially differentiate towards neural cells, while the formation of early cardiac mesoderm, and later cardiomyocytes, is repressed. Despite the high degree of enzymatic redundancy of DUSP6, DUSP7 and DUSP9, we have observed differences in severity of this shift during the differentiation, between cell lines depleted of individual phosphatases. This suggests the existence of spatiotemporal differences between studied DUSP during spontaneous differentiation.

PROTEOMIC ANALYSIS IDENTIFIES KEY PLAYERS IN THE PROCESS OF METASTASIS UNDER HYPOXIC CONDITIONS

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According to GLOBOCAN database for 2020, cancer is still at the top of the leading causes of death worldwide, with lung cancer ranking first. Patients with lung tumors, especially non-small-cell lung carcinoma (NSCLC), are often diagnosed at a metastatic stage and thus have a poorer prognosis. A critical factor in solid tumors that greatly affects the process of metastasis and hinders effective therapy is hypoxia. Understanding the effect of hypoxia, especially regarding tumor heterogeneity, would be beneficial in targeted treatment approaches not only in NSCLC.

Proteomic analysis revealed some interesting differences between two distinct subpopulations of lung adenocarcinoma cell line A549, namely resistant fraction with increased activity of BCRP transporter, called side population (SP), and the rest of cancer cells (nonSP). These differences were further exacerbated under hypoxic conditions, when a surprising decrease in BCRP protein with concomitant drop in the size of the SP was observed. Moreover, hypoxia has significantly changed level of proteins associated with cell cycle, metabolism, and, in particular, extracellular matrix organization (PLOD2, SERPINE1, LOXL2, P4HA1/2, BSG, AGRN) that promotes metastasis. Based on the latter, the migratory, invasive and clonogenic properties of SP and nonSP cells cultivated under hypoxia were also verified. Additionally, the effect of hypericin (HY), a promising natural anticancer substance, as well as competitive inhibitor of BCRP and violator of hypoxia regulator, HIF, has been also demonstrated. We have shown that HY not only decreased size of SP regardless of oxygenation, but also interfered with other features of cancer cells acquired in hypoxia, in line with the decrease of HIF-1α/HIF-2α.

To sum up, hypoxia, as a typical tumor microenvironment factor, can modulate resistant and aggressive phenotype of cancer cells, while HY could partially reverse some of these effects and moreover, could increase tumor sensitivity to some chemotherapeutics by predicted competitive inhibition of BCRP.

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CLINICAL CYTOMETRY

CYTOMETRIC FINDINGS UNCOVER IMMUNOLOGICAL CHANGES IN CHILDREN WITH RARE COMPLICATIONS AFTER SARS-COV2 INFECTION

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Virus SARS-CoV2 is notorious for causing severe disease in >10% of the elderly but not in children. We recently showed that majority of children is spared from severe course even when affected by anticancer treatment. In contrast with that, <1% previously healthy children develop a severe but treatable condition called Pediatric Inflammatory Multisystemic Syndrome (PIMS). Until now, factors leading to PIMS are not fully known but reports on immune changes emerge recently.

Here we study PIMS-related changes. In total, 36 patients with PIMS were included in the study. Findings were compared to control groups of children after COVID with no signs of PIMS (n=7) and of adult volunteers who had recovered from mild or moderate COVID infection (n=23) or who had had no proven SARS-CoV2 infection (n=6). Healthy SARS-CoV2 convalescents were investigated <1 month, 6 months and 1 year after COVID.

Peripheral blood was analyzed for blood count, extended lymphocyte subsets including functional maturation subsets of CD4 and of CD8 cells and for presence of anti-SARS-CoV2 antibodies. Mononuclear cells were cryopreserved for subsequent functional and genetic investigations (cytokine release after short term culture or after 2 weeks, repertoire of TCR V beta clones, and detailed analysis of TCR V beta 21.3+ cells). As of this abstract submission, results of fresh blood investigations are available, whereas only pilot investigations have been completed in cryopreserved cells.

Our data confirms recent reports that PIMS patients suffer from a significant lymphopenia (median+-standard deviation, PIMS and control children: 1.2+-1.8 and 2.9+-1.4 x10^9 per liter, respectively; p=.0047). Among the basic lymphocytic subsets, CD8 lymphocytes are disproportionally decreased in PIMS vs. control COVID children (18+-7.3% and 34+-9.5% of lymphocytes, respectively; p=.0031). PIMS patients have often T cell activation as judged by HLADR, however, this is not uniformly higher than in controls. Surprisingly, interferon gamma production among the first PIMS patients was markedly decreased, which contrasts with reports on its high plasma levels. Interestingly, a high proportion of TCR V beta 21.3+ was found among PIMS patients, confirming recent reports.

In summary, cytometry helps to discover details of immune dysregulation leading to PIMS.

ELEVATED PLATELET AND ENDOTHELIAL EXTRACELLULAR VESICLES IN CORD BLOOD OF PRE-TERM NEWBORNS WITH INFLAMMATION

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Analysis of extracellular vesicles (EV) is recognized as a potential diagnostic tool. Opposed to venous blood plasma, cord blood plasma is not extensively studied. Efforts are being made to standardize the cytometric analysis of EV to improve reproducibility of results. We have utilized number of recommended procedures to analyze EV from cord bloods of pre-term newborns using improved BD FACS Canto II cytometer. The cytometer was equipped with more powerful 405 nm laser and bandpass filter for violet side scatter (VSSC). Anticoagulated cord blood was centrifuged 2x 2800 g for 15 minutes to collect platelet free plasma which was snap frozen. For analysis, we labelled plasma with set of markers for platelets (CD36, CD41, CD62P) and endothelial cells (CD31, CD105, CD146). ApogeeMix beads were used to define EV acquisition gate. Size of EV in the gate was ranging from 200 nm to 2400 nm. We compared the numbers of measured EV in four experimental groups: Fetal inflammatory response syndrome (FIRS, n = 5), pre-term newborns with (PTIN, n = 18) and without inflammation (PT, n = 28) and full-term newborns (Control, n = 20). Platelet CD36+/CD41+ and CD36+/CD41+/CD62P+ EV counts and also endothelial CD31+/CD105+; CD31+/CD146+; CD105+/CD146+ and CD31+/CD105+/CD146+ EV counts were all significantly higher in PTIN than in PT ($P<0.05$). Additionally, CD31+/CD105+ EV were significantly lower in PT than in Control ($P<0.05$). In our study, we found significant differences in the number of platelet and endothelial EV between pre-term newborns with and without inflammation suggesting possible diagnostic potential of cord blood EV in pre-term infants complications.
This study was funded by the Ministry of Health of the Czech Republic, grant no. NV17-31403A, and Charles University, project GA UK No. 1322119 and Progress Q26/LF1/3.

IMPACT OF IVD REGULATION 2017/746 ON THE EUROPEAN HEALTH INSTITUTIONS

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In May 2017, the European Union published the In Vitro Diagnostic Medical Devices Regulation EU 2017/746 (IVDR) which repeals the In Vitro Diagnostic Medical Devices Directive 98/79/EC (IVDD) in order to establish a more robust, transparent, predictable and suitable regulatory framework for IVD Medical Devices [(1)].

The aim of IVDR is to guarantee patient safety by enforcing transparency, traceability and heightening the requirements to demonstrate clinical evidence and analytical performance of CE-IVD Medical Devices, which ensures a high level of safety and health whilst supporting innovation [(1)].

The IVDR affects not only economic operators of *in vitro* diagnostic medical devices (IVD), but also the health institutions as clinical laboratories. This new regulation will come into full effect on May 26th, 2022. From this day on, it will be mandatory to use CE-IVD products developed under commercially available IVD Regulation (IVDR) or the use of laboratory developed test (LDTs) when non-equivalent CE-IVD test developed under IVD Regulation is commercially available or does not fit specific clinical needs[(1)].

In addition, when developing LDTs, the clinical laboratories will need to justify their use and strictly comply all requirements related to the product and to the health institution [(1,3)].

Complying with the methodological requirements of the IVDR will be a challenge for many institutions and a correct approach and address will be necessary to comply with the requirements of the IVDR for LDTs.

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24-COLOR HUMAN IMMUNOPHENOTYPING PANEL USING 3 LASERS ONLY: CYTEK SPECTRAL CLINICAL FLOW CYTOMETER

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The Cytek full spectrum flow cytometry combined with the SpectroFlo software's real-time unmixing capability provides greater fluorochrome choice and panel flexibility and allows users to quickly visualize data and statistics. All this results in the ultimate flow cytometry solution for deep immunoprofiling, from 24 colors all the way to 40 colors. The detection of some fluorochrome combinations by conventional flow cytometry presents a challenge due to high amounts of peak emission spectral overlap. The Cytek Aurora and Northern Lights systems address this challenge by using differences in full emission spectrum signatures across all lasers to clearly resolve these dye combinations, even when conjugated to markers co-expressed on the same cell.

PLATELET-MONOCYTE AGGREGATES (PMA): IN VITRO FORMATION, AND DETECTION IN A COHORT OF HEALTHY INDIVIDUALS

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Metabolic disorders are highly prevalent conditions in the societies of developed countries such as the Czech Republic. These conditions can appear both due to genetic inheritance or as a consequence of the population lifestyle and alimentary habits. They include diabetes mellitus type I and II, the latter is associated with metabolic syndrom or isolated hypercholesterolemia. Their cardiovascular complications can be potentially life-threatening. Patients suffering from these disorders are known to have a higher ratio of activated platelets in comparison to healthy individuals. It is well known, that activated platelets are able to bind to circulating monocytes and give place to the occurence of platelet-monocyte aggregates (PMA), therefore, its detection can help quantify the amount of activated platelets in patient blood samples. PMA can be easily detected and analyzed by laboratory techniques such as flow cytometry, which can help identify individuals at risk without apparent symptoms. In the first part of this project, a flow-cytometry method was developed in which PMA formation was induced *in vitro* and the most sensible cluster of differentiation (CD) combinations for flow cytometry analysis was selected. The best combination showed to be CD41-FITC, for platelets, CD14-PE/Cy7 for monocytes and CD45-PerCP/Cy5.5, as a pan-leukocyte marker. In the second part of the project, we present the preliminary data of the analysis of a total of 32 individuals (15 male and 17 female) with ages ranging between 26 and 67 years. Healthy volunteers were enrolled among healthy workers and personnel from the University Hospital of Hradec Králové. All volunteers were healthy and whole blood was collected in sodium citrate-containing vials and analyzed within 1 hour to study the presence of PMA. After PMA detection, the values were compared to age, BMI and other biomarkers. Our results suggest a correlation between volunteer age and presence of PMA. Extension of the population sample is needed to confirm our preliminary data.

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IMMUNOPHENOTYPING OF T-ZONE LYMPHOMA IN DOGS USING MULTIPARAMETER FLOW CYTOMETRY

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T-cell lymphomas are diverse group of neoplasms with variable diagnostic features, pathophysiologies, therapeutic responses and clinical outcomes. In dogs, TLC includes indolent T-zone lymphoma (TZL) with long survival time despite generalized lymphadenopathy or high number of neoplastic cells in peripheral blood before treatment. Clinical response to chemotherapy is generally good, but in some cases, achieving of complete microscopic remission is not possible. The prevalence of TZL is estimated as 3 -14% of all canine lymphomas. TZL is characterized by the clonal expansion of T cell ($CD3^+$) and lacking expression of the pan-leukocyte antigen CD45. Another typical feature is a high level of expression of CD 21 in most of the cases. The presence of CD4 and CD8 T-cell markers is variable. The aim of this study was to determine frequency of various immunophenotype of TZL diagnosed in Veterinary Research Institute between years 2012 and 2020 using multiparameter flow cytometry in samples of canine lymph node aspirates. In this period, 10 aspirates (4 % from 235 clinical samples) were immunophenotypically consistent with TZL ($CD45^-CD3^+$). From that, the majority of samples (5 samples) were of $CD4^-CD8^-$ phenotype, followed by $CD4^+CD8^-$ (3 samples) and $CD4^-CD8^+$ (2 samples). All of the $CD45^-$ T-zone cells were also stained positive for MHCII, Thy1 and CD25. In two of the TZL cases staining for CD21 was negative. Due to unique immunophenotypic features of TZL, multiparameter flow cytometry is a useful tool in the objective diagnosis and further characterization of this type of lymphoma.

THE ROLE OF COMPLOSOME IN CD4 T-CELLS

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The serum complement system serves as an ancient humoral branch of innate immunity and represents the first line of defense against various pathogens. However, recent studies confirmed that also CD4 T-cells synthesize complement proteins intracellularly. This intracellular complement system refers to as complosome and regulates CD4 T-cell survival and Th1 differentiation.

Cell purification:

CD4 T-cells were separated from PBMC using a negative magnetic selection technique. The purity of cell suspension was typically over 93 % and was verified by a flow cytometry.

Cell stimulation:

CD4 T-cells were incubated in 96U well plates (150.000 cells/well). For the complosome activation, we stimulated cells with a combination of anti-CD3 (10ug/ml) + anti-CD46 (5ug/ml) + IL-2 (50U/ml) and with or without a Vitamin D ($1*10^{-7}$ M).

Complosome dynamics measurement:

For determination of period with maximal complosome changes, we incubated cells with or without stimuli for 0, 12, 36, 60 and 108 hours. Results showed that maximal complosome changes were detected after 60 hours of stimulation.

Complosome component detection:

Following complosome components were measured: CD46, C3aR, C5aR, C3b and cytokines INF-γ and IL-10. Cells were stained using a standard intracellular staining protocol with blocking of nonspecific monoclonal antibody binding using a monomeric human IgG (1ug/ml).

Flow cytometry:

The analysis of complosome components was measured in CD4+ positive cells after doublets and dead cells exclusion. The negativity for all measured CD markers was assessed using FMO controls.

Conclusion:

We were able to measure a complosome activation in CD4 T-cells, which drive their differentiation into Th1 proinflammatory phenotype (production of INF-γ). High dose of IL-2 initiate the transition from Th1 into regulatory Tr1 phenotype (production of IL-10). We also confirmed that addition of vitamin D into wells supported the transition into Tr1 phenotype. Because complosome dysregulation and positive effect of vitamin D treatment has been described in autoimmune disorders, we focused on potential complosome pathology and vitamin D effect in allergic asthma patients.

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INFLUENCE OF RADIATION DOSE ON THE CHANGE OF H2AX EXPRESSION

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Biological dosimetry plays an important role in estimating radiation exposure after radiation accidents or DNA targeted therapy, where physical dosimetry is not available. Ionizing radiation, as well as other harmful effects, induce the range of DNA lesions, the most serious of which are DNA double strand breaks (DSBs). After exposure to 1 Gy of gamma radiation, approximately 40 DSBs are formed in the cell and approximately 1% H2AX is phosphorylated. Each DSB induces phosphorylation of approximately 2,000 H2AX molecules. The phosphorylated version of H2AX is easily detectable by monoclonal antibodies and flow cytometry and H2AX gamma assay represents a useful biomarker for DNA DSBs monitoring.
The aim of the work was to evaluate the effect of gamma radiation escalating doses on DNA by monitoring of H2AX histone phosphorylation.

Methods:

The peripheral blood mononuclear cells (MNC) from 14 healthy donors were included in the study. The MNC fraction was cultured with mitogen L-PHA in RPMI 1640 culture medium for 24 h at 37°C and 5% CO2. The samples were then irradiated with 0.5 Gy, 1 Gy and 2 Gy dose of gamma rays. Irradiation was performed on a Gammacell® 1000 Elite caesium emitter. After irradiation, the samples were incubated for another 24 hours at 37°C and 5% CO2. Determination of H2AX gamma expression was performed on a flow cytometer (Navios, USA), following antibodies used in the assay: CD45 KO, CD3 PB (Exbio, Czech Republic) and anti-H2AX AF 488 (eBioscience, USA).

Results:

We detected statistically significant differences in H2AX gamma expression between individual un-irradiated samples and samples irradiated with a dose of 0.5 Gy (difference = 21.560, $p < 0.01$). Similar differences were observed also for dose of 1 Gy (difference = 25,480, $p < 0.01$) and dose of 2 Gy (difference = 25.535, $p < 0.01$).

Conclusion and discussion:

Gamma radiation causes double-strand breaks in eukaryotic cells. This serious DNA damage can be evaluated with H1AX gamma method. Several studies describe the association of radiation dose with the percentage expression of this histone. The formation of gamma-H2AX deposits occurs not only in cells in which DSBs have been induced, but also in response to DNA synthesis or cell apoptosis. Histone H2AX phosphorylation can be considered a reliable specific feature of DSBs and provide the basis for a sensitive assay of DNA damage. The next step of the study is to verify its performance on samples after subthreshold doses exposure (<0.5Gy).

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ACUTE LYMPHOBLASTIC LEUKAEMIA IN A DOG - DOCUMENTATION OF RAPID DISEASE PROGRESSION USING MULTIPARAMETER FLOW CYTOMETRY (A CASE REPORT)

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A 16-month-old female Portuguese water dog was referred for the acute onset of epistaxis, small focal area of necrotic gingivitis, nonregenerative anaemia and thrombocytopenia. No lymphoblasts were detected in the blood smear neither the flow cytometry of popliteal lymph node aspirate did not revealed changes suggestive for lymphoma (lymphoblasts less than 10 % of lymphoid cells). After antibiotic therapy and blood transfusion, the patient recovered, except of occasional episodes of epistaxis. In 1 month after the first presentation, the dog become apathetic and had severe leucocytosis and thrombocytopenia. Flow cytometry of peripheral blood showed the presence of lymphoblasts at level 70% (phenotype $CD45^{+}21^{-}79^{+}34^{+}25^{-}MHCII^{-}$). Despite corticosteroid therapy the health status deteriorated and the patient was euthanized. Flow cytometry confirmed diagnosis of B cell acute lymphoblastic leukaemia based on the presence of lymphoblasts in bone marrow aspirate at level 90 % with the same phenotype as in blood. Acute lymphoblastic leukaemia arises from the malignant transformation of lymphoid progenitors in bone marrow, which results in myelophthisis and subsequent invasion of peripheral tissues. Clinical signs are typically acute in onset, caused by the infiltrative and functional effects of the expanding burden of malignant cells, and are most commonly a consequence of disrupted haematopoiesis. In presented case, the clinical signs were firstly associated with epistaxis with no neoplastic cells in peripheral blood with further rapid progression and corticoid therapy resistance. Presented case showed the importance of flow cytometry and bone marrow sampling in the clinical diagnosis of lymphoproliferative disorders.

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ANALYSIS OF PLACENTAL EXTRACELLULAR VESICLES IN UMBILICAL CORD BLOOD OF PRE-TERM NEWBORNS

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Increased levels of extracellular vesicles (EVs) in peripheral blood were described in pregnant women with preeclampsia. EVs look like promising biomarker of different physiological and pathological conditions including pre-birth complications. In our work, we focused on levels of EVs in umbilical cord blood of pre-term newborns (gestational age <35 weeks) with inflammation compared to levels of EVs in cord blood of full-term newborns. We analyzed EVs in cord blood by flow cytometry on BD FACS Canto II. EVs were identified as events smaller than 1300 nm silica beads (ApogeeMix calibration beads) and positive for cellular markers - placental alkalic phosphatase (PLAP), CD105 (Endoglin), and Syncytin-1. Experimental groups were: pre-term newborns with fetal inflammatory response syndrome (FIRS; n=5), pre-term newborns with inflammation (PTIN; n=18), pre-term newborns without inflammation (PT; n=28), and full-term newborns as controls (n=20). Numbers of placental EVs were significantly increased in PTIN in comparison with PT in populations Syncytin-1$^+$PLAP$^+$ (15.5±9 vs. 7.5±8.6 EVs/µl; P<0.05), CD105$^+$Syncytin-1$^+$ (28±15.8 vs. 12.5±13.8 EVs/µl; P<0.05), CD105$^+$PLAP$^+$ (310±1287.2 vs. 149±90 EVs/µl; P<0.005), and also in Syncytin$^+$PLAP$^+$CD105$^+$ (14±6.7 vs. 5±7.5 EVs/µl; P<0.05). Numbers of EVs was then also increased in control group in comparison with PT in populations Syncytin-1$^+$PLAP$^+$ (15±7.1 vs. 7.5±8.6 EVs/µl; P<0.05), CD105$^+$PLAP$^+$ (358±1324.4 vs. 149±90 EVs/µl; P<0.0005), and Syncytin$^+$PLAP$^+$CD105$^+$ (11±6 vs. 5±7.5 EVs/µl; P<0.05). The specificity of EVs detection was confirmed by disappearance of the signal after addition of 0.1% Triton X-100. We found significantly increased number of placental EVs in cord blood of pre-term newborns with inflammation. Placental EVs may have diagnostic potential in complications connected with pre-term births.
Supported by GAUK 1322119, Progress Q26/LF1, SVV 260 520

GRAFT COMPOSITION VARIABILITY IN AUTOLOGOUSLY TRANSPLANTED MULTIPLE MYELOMA PATIENTS

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Multiple myeloma (MM) is a hematological malignancy of plasma cells and nowadays is the most common indication for high dose chemotherapy and autologous stem cell transplantation (ASCT). ASCT is an effective treatment method for malignancies such as lymphomas and myeloma and reaches the effective immune recovery in most cases. Thus, the aim of this study was: (1) to evaluate the variability of the graft composition in MM patients, and (2) to determine its correlation with the clinical parameters. Twenty-two patients with MM were enrolled in this study. A small part of the apheresis product, previously cryopreserved, was used in this study. The immune cells in this apheresis product such as B-regulatory cells (Bregs), T-cells (naive, Tregs), dendritic cells (DC) – myeloid (mDC) and plasmacytoid (pDC), NKG2D expression (proportion and mean fluorescence intensity – MFI) on NK and T-cells were investigated using flow cytometry. Our results showed that the proportion of all populations in grafts is very variable. T-cells subsets analyzed from all lymphocytes ranged within these proportions: naive T-cells 4-49 %, naive CD4+ 3-45 %, naive CD8+ 3-53 %, Tregs 0.34-2 %. Another similar regulatory subset – Bregs – varied from 0.021 to 0.408 %. NKG2D expression showed the range 13-52 % (on CD3 cells) and 22-86 % (on NK cells), also the level of expression estimated by MFI differed within patients and showed the range 42-597 on T-cells, and 103-1455 on NK cells. mDC showed the range 0.20-1.15 % and pDC showed 0.19-1.46 %, both from whole leukocytes. There was no correlation with the clinical data (such as progression-free survival (PFS), the depth of remission in apheresis period, and others). Overall, these results showed a large variability of the autologous graft composition. Their potential influence on treatment outcome needs to be evaluated on a larger group of patients.

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HEMATOLOGY

CONCEPT OF HIGHLY SENSITIVE 12-COLOR FLOW CYTOMETRY FOR DETECTION OF CLONAL PLASMA CELLS IN MULTIPLE MYELOMA

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Background: Detection of clonal plasma cells (PCs) is an essential part of diagnostic algorithms in malignant multiple myeloma (MM). Flow cytometry (FC) is able to identify even a small number of circulating PCs in peripheral blood in time of diagnosis and/or to detect clonal PCs in bone marrow in time of minimal residual disease (MRD) assessment. Standardized Euroflow protocol for PC detection is nowadays available, but it has disadvantage in a redundancy of some markers.
Aim: Development and verification of highly sensitive 12-color flow cytometric protocol for PC detection and clonality assessment in peripheral blood and/or bone marrow.
Methods: Peripheral blood (PB) of newly diagnosed MM patients was bulk-lysed and incubated with CD138-BV421, CD27-BV480, CD200-BV605, CD81-BV711, CD38-FITC, CD45-PerCP, CD28-PE, CD56-PEDL594, CD19-PC7, CD117-AF700. Cytoplasmic kappa-APC and lambda-APCC750 were added after fixation and permeabilization. Whole BM was also processed in some pair patients. Analysis was done on 4-lasers Omnicyt (Cytognos) and reanalysis by Infinicyt (Omnicyt).
Results: Total of 15 patients was analysed. Median volume of processed PB was 2 ml (range 1,0-4,0) with median of acquired 2,5x10^6 events. Circulating PC were detected in all cases with median 0,017 % (0,004-0,080) from nucleated cells and their phenotype and clonality was verified by BM analysis in selected cases. Evaluated 12-color protocol was created by expansion of 2nd generation of Euroflow protocol for PC dyscrasias, when next generation flow (NGF) approach was adopted. They share backbone markers with exception of BV510 fluorochrome what was replaced by BV480. The significant problem was undetectable CD117 due to the inappropriate combination with dim AF700.
Conclusion: Circulating PCs are usually present in very low number and share the same phenotype as clonal PCs in BM. Replacement of CD117, AF700 or change of whole protocol is matter of debate, although other markers work perfectly.
Supported by Ministry of Health of the Czech Republic, grant nr. NV18-03-00203.

PROGNOSTIC SIGNIFICANCE OF CIRCULATING PLASMA CELLS IN NEWLY DIAGNOSED MULTIPLE MYELOMA: CZECH VALIDATION COHORT

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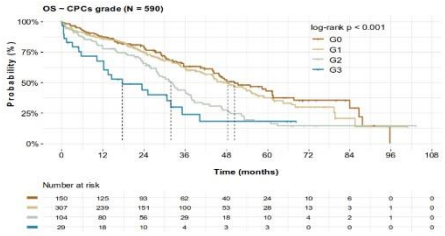
Background: Tumor burden in multiple myeloma (MM) has always been evaluated in the bone marrow; nevertheless it has never been implemented as a part of any risk stratification system due the lack of prognostic value. On the other hand the quantification of circulating plasma cells (cPCs) from peripheral blood (PB) may be used as a surrogate of tumor burden as well as a powerful diagnostic biomarker.
Aims: To validate the prognostic value of cPCs in newly diagnosed MM patients (as proposed by Garces, EHA 2021).
Methods: CPCs were analyzed in PB by flow cytometry (FC) in 590 MM patients diagnosed between 2012 and 2019 at University Hospitals Brno and Ostrava. Patients were treated in real-world setting and the clinical analysis was performed retrospectively based on data from the Czech Registry of Monoclonal Gammopathies.
Results: CPCs were detected in 74.6% (440/590) of newly diagnosed MM patients with applied threshold 20 cPCs and median of limit of detection 0.006% (range 0.0004-0.0391), sensitivity $10e^{-5}$. Patients were stratified into 4 groups based on the FC percentage of cPCs [0% (N=150), >0% to <0.24% (N=307), ≥0.24% to < 2.88% (N=104) and ≥ 2.88% (N=23)] that resulted in different progression-free survival (PFS) (21.2 vs. 18.0, 15.1 and 5.1months; P<0.001) and in different overall survival (OS) (50.3 vs. 48.4, 31.7 and 17.6 months; P<0.001). Quantification of cPCs was selected as the most powerful prognostic factor for survival by multivariable analysis including ISS, LDH and cytogenetics by Cox proportional hazard model.
Conclusion: Evaluation of cPCs by FC in PB is the most important diagnostic biomarker that may be used for risk stratification of newly diagnosed MM as we demonstrated, up to our knowledge, on the largest dataset worldwide.
Funding: Supported by grant AZV NV18-03-00203.

A



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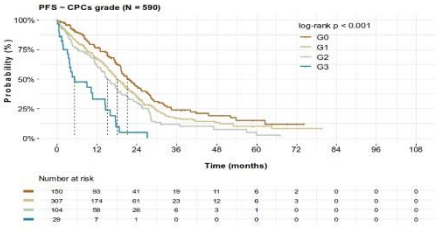

C

	Multivariable analysis (N=291)		
	N	HR (95% CI)	p-value
CPCs grade			
G0 - reference	53	–	–
G1	163	0.88 (0.55–1.42)	0.6004
G2	60	1.63 (0.97–2.73)	0.0664
G3	15	2.38 (1.05–5.38)	**0.0375**
ISS stage			
stage 1 - reference	81	–	–
stage 2	103	0.92 (0.58–1.45)	0.7151
stage 3	107	1.76 (1.10–2.80)	**0.0176**
LDH (µkat/l)			
normal (< 5 µkat/l) - reference	260	–	–
elevated (> 5 µkat/l)	31	1.64 (0.97–2.78)	0.0636
Cytogenetic abnormalities			
standard risk - reference	196	–	–
high risk	95	1.40 (0.98–2.01)	0.0650

D

	Multivariable analysis		
	N	HR (95% CI)	p-value
CPCs grade			
G0 - reference	53	–	–
G1	163	1.25 (0.83–1.88)	0.2795
G2	60	1.62 (1.03–2.54)	**0.0383**
G3	15	3.20 (1.49–6.85)	**0.0028**
ISS stage			
stage 1 - reference	81	–	–
stage 2	103	1.31 (0.90–1.91)	0.1625
stage 3	107	2.04 (1.39–2.99)	**0.0003**
LDH (µkat/l)			
normal (< 5 µkat/l) - reference	260	–	–
elevated (> 5 µkat/l)	31	1.87 (1.15–3.03)	**0.0109**
Cytogenetic abnormalities			
standard risk - reference	196	–	–
high risk	95	1.37 (1.01–1.85)	**0.0459**

CHRONIC INFLAMMATION DECREASES HSC FITNESS VIA HYPERACTIVATION OF THE DRUGGABLE IL-6/STAT3 SIGNALING PATHWAY

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Chronic inflammation is a hallmark of autoinflammatory disorders such as rheumatoid arthritis and inflammatory bowel disease. It is characterized by excessive production of cytokines and chemokines. Recent evidence showed that hematopoietic stem cells (HSCs) directly respond to these signaling molecules. In order to investigate how chronic inflammation may affect HSCs, we employed a mouse model suffering from chronic multifocal osteomyelitis (CMO). CMO mice exhibit a progressive autoinflammatory disorder that resembles human chronic recurrent multifocal osteomyelitis. CMO mice develop swollen paws, tail kinks, increased bone marrow (BM) cellularity, number of granulocytes, and production of cytokines such as IL-1□, IL-6, and MIP-1□. Remarkably, we observed expansion of HSCs already in asymptomatic mice. To assess whether the phenotypical expansion affects their functionality, we performed HSC limiting dilution assays and observed that CMO HSCs have significantly impaired engraftment ability compared to WT HSCs. Interestingly, by crossing MyD88-deficient mice with CMO mice, we observed that most of the CMO phenotype could be rescued, however, the HSC expansion was preserved. Furthermore, we generated chimeras to investigate the effect of the immune compartment and the BM niche on HSCs. Our results showed that both CMO compartments reduce HSC fitness. RNAseq data suggested that the loss of HSC function is mediated via upregulation of the IL-6/Jak/STAT3 signaling pathway. Accordingly, treatment of CMO mice with STAT3 inhibitor demonstrated a significant reduction of the HSC expansion. Altogether, our data indicate that chronic autoinflammatory conditions have a detrimental effect on HSCs, and highlight the possibility of adding clinically available STAT3 inhibitors to the current treatment to preserve stem cell functions.

DYSREGULATION IN TLR8 AND TLR7 SIGNALING LEADS TO AUTOIMMUNE HEMOLYTIC ANEMIA AND AUTOINFLAMMATION IN IDENTICAL MALE TWINS

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Toll-like receptors (TLR) 7/8 are endosomal sensors that play a crucial role in innate immune response against external pathogens and endogenous autoantigens. Interestingly, TLR8 acts against TLR7 and vice versa (Wang J Biol Chem 2006). A dysbalance between TLR7 and TLR8 in mice causes autoimmunity (Demaria JCI 2010), however in humans, the immunopathology of TLR8 and TLR7 remains unclear.

Recently, Aluri et al. described *TLR8* gain-of-function (GOF) mutation as a cause of immunodeficiency and bone marrow failure in 6 male patients (Blood 2021). Here, we present a novel X-linked c.1715G>T mutation in *TLR8* combining TLR8 GOF with TLR8/TLR7 dysregulation in male twins suffering from autoimmune hemolytic anemia, enteritis, arthritis, and CNS vasculitis. Their mother, a heterozygous carrier of the mutation, suffers from mild autoimmune symptoms.

The c.1715G>T mutation leads to a partial TLR8 protein deficiency caused by enhanced protein degradation. Interestingly, both the patients' and mother's cells displayed decreased response to TLR8 stimulation but augmented response to TLR7 stimulation detected as downstream NF-κB activation and IL-1β, TNFα, and IL-6 production. This phenomenon was replicable *in vitro* on mutant (mut) TLR8- or wild-type (wt) TLR8-tranfected HEK-Blue TLR7neg cells. Mut TLR8 caused increased activity of NF-κB upon stimulation with TLR8 ligand as well as cross-reactivity to diverse TLR7 ligands in comparison to wt TLR8. Moreover, co-transfection of mut TLR8 or wt TLR8 with TLR7 led to attenuation of TLR7 signaling in wt TLR8-transfected cells as described in the literature but not in mut TLR8-transfected cells. Taken together, mut TLR8 cross responded to TLR7 ligands and did not impair TLR7 signaling. This dysbalance between mut TLR8 and TLR7 introduced a bias towards TLR7-dependent pro-inflammatory signaling. Importantly, one of the twins is succesfully treated with hydroxychloroquine, a TLR7 antagonist, while the

other twin underwent a hematopoietic stem cell transplant in attempt to control the CNS inflammation.

In conclusion, we identified a novel Inborn Error of Immunity caused by TLR8 mutation that leads to a partial TLR8 protein loss and dysbalance between TLR8 and TLR7 signaling resulting in a complex phenotype with autoimmuity and autoinflammation.

NV19-05-00332, GAUK 362119

INFLUENCE OF *N*-ACETYLGALACTOSAMINYLTRANSFERASE 14 ON SURFACE GALACTOSE DEFICIENT IGA1 EXPRESSION

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IgA nephropathy (IgAN) is the most common cause of primary glomerulonephritis worldwide. It is characterized by glomerular deposition of IgA1-containing immune complexes, leading to kidney injury. IgA1 with some hinge-region O-glycans deficient in galactose (Gd-IgA1) is the main autoantigen in IgAN and plays a key role in the pathogenesis of the disease. Production of Gd-IgA1 is associated with dysregulated expression and activity of several key glycosyltransferases in IgA1-producing cells, but the role of O-glycosylation-initiating enzymes, N-acetylgalactosaminyltransferases (GalNAc-Ts) is not well understood.

Previously established immortalized IgA1-secreting cell lines derived from peripheral blood of patients with IgAN and healthy controls were transfected with bi-cistronic plasmid designated GT14-EGFP to drive expression of GalNAc-T14 and enhanced-green fluorescent protein (EGFP). Based on the EGFP fluorescence, the productively transfected cell population that also overexpressed GalNAcT-14 was analyzed for cell-surface Gd-IgA1 by flow cytometry using monoclonal GalNAcosylated hinge region specific antibody. ELISA was used to measure total IgA and Gd-IgA1 in the cell-culture medium.

Galactose deficiency of the cell-surface IgA1 was elevated in the cells overexpressing GalNAcT-14 compared to untreated cells. The elevation of cell-surface Gd-IgA1 was more profound in the cell lines from patients with IgAN than those from healthy controls. Secreted IgA1 exhibited the concordant phenotype.

Using IgA1-producing cell lines transfected with bi-cistronic plasmid encoding GalNAc-T14 and EGFP revealed that overexpression of GalNAC-T14 elevated cell-surface Gd-IgA1. These data suggest that overexpression of GalNAcT-14 in the IgA1-producing cells may elevate production of Gd-IgA1, a key autoantigen in the pathogenesis of IgAN.

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BLINATUMOMAB HAS BROAD EFFECT ON CELL COMPOSITION DURING B-CELL PRECURSOR LEUKEMIA THERAPY

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B cell precursor acute lymphoblastic leukemia (BCP ALL) is the most common acute leukemia subtype in children. Although the overall survival (>85%) makes it a relatively favorable malignancy, new treatments are developed both to further improve the outcome and to reduce side effects. A new targeted treatment with blinatumomab (bispecific anti-CD3 anti-CD19 antibody construct) was included in frontline treatment since 2019. The effect of the blinatumomab on defined cell populations and factors influencing treatment response are not completely understood.

We developed and optimized a multicolor combination for flow cytometry to analyze peripheral blood (PB) and bone marrow (BM) samples from BCP ALL patients using spectral cytometer Cytek Aurora in detail. The antibody combination includes 26 markers for cell immunophenotyping and 2 markers for PB and BM barcoding (in total in 27 fluorochromes). The combination contains markers for detailed analysis of T cells, B cells, NK cells, monocytes, dendritic cells, basophils. We selected time points in both blinatumomab and control treatment arms to see dynamics of features before and after the treatment.

Since 10/2020, 46 BCP ALL samples were measured (n=22 combinations of PB+BM, n=2 PB, n=22 BM). Out of them, 39 of samples were prior and 7 after blinatumomab treatment. We were able to detect all the populations. However, separation was better for PB as the panel did not include markers for discrimination of maturing myeloid cells. In 4 out of 6 patients on day 29 after blinatumomab administration, we observed complete and persistent loss of CD19 cells. Interestingly, two other patients showed B cell regeneration on day 29. Both of them were treated in medium risk arm, unlike the 4 patients with a complete loss treated in high risk arm. CD8pos T cells in the two patients with regeneration were not activated using HLA DR expression (<10%), in contrast to the 4 other patients.

In summary, contemporary flow cytometry enables us to measure >20 parameters at once and to study broad variety of cell populations in hypocellular samples. Our 27-color panel shows an effect of immunotherapy in BCP ALL patients. Preliminary results show that the response to therapy is not uniform and diverse immune cell populations might be relevant for the evaluation of blinatumomab effect.

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COMPREHENSIVE IMMUNOMONITORING OF PATIENTS AFTER HEMATOPOIETIC STEM CELL TRANSPLANTATION

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Hematopoietic stem cell transplantation (HSCT) represents the standard curative treatment for a variety of haematological malignancies, i.e. AML and MDS. However, it is still associated with high treatment-related mortality, caused mainly by relapse of the original disease and infectious complications. All these complications are caused by improper immune system function. To further deepen the understanding of the underlying post-HSCT immune dysfunction we developed an optimized 29-colour immunophenotyping panel allowing us to monitor the reconstitution of NK cell and T cell subpopulations as these represent major drivers of anti-leukaemia and anti-pathogen responses as well as gatekeepers of tissue tolerance. The aim of our project is to determine immune signatures of high-risk patients, that could in the future be used for identification of individuals requiring more intensive post-HSCT care or would be suitable for immunotherapeutic interventions.

Our panel includes markers for all major NK cell and T cell subsets and for analysis of their quantitative and qualitative properties. In the NK cell compartment, we focus mainly on the expression of activating (NKG2D, DNAM-1) and inhibitory receptors (NKG2A, TIGIT). Regarding T cells, we analyse the emergence and properties of major T cell populations with a particular interest in CD8, Th1, ThCTL and Treg subsets. Besides that, we use the detection of CD4+ recent thymic emigrants as a marker of thymus function.

In conclusion, we have established a comprehensive immune system monitoring project with the aim to identify predictive risk factors on transplant outcomes that would allow us to suggest potential immunotherapeutic interventions.

Research Funding

European Regional Development Fund and the state budget of the Czech Republic (project AIIHHP: CZ.02.1.01/0.0/0.0/16_025/0007428, OP RDE, MEYS).

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LIMITED EFFICACY OF DARATUMUMAB IN MULTIPLE MYELOMA WITH EXTRAMEDULLARY DISEASE

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Aims

Even though novel agents have been incorporated into therapeutic strategies of multiple myeloma (MM), the options for patients with extramedullary myeloma disease (EMD) remain undefined and unsatisfactory. Daratumumab, monoclonal antibody targeting CD38 antigen, has been rapidly implemented into the standard of care regimens due to its unprecedented efficacy. No data regarding the activity of daratumumab in EMD are currently available; therefore we aimed to shed light on its efficacy in this setting.

Methods

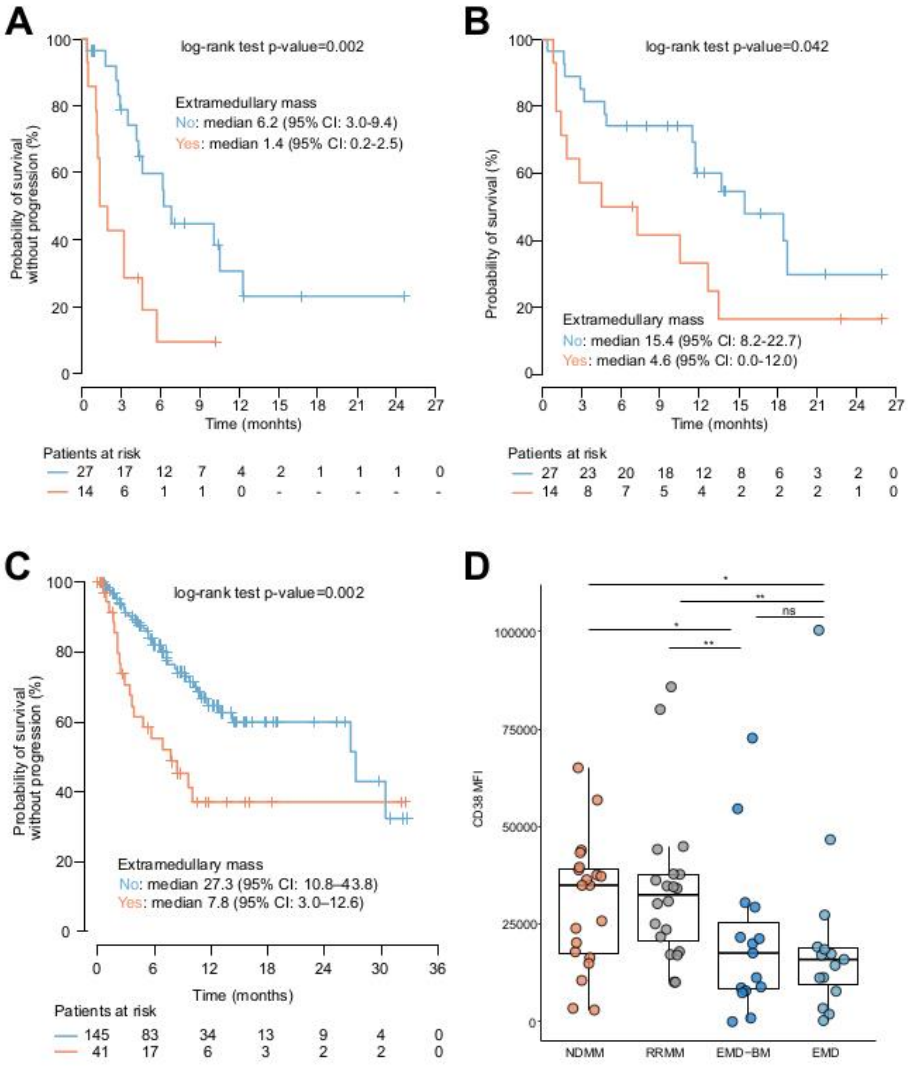
Relapsed-refractory myeloma (RRMM) patients both with and without EMD treated with daratumumab were retrospectively analyzed. After demonstrating agent's lower efficacy in EMD, we decided to use multicolor flow cytometry and RNAseq to quantify the expression of CD38 antigen.

Results

The therapeutic outcome of patients with EMD was significantly worse compared to the ones without EMD when treated with daratumumab monotherapy (n=41) - ORR 21.4% vs. 50.0%, mPFS 1.4 months vs. 6.2 months (p=0.002) (A), mOS 4.6 months vs. 15.4 months (p=0.042) (B) as well as with combined regimen of daratumumab, lenalidomide, dexamethasone (n=186) - ORR 57.7% vs. 85.4% (p=0.0048), mPFS 7.8 months vs. 27.3 months (p=0.002) (C). The underlying mechanism of lower efficacy is unknown but we observed decreased expression of CD38 on PCs isolated from EM tissue (n=15) compared with the bone marrow (BM) PCs from unpaired newly diagnosed myeloma (n=20) and RRMM patients (n=20) where median mean fluorescence intensity counted 15 946 vs. 34 958 (p= 0.03) and 15 946 vs. 32 500 (p=0.0068), respectively. These results were confirmed on five EMD samples and their paired BM samples from the time of MM diagnosis where normalized read count median was 716.5 and 1525.1, respectively (D).

Conclusion

We demonstrated that daratumumab is significantly less active in patients with EMD which can be, at least in part, explained by decreased CD38 expression on EM PCs.


A
log-rank test p-value=0.002
Extramedullary mass
No: median 6.2 (95% CI: 3.0-9.4)
Yes: median 1.4 (95% CI: 0.2-2.5)
Probability of survival without progression (%)
Time (monhts)
Patients at risk
27 17 12 7 4 2 1 1 1 0
14 6 1 1 0 - - - - -
B
log-rank test p-value=0.042
Probability of survival (%)
Extramedullary mass
No: median 15.4 (95% CI: 8.2-22.7)
Yes: median 4.6 (95% CI: 0.0-12.0)
Time (monhts)
Patients at risk
27 23 20 18 12 8 6 3 2 0
14 8 7 5 4 2 2 2 1 0
C
log-rank test p-value=0.002
Probability of survival without progression (%)
Extramedullary mass
No: median 27.3 (95% CI: 10.8–43.8)
Yes: median 7.8 (95% CI: 3.0–12.6)
Time (months)
Patients at risk
145 83 34 13 9 4 0
41 17 6 3 2 2 0
D
CD38 MFI
ns
NDMM
RRMM
EMD−BM
EMD

NECESSITY OF FLOW CYTOMETRY ASSESSMENT OF CIRCULATING PLASMA CELLS AND ITS CONNECTION WITH CLINICAL CHARACTERISTICS OF PRIMARY AND SECONDARY PLASMA CELL LEUKEMIA

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Background: Plasma cell leukemia (PCL) is a rare and very aggressive plasma cell disorder characterized by the presence of circulating plasma cells (cPC) in peripheral blood (PB). Dismal outcome of PCL requires early diagnosis with appropriate analytical tools. Development of flow cytometry together with some newly analysed antigens may reveal some marker affecting the prognosis of PCL patients.
Aim: Analysis of phenotypic profile of cPC/PCs to find association with clinical outcomes and to evaluate the prognostic value of analyzed markers.
Methods: Total of 33 primary and secondary PCL patients were investigated and circulating plasma cells (PCs) quantity and phenotype profile was analysed by polychromatic flow cytometry.
Results: Flow cytometry is an excellent method for circulating PCs identification as a significantly higher number was identified by flow cytometry than by morphology (26.7 vs 13.5%, p=0.02). None of secondary PCL cases expressed CD19 or CD20. Low level of expression with similar positivity of CD27, CD28, CD81, and CD117 were found in both PCLs. Decrease of CD44 expression was detected only in secondary PCL. Expression of CD56 was present in more than half of PCL cases as well as cytoplasmic nestin. Decreased level of platelets, ECOG score 2-3 and lack of CD20+ PC were associated with higher risk of death.
Summary/Conclusion: Flow cytometry should be incorporated in PCL diagnostics as not only providing number of circulating PCs, which is in addition overcoming morphology assessment. Moreover, PCL phenotype profile could be connected to patient's diagnosis and possible prognosis.
Funding: Supported by grant AZV NV18-03-00203.

EFFECT OF DARATUMUMAB-CONTAINING INDUCTION ON CD34+ HAEMATOPOIETIC STEM CELL SUBSETS BEFORE AUTOLOGOUS STEM CELL TRANSPLANTATION IN MULTIPLE MYELOMA

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Background: Daratumumab is an anti-CD38 monoclonal antibody representing a novel treatment approach for multiple myeloma (MM). Unfortunately, impairment of CD34+ hematopoietic stem cell (HSC) mobilisation was reported, including lower yields of CD34+ cells after the therapy (Hulin et al., 2021). Impact of anti-CD38 treatment on the mobilisation process remains poorly understood even though it is a crucial step for a successful HSC transplantation.

Aims: To compare the effect of anti-CD38 (Daratumumab-Bortezomib-Dexamethasone [D-VCd]) and conventional (Bortezomib-Thalidomide-Dexamethasone [VTd]) treatment on CD34+ HSCs.

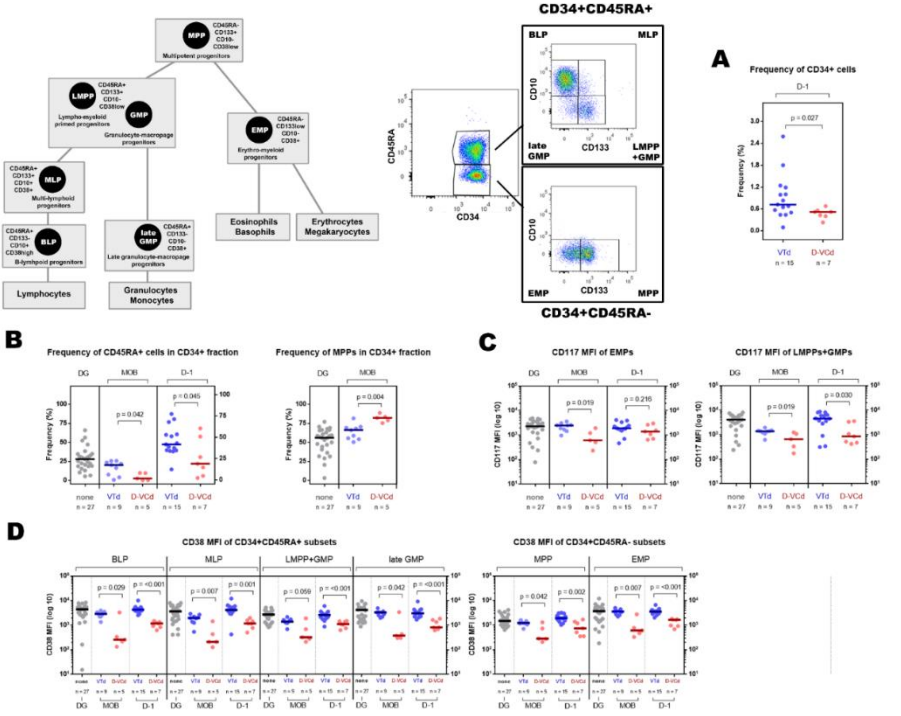
Methods: An 8-color panel (CD10, CD34, CD38, CD44, CD45RA, CD117, CD133, CD184) was designed for a flow cytometry screening of CD34+ subsets at three different time points (diagnosis [DG], mobilisation [MOB] and a day prior to transplantation [D-1]), either in the bone marrow (BM) at DG and D-1 or in the apheretic product (AP) of mobilised HSCs.

Results: Overall DG n = 27, AP VTd n = 9 vs D-VCd n = 5 and D-1 BM VTd n = 15 vs D-VCd n = 7 were compared. Frequency of CD34+ cells in cell fraction (figure 1 A) was decreased in D-VCd D-1 BMs (p = 0.027). Changes in the subset distribution were detected in the CD34+ pool (figure 1 B), including diminution of CD34+CD45RA+ lympho-myeloid compartment in both D-VCd APs (p = 0.042) and D-1 BMs (p = 0.045), together with greater frequencies of CD45RA-CD133+CD10- multipotent progenitors (MPPs) in D-VCd APs (p = 0.004). Median fluorescence intensity (MFI) of CD117 (figure 1 C) decreased in D-VCd AP EMPs (p = 0.019) and in the LMPP+GMP pool of both D-VCd APs (p = 0.019) and D-1 BMs (p = 0.030). CD38 MFI was diminished in all CD34+ subsets in D-VCd APs and D-1 BMs (figure 1 D).

Summary: Our study provides first insights into the mechanistic impact of daratumumab on CD34+ HSCs and on the mobilisation process.

Keywords: daratumumab, anti-CD38 therapy, mobilisation, hematopoietic stem cells, multiplemyeloma, phenotypisation, flow cytometry

1. Hulin C, Offner F, Moreau P, et al. Stem cell yield and transplantation in transplant-eligible newly diagnosed multiple myeloma patients receiving daratumumab + bortezomib/thalidomide/dexamethasone in thephase 3 CASSIOPEIA study. *Haematologica*. Published online March 4, 2021. doi:10.3324/haematol.2020.261842



FLOW CYTOMETRY DETECTION OF TUMOR SPECIFIC MICROVESICLES.

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Malignant cells release very small tumor-specific signaling particles, microvesicles, into the patient's bloodstream. We present a methodology for the detection of tumor microvesicles in chronic lymphocytic leukemia (CLL) using multicolor flow cytometry and novel method of staining using CFDA dye. Pilot analysis was performed on 76 patients in various stages of CLL disease. Typical coexpression of CLL markers CD19,CD5 and CD52 was found on the surface of CLL derived microvesicles. A correlation between the population of tumor microvesicles and the proportion of malignant B-lymphocytes in the peripheral blood has been demonstrated. Furthermore, the Pearson correlation coefficient (r = 0.88) was determined, which shows that the proportion of MP is directly dependent on the number of tumor B - lymphocytes. Every 10% increase in malignant B-cells doubles the number of tumor microvesicles. Amount of tumor microvesicles is not affected by modern therapeutics, such as BCR signaling inhibitors and therapeutic anti-CD 20 monoclonal antibodies. Based on our analyzes, a predictive model of CLL tumor load was formulated based on the number of tumor-specific microvesicles in the peripheral blood of patients.
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SUITABILITY OF FLOW CYTOMETRY FOR MINIMAL RESIDUAL DISEASE ASSESSMENT IN PEDIATRIC PATIENTS WITH ACUTE MYELOID LEUKEMIA

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Acute myeloid leukemia (AML) in children is a heterogeneous disease with overall survival around 60%. In contrast to acute lymphoblastic leukemia, minimal residual disease (MRD) assessment is not yet included in the current treatment protocols except for minority of patients with promyelocytic AML. Current AML MRD methods comprise detection of fusion gene transcripts on RNA level; quantification of mutations on DNA level and flow cytometry (FC) detection of cells with atypical phenotype. FC MRD examination is fast, provides precise quantification and does not need laborious optimization. Nevertheless, it is complicated by AML heterogeneity, immunophenotypic shifts on treatment and regenerating nonmalignant myeloid cells influencing the sensitivity of the examination. In our laboratory, we started to prospectively analyze FC MRD in AML patients since 04/2018. Since then, 56 primary pediatric AML patients were diagnosed in Czech Republic: 17 patients with immature AML (M0-2 according to the French-American-British classification), 3 patients with promyelocytic AML (M3), 23 patients with myelomonocytic/monocytic AML (M4-5) and 12 patients with megakaryocytic AML (M7). In follow-up samples, we measured FC MRD using 8- to 12-parameters designed for initial immunophenotype. Additionally, we aimed to analyze granulocytic, myeloid and B lymphoid compartment.
On d28 of the treatment, 22/47 patients have MRD>0.1% by PCR. In 11 of them (50%), we were not able to detect MRD by FC. The false negativity was distributed across the morphologic subtypes. Interestingly, in 7 out of 11 FC MRD positive patients, we observed immunophenotypic shift. Three of them (all AML M3) were treated by retinoid acid. One patient with M4eo FAB subtype presented with more mature monocytic phenotype on d28. In other 3 patients with initial immature immunophenotype (M1/M2) we observed atypical immature granulocytic population, with significant reduction of immature blasts.
In conclusion, FC AML MRD remains a challenge in the era of ≥8-color FC. Comparison with precise genomic MRD evaluation showed that approximately 50% of samples after induction treatment are false negative. Immunophenotypic shifts are frequent and concurrent analysis of other myeloid populations may be necessary.

Supported by grants NU20J-07-00028, NV18-07-00430

CELL THERAPY

SELECTION, EXPANSION, AND UNIQUE PRETREATMENT OF ALLOGENEIC HUMAN NATURAL KILLER CELLS WITH ANTI-CD38 MONOCLONAL ANTIBODY FOR EFFICIENT MULTIPLE MYELOMA TREATMENT

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Following promising results in various clinical trials with chimeric antigen receptor T cells, cellular immunotherapy is emerging as a new pillar in cancer treatment. However, there are some drawbacks to this novel therapy, including off-tumor toxicity, cost, and tumor recurrence in heterogeneous tumors. To overcome these constraints, we take advantage of the unique anti-tumor properties of natural killer (NK) cells. Our study's goal was to obtain a clinically relevant number of allogeneic NK cells derived from peripheral blood (median of 14,050 million cells from a single donor) to target a wide range of solid and liquid tumor types. Allogeneic NK cells were combined with the approved anti-cluster of differentiation 38 (CD-38) monoclonal antibody Daratumumab to achieve a synergistic therapeutic effect against incurable multiple myeloma. To avoid unwanted fratricide, the combination treatment was refined with CD16 polymorphism donor selection and uncomplicated novel in vitro pretreatment, increasing the in vitro specific lysis by more than 20 % against the CD-38 positive multiple myeloma cell line. After time-lapse imaging of mice with subcutaneous human multiple myeloma xenografts, we discovered that combining selected and pretreated NK cells with Daratumumab resulted in tumor volumes that were 43-fold smaller than controls. With an allogeneic source of fully functional NK cells, combined treatment may be helpful in future clinical settings to avoid the low therapeutic efficacy of monoclonal antibodies in MM patients due to NK cell dysfunctionality.

STIMULATION OF MURINE EMBRYONIC STEM CELLS WITH PEDOT:PSS BASED DEVICE

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Electric stimulation of stem cell represent a potentially promising tool for regenerative medicine. Electric stimulation positively influence stem cells differentiation. Various devices are used to stimulate cells, but in our work we focused on devices based on an conductive organic polymer poly(3,4-ethylenedioxythiophene):polystyrene sulfonate (PE-DOT:PSS). Further, organic conductive materials appear to be an excellent alternative to commonly used inorganic materials (e.g., gold or platinum).
In our work, the hypothesis if a platform based on conductive polymer poly(3,4-ethylenedioxythiophene):polystyrene sulfonate (PE-DOT:PSS) can be more efficient for differentiation of murine embryonic stem cells (ES) compared to gold-based one was tested.
The embryoid bodies were stimulated with the pulsed DC electrostimulation mode (1 Hz, 200 mV/mm, 100 ms pulse duration) on platform with PEDOT:PSS and compared to the gold one.

It was clearly demonstrated that the observed upregulation of differentiation to cardiomyocytes and potential inhibition of neurogenesis was connected to PEDOT:PSS material itself with minor contribution of electrostimulation.

EFFECT OF PSEUROTIN D ON MEC-1 HUMAN LYMPHOMA CELLS

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Chronic lymphocytic leukemia (CLL) is the most prevalent lymphoid malignancy in many geographical regions of the world. The current treatment consists mainly of a combination therapy, which is focused on inhibition of DNA replication, BCR signalization, induction of apoptosis and, more recently, mitochondrial metabolism. Pseurotin D is a secondary metabolite from the pseurotin family of bioactive natural products with a newly ascribed range of interesting biological activities. The purpose of this study was to bring new insights into the mechanism behind the effects of pseurotin D on CLL cells, with a particular focus on targeting mitochondria metabolism.
The effect of pseurotin on viability was measured by counting number of cells and MTT assay. Apoptosis was detected by flow cytometry (ANX/PI, active casp3/PI staining), Western blot (pro-apoptotic proteins) and DNA fragmentation. Cell cycle was measured by flow cytometry (Clicl-iT EdU kit) and Western blot (cyclins expression). We measured mitochondrial respiration by Aligent Seahorse analyzer and BIOLOG MitoPlates. Mitochondrial reactive oxygen species (ROS) production was measured by flow cytometry (MitoSOX and DHR123 probes) and fluorimetry (AmplexRed). Flow cytometry was also used for detection of mitochondrial membrane potential (TMRE probe). Phosphorylation of STAT, p70 S6 and MAPK proteins was determined by Western blot.
Our results showed that pseurotin D was able to significantly inhibit the proliferation of CLL cells, arrested them in the G2/M cell cycle phase and induced apoptosis. We found that these effects were associated with a change in mitochondrial membrane potential and the production of mitochondrial ROS. We showed for the first time that pseurotin D suppresses CLL cell proliferation and induces apoptotic cell death via induction of the collapse of the mitochondria respiratory chain and the ROS-related caspase pathway. Therefore, they could represent a new potent group of drugs for therapeutic treatment associated with lymphomas.

IMMUNOPHENOTYPING OF DLBCL AND B-ALL PATIENTS RECEIVING ANTI-CD19 CAR T CELL THERAPY

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Immunotherapies based on T cells modified with chimeric antigenic receptors (CAR T) have proven themselves highly effective for treating CD19+ B cell malignancies. To date, two therapies have been approved for commercial use in the US and EU to treat patients with relapsed/refractory diffuse large B-cell lymphoma (DLBCL) and acute lymphoblastic leukemia (ALL). However, effective antitumor therapy is dependent on expansion and long-term persistence of CAR T cells. Thus, investigating the immune cell phenotypes linked with the success of CAR-T-cell therapy is essential.

We developed a multi-color flow cytometry panel to determine presence (expansion) of CAR T cells, their differential phenotype, and expression of inhibitory receptors. This panel was utilized to stain PBMCs isolated from the whole blood of DLBCL and B-ALL patients (n=25) treated with Tisagenlecleucel (Novartis) at UHKT, VFN, and FN Motol. From each patient, samples were collected from: 1) apheresis used for CAR T manufacturing, 2) CAR T-cell product from infusion bag, 3) whole blood of patients following CAR T infusion at three-time points – day 2-3, day 10-14, and day 30+.

CAR T cell product consisted of around 15-30% CAR+ T cells, with a majority of CD4+ cells. We compared data of patients with complete response (CR) and non-responders (NR). NR had minimal detected CAR T cells in peripheral blood, while in CR, CAR T cells significantly expanded and were detected in peripheral blood around day 10-14.

HIGH OUTGROWTH PLATING OF SUSPENSION CHO CELLS

Alaynick W.

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Suspension cell lines are critical for the scaled-up production of proteins and biologics intended for research and clinical use, respectively. Suspension cells producing biologic drugs need to be grown serum-free to avoid using inconsistent animal derived serums, having to use purification methods to eliminate serum proteins during downstream processing, and to avoid the potential of introducing harmful microbes, viruses or prions. With the WOLF or WOLF G2 Cell Sorter and the N1 Single-Cell Dispenser, users can rely on an easy-to-use platform for suspension cell outgrowth that relies on serum-free preparation while maintaining high viability and monoclonality.

DEVELOPMENT AND OPTIMIZATION OF MATRIGEL-BASED MULTI-SPHEROID 3D TUMOR ASSAYS USING REAL-TIME LIVE-CELL ANALYSIS

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The tumor-associated extracellular matrix (ECM) provides critical biochemical micro-environment cues, as well as an essential structural scaffold, for solid tumors to survive and grow (see Pickup et al. 2014 for review). With a view to enabling more translational and turnkey 3D in vitro assays for cancer biology, we have developed and optimized techniques for seeding, growing and automatically quantifying the properties of multiple tumor spheroids in ECMs in 96-well format using real-time live-cell analysis. Using a custom autofocusing method, phase contrast, bright-field and fluorescence images (10x) were captured every 6h for 7 days from within the cell incubator (IncuCyte S3 live-cell analysis system). Typically, 20 - 80 spheroids were analyzed in each well. All four cell types formed multiple cell aggregates within the first 3 days, ranging in diameter from 30 - 80 µM. A549, SKOV-3 and MCF-7 multi-spheroids grew as round aggregates while MDA-MB-231 spheroids displayed stellate branching characteristic of an invasive morphology. At Matrigel volumes less than 40 µL or concentrations less than 3 mg mL-1, cells penetrated to the base of the plate and grew as ‘flat 2D' cultures. Using a novel bright-field image analysis algorithm, the number, area and average size of the spheroids could be computed over time non-invasively and without the use of fluorescent labels. The combination of protocol developments, novel image acquisition/analysis algorithms and cell health reporters creates an integrated solution for measuring growth and vitality of multiple small spheroids in a relevant and 3D bio-matrix over time. This approach should be applicable to primary- and patient-derived organoid tumor samples as well as cancer cell lines.

DATA ANALYSIS

FLOW-SORTED MITOTIC CHROMOSOMES AS A TOOL TO UNCOVER THEIR 3D ARCHITECTURE

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Despite the chromosomes were observed and described in 1882, their top level organization and 3D topography are being unravelled in detail in the recent years. The ability to flow-sort millions of mitotic chromosomes enabled performing chromosome conformation capture study that revealed their helical organization and allowed precise calculation of chromatin turn length along the chromosome body. The flow-sorted chromosomes can also be highly purified for observation by environmental scanning electron microscopy – a method that allows direct observation of their surface structures without any metal coating in conditions that keep the chromosome as close to its native state as possible, bringing view that challenges previous observations.

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BIVARIATE FLOW CYTOMETRY OF WHEAT-AEGILOPS ADDITION LINES FACILITATE DISSECTING THE GENOMES OF AE. GENICULATA AND AE. BIUNCIALIS INTO INDIVIDUAL CHROMOSOMES

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Allotetraploid goatgrasses *Aegilops. geniculata* and *Ae. biuncialis* are attractive sources of beneficial traits for wheat improvement. However, transfer of favorable traits from these species into wheat has been hampered by poor knowledge of their genomes and scarcity of molecular tools for efficient screening of hybrid progenies. Purification of individual chromosomes by flow cytometric sorting would make it possible to sequence the complex genomes chromosome by chromosome and develop chromosome-specific DNA markers. However, the ability to sort individual chromosomes relies on a possibility to discriminate them. In this work, we performed bivariate flow karyotyping of chromosome suspensions prepared from both species. The chromosomes were labeled with FITC-conjugated probe for $(GAA)_7$ microsatellite and their DNA was stained by DAPI. Flow karyotypes of *Ae. biuncialis* (Figure 1) and *Ae. geniculata* (Figure 2) comprised nine and fourteen chromosome populations, representing one to five and three to five chromosomes, respectively. In order to purify single chromosomes, bivariate analysis of DAPI vs. FITC fluorescence was performed with suspensions of chromosomes prepared from wheat-*Ae. biuncialis* and wheat-*Ae. geniculata* chromosome addition lines. Individual chromosomes of the two species could be sorted at purities ranging from 74.5 to 96.6% and from 87.8 to 97.7%, respectively. Flow-sorted chromosomes will streamline genome analysis of *Ae. biuncialis* and *Ae. geniculata*, facilitate gene cloning and enable development of molecular tools to support alien introgression breeding of wheat.

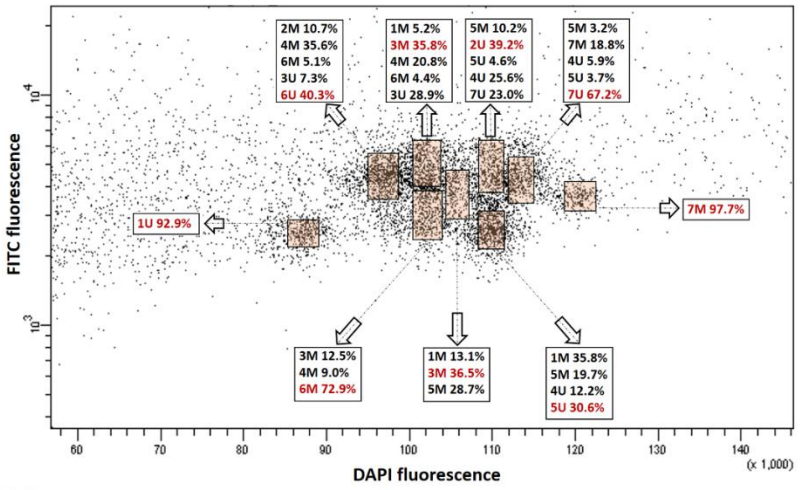

Fig. 1: Bivariate flow karyotyping and sorting of mitotic chromosomes from Ae. biuncialis. FISHIS with probes for GAA resolved nine chromosome groups (colored regions).

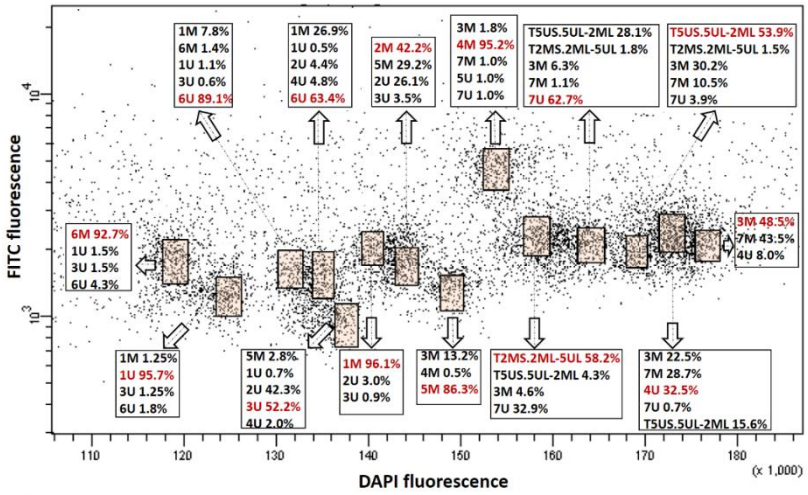

Fig. 2: Bivariate flow karyotyping and sorting of mitotic chromosomes from Ae. geniculata. FISHIS with probes for GAA resolved fourteen chromosome groups (colored regions).

Acknowledgements: Czech Ministry of Education, Youth and Sports and the European Regional Development Fund (ED0007/01/01) and OTKA K135057.

UNSUPERVISED AUTOMATED POPULATION DETECTION AND IMMUNOPHENOTYPISATION OF ACUTE LYMPHOBLASTIC LEUKAEMIA CYTOMETRIC DATA

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Multiparameter flow cytometry (MFC) is a major diagnostic and disease monitoring method for acute lymphoblastic leukaemia (ALL). While multiple computer tools for MFC have been shown to reliably detect cell populations, their clinical use is limited. Some of the limitations of computer assisted approaches are their scope (e.g. populations are detected, but their immunophenotype is not derived) and relative difficulty of their usage. We have developed automated computer-assisted analytical approach to immunophenotype major cellular populations in any given FCS file. Resulting tool analyses cytometric files without any human input, including data preprocessing, population identification and immunophenotypisation of the detected populations. For population identification we tested three clustering approaches so far (hierarchical clustering (Fišer et al., 2012), flowSOM (Van Gassen et al., 2015) and Phenograph (Levine et al., 2015)). We have developed a new method for immunophenotyping of cellular populations. The generated report includes graphical output and phenotypes in clinically used Flow Diagnostics Essential (FDE) code and several quantitative metrics.
We have tested our approach on 101 diagnostic files measuring the same antibody panel and we identified major populations including candidate malignant populations. Based on the clustering results we also automatically defined negative reference needed for phenotypisation. As part of the analytical framework all FDE phenotypes were reported.
We have also used another cohort of 19 diagnostic ALL files to compare our automated approach with expert generated report. We have achieved absolute match of automated and expert FDE in 75.7 % of markers from all files. Additional 20.9 % of automated single-marker-phenotypes were one step from the expert report (weak vs strong or negative, or vice versa).
To conclude, we build software tool to automatically phenotype cytometry data using established clustering methods and newly developed immunophenotyping method. We show that computer generated immunophenotypes are comparable to expert reported immunophenotypes.

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USING CROSS-STAIN INDEX TO ASSESS SPREADING ERROR IN SPECTRAL FLOW CYTOMETRY

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Recent developments in spectral flow cytometry and the introduction of novel fluorochromes has allowed building multicolor panels with over 40 parameters. However, combination of many fluorochromes, especially those with highly overlapping spectra, result in spreading error which can limit the sensitivity for individual fluorochromes. The aim of this study was to assess the spreading error in spectral cytometers using a previously described cross-stain index (CSI).
Human PBMCs were stained as single-stained controls with CD4 antibodies conjugated to 25 different fluorochromes and measured using spectral cytometers Sony ID7000 and Cytek Aurora, both equipped with 5 lasers.
CSI was calculated and CSI matrix was generated using FCS Express. The effect of addition or removal of individual fluorochromes from the panel on the resulting stain index in individual fluorescence channels was assessed.
Identification of fluorochromes and combinations which cause significant spreading error is an important step in designing multicolor panels in flow cytometry.

USING ARTIFICIAL INTELLIGENCE AND IMAGING FLOW CYTOMETRY TO ANALYZE MICRNONUCLEUS ASSAY

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The in vitro micronucleus (MN) assay is a standard test to assess chromosomal damage caused by genotoxic substances or radiation. Several methods have been developed to score the MN assay, including manual and automated microscopy, and conventional and imaging flow cytometry. Previously, imaging flow cytometry using the ImageStream® has been applied to develop a rapid and automated MN assay based on feature-based image analysis in the IDEAS® software. However, the analysis strategy required rigorous optimization across chemicals and cell lines. To overcome the complexity and rigidity of feature-based image analysis, we used the Amnis® AI software to develop a deep-learning method based on convolutional neural networks to score IFC data in both the cytokinesis-blocked and unblocked versions of the MN assay. We show that the use of the Amnis AI software to score imagery acquired using the ImageStream® compares well to manual microscopy while facilitating full automation of the MN assay.

MIHA CELL MODEL OF IMMORTALIZED HEPATOCYTES AND ITS POSSIBLE APPLICATION FOR DETECTION OF LIPOGENIC EFFECTS OF DRUGS/CHEMICALS

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Dysregulation of liver function can lead to accumulation of lipids in the liver resulting in the development of non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis. Disruption of lipid metabolism leading to NAFLD has been suggested to affect over 25% of the global population, and both occupational and environmental chemical exposures have been suggested to contribute to the development of NAFLD. Therefore, there is a growing need for human in vitro cellular models allowing evaluation of the effects of both endogenous and exogenous compounds disrupting lipid metabolism. Here, we analyzed effects of amiodarone (AMD), a *commonly used* anti-arrhythmic drugs and a model inducer of lipid accumulation, on MIHA cell line, immortalized human hepatocytes. For analysis of lipid accumulation, we performed flow cytometric and confocal-microscopic analyses, using a wide range of lipid-specific dyes (such as LipidTOX™ Neutral Lipid Stain, LipidTOX™ Phospholipidosis Stain, BODIPY™ 493/503, Nile Red, and others). We compared different approaches to their quantification, as well as evaluated expression of lipid metabolism genes, primarily focusing on those governing fatty acid synthesis, transport and formation of lipid droplets. Finally, we compared short-term and long-term exposures to AMD and the versatility of MIHA cells as a model for screening of lipogenic effects. Our results suggest that MIHA cells can be potentially used as an alternative model for evaluation of accumulation of lipids in hepatocyte-like cells; however, future studies should address its sensitivity as compared with other well-established 2D models of liver cells. [This study was supported by OBERON, a collaborative project funded by the EU Framework Programme for Research and Innovation Action (RIA), Horizon 2020, under grant agreement no 825712. This output reflects only the author's view and the European Union cannot be held responsible for any use that may be made of the information contained.]

IMPROVING DATA QUALITY WITH CYTEK SPECTRAL FLOW CYTOMETERS AND AUTOFLUORESCENCE EXTRACTION

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Autofluorescence is the glow that is emitted by cells and is caused by the natural tendency for some biological structures, such as mitochondria and lysosomes, to fluoresce under light. Much of this fluorescence comes from NADH, riboflavins, and flavin coenzymes1. Autofluorescence presents challenges in flow cytometry because high autofluorescence can inhibit resolution of positive populations of interest. While this is not a problem when using fluorescent molecules that are much brighter than the autofluorescence emitted by the cell, it can be problematic when the magnitude of the autofluorescence emission approximates that of the fluorescent molecule. This is where the Aurora and Northern Light's full spectrum technology can help. The emission optics of the system are able to detect the full spectrum of the autofluorescence of the unstained samples just like it does for any dye. The unmixing algorithm in SpectroFlo software treats this autofluorescence spectrum as a separate parameter and extracts it from the fluorescence data, if desired.

QUANTIFICATION OF DNA IN ADHERENT CELL CULTURES: COMPARISON OF TWO LABORATORY METHODS

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The most frequently used flow cytometric (FCM) DNA content analysis in vertebrate cells relies on ethanol fixation followed by RNA digestion and propidium iodide intercalation into DNA. This is a relatively laborious and time-consuming technique, which, due to washing procedures contained in the preparation process, suffers from inherent systematic error influencing data reliability. In addition, adherent cell lines must be detached from laboratory plastic by mechanical or enzymatic treatment and washed prior to processing, which is also time consuming and inevitably results in cell losses that may be selective depending on supernatant discarding method and/or cell subset fragility and sedimentation density. An ideal method for adherent cell preparation for FCM determination of DNA content would include no washing and no cell transition steps. In addition, high throughput assays required in e.g. pharma industry for drug screening on adherent cell lines require minimal workload, maximal automation and, if possible, significant reduction of individual sample size. Here we show how a well-established commercial kit – BD Cycletest Plus DNA Kit - designed for cell cycle quantification and aneuploidy qualification in clinical samples can be used to fulfill all such criteria. In our experiments, we used wild type HeLa cells and its knockout PUMA protein BBC3 counterpart. Cell lines were, in parallel, seeded at different densities into culture plates of different sizes, cultured for 24 hours, treated by low and high dose gamma irradiation (2 Gy and 10 Gy, respectively), cultured overnight and relative proportions of cells in G0/G1, S and G2/M phases of the cell cycle were compared. Data analysis has clearly shown that the use of the BD Cycletest Plus DNA kit is superior to the conventional method because it is faster, more accurate and more reproducible. In addition, the conventional technique could not be used in 96 well plates due to cell losses during processing while the newly designed approach worked very well. Another data sets ran in collaboration with other laboratories have proven that wells as small as those in 364 well in plates can be used and sample loaders further automate our newly designed assay. The work was supported by the Ministry of Education, Youth and Sports of the Czech Republic (project SV/FVZ202003) and Ministry of Defence of the Czech Republic (project DZRO-ZHN-2017).

IMMUNOLOGY

HUMAN MYELOID-DERIVED SUPPRESSOR CELL EXPANSION DURING SEPSIS IS REVEALED BY UNSUPERVISED CLUSTERING OF FLOW CYTOMETRIC DATA

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Myeloid derived suppressor cells (MDSCs) are important regulators of immune processes during sepsis in mice. However, confirming these observations in humans has been challenging due to the lack of defined preparation protocols and phenotyping schemes for MDSC subsets. Thus, it remains unclear how MDSCs are involved in acute sepsis and whether they have a role in the long-term complications seen in survivors. Here, we combined comprehensive flow cytometry phenotyping with unsupervised clustering using self-organizing maps to identify the three recently-defined human MDSC subsets in blood from severe sepsis patients, long-term sepsis survivors, and age-matched controls. We demonstrated the expansion of monocytic M-MDSCs and polymorphonuclear PMN-MDSCs, but not early-stage (e)-MDSCs during acute sepsis. High levels of PMN-MDSCs were also present in long-term survivors many months after discharge, suggesting a possible role in sepsis-related complications. Altogether, by employing unsupervised clustering of flow cytometric data we have confirmed the likely involvement of human MDSC subsets in acute sepsis, and revealed their expansion in sepsis survivors at late timepoints. The application of this strategy in future studies and in the clinical/diagnostic context would enable rapid progress towards a full understanding of the roles of MDSC in sepsis and other inflammatory conditions.

ACCELERATED AGING OF IMMUNE CELLS IN CHILDHOOD AND ADOLESCENT CANCER SURVIVORS - CLINICAL PHENOMENON AND SEARCH FOR MOLECULAR MECHANISM

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The therapy of neuroblastoma, the most frequent extra-cranial solid tumor in early childhood, is targeting important functions of tumor cells. While replication arrest and induced differentiation of these cells are the therapeutic goals, the damages of other cell types form an eventual risk in long-term perspective.
Senescence of immune cells is characterised by decline of a plethora of immune cell functions including adaptive as well as innate responses and is associated with a number of pathologies linked to aging, such as higher susceptibility to infections and cardiovascular diseases. Moreover, frailty, a condition linked to immunosenescence and accelerated aging, has been already described in childhood cancer survivors. Therefore, we hypothesize that intensive therapy including treatment with 13-cis-retinoic acid and topotecan and/or inflammatory burden caused by acquired comorbidities, serve as inducers of accelerated aging of immune system.
In this study we focused on immunosenescent phenotype in high-risk neuroblastoma patients and on effects of 13-cis-retinoic acid and topotecan, used in neuroblastoma therapy, on monocytes and hematopoietic stem and progenitor cells (HSPCs). The analysis of immunosenescent phenotype in high-risk neuroblastoma patients revealed a senescence-like phenotype in CD8 T cell subsets early after therapy but this phenotype normalized in later follow-up. Nevertheless, the monocyte activation status and phagocytosis remain heightened in the later follow-up. On top of that, monocytes from healthy donors showed higher frequency of phagocytes and cytokine production after 13-cis-retinoic acid treatment. Regarding the HSPCs, the differentiation profiles were altered and the expression of cyclin-dependent kinases' genes related to senescence was ongoing after in vitro expansion. Although only transient senescence-like phenotype was detected in CD8 T cells in high-risk neuroblastoma patients, remaining alterations to monocytes and phagocytes, and altered differentiation/expression profiles found in HSPCs in vitro may represent a long-lasting damage to HSPCs that might potentially immunocompromise survivors later in life.

IMMUNOMETABOLIC STATUS OF ACTIVATED HUMAN MONOCYTES IS ALTERED BY IMMUNOSUPPRESSIVES.

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Energy metabolism of monocytes in steady-state relies on fatty acid oxidation (FAO), while exposure to environmental stimuli followed by monocyte activation leads to a metabolic switch, represented by increased glycolysis linked with anabolic processes. In contrast, the late phases of inflammation are mainly catabolic, fueled by oxidation of fatty acids again. The immunometabolic status and its subsequent transformations are regulated by various signaling pathways. mTOR is well-known regulator of various metabolic processes as de novo protein synthesis for membrane reconstruction or synthesis of nucleotides. The changes of metabolic program are regulated also by glycolysis-controlling genes under control of HIF-1α, whereas the late phase of activation relying again on FAO and OXPHOS, is under PGC-1 supervision.

Immune metabolism may be altered by various pharmacological treatments (e.g. immunosuppressive drugs), which alter the status of immune cells activation, including the changes in signaling pathways. These changes may affect also effector functions of immune cells. We observed changes in functionality of monocytes induced by immunosuppressive treatment and show significant decrease in ROS production. This might lead to the impairment of important immunoprotective function of myeloid cells as monocytes use ROS for killing of phagocyted pathogens.

In summary, we showed a novel aspect of the immunosuppressive therapy in the monocyte metabolism and their effector functions, indicating possible role of immunosuppressive treatments in increased susceptibility to various infections.

CYTOKINE-ANTIBODY SINGLE-CHAIN FUSIONS FOR CANCER IMMUNOTHERAPY

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Interleukin-2 (IL-2) is a multifunctional cytokine that is able to potently stimulate immune effector cells (e.g., $CD8^+$ T and NK cells). Unfortunately, its concurrent promotion of regulatory T cells (T_{reg}) and harmful off-target effects have limited its clinical efficacy. Boyman (*1*) reveal methods with which to mitigate these issues by complexing mouse IL-2 to anti-IL-2 mAb S4B6. These IL-2 complexes are superior to free IL-2, they manifest selective stimulatory activity for memory $CD8^+$ T and NK cells and possess significant antitumor activity. However, the potential clinical use of these complexes is limited due to the mouse origin of IL-2 and the dissociation of the complexes at low concentrations. Based on our previous studies, we designed, engineered and produced translationally relevant protein chimera (immunocytokine, IC) consisting of hIL-2 linked through a flexible oligopeptide spacer to light chain of anti-hIL-2 mAb MAB602, either in unmodified or mutated version, functionally similar to scIL-2/S4B6 immunocytokine (*2*). This approach circumvents disadvantages of IL-2/S4B6 mAb complexes and exerts sufficient biological activity. We demonstrate that this IC we produced contained both IL-2 and mAb in a single molecule and IL-2 interacted with binding site of mAb. We also demonstrate its biophysical characteristics related to IL-2 receptor, its biological activity in vitro and in vivo and also therapeutic potential to eradicate experimental tumors.

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2. J. Tomala *et al.*, Chimera of IL-2 linked to light chain of anti-IL-2 mAb mimics IL-2/anti-IL-2 mAb complexes both structurally and functionally. *ACS Chem Biol* 8, 871-876 (2013).

A DIAGNOSTIC CONUNDRUM: IDENTIFICATION OF A CAUSAL MUTATION IN A PATIENT WITH SEVERE NEONATAL-ONSET BOWEL DISEASE

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The advent of next generation sequencing has largely facilitated discovery of novel disease-causing mutations. The sequencing data must be interpreted with caution though, especially in case of previously undescribed variants, where functional studies are required to prove their pathogenicity. We present a case of a boy with severe neonatal-onset bowel disease. After excluding the most common monogenic enteropathies, PIDs and metabolic disorders, whole exome sequencing (WES) was performed and a novel heterozygous previously undescribed variant in TNFAIP3 gene encoding an important negative regulator of NF-κB pathway was identified. Patients with different mutations in the same gene and a similar phenotype have been reported previously. To study the NF-κB pathway in our patient, we stimulated patients PBMCs with TNFα and measured the activation of NF-κB pathway in different cellular subsets by flow cytometry. Indeed, the pathway hyperactivation was observed in patient´s monocytes and T cells when compared with healthy age-matched controls. To validate the phenotype, we created a cellular model with TNFAIP3 gene knock-out in THP-1 and 293T cell lines using CRISPR/Cas9. The NF-κB pathway in the model cell lines was activated by different ligands (TNFα, LPS) and phosphorylation of multiple key molecules of the pathway (p65, IkB, IKK, p38) was determined both by flow cytometry and western blot. However, the results of the functional tests did not confirm pathogenicity of the TNFAIP3 variant. After further re-analysis of patient´s clinical status and WES data, another mutation was found in the AP1S1 gene suggesting MEDNIK syndrome. The damaging character of the mutation has been confirmed by proteomic functional experiments that corresponded with previously published data. We believe this case shows the importance of multidisciplinary approach in both diagnostics and treatment of patients with monogenic diseases and highlights the need for strong reliable experimental evidence to confirm the pathogenicity of newly identified variants.

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LUNG FIBROSIS, IONIZING RADIATION AND BIODEGRADABILE HYALURONAN NANOPARTICLES

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Lung fibrosis is an end-stage tissue disorder characterized by an excessive and self-sustaining process of extracellular matrix proteins, fibronectin and collagen accumulation leading to physiological tissue damage and organ dysfunction. From another point of view, lung fibrosis has been described as a dysregulated wound-healing response. Progression into lung fibrosis may be induced by various conditions, the most common triggers e radiation therapy, pharmacological substances, bacterial or viral infection or inflammatory lung disease. Radiation-induced pulmonary fibrosis (RIPF) can develop as a late effect of radiation therapy due to tissue exposure to ionizing radiation. RIPF is described as a slow, irreversible process and recent studies propose that it is not necessarily a fixed process.
Over the past few decades research of the role of hyaluronic acid (HA) in pulmonary homeostasis and pathobiology has shown that HA is one of the key factors in lung tissue. HA is an important regulator of inflammation, restoration of homeostasis after insult and repair of the injured lung.
Our main aim was to identify whether treatment of C57Bl/6J mice with hyaluronic acid could attenuated the effects on lung tissue after irradiation. However, we did not use linear structure of HA, although the molecule was self-assembled into form of nanoparticles (HANPs). Our study is the first of its kind to confirm that intramolecularly cross-linked HA into HANPs prevents ionizing radiation defragmentation.
The results suggest that HANPs in our experimental model significantly contribute to mitigation of the process of RIPF. The most significant effects were observed in molecular and cellular patterns. In the blood, population of B-lymphocytes and neutrophils were significantly changed. On the other hand, level of TGF-β, crucial factor of lung tissues fibrosis after irradiation, were significantly affected by HANP-s treatment and population of T helper and neutrophils in the lung during intermediate and fibrotic phases. According to our findings HA-NPs can control and modify the outgoing fibrotic response in lung tissue, mainly during chronic, fibrotic phase.

The work was supported by the Ministry of Defence of the Czech Republic “Long Term Development Plan – Medical Aspects of Weapons of Mass Destruction” of the Faculty of Military Health Sciences Hradec Kralové, University of Defence.

FLOW CYTOMETRIC CHARACTERISATION OF MONOCYTE-MACROPHAGE SUBSETS IN SYNOVIAL FLUIDS IN KNEE OSTEOARTHRITIS

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Monocyte-macrophage lineage (MON-Mϕ) cells are key players in knee osteoarthritis (KOA), mainly due to their ability to produce spectrum of inflammatory and degradative mediators. Immunophenotyping of this lineage in synovial fluid (SF) by 14-colour flow cytometry is important when determining the status of the joint, but the classical gating strategy is insufficient due to overlapping CD14/CD16 subpopulations.
Therefore, automatic clustering approaches by the Phenograph plugin (FlowJo v10.7) was used for analysis of MON-Mϕ lineage cells in SFs obtained in KOA patients with/without infection subdivided according to the major immune cell populations prevalence (lymphocytes, neutrophils, MON-Mϕ). MON-Mϕ lineage was primarily stratified by the classical gating strategy into the CD14+CD16+, CD14+CD16-, CD14-CD16- subsets and further characterised by the expression of seven chemokine receptors (CXCR4,5/CCR1,3,5-7).
The Phenograph plugin discovered 28 clusters of MON-Mϕ lineage among four studied KOA groups. The most different profile was obtained in SF samples from KOA patients with infection comparing to KOA samples without infection, mainly due to low abundance of CD14-CD16- subset. Samples with prevalence of MON-Mϕ (>50%) contained high abundance of CD14+CD16+ subset with high CCR7, a marker for M1 polarised macrophages. Surprisingly, samples without prevalence of any major cell population (mixed phenotype) were highly similar to samples with prevalence of MON-Mϕ, with exception of low abundance of CD14-CD16- cluster expressing high CXCR4 and CCR5. High lymphocytic samples (>50% LYM) were typical by high abundance of CD14-CD16- clusters expressing CCR5 and CCR6 receptors. On the other hand, classical gating strategy showed only decreased expression of CCR7 on CD14+CD16- subset in infection KOA but could not distinguish other sample types.
In conclusion, our data highlights the advantage of clustering approaches for characterisation of subpopulations with overlapping marker expression, as shown on example of complex SF samples in KOA.
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USE OF FLOW CYTOMETRY TO ASSESS AND CHARACTERISE SYNOVIAL FLUID PHENOTYPES OF KNEE OSTEOARTHRITIS PATIENTS

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Introduction**:** Knee osteoarthritis (KOA) is a degenerative joint disease with heterogeneous manifestation and frequent synovial fluid (SF) abundance in some patients. Although the cellular composition of SF and soluble biomarker levels may reflect the disease course, classification into phenotypes with the use of its knowledge is surprisingly underused.
Aim**:** To utilize flow cytometry and analytical tools provided by FlowJo software v10.7.1 to assess and describe KOA phenotypes.
Material and Methods**:** SF obtained from 39 patients with KOA was investigated for immune cell composition and levels of 13 pro/anti-inflammatory molecules **(**Macrophage/Microglia Panel, BioLegend) using flow cytometry. Multivariate network analysis was performed to identify KOA phenotypes, where the TriMap algorithm was used to assess chemokine receptor-pattern (CXCR4,5/CCR1,3,5-7) on cells from the monocyte-macrophage lineage (MON-Mϕ).
Results**:** Multivariate network analysis revealed five different KOA phenotypes. With the use of TriMap algorithm, MON-Mϕ lineage was stratified into 8 clusters, mainly differing in chemokine receptor-pattern on macrophages and myeloid dendritic cells (mDC). "Healing" phenotype (n=11) had a higher percentage of Treg, CD4+ T cells and cluster of macrophages expressing all studied chemokines, yet low representation of mDC clusters. "Pro-inflammatory" (n=7) and "regenerative" (n=9) phenotypes, both typical by a high percentage of activated CD8 T-cells, were similar in macrophage chemokine-clusters, but differing in mDC clusters. "Exhausted" phenotype (n=4) was characterised by high representation of low-activated CD4+ and CD8+ T-cells and macrophage subpopulations expressing all studied receptors. Last, the "immune cell-deficient" phenotype (n=8) was typical by low T-cell activation and clusters of low-activated macrophages and mDCs.
Conclusion**:** Pilot study revealed five KOA phenotypes characteristic by immune cell composition, biomarker levels and chemokine receptor patterns on macrophages and mDCs. Patient stratification based on the assessment of KOA phenotypes by flow cytometry may refine the precision orthopaedics approach.
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CONTRIBUTION OF HYALURONIC ACID NANOPARTICLES FOR MODULATION OF RADIATION-INDUCED PULMONARY FIBROSIS

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Radiation-induced lung injury (RIPI) is a dose-limiting side effect of thorax or total body irradiation. The thoracic irradiation is performing frequently, especially in cases of lung, breast, mediastinal, and certain rare forms of heart cancer. A better understanding of the pathophysiological sequence of RIPI may help to prevent misdiagnosis, create a novel treatment alternative and possibly improve patient prognosis.
Hyaluronic acid (HA) has excellent biological properties such as biocompatibility, biodegradability and non-toxicity. Over the past few decades research of the role of HA in pulmonary homeostasis and pathobiology has shown that HA is one of the key factors in lung tissue. HA is an important regulator of inflammation, restoration of homeostasis after insult and repair of the injured lung. Additionally, the molecule can be self-assembled into HA-NPs. These nanoparticles keep their biocompatibility and receptor-binding properties. The purpose of our study was to determine whether treatment of C57Bl/6J mice with hyaluronic acid nanoparticles (HA-NPs) could attenuated the acute and / or chronic radiation effects on lung tissue.
In our study, female C57BL/6J mice were whole thorax irradiated by 17 Gy. Nanoparticles of two different sizes (84.5 and 124 nm) were delivered by intratracheal instillation directly into lung 1 hour before irradiation. Samples of blood and lung tissue were collected 113 (acute phase of RIPI), 155 (intermediate phase) and 180 day (chronic phase of RIPI) after irradiation. Blood count, immunophenotypization and cytokine profile were measured in blood. In lung, immunophenotypisation, cytokine profile and histopathology were evaluated.
The results suggest that HA-NPs in our experimental model significantly contribute to the process of radiation induced changes in blood and lung tissue. The most significant effects were observed in molecular and cellular patterns. In the blood, population of B-lymphocytes and neutrophils were significantly changed. On the other hand, level of TGF-β, crucial factor of lung tissues fibrosis after irradiation, were significantly affected by HANP-s treatment and population of T helper and neutrophils in the lung during

intermediate and fibrotic phases. According to our findings HA-NPs application seems promising to attenuate RIPI, mainly during chronic, fibrotic phase.

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MACROPHAGE POLARIZATION AS A RESPONSE TO AMINE PLASMACHEMICALLY FUNCTIONALIZED SURFACES

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It is required to evaluate biocompatibility and immunocompatibility of the biomaterial considered for medical application. The immune system response depends on many cues, e.g. material wettability or hardness[a].
In this work, we assessed immunocompatibility of surfaces coated by thin films of cyclopropylamine plasma polymer (CPA PP), that can be used as vascular prostheses or the wound dressings. Previously we found out the surface coating increased hydrophilicity and provided variety of functional groups such as amines, imines, etc. This could be the reason for improved cell adhesion and higher trypsin resistance of non-endothelial cell lines[b,c].
The immune response to biomaterials is very complex, but macrophages can control the whole immune response to biomaterial and promote either pro-inflammatory or pro-regenerative processes by activating T-lymphocytes and promote their differentiation into Th1 or Th2 lymphocytes[d]. Similarly, macrophages can also be divided principally into subsets – pro-inflammatory M1 and beneficial pro-regenerative M2 macrophages. These two types differ in some surface markers, genes, and secreted cytokines[e].
We assessed THP-1 macrophage polarization on various CPA PP surfaces that contain nitrogen and oxygen moieties depending on the deposition conditions. We found out profound differences. The higher the energy invested into the plasma-chemical processes, lower the amount of nitrogen functionalities and higher the polymer cross-linking. It led to less pro-inflammatory phenotype of macrophages, as determined by expression of particular cytokines, namely CCR7, IL-1β and TNF-α. This finding was confirmed by increased level of CD206 expression, which is a marker of pro-regenerative M2 macrophages. These outcomes indicate that just small surface modifications result in big changes of their immunocompatibility.

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A MAP OF REGENERATING IMMUNE SYSTEMS OF HSCT PATIENTS USING GROWING QUADTREE-STRUCTURED SOMS

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Recent advancement in single cell cytometry has enabled researchers to understand the dynamics of the immune system. Patients after hematopoietic stem cell transplantation (HSCT) represent a significant group which can benefit from immune system dynamics studies as they can help stratify patients base on quality of immune system reconstitution and identify, those in need for additional therapeutic interventions. However, analysis of the resulting vast amounts of high dimensional datasets using current dimensionality reduction algorithms remains challenging as they suffers from overcrowding of clusters and run time issues. Our recent updates in EmbedSOM algorithms1 includes Growing QuadTree-structured SOMs (GQTSOMs) which overcomes these issues and improves the description of rare and unique cell types.

An exploratory cytometry panel consisting 29 phenotypic markers for identifying NK and T cells, their differentiation, activation and inhibition status was designed. PBMCs were collected from whole blood of healthy controls and patients after HSCT. Cells were stained and acquired using BD FACS Symphony A5 cytometer. Following the data acquisition, FCS files were analysed using GQTSOMs generated landmarks from EmbedSOM R package.

Our study shows that GQTSOM embedding helps to identify dynamics of the immune system during reconstitution phase and the influence of clinically important infections such as HCMV on emergence of unique populations of NK cells (NKG2D, NKG2C positive) compared to healthy controls.
This proof-of-concept study illustrates that system-level immune cell analysis of HSCT patients using landmark based embedding holds the potential for identifying immune system reconstitution trajectories.

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FLOW CYTOMETRY AS A TOOL OF PRACTICE BIODOSIMETRY

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Introduction

The aim of biodosimetry is to gather information about biological changes induced by ionizing radiation and based on it to estimate the dose of irradiation and to predict the clinically relevant response. Peripheral lymphocytes are known as circulating small dosimeters. Flow cytometry belongs to methods enabling various types of lymphocyte analyses and, therefore, flow cytometry is successfully used in biodosimetric reserch.

Method

In our work, we used minipigs as a mammalian experimental model for whole-body homogeneous irradiation of 0 - 4 Gy and flow cytometry for determination of changes in haematological parameters occurring during the first 15 days after irradiation (especially changes in total lymphocytes and neutrophils in the peripheral blood). Animals were divided into five categories H0, H1, H2, H3 and H4, within which the proposed treatment and prognosis differ. Consequently, the indicators of nuclear DNA damage (histone H2AX phosphorylation), induction of cell death (caspase 3 activation) and changes in relative and absolute values of T and B lymphocytes in peripheral blood were monitored by flow cytometry in all individuals.

Results

Minipigs represent a convenient large animal model for *in vivo* studies on peripheral blood leukocyte cell sensitivity with possible implications in practical biodosimetry. Based on our measurements, it is possible to classify individuals into one of the five categories within 48 hours after irradiation and thus ensure the timely initiation of adequate treatment.

Conclusion

The objective of the Department of Radiobiology of the Faculty of Military Health Sciences was to design biodosimetric methodology, efficient for practical usage for the civilian population and for military personnel exposed to such emergencies. Here we have shown that flow cytometry can be successfully used for fast triage of irradiated individuals. In addition, it is easy and user friendly method which allows broad military as so as civilian use.

Acknowledgement

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METABOLIC CHANGES OF IN VITRO EXPANDED NK CELLS

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Introduction

NK cells are an important part of innate immunity. When activated by cytokines and through activation receptors, NK cells are able to kill tumor or pathogen infected cells without prior stimulation. Diferentiation, proliferation and effector functions of NK cells are strongly influenced by its cell metabolism. The main energy source of steady state NK cells is OXPHOS, however activated NK cells switch their metabolism towards glycolisis. The aim of this project is to define the effect of immunometabolism on NK cell expansion and cytotoxic function in a context of cell based immunotherapy of hemato-oncological diseases.

Methods

Freshly isolated NK cells (NK Cell Isolation Kit, Miltenyi) were expanded 14 days *in vitro* in a presence of cytokines IL-2 and IL-15. The metabolic profile (Seahorse), cytotoxicity (Calcein AM) and expression of effector molecules (Granzym B) were measured. Phenotypization of CD markers (CD3, CD16, CD56, CD226, CD335, CD337) was measeured by flow cytometry (Cytek Aurora). Production of cytokines and effector molecules was measured by ELISA and qPCR.

Results

In vitro expanded NK cells showed better cytotoxicity depending on their spare respiratory capacity (SRC). Based on this data we choosed candidate metabolic genes for qPCR analysis. We have also observed differences between freshly isolated and 14 days cultivated NK cells in a production of Granzym B and INF-γ. We have proved that metabolic status of NK cells correlates with their cytotoxicity. The results also shows, that variabiliy of NK cells among donors has a influence on their ability to expand.

Conclusions

Freshly isolated and in cell culture expanded NK cells do differ in a production of effector molecules, mainly granzym B. We showed, that cytotoxicity correlate with the metabolic state of NK cells. These data will help to optimize NK cell based immunotherapeutic protocol.

PSEUROTIN AS AN INHIBITOR OF SPECIFIC IMMUNITY

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One of the bioactive natural compounds with high potential as pharmaceutical agents are pseurotin family. Pseurotins are a class of structurally unique yet underexplored bioactive natural products. Natural pseurotins are a secondary metabolite produced by many species of fungi, mainly by Aspergillus sp. and Penicillium sp. Pseurotins have antimicrobial and antiparasitic activity. Interestingly, a few studies suggested effects of pseurotins in eukaryotes, such as antiangiogenic activity, inhibition of activation of B-cell and IgE production, or osteoclastogenesis. In this study, we focused on effects of pseurotin on physiological functions of immune cells.

The first cells tested were mouse B-lymphocytes (isolated from spleen based on CD19 positivity). Interestingly, pseurotin inhibited IgE and IgM production of B-lymphocytes activated by a combination of E.coli endotoxin and IL-4. Our experiments demonstrated, for the first time, that pseurotin D inhibits the activation of B-cells via the STAT signaling pathway, resulting in decreased proliferation and differentiation of B-cells. Furthermore, pseurotin D reduced delayed-type hypersensitivity response in vivo that suggests reduced adverse response to ovalbumin in pseurotin D treated mice.

The second cells tested were human T- and B-lymphocytes. Pseurotin D significantly inhibited the activation of both CD4+ and CD8+ human T-lymphocytes (activation markers CD69 and CD25) complemented by the inhibition of TNF-α production without significant acute toxic effects. The Pseurotin D-mediated inhibition of T-lymphocytes activation was accompanied by the induction of the apoptosis of T-lymphocytes. This corresponded with the inhibited phosphorylation of STAT3 and STAT5. In human B-lymphocytes, pseurotin D did not significantly inhibit their activation; however, it affected their differentiation (differentiation markers CD19, CD20, CD27, CD38, and IgD).

Our results advance the current mechanistic understanding of the pseurotin-induced inhibition of lymphocytes and suggest pseurotins as new attractive chemotypes for future research in the context of immune modulatory drugs.

EFFECT OF 4-METHYLUMBELLIFERONE ON THE IMMUNE RESPONSE OF MACROPHAGES

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4-methylumbelliferone (4-MU) is an active substance of a drug called Hymecromone used to treat biliary spasm. At the intracellular level, 4-MU inhibits the synthesis of hyaluronan (HA), a prominent component of the extracellular matrix. There is an increasing number of studies utilizing 4-MU to inhibit HA synthesis for experimental purposes in both in vitro and in vivo models. Interestingly, some of these recent publications demonstrated that when HA synthesis is inhibited using 4-MU, beneficial effects are observed in several diseases related to pathological immune response such as type 1 diabetes. Some studies show the regulatory effects of 4-MU on specific immunity, but the effect of 4-MU on nonspecific immunity cells, especially phagocytes, is not known yet.
In this study, the effects of 4-MU on the innate immune response were characterized, employing mouse macrophages RAW 264.7. 4-MU significantly decreased production of nitric oxide and the expression of nitric oxide synthase as well as cyclooxygenase-2 by macrophages in response to lipopolysaccharide stimulation. Similarly, 4-MU decreased lipopolysaccharide mediated production of pro-inflammatory cytokines, namely, interleukin-6 and tumor necrosis factor alpha.
Overall, it is concluded that 4-MU has great potential to be used as an anti-inflammatory drug.

THE EFFECT OF PSEUROTIN D ON MOUSE IMMUNE RESPONSE

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The goal of the study: Secondary fungi metabolites from the pseurotin family were reported to be affect specific immune response. However, the mechanistic details underlying these effects are not well understood. The aim of the current study was to examine the effects of pseurotin D on specific immune response especially on delayed-type IV hypersensitivity reaction and interpret its underlying mechanism.
Methods**:** Naive T lymphocytes were isolated from mouse lymph nodes and sorted by specific features of $CD4^{+}$ $CD44^{-}$ $CD62L^{+}$ using BD FACS Aria II sorter. Subsequently, activated using anti-CD3 and anti-CD28 antibodies and treated with pseurotin D. Naïve T cells were differentiated using specific cytokines into Th1, Th2 subpopulations during 6 days. Mice were sensitized with chicken ovalbumin for 4 days. After that were treated mice intravenously with pseurotin and dexamethasone one hour before second booster of chicken ovalbumin into right food pad.
Results: Data revealed that natural Pseurotin D decrease proliferation of Th1, Th2 lymphocytes *in vitro* accompanied by inhibition of gene expression (GATA3, tbx21) and effector cytokine production (IFN-γ, IL-13) specific for each Th population. The paw swelling were significantly reduce after pseurotin treatment.
Conclusions: Natural pseurotin D can downregulate specific immune response. On the other hand pseurotins could be seen as potential drugs for treatment of overwhelming immune response such as hypersensitivity reaction.

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PRELIMINARY VALIDATION OF MONOCLONAL ANTIBODIES GENERATED AGAINST TWO RABBIT CD34 SYNTHETIC PEPTIDES

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CD34 surface protein is considered to be a marker of hematopoietic stem and progenitor cells (HSC/HPCs) in mammals. Antibodies against CD34 are commonly used to identify and isolate the HSC/HPCs from human, mouse, dog etc. On the other hand, the rabbit HSC/HPCs have not been characterize yet, mainly due to the lack of specific anti-rabbit CD34 antibodies. Therefore, during our research projects several mouse monoclonal antibodies were generated against synthetic rabbit CD34 peptides (peptide 1 and peptide 2). The aim of this study was to test and validate the specificity of selected novel antibodies for the detection of rabbit CD34 protein in peripheral blood and bone marrow samples. Briefly, mononuclear cells from peripheral blood (PBMCs) and bone marrow (BMMCs) were double stained with the specific subclone of CD34 antibody and specific anti-CD45 antibody in order to determine primitive hematopoietic cell population ($CD34^+CD45^-$) by flow cytometry. According to the positive detection of $CD34^+CD45^-$ cells within PBMCs and BMMCs, three antibodies against each peptide (peptide 1: 16/94, 114/3/12, 204/42 and peptide 2: 58/47, 58/47/26, 58/47/34) were chosen for the subsequent Western blot (WB) analyses. Antibodies against peptide 1 did not detect any specific CD34 protein in samples. However, antibodies against peptide 2 detected a specific protein with the size about 40 kDa, which agree with the predicted size of rabbit CD34 protein. In conclusion, the presented antibody subclones seems to be specific for rabbit CD34 protein. However, further analyses are required in order to fully validate their specificity.

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LIPOPOLYSACCHARIDES FROM CYANOBACTERIAL WATER BLOOM-FORMING SPECIES POSSESS PRO-INFLAMMATORY EFFECTS ON EPITHELIAL AS WELL AS IMMUNE CELLS

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Cyanobacterial water blooms (cyanoWB) are a worldwide problem of freshwater reservoirs where they pose a significant risk to human health. In addition to highly toxic cyanotoxins, they also produce mixture of cyanobacterial and also bacterial lipopolysaccharides (LPS). LPS from G- bacteria is well known to be pro-inflammatory agent but effects of cyanobacterial LPS (cyanoLPS) are almost unknown, especially those of cyanoWB-forming species.

To study their ability to induce pro-inflammatory responses, cyanoLPS extracts from axenic cultures of selected species were prepared. First, pyrogenicity of these LPS was tested. Very low effects were observed in most samples. Whereas contact with various epithelial cells is common during recreational exposure, HaCaT cells (keratinocytes) and differentiated Caco-2 cells (enterocytes) were chosen for further studies. Interestingly, all samples activated wound-healing of scratched HaCaT monolayer with exception of *Microcystis aeruginosa* (the strongest sample in terms of pyrogenicity). Although each sample was active in at least one endpoint studied in further experiments, Aphanizomenon flos-aquae showed pro-inflammatory activity across cell types. It activated production of pro-inflammatory cytokines in keratinocytes as well as intestinal epithelial cells. Moreover, mice macrophages RAW264.7 were used to see effects on immune cells and *A.* flos-aquae cyanoLPS induced production of TNFα, NO and IFNβ by the cells. It also activated NF-κB and SAPK/JNK pathway.

Considering TLR4 as the main receptor for bacterial LPS, its inhibitor was used to see if it works also for cyanoLPS. Surprisingly, effect of none of the samples was affected by this inhibition. On the other hand, TLR2 neutralizing antibody caused significant decrease of TNFα production after exposure to *A.* flos-aquae cyanoLPS.

Our data suggest that cyanoLPS can have pro-inflammatory effects on different types of cells. Considering that cyanoWB are mixtures of various toxins, effects of cyanoLPS and their interactions with other toxins should be deeply studied to understand adverse human health effects of cyanoWB exposures documented worldwide.

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INTRAVENOUS IMMUNOGLOBULIN MODULATES THE NUMBER AND FUNCTION OF LOW DENSITY NEUTROPHILS IN PATIENTS WITH COMMON VARIABLE IMMUNODEFICIENCY

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Common variable immunodeficiency disorder (CVID) is the most common form of clinically significant primary immunodeficiency. Majority of patients receive intravenous immunoglobulin (IVIg) replacement therapy with potent immunomodulatory properties on individual components of the immune system. CVID is associated with chronic granulocytic activation and an increased percentage of Low Density Neutrophils (LDN) observed in the layer of peripheral blood mononuclear cells (PBMC).
Changes in the percentage of LDN in PBMC and expression of their surface markers were studied in 25 CVID patients and 27 healthy donors (HD) in a whole blood sample and after in-vitro stimulation of whole blood with IVIg.
We confirmed an increased number of LDN in the PBMC layer in a fresh blood sample from CVID patients. The LDN of patients and a HD group consisted of mature and immature neutrophils distinguished based on the expression of CD10 and CD16. Stimulation of whole blood with IVIg caused an increase of LDN in both CVID patients and HD. This enhancement in LDN was caused by mature neutrophils $CD16^{+}CD10^{+}$ in both groups. The suppressive effect of induced LDN on T-cell proliferation was confirmed. Flow cytometry measurements revealed altered expression of CD274 and CD10 on mature LDN of CVID patients. The addition of IVIg inhibited the expression of CD11b on the mature LDN in both experimental groups.
These results indicate that IVIg supports the formation of LDN, which can negatively affect the immune response and can significantly contribute to the chronic inflammation observed in patients with CVID.
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IMMUNE RESPONSE IN ZINC ADMINISTRATION AND ASCARIDIA GALLI INFECTION

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In organic poultry production using of floor and aviary systems with free-range areas resulted in a renewed importance for helminthoses of which Ascaridia galli with Heterakis gallinarum are the most widespread species. The regular use of antihelmintic treatment is not compatible with organic regulations and alternative control strategies are required. In connection with *A. galli* and zinc the effects of different forms and doses on treatment of ascaridiosis were studied to result in lower warm burden. Infected poultry have higher demands on feed with essential elements including zinc. Immunobiological aspect of zinc as a second messenger in signal transduction, the impact on immune cell function, and the competition for zinc between host and pathogen, are known as nutritional immunity. Zinc deficiency affects development of acquired immunity by preventing both the outgrowth and certain functions of T lymphocytes such as activation, Th1 cytokine production, and B lymphocyte help. The aim of this study was to investigate the effect of inorganic zinc on the immune response of a host infected with *A.* galli parasite. Experiment lasted 14 days, 24 broiler chickens 35-days-old COBB 500 breed were divided into 4 groups: C- control, Zn- individually perorally 12 days administered by water solution of $ZnSO_4$ (50mg/0.5ml), Ag- individually per os inoculated (single dose of 500 embryonated *A.* galli eggs) on day 3 of experiment, Ag+Zn- combination of *A.* galli eggs and $ZnSO_4$ solution. The immune response was monitored by 2 samplings (7 and 14 d) through evaluation of haematological parameters and quantitative changes of immunocompetent cells in blood and jejunum (IEL, LPL). Haematology appeared at 1 sampling the increase of leukocytes, lymphocytes (Zn), heterophils, monocytes (Zn, Ag+Zn). Eosinophilia in infected groups (Ag, Ag+Zn) confirmed presence of parasite at both samplings. Immunocompetent cells, evaluated by flow cytometry, showed in blood the activation of cell mediated immunity by $CD8^+$ T-lymphocytes increase in zinc groups (Zn, Ag+Zn) of both samplings. Jejunal IEL IgA^+ improving (Zn, Ag+Zn, 2 s) tended to the activation of mucosal immunity. Growing of $CD4^+$ IEL and LPL (Zn) at 1 sampling referred to beneficial effect of administrated inorganic zinc form to chickens. Financial support: VEGA 1/0355/19, VEGA 1/0107/21.

IMMUNOMODULATORY AND GUT-PROTECTIVE EFFECTS OF HUMIC SUBSTANCES IN BROILER CHICKENS

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Humic substances (HS) possess a wide range of positive health effects. In this study we focused on the effect of administration of HS on the cellular immune response and the protection of intestine in broilers. Cobb 500 male broilers (n=100) were involved in the experiment, whilst day-old chicks were divided into two groups: the control (C; n=50) and the experimental group (H; n=50). Throughout the fattening period, the diet of the H group contained HS (originated in leonardite) in 0.8% concentration. At day 38, 15 chickens were randomly selected from both groups and they were subjected to subsequent immunological and microbiological analysis. In peripheral blood, a flow cytometric analysis of phagocytic activity and percentage of selected subpopulations of lymphocytes was performed. Samples of tissues from the caudal part of the cecum were processed and used to determine the expression of genes for IgA, intestinal mucin 2 (MUC-2), insulin like growth factor 2 (IGF-2) and avian beta defensin 2 (AvBD2). The intestinal contents were used for microbiological analysis. In the H group, we noted the stimulation effect of HS on phagocytic activity as well as the engulfing capacity of phagocytes, however the level of oxidative burst of phagocytes was not significantly influenced. We observed significantly increased proportion of CD4+ lymphocytes, decreased CD8+ lymphocytes, and only minimal effect on the percentage of double-positive CD4+CD8+ lymphocytes in the H group. The resulting ratio of CD4+ and CD8+ lymphocytes as an indicator of immune stimulation was significantly increased. HS did not significantly affect the expression of the IgA gene. In contrast, we observed a significant increase in expression of the MUC-2 gene, and expression of the IGF-2 and also AvBD2 genes was lower in the experimental group. In microbiological analysis, we recorded a significant decrease of enterobacteria counts in small intestine and cecum, while the numbers of lactic acid bacteria were slightly increased after addition of HS. The resulting ratio of enterobacteria and lactic acid bacteria shows positive effect on intestinal microbiota. Despite the increase of lactic acid bacteria numbers, we did not record a decrease in pH values in the intestine. We confirmed a gut-protecting and an immunostimulatory effect of HS in broiler chickens.

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EFFECTS OF WATER BLOOM LIPOPOLYSACCHARIDES ON CO-CULTURE INTESTINAL MODEL

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Cyanobacterial water blooms represent a global ecological problem. They are a source of wide range of toxins including lipopolysaccharides (LPS), which are a part of cyanobacterial cell wall. We focused on the effect of LPS on the intestinal epithelium as intestine is one of the main interfaces getting to the direct contact with these endotoxins. Caco-2/PBMC (peripheral blood mononuclear cells) co-culture was used as a simplified model of intestine. We studied possible pro-inflammatory effect of LPS isolated from four cyanobacterial water blooms predominated by diverse cyanobacteria species (Microcystis aeruginosa, Dolichospermum flos-aquae, Planktothrix aghardii, and Aphanizomenon flos-aquae, respectively). Levels of pro-inflammatory mediators, specifically interleukin (IL)-6, IL-8, chemokine (C-C motif) ligand (CCL)-2, and CCL-20, were measured by ELISA. Tight junction proteins, such as zonula occludens (ZO)-1, ZO-3, claudins 2 and 4, were visualized by confocal microscopy and their amount was determined by western blot. The changes in leukocyte populations and their activation were observed by flow cytometry. We detected particularly chemokines (IL-8, CCL-20) to be elevated, although the effect was smaller than if Escherichia coli LPS (positive control) was used. Confocal microscopy has not revealed any changes in tight junction proteins but we saw shifts in claudin 2 and ZO-1 amounts measured by western blot. We observed elevation of activation marker CD16 in the case of monocyte population. In conclusion, the effect of water bloom LPS on Caco-2/PBMC co-culture model was weaker than effect of E. coli LPS but not negligible. In combination with other cyanotoxins, such as microcystins, water bloom LPS could have adverse effect on intestinal tract and thus pose a risk to human health.

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FORSKOLIN-MEDIATED MODULATION OF HUMAN T HELPER CELL DIFFERENTIATION

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Forskolin (FSK) is a labdane diterpene that is suggested as a potential immunomodulator. It is well known as an activator of the cAMP signaling pathway. However, the specific effects of FSK-mediated cAMP elevation on T helper (Th) cell differentiation and functions are still unclear. We speculate that increased levels of cAMP in Th cells may have distinct effects on each differentiation program. Our study is therefore aimed to clarify this effect on Th1, Th2, and Th17 subsets. Modulation of Th cell differentiation via stimulation of the cAMP signaling pathway can contribute to the identification of novel therapeutic targets for the treatment of Th cell-related pathological processes.

Human Th naïve cells were sorted and stimulated by selected activators and cytokines to induce differentiation into Th1, Th2, and Th17 subsets. Subsequently, part of the samples was treated with FSK during the differentiation process. Selected samples underwent RNA sequencing analysis on a single cell scale (scRNAseq) which provided detailed transcriptomic data for each subset. To cover the heterogeneity of donors, data from scRNAseq analysis was combined with analyses of expression of Th subset-specific genes by RT-qPCR and production of effector cytokines using ELISA.

The data clearly show the differences of FSK influence on the differentiation of distinct Th subsets. FSK mediated elevation in Th1-specific markers and supported their natural immunophenotype. The differentiation of Th2 was not changed by FSK, though metabolism was negatively affected. On contrary, the Th17 immunophenotype was severely suppressed leading to highly specific upregulation of the CXCL13 gene and suggesting the possibility of Th17/Treg plasticity.

INFLIXIMAB POLYMER CONJUGATES FOR TREATMENT OF RHEUMATOID ARTHRITIS

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Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease which is causing irreversible damage of inflamed joints. Pro-inflammatory mediators such as TNF-α produced by activated immune cells participate in maintenance of inflamed environment in joints. Biological therapy (e.g., therapeutic antibodies) is considered to be the most potent treatment for RA. This therapy targets mediators involved in the pathogenesis of RA such as TNF-α (e.g., therapeutic antibody infliximab (IFX)). However, their systemic application can increase the risk of immunogenicity and promote pathological immune reaction leading to the treatment failure. Conjugation of therapeutic antibodies to hydrophilic polymers may decreased these limitations due enhanced accumulation in inflamed site. Therefore, we aim to attach the widely used therapeutic antibody IFX to polymeric delivery system and evaluate its biological activity.
There were performed experiments both in vitro and in vivo and the effect of IFX polymer conjugates was evaluated. THP-1 cells activated by TNF-α were used for in vitro experiments. Inflammatory response was triggered by injection of human TNF-α into mice and effects of IFX conjugates on this stimulus were evaluated. None of the tested compounds was toxic for the cells and conjugation of IFX to polymer did not change its biological activity.

DEXAMETHASONE BASED POLYMER THERAPEUTICS FOR TREATMENT OF RHEUMATOID ARTHRITIS

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Rheumatoid arthritis is a chronic inflammatory autoimmune disease caused by alteration of both the innate and adaptive immune system. Current therapy includes glucocorticoids, nonsteroidal anti-inflammatory drugs. Unfortunately, it is often connected with various side effects. The conjugation of the drug to polymeric delivery systems improves therapeutic properties of the carried drugs. In some cases, the drug must be derivatized prior the attachment to polymer with the aim to introduce suitable functional groups for the attachment via stimuli sensitive spacers. Therefore we aimed to evaluate the biological properties of dexamethasone (DEX) modification intended for synthesis of drug delivery systems targeting the affected joints. The derivatives of DEX were prepared by reaction of hydroxyl group of DEX with different oxo-acids. These derivatives were afterward attached to water-soluble linear N-(2-hydroxypropyl) methacrylamide copolymer platform. The biological effects of dexamethasone derivatives were evaluated using murine peritoneal macrophages RAW 264.7 activated by lipopolysaccharide (LPS). The toxicity was evaluated by measuring of lactate dehydrogenase release. The anti-inflammatory effects were studied by measurement of nitric oxide and cytokine production. The modifications of dexamethasone did not cause significant acute toxicity. The derivatives of the DEX showed at least similar inhibitory effects on inflammatory response of RAW 264.7 macrophages activated by LPS as free DEX. The data demonstrate that anti-inflammatory activity of DEX was retained after its modification. The data suggest that the modification of DEX did not affect its biological activity. Therefore, these derivatives are suitable for preparation of drug delivery system.

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INTERCELLULAR COMMUNICATION IN THE DEVELOPMENT OF PERITONEAL ADHESIONS

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Peritoneal adhesions represent one of the major complications following intra-abdominal surgery leading to symptoms such as abdominal pain, bowel obstruction, and infertility. Adhesion formation is primarily caused by activation of mesothelial layer and underlying tissues in peritoneal membrane resulting in transition of mesothelial cells and fibroblasts to pro-fibrotic phenotype. Communication between immune cells, mesothelial cells, and fibroblasts is of fundamental importance in this process. Extracellular vesicles (EVs) are membrane-derived particles that can reflect their cellular origin and functional state through the bioactive cargoes they carry. We hypothesize that EVs together with soluble mediators represent crucial modulators in the development of peritoneal adhesions.
Two different subpopulations of EVs (small and large) were isolated by multistep ultracentrifugation from peritoneal fluid of healthy C57Bl/6J mice and mice with induced peritoneal adhesions. Further analyses including characterization of protein expression by western blotting (vinculin, CD63, CD81, TSG101), staining of lipids in EVs membranes by PKH67, and visualization by cryo-electron microscopy were performed. Selected inflammatory cytokines (IL-1beta, IL-6), pro-fibrotic mediators (TGF-beta, SDF-1), and fibrinolytic factors (tPA, PAI-1) were measured by ELISA in peritoneal lavage fluid. An increase in soluble mediators' levels and the EVs production was observed in the peritoneum of fibrotic mice, especially 1 and 3 days after having induced adhesions. TGF-beta levels were higher in the EVs fractions isolated from mice 1 day after adhesion induction when compared to healthy controls.
These results point out the importance of cell communication, particularly in the early phases of peritoneal adhesion development. Our results help to understand the exact role of EVs in the development of fibrosis and will advance therapeutic methods targeting the prevention of peritoneal adhesion formation and protecting physiological functions of the peritoneal membrane.

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CN-NFAT INHIBITORS IMPAIR PTX-3 PRODUCTION IN HUMAN NEUTROPHILS

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Calcineurin (CN) - Nuclear factor of activated T-cells (NFAT) inhibitors are commonly used immunosuppressive drugs, but they frequently leave patients vulnerable to fungal infections. Pentraxin-3 (PTX-3), soluble pattern recognition receptor (PRR), recognizes and binds fungal conidia, which allows clearing the infection. We have previously shown that the expression of PTX-3 in mouse dendritic cells and neutrophils and in human monocytes is co-regulated by CN-NFAT. Here we address the role of CN-NFAT in expression control of PTX-3 in human neutrophils, which are indeed the major source of PTX-3. The role of CN-NFAT pathway in human neutrophils remains elusive.

Using neutrophils (CD16+ and CD66b+) isolated from peripheral blood of healthy donors, we show that CN-NFAT pathway is active and that its inhibition perturbs the expression of PTX-3. Isolated neutrophils were activated by different PRR ligands in the presence or absence of CN-NFAT inhibitors (Cyclosporine A and Tacrolimus) and the changes in PTX-3 expression were measured using qPCR and ELISA. Zymosan, LPS and heat-killed Candida albicans induced PTX-3 expression in human neutrophils as detected on mRNA and protein levels, interestingly this trigger induced expression was downregulated in presence of CN-NFAT inhibitors.

Results show, that the CN-NFAT pathway is an important regulator of PTX-3 production in human neutrophils and PTX-3 expression is impaired in the presence of CN inhibitors.

EFFECT OF TNF INHIBITORS ON GD-IGA B CELLS IN PERIPHERAL BLOOD OF IGAN PATIENT – A CASE STUDY

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IgA nephropathy (IgAN) is one of the most abundant kidney diseases worldwide. Kidneys of IgAN patients are negatively affected by deposits of galactose-deficient IgA1 (Gd-IgA1) containing immune complexes, leading often to renal failure. One of the crucial factors in IgAN affecting Gd-IgA1 production is IL-6 cytokine. Effective IgAN treatment consists mainly of corticosteroids (CS). Here we show an enormous effect of TNF inhibitors, prescribed for ulcerative colitis treatment, on Gd-IgA1+ B cells in PBMC of IgAN patient.
PBMC of patient treated by Adalimumab for ulcerative colitis and Prednisone for IgA nephropathy were analysed for Gd-IgA1+ B cells. At 3 different stages of treatment (anti-TNF+CS, CS alone, and no administration of anti-TNF and CS) PBMC were stimulated by IL-6 and analysed for immune response of CD19+, IgA+ and Gd-IgA1+ populations by multiparameter spectral cytometry.
Our data show that anti-TNF therapy moderately increases populations of CD19+ and IgA+, but not Gd-IgA1 cells. After IL-6 stimulation during anti-TNF therapy, the CD19+ population is reduced, simultaneously the IgA+ subpopulation is slightly increased, showing the B cells differentiation to antigen producing cells. In contrast the Gd-IgA1+ subpopulation of PBMC sampled during anti-TNF therapy exhibited a robust increase (up to 92,4% of all IgA+ cells) in contrast to average response (30%) of PBMC of IgAN patients. After termination of anti-TNF treatment, populations of CD19+, IgA+, and also Gd-IgA+ cells after IL-6 are strongly reduced (CD19+ 2x, IgA+ 4x and Gd-IgA+ 2,5x), which corresponds to immune responses of IgAN patients after IL-6 stimulation. Our results show that anti-TNF treatment routinely prescribed by ulcerative colitis massively affects Gd-IgA1+ B cells as the key population responsible for pathogenic Gd-IgA production in IgA nephropathy.
Supported by the Ministry of Education, Youth and Sport, Czech Republic grant CEREBIT CZ.02.1.01/0.0/0.0/16_025/0007397 (MR, JT, PM) and Palacky University Olomouc grant IGA_LF_2021_015.

COMPREHENSIVE IMMUNE MONITORING OF T CELL SUBPOPULATIONS GIVES RELEVANT INFORMATION TO IMPROVE PATIENT CARE

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The adaptive immune system has a crucial role in the response to infections and oncology(1). T cells are considered key immune cells against pathogens and also, maintain immunological memory and self-tolerance(2). Thus, the immune monitoring of these cells has become increasingly useful to evaluate the response to treatment of certain diseases. In this review, we present several studies that demonstrate the importance of T cell immune monitoring in oncological patients, response to infections (bacterial or virical) and to evaluate immunetherapies.

Studies suggest that patients with non-small cell lung cancer show increased percentage of CD8+ effector T cells and reduced percentage of CD8+ effector memory cells when compared to the healthy donors(3). Although this analysis of T cell memory immunophenotype is currently not part of the diagnostic or prognostic processes in oncology, it could be implemented easily as a simple and fast method to monitor the immune response to tumor or treatment.

Other studies have already proven the utility of T cells for measuring the response to bacterial infections(4). In this case, a significantly increase in the absolute numbers of naïve and a decrease in the absolute numbers of central and effector memory CD4+ T cells in comparison to uninfected individuals has found.

In regards to viral infections, the protective immunity of the vaccines to SARS-CoV-2 is currently unclear. However, there are recent studies that demostrate that the magnitude of T cell responses were significantly higher in patients with severe disease as compared with mild cases(5). This data emphasize the importance of T cell immunity in monitoring patients with COVID-19 or response to vaccination.

Finally, T cells are of pivotal importance in the monitoring of immunotherapies, such as CAR-T cells. In the last few years, it has been proved that CAR-T products that are rich in central memory T cells are long-lasting and more effective in tumor clearance. Thus, the selection and ex vivo expansion of these specific memory T cells among the CART-T cells allows a more effective therapy(6,7).

This review shows the increased importance of measuring by multiparametric flow cytometry, the treatment- or disease-induced effects to obtain valuable information from a clinical point of view.

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PROTOCOL SETTINGS OF PHAGOCYTIC ACTIVITY OF MONOCYTE SUBPOPULATIONS

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Introduction:
Monocytes, which serve as precursors of various subsets of macrophages and dendritic cells, play a key role in effector mechanisms, helping to regulate both innate and adaptive immune responses. One of the main function is phagocytosis. Monocyte cells are divided into different subpopulations based on phenotype and cell function – classical monocytes (CD14+CD16-), intermediate monocytes (CD14+CD16+) and non-classical monocytes (CD14lowCD16+). CD16 positive subsets are an important source of multiple cytokines. It is still not clear, how the phagocytosis level can be changed in individual monocyte subsets.

Patients and methods:
In this pilot study we tried to set up a functional test for detection of phagocytic activity of individual monocyte subpopulations.
We used FagoFlowEx Kit, which is based on measuring of oxidative burst after stimulation by inactivated E. coli. Fluorescent product Rhodamin 123 was detected by flow cytometry and fluorescent staining of CD14 and CD16 was supplemented.
We collected samples of peripheral blood. Both patients and healthy donors were included.

Results:
Peripheral monocytes were divided into three subsets according to CD14 and CD16 expression.
We detected significant lower phagocytic activity in the group of nonclassical monocytes. No differences between healthy donors and patients were observed, but it could be due to the low number of samples.

Conclusion:
We set up a protocol for detection of phagocytosis activity in different monocyte subpopulations. After this pilot study, we would like to collect samples from patients after kidney transplantation and measure, if the change of phagocytic activity could be associated with acute rejection of kidney allograft.
As we assumed, the subpopulation of nonclassical monocytes shows lower phagocytic activity. It points out their regulatory function at the expense of phagocytosis.

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COVID-19 INFECTION AND SELECTED IMMUNOLOGICAL PARAMETERS IN PATIENTS WITH SEVERE COURSE – OUR FIRST EXPERIENCES

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Severe acute respiratory syndrome coronavirus 2 (SARS-CoV2) is currently one of the most widespread infectious disease worldwide and poses potential biological threats to human. The majority of infected individuals have an asymptomatic or mild course, but some patients have a severe course and need hospitalization and intensive care.
One of the most important tasks recently is to understand the immune response to the COVID-19. Clinical studies suggest that non-specific and specific immune responses are included. Significant changes in selected immunological parameters have been reported in many scientific publications among patients with severe course of infection.
The aim of our work was to compare selected immunological and biochemical parameters in patients with severe course of infection with the published data of available studies. We particularly focused on the parameters of specific cellular immunity (lymphopenia, decreased T cells, changes in T regulatory cells and naive T cells, cytokine release syndrome). We also discuss some possible prognostic parameters.
Key words: COVID-19, immune response, lymphopenia, T cells, cytokine release syndrome

ROLE OF FAM83H IN IMMUNE SYSTEM HOMEOSTASIS

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Immune system homeostasis can be defined as the mechanisms that maintains an organism healthy and stable. Numerous genes could be important for this and a recent study suggests that Family with sequence similarity 83 member H (Fam83h) could be one of them. FAM83H was first characterized in Autosomal-dominant hypocalcification amelogenesis imperfecta (AI) which is a soft enamel disease. Fam83h has been suggested to be responsible for intracellular molecular transport, regulation of cytoskeletal networks, and enamel formation. Fam83h is quite ubiquitously expressed mainly in epithelial cells. Interestingly, two AI patients in one family in Czech Republic with confirmed mutation in FAM83H showed also symptoms of juvenile arthrosis. The roles of FAM83H in these diseases and in immune system homeostasis still remain elusive!

We show that Fam83h is ubiquitously expressed across different tissues with highest expression in ileum, colon and ameloblasts. In contrast to previous reports we show Fam83h to be expressed in immune organs (spleen, lymph nodes, thymus) and bone marrow, where the source of its expression are likely stromal cells. Fam83h deficient mice are subviable with the frequency 7% of KO animals in F2 litters, display growth retardation and have swollen joints and fingers. Data from the IMPC screen show increased levels of potassium, ALT and urea in blood of KO mice, attesting for kidney and liver damage. Fam83 KO pups are smaller, neutrophilic, lymphopenic and show the highest degree of arthritis-like disease. Interestingly, weight and leukocyte counts return back to normal levels as animals age. The disease presents clearly an early onset and only few animals survive till week 16 of age.
The goal of the study is to reveal the mechanism of autoimmune disease in Fam83h KO mice. Our results will contribute to the understanding of the role of Fam83h in immune system homeostasis.

QUANTIFYING IMMUNE CELL SUBSETS IN LIVING CULTURES OVER TIME USING LIVE-CELL ANALYSIS

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Here we describe a novel labelling and analysis strategy to enable long-term, non-invasive quantification of immune cells based on IncuCyte® live-cell imaging. An Fc-targeted anti-mouse Fab fragment conjugated to a green-emitting fluoroprobe (IncuCyte FabFluor-488) was used to tag antibodies to cell surface markers. Addition of the FabFluor488-antibody complex to living cells, including OptiGreen background suppressor, produced fluorescent labelling that was bright and stable without perturbing cells. With new image analysis and visualisation tools, individual cells were segmented from the phase image and quantified cell by cell for fluorescence. In PBMCs, anticipated frequencies of lymphocyte subpopulations (CD4, CD8) were detected using this method. Cell subsets can be classified for analysis determining dynamic changes in response to stimuli. This method is powerful in analyses on dynamic heterogeneous cell models and cell-cell interactions.

HOW IMAGING FLOW CYTOMETRY CAN ILLUMINATE IMMUNE CELL INTERACTIONS

Hall B.

Luminex Corporation, Austin, Texas, United States

Compared to conventional cytometers, imaging flow cytometry delivers higher fluorescence sensitivity and enables visual verification of cells and populations as well as quantitative image analysis. Together, these features open the doors for applications that are impossible with standard flow cytometry or microscopy alone. Imaging flow cytometry applications include analysis of cell signaling, co-localization, cell-cell interactions, targeted immunotherapy, stem cell differentiation, and much more. Using traditional microscopy to study these rare events can be difficult and time-consuming, and it's often plagued by low statistical significance. With imaging flow cytometry, though, it is much more feasible to study these important elements. In this project, our team used the Amnis® FlowSight® Imaging Flow Cytometer and IDEAS® Software to isolate T cell-APC conjugates, identify the region of cell contact by F-actin localization, and evaluate the percentage of T cells with organized immune synapses. We also used Amnis® AI Image Analysis Software for immune synapse image classification, including criteria to identify subtle morphological differences and improve the classification model efficacy. Artificial intelligence was an important part of this project, and we think it adds value to any imaging flow cytometry workflow. Our tools incorporate AI by seamlessly adding machine learning and deep learning models to simplify image analysis, classify data based on pre-existing models, perform AI-assisted tagging, and quickly generate reports. For this effort, we trained an AI model by merging data files from five independent experiments and creating "truth" populations for the types of cells and interfaces we were looking for. In our hands, the model proved 91% accurate in classifying control cells and those treated with Staphylococcal enterotoxin B.

ULTRA-HIGH PARAMETER EXPERIMENTS WITH CYTEK AURORA SPECTRAL FLOW CYTOMETER : 40 COLORS, ONE TUBE

Brewińska-Olchowik M.

Cytek Biosciences inc., 46107 Landing Pkwy, Fremont CA, 94538

Until recently, developing fluorescence-based flow cytometry assays with 40 colors has been merely aspirational, with many turning to alternative technologies for high-parameter applications. No longer. With 64 fluorescence detectors and only 5 lasers, the Cytek Aurora now has the capability to resolve up to 40 colors in combination. Cytek has developed a 40 color human immunophenotyping panel acquired from just a single tube, with outstanding resolution. FlowSOM and t-SNE-CUDA analysis was performed using OMIQ software (www.omiq.ai). Doublets, aggregates, and dead cells were excluded from the analysis. 45 metaclusters were identified using FlowSOM, shown below. t-SNE-CUDA plots colored by marker expression are presented at the right; markers are organized by major cell subsets.

EX VIVO PHENOTYPING AND POTENCY MONITORING OF CD19 CAR T CELLS WITH A COMBINED FLOW CYTOMETRY AND IMPEDANCE-BASED REAL TIME CELL ANALYSIS WORKFLOW

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Evaluation of the potency of CAR T cells is essential for effective immunotherapies. Here we used a combined impendence-based Real Time Cell Analysis (RTCA) and flow cytometry workflow for ex vivo cytolytic potency monitoring of CD19 CAR T cell (CART19), as well as examination of phenotypic and functional responses to antigen exposure over time. This powerful workflow can be used to easily measure the cytolytic capacity of CAR T cells with in-depth analysis of T cell cytokine production, cell differentiation, and activation state. Next, the expression of inhibitory receptors PD-1, TIM-3, and LAG-3 was examined as well as activation markers CD25 and CD127. Ex Vivo Phenotyping and Potency Monitoring of CD19 CAR T Cells With a combined Flow Cytometry and Impedance-based Real Time Cell Analysis Workflow Advancements in immunotherapy have altered the available treatments for cancer, by using the specific ability of the immune system to recognize and kill cancer cells. One's T cells can be directed to target cancer cells by engineering them into chimeric antigen receptor (CAR) T cells. CAR T cells specifically bind and become activated by a cancer antigen. While they hold tremendous promise for cancer treatment, significant challenges remain in the clinical translation to many cancers and control of side effects. Unlike other cytolytic endpoint assays, the Agilent xCELLigence RTCA continuously monitors CAR T cell cytolytic activity in real-time over days. Also, orthogonal assays that evaluate T cell activation, differentiation, and exhaustion are useful to fully determine the quality of the CAR T cells under investigation. Here, we study the potency of CAR T cells using a combined impendencebased Real-Time Cell Analysis (RTCA) and flow cytometry workflow for ex vivo cytolytic potency monitoring of CD19 CAR T cells (CART19).

T CELL EXHAUSTION IN PRIMARY IMMUNODEFICIENCIES, INCLUDING CVID AND 22Q11.2 DELETION SYNDROME

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Primary immunodeficiencies (PIDs) are a diverse group of disorders characterized chiefly by the impaired functionality of the immune system, susceptibility to infections or development of non-infectious complications. Common variable immunodeficiency (CVID) is the most common symptomatic PID, hallmarked by impaired antibody response and traditionally associated with dysfunctional development of B cells. Many patients with CVID display noninfectious complications such as enteropathy, interstitial lung disease or autoimmune cytopenias and early reports also show previously hidden changes within their T cell compartments. On the other hand, the immunopathology of 22q11.2 deletion syndrome (22q11.2DS) is driven by various degrees of thymic dysplasia, which impacts maturation of T cells directly. We thus sought to evaluate the maturation, differentiation and features of senescence and exhaustion, as well as their association with (non-)infectious complications in these two PIDs.
We evaluated 17 pediatric patients with 22q11.2DS with 17 healthy age-matched donors, and 40 adult patients with CVID (10 infection only (CVIDio), 30 complicated disease (CVIDc)) with 17 healthy adult donors, using flow cytometry and mass cytometry by time-of-flight (CyTOF).
We saw a significantly accelerated maturation of T cells both within the 22q11.2DS and CVID, which was more pronounced in the CVIDc cohort, with expansion of effector memory and TEMRA T cells. Further, these changes were accompanied by increased expression of inhibitory receptors including PD-1, TIGIT, Tim3, KLRG1 and senescence marker CD57. In CVID, the loss of IL-7 receptor CD127 responsible for long-term survival of T cells and the inhibitory marker TIGIT was strongly associated with development of non-infectious complications such as interstitial lung disease or autoimmune cytopenias, whereas T cell activation measured as expression of HLA-DR and CD38 was associated with enteropathy.
In summary, we present novel findings showcasing the dysregulated maturation and exhausted phenotype of T cells in CVID and 22q11.2DS.

EFFECT OF EARLY POSTNATAL PROBIOTIC SUPPLEMENTATION ON IMMUNE SYSTEM MATURATION AND COLITIS DEVELOPMENT

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The mutual homeostatic interactions between microbiota and host immune system are important to prevent aberrant immune reaction leading to development of immune mediated disorders. Early postnatal events are critical in maturation of neonatal immune system. In children delivered by caesarean section or treated by antibiotics, development of both microbiota and immune system is impaired making these individuals more prone to development of immune mediated disorders. Probiotics supplementation seems to be reasonable measure correcting for dysbiosis and mutual interaction between microbiota and host immune system.

To test the capacity of probiotics to promote immune system maturation and homeostatic interaction, probiotic strain of E. coli O83:K24:H31 (EcO83) was administered to pups within 48 hours after delivery. Selected immune parameters were followed (e.g. innate lymphoid cells (ILC), regulatory T cells (Treg), neutrophils) by flow cytometry. To test the capacity of early postnatal probiotic supplementation to prevent colitis development, trinitrobenzene sulfonic acid (TNBS) was administered intrarectally to mice at the age of 8 weeks. To evaluate the potential of EcO83 to renew homeostatic interactions between microbiota and host immune system, dysbiosis was induced by antibiotics (ATB) administration followed by EcO83 supplementation.

Early postnatal EcO83 supplementation promoted immunoregulatory function by increasing IL-10+Treg. Increased gene expression of IL-10 was detected in small intestine and colon of EcO83 treated mice and dendritic cells stimulated by EcO83 in vitro. Early postnatal EcO83 administration lowered the severity of TNBS induced colitis in adult mice. EcO83 was able to lower the proportion of CXCR2+CD62L+neutrophils (to the level observed in the control group) in comparison with the group of mice with ATB induced dysbiosis.

Our data suggest that early postnatal administration of EcO83 is promoting immune system maturation together with setting immunoregulatory functions.

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REGULATION OF T CELL RESPONSE WITH SELECTIVE CASEIN KINASE 1 FAMILY INHIBITORS

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Rheumatoid arthritis (RA) is an autoimmune disease that results in persistent inflammation and bone destruction in joints and can also affect other organs as well. A main hallmark of RA pathogenesis is lymphocyte infiltration and the imbalance between immune cells presented in joint synovium. Although different cells are involved in the pathological process, CD4 T cells, the most abundant lymphocytes in the synovial infiltrate, seem to be central by secreting cytokines and by cooperating with other cells in the affected tissue. CD4 T cell subpopulations penetrate the synovial membrane where they release proinflammatory cytokines that contribute to bone destruction and thickening of the synovial membrane. Some of them can promote B cell differentiation into plasma cells producing antibodies like rheumatoid factor, which target joint as well. Moreover, released proinflammatory cytokines can activate macrophages, neutrophils, and synovial fibroblasts, causing transformation of these cells into tissue-destructive effector cells. Members of the casein kinase 1 (CK1) family belonging to the serine/threonine kinases have been shown to phosphorylate key regulatory molecules involved in T cell signaling pathways. Mouse Th naive cells were sorted from lymph nodes and stimulated by selected activators and cytokines to induce differentiation into Th1, Th2, Th17, and Treg subsets. Subsequently, part of the samples was treated with selective CK1 inhibitors. To characterize T cell subsets, analysis of specific gene expression by RT-qPCR and production of effector cytokines by ELISA were performed. Obtained data suggest that CK1 inhibition significantly affects Th cell differentiation. Therefore, regulation of CK1 activity by using of specific inhibitors could diminished negative effects of Th cells during pathological immune response.

SERUM-REDUCED CULTURE OF PORCINE MONOCYTE-DERIVED MACROPHAGES

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The aim of the present study was to establish a cell culture system for the generation of porcine monocyte-derived macrophages (MDM) under serum-reduced conditions. Corning® Nu-Serum™ Growth Medium Supplement (NUS) was compared with classical fetal bovine serum (FBS) based culture, FBS from two different providers were included. Macrophages were derived by plastic adherence from enriched CD14+ blood monocytes during 5-day cultivation. In order to confirm that serum-reduced MDM culture is suitable for porcine macrophage functional studies in vitro, their morphology, yield, surface markers expression, respiratory burst, phagocytic activity and cytokine production was assessed. Significant impact of culture conditions were found within all supplements including each type of FBS. Serum-reduced macrophage culture showed increased levels of typical M1 markers IL-1b and CD86. Their cytokine profile was pro-inflammatory including IL-23p19, CXCL10 and CCL5 increased mRNA expression. Disadvantage of the NUS culture was lower yield of MDM after 5-day differentiation. Phagocytic and respiratory burst capacity was not negatively affected by lacking of FBS. Macrophage culture supplemented with FBS from first provider gave rise to macrophages with higher surface level of CD14, CD16, CD163, lower CD80 mRNA expression and increased IL-10 production as typical attributes of M2-polarized macrophages. Macrophages culture supplemented with second FBS did not share M2-related markers accompanied by increased levels of IL-1b and CD86. In conclusion, serum-reduced condition is useful tool for porcine MDM culture which follows current trends enhancing reduction of FBS use across biological research. The project was supported by the Ministry of Agriculture (QJ1910311 and RO0518).

THE ROLE OF PATTERN RECOGNITION RECEPTOR-INDUCED TRANSCRIPTION FACTOR SIGNALLING IN MONOCYTE RESPONSES

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Detection of microbial structures through pattern recognition receptors (PRRs) is crucial for proper function of innate immunity. Pathogen recognition induces several signalling cascades, resulting in activation of transcription factors (TFs). Efficient immune response requires strictly regulated cooperation of TFs. While a number of studies have demonstrated the contribution of nuclear factor of activated T-cells (NFAT) to myeloid cell responses, the exact role of NFAT in transcriptional program in activated monocytes is still unknown.

We have analysed the transcripts regulated by NF-κB and NFAT in stimulated human monocytes, enriched from the blood of healthy donors. Similarly, we evaluated the crosstalk of NF-κB and NFAT in established THP-1 luciferase reporter lines. Next, we analysed nuclear translocation of NF-κB and NFAT in monocytes using confocal microscopy. We optimized intracellular staining (ICS) protocol using commercially available kits for flow cytometry in order to show the translocation of TF from cytoplasm to nuclei. Using this high-throughput method to assess the TFs activity, we obtained preliminary data suggesting the crosstalk between NF-κB and NFAT signalling pathways in non-classical subset of monocytes. While further tests are being carried out, we believe that this method could provide a useful tool for monitoring the activity of multiple signalling pathways at the same time within specific cells by flow cytometry.

MICROFLUIDICS

MICROFLUIDIC CHIP AS A TOOL FOR GENERATION OF UNIFORM ORGANOIDS

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Organoids dramatically changed the field for creating biological models with 3D cellular organization due to their ability to recapitulate pato/physiological properties of human tissues or organs. Methodological obstacles lie in organoid formation and following long-term cultivation and differentiation. Recent preparation and maintenance of organoids is labor-intensive, medium exchange is discontinuous, and individual organoids are highly heterogenous in size, morphology, and cellular composition. Microfluidics is promising platform to overcome some of these disadvantages. Due to automation and proper design of chips, microfluidic systems are capable of continual medium flow in space and time, which allows to create better controlled microenvironments for cells.
In this work, we present utilization of microfluidic system for uniform formation of aggregates with low divergence of size and shape. System enables a parallel perfusion culture of large amount of cell spheroids as well as long term cultivation and cell differentiation. Upon in silico simulations, we optimized chip designs and characterized several specific conditions in a microwells including sufficient nutrient exchange in microwells. We characterized cell behaviour of human induced pluripotent stem cells, such as proliferation, morphology and differentiation to cerebral and retinal organoids.
This work was supported by OPVVV INBIO CZ.02.1.01/0.0/0.0/16_026/0008451 and project from Masaryk University (MUNI/A/1689/2020).

IN VITRO FLUIDIC MODELS TO STUDY CARDIOVASCULAR DISEASES

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Cardiovascular diseases (CVD) are the most important causes of mortality and morbidity worldwide. In many cases, they originate from an imbalanced interplay among blood flow, vascular endothelial cells and inflammatory stimuli. Over time, such imbalance can develop into life threatening pathologies like atherosclerosis, myocardial infarction or stroke.
The focus of our group is to study aspects of CVD using in vitro vasculature models. 3D, 2.5D and 2D models are used to replicate processes in vasculature. The models are being prepared by approaches including lithography, 3D printing and polymer (PDMS) casting.
3D patient-derived fluidic models are included in a study of hemodynamics of cerebral aneurysms and stenoses. The idea of the study is to establish hemodynamics as a predictive tool to evaluate aneurysms and stenoses development. The in vitro models are used as a feedback for computational flow modeling.
2.5D fluidic model of middle cerebral artery has been used in determination of the role of collateral circulation in thrombolysis and vessel recanalization during ischemic stroke. The data recapitulated more rapid recanalization in presence of collateral circulation but thrombolysis was not affected.
2.5D and 2D fluidic models have been used in determination of the impact of shear stress and other stimuli onto vascular endothelial cells status and the signaling mechanisms behind.

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DISCOVERY OF NEW BIOCATALYSTS ACCELERATED BY MICROFLUIDICS

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Next-generation sequencing technologies have led to the doubling of genomic databases every 2.5 years. The collected sequences represent a rich source of novel biocatalysts. However, the accumulation of sequence data exceeds functional characterization, calling for acceleration and miniaturization of biochemical assays [1]. Here, we present an integrated platform for the exploration of unmapped sequence space and discovery of novel industrially relevant biocatalysts [2].
The platform is based on bioinformatics, microanalytics, and microfluidics. The automated in silico analysis uses EnzymeMiner for identifying a set of promising sequences in the sequence database [3]. The high-throughput in vitro part consists of two microfluidic platforms for systematic characterization of substrate specificity, temperature profiles, enzyme kinetics, and thermodynamics. The microfluidic methods provided increased throughput up to 20,000 reactions per day with 1,000-fold lower protein consumption than conventional assays, making this systematic functional analysis fast and cost effective.
Targeting dehalogenases as a model enzyme family, we obtained 2,905 new sequences, of which 45 representative enzymes were selected for experimental characterization. Robust high-throughput expression analysis delivered 24 variants with sufficient yield and solubility for further biochemical characterization in a single run. This is roughly the same number of new biocatalysts as the conventional methods delivered over the last three decades. These new biocatalysts have several industrially interesting characteristics and 6 of them significantly surpassed all existing family members in terms of their overall activity. The strategy is generally applicable to other enzyme families, paving the way towards accelerating the process of identification of novel biocatalysts for industrial applications and the collection of homogenous data for machine learning [4].

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CONVOLUTIONAL NEURAL NETWORKS FOR AUTOMATED COUNTING OF SINGLE PHOTON-UPCONVERSION NANOPARTICLES IN MICROFLUIDIC CHIPS

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Methods that allow the counting of single molecules advances current analytical chemistry. The reason is reaching the ultimate limit of detection, which a single molecule is. Previously, we have shown that photon-upconversion nanoparticles are excellent luminescence labels for single-molecule immunochemical assays and applicable for droplet microfluidics.[1-3] Here, we discuss current advancements of instrumentation, which are important for integrating single-molecule immunochemical assays of protein markers with microfluidics. The experimental device utilizes a laboratory-built epiluminescence microscope. High-intensity near-infrared excitation source enables imaging of single photon-upconversion labels, which are emitting visible wavelengths. The image data are recorded by an sCMOS camera up to 120 frames per second. Convolutional neural networks automatically process images and localize nanoparticles. The possibilities for multiplexing are discussed.

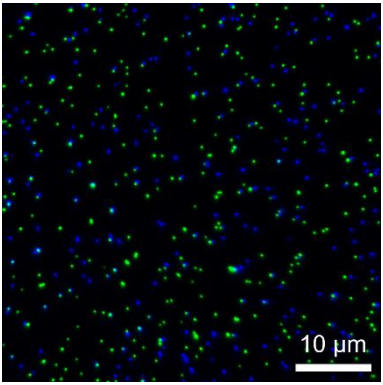

Figure 1. Two types of photon-upconversion nanoparticles on a glass substrate (sizes ~25 nm; nanoparticles doped either with Er^{3+} (emission 550 nm, green) or Tm^{3+} (emission 475 nm, blue); excitation 980 nm).

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ANALYSIS OF BACTERIOPHAGE-HOST INTERACTION BY RAMAN TWEEZERS

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Bacteriophages, or "phages" for short, are viruses that replicate in bacteria. The therapeutic and biotechnological potential of phages and their lytic enzymes is based on their ability to selectively destroy pathogenic bacteria, including antibiotic-resistant strains. Introduction of phage preparations into medicine, biotechnology, and food industry requires a thorough characterization of phage-host interaction on a molecular level. We employed Raman tweezers to analyze phage-host interaction of Staphylococcus aureus strain FS159 with a virulent phage JK2 (812K1/420) of the Myoviridae family and a temperate phage 80α of the Siphoviridae family. We analyzed the timeline of phage-induced molecular changes in infected host cells. We reliably detected the presence of phages in bacterial cells within five minutes after infection. Our results lay the foundations for building a Raman-based diagnostic instrument capable of real-time, in vivo, in situ, nondestructive characterization of the phage-host relationship on the level of single cells, which has the potential of importantly contributing to the development of phage therapy and enzybiotics.

RAMAN MICROSPECTROSCOPIC ANALYSIS OF SELENIUM BIOACCUMULATION BY GREEN ALGA CHLORELLA VULGARIS

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Selenium (Se) is an element with many commercial applications as well as an essential micronutrient. Dietary Se has antioxidant properties, and it is known to play a role in cancer prevention. However, the general population often suffers from Se deficiency. Green algae, such as Chlorella vulgaris, cultivated in Se-enriched environment may be used as a food supplement to provide adequate levels of Se. We used Raman microspectroscopy (RS) for fast, reliable, and non-destructive measurement of Se concentration in living algal cells. We employed inductively coupled plasma-mass spectrometry as a reference method to RS and we found a substantial correlation between the Raman signal intensity at 252 cm^{-1} and total Se concentration in the studied cells. We used RS to assess the uptake of Se by living and inactivated algae and demonstrated the necessity of active cellular transport for Se accumulation. Additionally, we observed the intracellular Se being transformed into an insoluble elemental form, which we further supported by the energy-dispersive X-ray spectroscopy imaging.

PLASMON-ENHANCED OPTICAL FORCE AGGREGATION OF GOLD NANORODS AND SERS IN A MICROFLUIDIC CHIP

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Optical printing of metal-nanoparticles-proteins complexes in microfluidic chips is of particular interest in view of the potential applications in biomolecular sensing. Optical manipulation techniques play an important role as a method that form and lead the aggregation process of metallic microclusters. Such a cluster can serve as a source of surface enhanced Raman spectroscopy hot-spots. SERS-active aggregates are formed when the radiation pressure pushes the particle-protein complexes on an inert surface, enabling the ultrasensitive detection of proteins down to pM concentration, in very short times (minutes). On the basis of an optical forces calculation and experimental results we introduce a procedure to microfluidic SERS substrate fabrication that could serve as a detection part of microfluidic bioassay or a lab-on-a-chip device. Described procedure constitutes fabrication of functional SERS microfluidic platform directly by user in the required area.

A HIGHLY CUSTOMIZABLE FLUIDICS CONTROL MODULE FOR FLOW CYTOMETRY

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Background: Highly sensitive cytometers and microfluidic devices require unique fluidics specifications and flexibility is often not achievable with the fixed layout fluidics packages included with most commercial analyzers. We have designed highly customizable fluidics module that can be adapted for any analyzers and enable the user to achieve an unprecedented level of customizability to allow them to meet their unique requirements.

Methods: We have designed the Shasta Fluidics Control Module, driven by dual hydrostatic pressure to separately control sample and sheath pressures, to support essentially unlimited flow rate requirements. The front panel enclosure allows easy access for the user to monitor and fine tune system pressures, optimizing fluid flow rates to meet their specific application needs. The system operates in five different modes—drain, fill, run, standby, and backflush—which control movement of the fluids through the system via different paths depending on user needs. The module utilizes quick connectors, allowing for easy attachment or removal of tubing. Importantly, the system is fully customizable and is designed to be retrofitted to virtually any cytometer, allowing users expanded fluidics functionality on their existing instruments. **Results.** We will present the results of instrument characterization, including flow rates (as low as 0.3 µL/min and as high as 130 µL/min) and transit times, and flow rate stability. Additionally, we will demonstrate the utility of the Shasta by demonstrating performance in applications that are uniquely enabled by the Shasta's flexibility and customizability.

Conclusion: For certain research applications, a wide degree of fluidics customization is required that cannot be achieved using standard off-the-shelf solutions. The Shasta is expected to enable improved performance in difficult assays when adapted to an existing flow cytometer or microfluidic device.

RETINAL ORGANOIDS IN MICROFLUIDIC SYSTEMS – FORMATION AND EARLY-STAGE DIFFERENTIATION

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Organoids as self-organizing stem-cell derived 3D constructs bring the potential to go beyond what is possible in 2D in vitro models by mimicking in vivo structure and function. Since the development of the first retinal organoids roughly one decade ago, methods for retinal organoid cultivation have been modified to give rise to all major cell types of the neuroretina, including mature photoreceptors. However, several challenges remain, such as heterogeneity between organoids, poor photoreceptor maturation and the degradation of retinal ganglion cells in long time cultures. Microfluidic platforms have the potential to help overcome some of these by allowing for automation and a continuous precise control of the microenvironment. In this work we present the application of a microfluidic system for the formation of retinal organoids and cultivation for more than 20 days. The presented platform allows for uniform aggregate formation and automated continuous perfusion of medium as well as cell differentiation and cultivation of retinal organoids. We characterized cell behaviour of human induced pluripotent stem cells, including proliferation, differentiation, and morphology of developing retinal organoids.

PRODUCTION OF A MICROFLUIDIC CHIP TO STUDY THE CD44-HIPPO PATHWAY CROSS-TALK

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During the last decade, demand for physiologically relevant 3D in vitro models has arisen since these models are capable to better recapitulate complex cell behaviour than 2D cell cultures as well as reduce the need for animal testing. However, commercially available chips usually have a rectangular cross-section of the channels, which may cause non-physiological behaviour of the cells due to disturbances in shear stress. It is now well established that laminar flow generating constant shear stress poses a protective effect on vasculature. On contrary, disturbed flow is responsible for the pathologies. Among many mechanosensitive pathways, the Hippo pathway was shown to sense the shear stress in endothelial cells. CD44 is a promiscuous mechanoreceptor that senses the ECM components in addition to other functions. Literature has indicated that it may interact with the Hippo pathway.
To overcome problematic rectangular cross-section, we designed a microfluidic chip with a circular cross-section of the channels that generates uniform shear stress. The chip was made of polydimethylsiloxane (PDMS). The surface of native PDMS was plasma oxidized and further coated by collagen IV to overcome its limiting hydrophobicity that strongly limits cell adhesion. Channels with modified surface were then seeded with endothelial cells that were cultivated under flow. Using this chip, we were able to repeatably get a uniform layer of highly viable endothelial cells having physiological behaviour.
The monitoring of the Hippo pathway was optimised in a static HUVEC cell culture. First, a fluorescence reporter of Hippo pathway-related transcription was introduced. It, however, worked well with confocal microscopy only. The expression of Hippo pathway downstream genes was determined by RT-qPCR, reference gene for HUVECs was optimised. Hippo pathway activation was further examined using western blotting. Next, the Hippo pathway activity will be studied under flow using our model with unique surface chemistry.

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DROPLET MICROFLUIDICS WITH PHOTON-UPCONVERSION LUMINESCENCE DETECTION: TOWARDS AUTOMATED SINGLE-CELL ASSAYS

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Droplet-based microfluidics uses a two-phase system of aqueous microdroplets (1 pL to 1 µL) embedded in immiscible oil. Individual droplets can be manipulated and analyzed independently, which allows for developing high-throughput analytical methods.
Visible emission of photon-upconversion nanoparticles (UCNPs) can be excited by near-infrared light (typically 980 nm). Advantages of UCPNs include multiple and narrow emission bands, negligible autofluorescence, and high photostability making them an ideal luminescent label for use in droplet barcoding[1,2] and multiplexed single-nanoparticle imaging[3].
Here, a droplet-based microfluidic chip with luminescence photon-upconversion detection was used for indexing the chemical content of microdroplets (Figure 1). The technique is used for the assay of β-glucosidase that hydrolyzes 4-methylumbelliferyl β-D-glucopyranoside into fluorescent 4-methylumbelliferone. Further, we discuss the applicability of the presented instrumentation for single-cell analysis utilizing either enzymatic or immunochemical methods with capabilities for single-molecule detection.

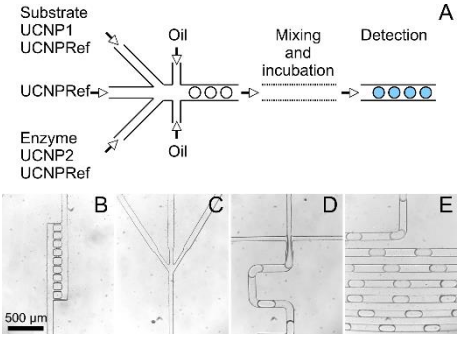

Figure 1. Microfluidic chip for photon-upconversion barcoding. (A) The scheme of a microfluidic chip for the β-glucosidase assay. Micrographs of: (B) filtration, (C) co-fluence, (D) flow-focus droplet formation, and (E) incubation part of the chip.

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SYNTHESIS OF PHOTON-UPCONVERSION NANOPARTICLES FOR MICROFLUIDIC APPLICATIONS

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Photon-upconversion nanoparticles (UCNPs) are luminescent lanthanide-doped nanocrystals that can be excited by near-infrared light and emit light of shorter wavelengths. Photon-upconversion luminescence appears without a background autofluorescence, which makes UCNPs extremely suitable for single-molecule immunochemical assays. One of the potential applications of UCNPs may be in droplet-based microfluidics. This technique utilizes a two-phase system of aqueous microdroplets in immiscible oil for high-throughput assays down to the single-cell level.

Here, we describe the development and optimization of photon-upconversion nanoparticles, which will be suitable for automated single-molecule immunoassays for single-cell experiments in droplet microfluidics. We present UCNPs with $NaYF_4$ or $NaGdF_4$ crystal matrices doped with lanthanide ions (Er^{3+}, Eu^{3+}, Tm^{3+}). The composition and internal architecture of UCNPs are optimized to provide bright and colored emission (Figure 1).

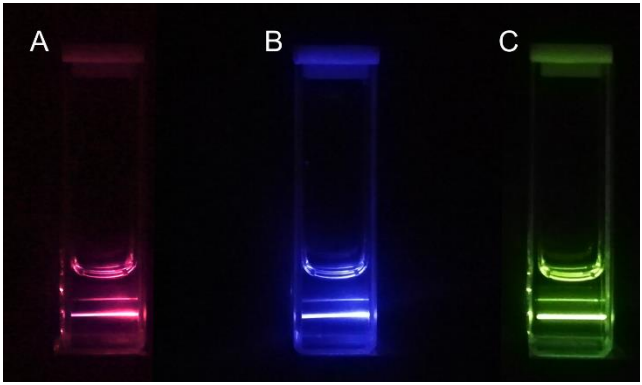

Figure 1. Photon-upconversion luminescence. Dispersions of photon-upconversion nanoparticles doped with: (A) Eu^{3+}, (B) Tm^{3+}, (C) Er^{3+}. Excitation wavelength 980 nm.

Acknowledgment:
We acknowledge financial support from the Czech Science Foundation (21-03156S) and Institutional support RVO:68081715.

Reference:

1) Hlaváček A, Mickert MJ, Soukka T, et al (2019) Anal Chem 91:1241–1246

IMAGE CYTOMETRY

3D IN VITRO MODEL OF HUMAN ENTEROCYTES

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Currently, 3D in vitro models are being widely employed in modern cell and molecular biology experiments. Spheroids with necrotic cores and oxygen gradient zonation are frequently used in cancer research or disease modelling. Nevertheless, to mimic healthy intestinal epithelium, luminal spheroids made by monolayer of intestinal epithelial cells were needed to be evaluated in our laboratory.
To fulfil this aim, the sacrificial micromolding method was optimized for Caco-2 cells. Agarose mold with defined microwells was made using specialized wafer to prepare high amount of cell clusters with roughly the same number of cells. This enables us to obtain homogenic population of spheroids with similar degree of differentiation. After 24 hours, the cell clusters were brought onto Matrigel which mimics the extracellular matrix and gives the cells impulse to form a polarized and differentiated monolayer. After a 6-day cultivation, differentiated intestinal spheroids have been obtained.
The spheroid structure and cell differentiation has been assessed using confocal microscopy. DAPI staining of the nucleus shows monolayer of cells surrounding a hollow lumen like intestinal epithelium in vivo. Antibodies conjugated with a fluorescent dye have been used to visualise proteins essential for cell-to-cell interactions (actin filaments and zonula occludens-1). Moreover, brush border protein ezrin was also detected. Expression and apical localization of these proteins is a marker of differentiated intestinal epithelium.
This study presents an optimised protocol for intestinal spheroids preparation. This model is well suitable for studying the responses of the healthy intestine after stimuli coming from the blood flow. To assess the response to apical stimuli, treatment would need to be injected to the lumen of the spheroid.

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VISUALIZATION OF TETRASPANIN ENRICHED MICRODOMAINS USING STRUCTURED ILLUMINATION MICROSCOPY

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One of the identified molecule linked with cellular plasticity and tumor dissemination is Tumor-Associated Calcium Signal Transducer 2 (Trop-2, TACSTD2) transmembrane glycoprotein [1]. Tetraspanin family members CD9 and CD63 are other cell membrane receptors that are associated with increased proliferation, invasiveness and adhesion [2, 3]. All these proteins generate a scaffold of receptors in the cellular membrane, which spatial organization is essential for homeostasis and frequently deregulated in a malignancy state.
Post-translational modification of tetraspanins forms areas on the membrane with increased accumulation of receptors, so-called tetraspanins-enriched microdomains, where they specifically interact with other tetraspanins, integrins and cell-specific receptors and hence might affect the intracellular signalling and cellular functions [4].
For this study, we utilized cell clones of MCF-7 breast cancer cell line with deleted CD9 or CD63 expression. To reveal nanoscale organization, pattern and colocalization of regulators of cancer dissemination and to study the unknown link between Trop-2 and tetraspanins, we have established a 4-color immunofluorescence protocol for super-resolution microscopy. By the methods of image processing and 3D segmentation, we show for the first time 3D models of identified microdomains in breast cancer.

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STUDY CELL FREE DNA HISTONES AND NUCLEOSOMES BY IMAGING FLOW CYTOMETRY

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Cell free DNA and histones are constantly released into the human blood stream both in health and in disease, upon cell death and making them excellent candidates for diagnosis and prognosis of various diseases. Intact nucleosomes, released from dying cells into the blood circulation, are found elevated in several types of cancers (pancreatic, lung, colorectal and breast), and in acute conditions such as stroke, trauma, and sepsis.
Here, we use immunofluorescence labelling and imaging cytometry for screening blood samples for the composition of histone complexes. Imaging cytometry is technology combining speed and statistical power of flow cytometry with imaging features of fluorescence microscopy. The capability to image large quantity of cells at high resolution significantly improves quality of collected information from flow cytometric analysis. We are able to visualize more than 5000 acellular objects in a volume of 0.3 µl within minutes. The identity of the acellular complexes as true histone complexes was further confirmed through positive controls utilizing recombinant core histones or intact nucleosomes.
Our results allows a deep understanding of the variability and combinations of histone complexes, or nucleosome composition, and of their detection specificity for specific diseases such as cancer versus healthy state.