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BOTANY AND ZOOLOGY

THE GLOBAL BIOGEOGRAPHY OF ANGIOSPERM GENOME SIZE IS SHAPED BY CLIMATE AND RANGE SIZE

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The size of an organism's genome is a fundamental characteristic that is relatively stable across species, while geographic range is a key feature that reflects a network of biotic and abiotic interactions shaping species evolution. Recent advances in flow cytometry have allowed for the standardized measurement of genome size in >5% of known angiosperm species, enabling the examination of hypotheses on the links between genome size, distribution, and environment at a global scale. In our study, we used consistent geographic data from the Plants of the World Database to investigate three hypotheses on the relationship between genome size and geographic range in flowering plants: the large genome constraint hypothesis, the polyploidy-mediated hypothesis, and the climate-mediated hypothesis. Our analysis revealed that large-ranged angiosperm species only have small genomes, supporting the large genome constraint hypothesis. We also identified a unique association between the global latitudinal gradient and genome size, with climate having a strong effect on the latitudinal pattern in genome size. In contrast, the effect of polyploidy was small. Furthermore, we found that the increase in genome size of flowering plants from the equator to 40–50°N/S is likely mediated by different mechanisms than the decrease in genome sizes from 40–50°N northwards. Overall, our findings suggest that species range sizes and climate are key factors shaping the global distribution of genome sizes in flowering plants. By resolving conflicts in earlier genome size studies, our study provides important insights into the evolution of this fundamental biological trait.

USE OF FLOW CYTOMETRY TO STUDY MICROALGAE

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Microscopic algae are a remarkably diverse group of organisms with a major impact on global ecosystems and are receiving increasing attention as a potential source of next generation biofuels or usable metabolites. In many areas of microalgal research, the use of flow cytometry (FCM) could have great potential, yet its application is still in its infancy. Unfortunately, FCM of microalgae is often laborious compared to plant and animal studies, due to the difficulties in obtaining large amounts of biomass, protoplast extraction (due to a variety of cell wall components), and the common presence of various pigments and secondary metabolites that can interfere with fluorescent staining. I provide general recommendations for successful protoplast extraction and FCM analysis of various microalgae. By overcoming these issues, FCM of microalgae can be applied to the determination of chlorophyll content or cell viability, the detection of harmful algal blooms, or the identification of different life cycle stages. For example, by alternating two ploidy stages, both of which are capable of mitotic propagation and long-term survival in culture, I have revealed an isomorphic haploid-diploid life cycle of golden-brown algae. Furthermore, unicellular microalgae, due to their short generation time, immediately apparent phenotypic changes, and easy manipulation under experimental conditions, represent a promising model group to study genome size evolution.

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GENTLE ISOLATION OF PLANT PROTOPLASTS, NUCLEI, AND GROWTH OF TRANSGENIC MICROALGAE USING THE WOLF CELL SORTER

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Cell isolation and cloning via gentle cell sorting are needed for numerous biological workflows in plant biology including gene characterization and function, proteomics and transcriptomics studies, single cell genomics, improved cell line development through gene engineering/CRISPR Cas 9 editing, and breeding in the microalgae space. Fluorescence activated cell sorting can be used to enrich desired populations of microalgal isolates in fundamental research, such as the discovery and isolation of new species, and in the development of stable engineered strains for various biotechnology applications Sorting plant protoplasts, nuclei, and microalgae is highly challenging with traditional droplet cell sorters due to the high pressures, shear stress and osmotic changes which damage or lyse the cells and nuclei making them unusable for downstream cell culture or genomic applications Traditional cell sorters limit the use of custom sheath buffers, which quickly degrade the fluidics components in the instruments, but are needed to support cell viability during and post sort Lastly, traditional cell sorters are restricted to sorting smaller cell sizes (mostly 30 µm), due to the inability to hold a stable drop delay with larger nozzles 200 µm or larger) and low sheath and sample pressures 10 psi) Plant protoplasts, nuclei, and microalgae share sensitivity and stress induction when enriched via traditional cell sorting instrumentation. The WOLF® cell sorter offers a solution that efficiently enriches for populations of interest with very low shear stress and supports cell viability by enabling scientists to use culture medium as sheath fluid. Herein we demonstrate isolation and successful separation of tomato mesophyll protoplast classes, plant nuclei from the leaves of Roma tomato and green bell pepper plants, and enrichment and growth of transgenic Chlamydomonas reinhardtii via bulk and single cell sorting.

RADIATION FIBROSIS MODULATION BY ANTI-CD25 HYALURONIC ACID NANOPARTICLES

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Radiation-induced damage to the pulmonary tissue triggers a cascade of molecular events, including reactive oxygen species production and DNA damage, resulting in an early immune response to initiate wound tissue healing processes. Expect fibroblast and their massive transforming growth factor-β (TGF-β) production. Another cellular component responsible for the fibrotic process is a subset of CD4+ T lymphocytes – regulatory T cells (Tregs). Tregs contribute to fibrotic diseases in the lung by promoting a pro-fibrotic microenvironment by producing TGF-β and promoting β-catenin, which are mechanisms driving epithelium-to-mesenchyme transition (EMT). We have previously shown the effect of hyaluronic acid nanoparticles (HANPs) on radiation-induced processes in lung tissue, potentially decreasing the extracellular matrix degradation and suppressing the production of TGF-β leading to lung fibrosis.
Our study aimed to design a new structure of hyaluronic acid nanoparticles (HANPs) to suppress the population of Tregs in lung tissue and determine the effect of acute and/or chronic phase of radiation damage to lung tissue.
The surface of HANPs was modified with anti-25 CD mAb (anti-CD25 HANPs) to deplete Tregs. Subsequently, anti-CD25 HANPs were applied by intratracheal instillation to C57BL/6J before the whole thorax irradiation by 17 Gy. Blood and lung tissue samples were collected 21, 60, 105, 155, and 190 days after irradiation. Blood count and immunophenotypisation were measured in blood. In the lung, immunophenotypisation, cytokine profile, and histopathology were evaluated. The efficiency of Tregs depletion was determined by flow cytometry.
In summary, the application of anti-CD25 HANPs did not have any significant effect on mice survival, nor improve their survival. The results showed that our treatment anti-CD25 HANPs haven't fully depleted the Tregs population but caused substantial changes in leukocytes, mainly lymphocyte population, in blood and lung tissue. Invariably to cell alternation, the levels of total protein and cytokines were detected. We concluded that even though Tregs weren't fully depleted with anti-CD25 HANPs, this treatment still efficiently mitigated radiation damage to lung tissue.

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LATITUDINAL PATTERNS OF REPRODUCTIVE STRATEGIES IN ALPINE PLANTS OF THE NEW WORLD

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Flow cytometry is a powerful tool for biosystematics and microevolutionary studies of plants. Namely Flow cytometry seed screening (FCSS) is used to investigate reproductive strategies of Angiosperms (identification of various apomixis subtypes). Apomictic reproduction, which is strongly connected with polyploid and hybrid speciation, is supposed to be restricted predominantly to the temperate and boreal zone of the Northern Hemisphere. The continuous range of the American Cordillera provides unique settings for broad-scale evolutionary studies. Especially South America is almost untouched by the flow cytometry research. The presented lecture aims to show how flow cytometry can contribute to the solution of microevolutionary questions of plant evolution especially in alpine plants of the Andes. Based on our research, we will show how to study the reproductive modes of plants and how their latitudinal patterns correspond with published hypothesis. Moreover, flow cytometry contributed to elucidate a unique type of endosperm formation as was proved in the example of the model genus *Azorella*.

GENOME SIZE MEASUREMENTS IN INSECTS

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The flow cytometry method (FCM) has its very important and irreplaceable place in medicinal diagnostics. However, it can also be a very powerful tool for study of plant or animal evolution. Morphological traits or DNA sequences are traditional features used by all modern taxonomists. And what about the genome size of each species? This distinct characteristic also changes during the evolution of species. Sometimes sister species have almost the same genome size, sometimes their genomes are absolutely different. Why?
Evolution happens through changes on DNA molecules and these changes could be really huge in the meaning of DNA volume difference. And this is precisely the momentum which can be observed by FCM, moreover, we can connect FCM with classical karyotype analysis (can be enriched by FISH). Then, we can track down what happened, on the level of chromosomes or genomes, between closely related groups of organisms or even ancient changes in deep evolution.
I studied chromosomal variability of 200 human bed bugs (*Cimex lectularius*) from 50 localities, where the basic male karyotype consists of 2n = 26+XXY and the known chromosome number variation ranges up to 2n = 26+20XY. The results of combined analysis (FCM and classical cytogenetics) showed that individuals with additional chromosomes have much broader range of nuclear DNA volume (28% higher (2C = 2.72 pg) or 12% lower (2C = 1.87 pg)) than the specimens with basic karyotype (average 2C = 2.12 pg). That suggests more chromosomal rearrangements like duplication or deletion accompanied the development of high chromosome number karyotype beside the supposed simple sex chromosome X fragmentation.

RABBIT SPERM QUALITY ASSESSMENT USING TWO DIFFERENT FLOW-CYTOMETRIC GATING STRATEGIES

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Rabbit semen is known to comprise great number of seminal granules which presence within the sperm population might make difficult to objectively evaluate the sperm quality attributes using flow cytometry. The aim of this study was to evaluate sperm quality based on common FSC/SSC gating or using DRAQ5 dye for gating of nucleated cells. Three heterospermic doses from 10 different bucks were used for each flow-cytometric staining panel. In both panels, sperm viability (SYBR-14), apoptosis (YO-PRO-1 and Caspase 3/7), acrosomal status (PNA), membrane mitochondrial potential (MMP; MitoTracker Green), capacitation (FLUO-4) and ROS generation (CellROX Green) were analysed. In first panel, co-staining with DRAQ7 dye was performed to detect the dead spermatozoa concurrently with the analysed viability, apoptosis, etc. within the FSC/SSC sperm gate. In the second panel, co-staining with DRAQ5 dye concurrently with the analysed sperm attributes was performed to gate only nucleated cells. At least 10,000 events were acquired for each sample using FACSCalibur flow cytometer and assessed using FlowJo Software (BD Biosciences). Obtained data were statistically evaluated using GraphPad Software. Results are expressed as mean ± SD. In first gating strategy (panel), about 30 – 50% of events were gated subjectively on FSC/SSC dotplot according to our previous experiences. We observed 54±16% of SYBR-14$^+$DRAQ7$^-$, 20±6% of YO-PRO-1$^+$ and 31±4% of Caspase 3/7$^+$, 29±9% of PNA$^+$, 66±10% of MitoTracker Green$^+$DRAQ7$^-$, 8±2% of FLUO-4$^+$ and 65±11% of CellROX Green$^+$ sperm. In second gating strategy, about 50% of events were gated based on DRAQ5 positivity. DRAQ5$^+$ sperm comprised 80±4% of SYBR-14$^+$, 21±7% of YO-PRO-1$^+$ and 22±8% of Caspase 3/7$^+$, 17±2% of PNA$^+$, 29±13% of MitoTracker Green$^+$, 22±5% of FLUO-4$^+$ and 50±4% of CellROX Green$^+$ cells. In conclusion, DRAQ5 gating successfully omit seminal granules and cell debris from the analysis.

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FLOW CYTOMETRY APLLIED FOR THE STUDY OF LICHEN ECOLOGY AND EVOLUTION

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Genome size has a huge impact on organismal morphology, development, and life strategy, as it is positively correlated with cell size and negatively correlated with the cell division and growth rates in eukaryotes. Flow cytometry has made it possible to estimate the genome sizes of a large number of species. However, this method has not yet been used in lichenology due to methodological problems associated with their small genome sizes. We optimized the flow cytometry method and measured genome sizes of 146 lichen species.
Lichen genome sizes are partly affected by their phylogenetic history, which is clearly seen in the order Peltigerales, which includes species with greatly increased genome sizes. However, after subtracting out the effect of phylogenetic relationships among lichens, we also found that genome size is linked to lichen ecology. Substrate preferences belong among the statistically significant traits. Epiphytically growing lichens have relatively smaller genome sizes than terricolous or saxicolous lichens. At the same time, species that prefer disturbed habitats and produce large amounts of small conidia have smaller genome sizes than species with bigger conidia growing on stable habitats.
In conclusion, we consider flow cytometry to be a suitable method for genome size measurements in lichens. It brings otherwise unavailable data and enables a new perspective on the lichen ecology and evolution.

CANCER BIOLOGY

THE ORION PLATFORM FROM RARECYTE ENABLES SAME-DAY 21-PLEX FLUORESCENCE TISSUE ANALYSIS

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Tissue consists of heterogenous cell types, each with diverse functions and functional states, arranged spatially in a way that impacts patient health status. Resolving this complexity at the subcellular level has historically been challenged by fluorescence overlap, a limited number of targets that can be simultaneously assessed, and low throughput. Orion technology breaks these barriers, providing rapid, straightforward, highly multiplexed whole slide tissue analysis. The recently announced Orion platform rapidly generates high resolution images in up to 21 channels to enable comprehensive phenotypic profiling and characterization of tissue architecture including micro-anatomy, analysis of tumor heterogeneity and the complex tissue microenvironment. FFPE tissue samples were stained with a 20-plex TissuePlex™ panel and whole slides were imaged on the Orion Instrument. Resulting datasets were reviewed by a pathologist to verify staining specificity. The TissuePlex core panel includes SYTOX™ Blue stain and antibodies against PCNA, CD20, SMA, CD31, CD68, CD45, CD4, FOXP3, CD8A, CD11B, PD-L1, CD11C, CD163, E-Cadherin, PD-1, Ki67, CD3d, CD45RO, and pan-cytokeratin (Pan-CK). The TissuePlex core panel was validated against single marker IHC.

EXTRACELLULAR VESICLES IN OVARIAN CANCER ASCITES

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Ovarian cancer (OC) ranks among the deadliest cancers in women. Lack of symptoms, rapid metastases and common chemoresistance contribute to unfortunate fate of majority of OC patients, especially those having high-grade serous carcinoma of the ovary, fallopian tube and peritoneum (HGSC), the most common and most aggressive type of OC. Many HGSC patients have excess fluid in the peritoneum called ascites. Ascites is basically a tumor microenvironment (TME) containing various cells, proteins and extracellular vesicles (EVs). EVs are small membrane-bound particles that convey proteins, lipids and nucleic acids between cells and their cargo reflects the cell of origin. EVs play important role in cancerogenesis and hold great promise as disease biomarkers. Small size and polydispersity of EVs bring various challenges to their isolation and characterization, including method-dependent enrichment of different EV subtypes as well as contaminants.
In the presented study, we isolated EVs from HGSC ascites by two orthogonal methods and analyzed them by mass spectrometry. We identified set of "core ascitic EV proteins", which contains typical EV markers, is devoid of proteins routinely contaminating EV isolates and largely covers interpatient heterogeneity. We compared them with proteins of EVs from related control fluids and described proteins present only on EVs from HGSC patients. We believe this list contains important players of HGSC progression as well as potential biomarkers. Using single cell RNA sequencing data, we mapped the origin of EVs to different types of cells present in malignant ascites. Our results suggest that EVs in ascites do not come predominantly from tumor cells, but rather from variety of non-malignant cell types. Analysis of cellular composition of ascites by spectral flow cytometry in combination with the analysis of EV cells of origin point to the critical contribution of macrophages to the ascitic TME, including the potential to predict patient prognosis. This emphasizes the recently appreciated role of TME in progression of HGSC.
To conclude, this is the first study attempting to link EV composition to the cell types producing it. As such it opens numerous avenues both for better understanding of EV role in tumor promotion/prevention and for the improved HGSC diagnostics.

L-ASPARAGINASE BY-PRODUCT GLUTAMATE IMPEDES THE EFFECT OF CHEMOTHERAPY BY AUGMENTING GLUTATHIONE BIOSYNTHESIS

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L-asparaginase (ASNase) is one of the crucial components of acute lymphoblastic leukemia (ALL) therapy. ASNase transforms asparagine (Asn) and glutamine (Gln) to aspartate (Asp) and glutamate (Glu), respectively. High Asp and Glu extracellular concentrations are considered to be a by-product of ASNase treatment without any known consequences. However, our results show that even though TCA cycle is diminished after ASNase treatment, leukemic cells are able to maintain intracellular Asp and Glu levels. We evaluated the role of Asp and Glu in the resistance mechanism of leukemic cells to ASNase.

Using stable isotope tracing with U^{13}C-Asp and U^{13}C-Glu we discovered that leukemia cell lines were able to import Asp and Glu from the culture media. Noteworthy, leukemia cell lines and also primary leukemia cells expressed the genes coding for Asp/Glu transporters. Moreover, inhibitor of Asp/Glu transporters moderately sensitized NALM6 cell line to ASNase.

Next, we discovered that high Glu doses, but not high Asp doses, helped the cells to survive in Asn-/Gln-depleted conditions. Furthermore, in high Glu conditions, using flow cytometry, we detected elevated intracellular ROS compared to Asn-/Gln-depleted media. Since GSH is the main antioxidant molecule in the cells and Glu is one of its three amino acids, we pursued the idea that cells under Asn-/Gln-depleted conditions use Glu to synthesize GSH and by that overcome the nutrient stress. Indeed, we discovered that leukemia cells used imported Glu to biosynthesize GSH. Preliminary data with ROS scavenger showed that ROS scavenging further improved leukemia cell proliferation in high Glu conditions. Together with the fact that imported Glu also fueled TCA cycle, GSH biosynthesis probably partially rescues the cells from oxidative stress caused by respiration. When we used GSH synthesis inhibitor BSO, proliferation of NALM6 cells was significantly more affected in high Glu condition compared to media without Glu.

Overall, this is the first study describing the transport of Glu into leukemic cells and its survival advantage after ASNase treatment.

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ULTIMATE CLARIFICATION OF THE RELATIONSHIP BETWEEN ASPARAGINE SYNTHETASE ACTIVITY AND SENSITIVITY OF LEUKEMIA TO L-ASPARAGINASE

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Backround: L-asparaginase (ASNase) is one of the crucial drugs used in treatment of childhood acute lymphoblastic leukemia (ALL). ASNase administration *in vivo* depletes circulating Asn. Leukemia cells are sensitive to ASNase because they have lower asparagine synthetase (ASNS) level compared to healthy cells. However, correlations of ASNS gene expression or enzyme production with sensitivity of leukemia to ASNase are inconsistent.
Aims: The main aim is to evaluate the role of ASNS enzymatic activity in predicting sensitivity of ALL cells to ASNase (Asn-depletion).
Methods: We employed stable isotope tracing to study the activity of ASNS. Sensitivity of cells to Asn-depletion and chloroquine (CQ) was measured using MTS assay. Protein levels were detected using western blot (WB).
Results: We measured the activity of ASNS together with ASNS protein levels in ALL cell lines. Six of them had ASNS detectable on WB (ASNS-plus). When Asn was limited in the media, ASNS-plus cells were able to synthesize Asn whereas ASNS-null cells were not.
Then, we measured the sensitivity of all studied cell lines to Asn-depletion. In B-ALL, all ASNS-plus cell lines were less sensitive to Asn-depletion compared to ASNS-null cell line. Interestingly, in T-ALL, ASNS-plus cell lines together with one ASNS-null cell line (ALL-SIL) were less sensitive to Asn-depletion compared to the second ASNS null cell line (DND-41). Since autophagy could provide Asn when it is limited in the media, we looked into autophagy activity. ALL-SIL cells were more sensitive to autophagy inhibitor CQ and also had higher autophagic flux compared to DND-41 cells. In accordance with that, we detected higher mTOR activity in DND-41 than in ALL-SIL cells.
Summary: Altogether, our study for the first time determined ASNS activity in leukemia cells under different nutrient availability. Importantly, ASNS activity correlates with ASNS protein levels in Asn-deplete conditions. In general, ASNS-plus cells are less sensitive to Asn-depletion than ASNS-null cells. However, the second mentioned could overcome ASNase treatment by high autophagy state and therefore bias the correlation between ASNS protein level and sensitivity to ASNase.

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TURNING ASPIRATIONS INTO REALIZATIONS: 40 COLORS, ONE TUBE WITH CYTEK SPECTRAL TECHNOLOGY

Jaimes M.

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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 performer 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.

BioID AS A TOOL FOR IDENTIFICATION OF HASPIN KINASE'S INTERACION PARTNERS

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Haspin is an atypical Ser/Thr kinase that is active during mitosis. Specifically, Haspin has been described to regulate chromosome alignment and timely segregation of sister chromatids. Interestingly, while all of Haspin's known functions are confined to mitosis, the kinase is present in the cell during the whole cell cycle, offering the possibility that Haspin could also be involved in other phases of the cell cycle.
One of the options to uncover new functions of a protein is to describe its interactome – a set of protein-protein interactions involving the protein of interest. The method we utilized in analysis of Haspin's interactome, BioID, relies on ligation of biotin to potential interaction partners based on their proximity to the investigated protein. After utilizing the BioID method in HeLa cells, mass spectrometry and subsequent bioinformatical analyses of biotin-tagged proteins identified 127 proteins as likely interaction partners of the Haspin kinase. While some of the proteins are established interaction partners of Haspin (e.g., Pds5b), the overwhelming majority has no published connection to the kinase. Furthermore, a subset of the identified proteins were found to differ between samples representing different points of the cell cycle, suggesting that changes in the interactome can depend on cell cycle progression.
In follow-up experiments we plan to carry out BioID in cells carrying the Fucci2 reporter system which will allow us to employ cell sorting according to cell cycle phases before the proteomic analysis.

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ESTABLISHMENT AND CHARACTERIZATION OF PRECLINICAL MODELS DERIVED FROM CIRCULATING TUMOR CELLS FOR BREAST CANCER DISSEMINATION AND DRUG SCREENING

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Circulating tumor cells (CTCs) are mediators of tumor dissemination, entering the peripheral circulation from the primary tumor and metastases and being transported to distant sites in the body. Due to their easy availability from the peripheral blood, CTCs have been extensively studied as a possible insight into the metastatic cascade. CTCs-derived preclinical models such as xenografts (CDXs) and *in vitro* cell cultures, are urgently needed to understand the biology of CTCs, their role in dissemination and to find potential drugs targeting CTCs. In this work, we present the establishment and characterization of an *in vivo* CDX model and CDX-derived *in vitro* cell culture of progressive breast cancer. A CTCs-enriched fraction was able to form a tumor (CDX) under the renal capsule 6 months after implantation and formed CDX was propagated subcutaneously in several passages. Next, a single cell suspension from formed tumor xenograft was stained for human CD298 marker and $CD298^+$ cells were sorted using a cell sorter and propagated in 3D spheroids *in vitro*. We further evaluated the stem-like potential of established cell culture in a clonogenic assay. To examine the tumorigenic and metastatic potential of the established cell culture, we performed subcutaneous implantation and implantation into the mammary fat pad. Finally, we evaluated several surface markers by spectral flow cytometry to describe epithelial/mesenchymal phenotype of each model. In summary, we have established an *in vivo* and *in vitro* model of progressing breast cancer derived from CTCs. Characterization of these models may help us understand the plasticity and behavior of CTCs during tumor progression and test potential CTCs-targeted therapies.

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EVALUATING METABOLOMIC PARAMETERS USING FLOW CYTOMETRY: A NOVEL APPROACH TO IDENTIFY AND CHARACTERIZE CANCER STEM CELLS

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Cancer stem cells (CSCs) are important for tumor growth and metastasis. In addition, whilst CSCs generally represent only a small fraction of the tumor (often less than 0.1%), they are relatively quiescent and resistant to conventional anticancer therapies. CSC phenotypes may fluctuate, and not all CSCs in a tumor are identified by flow cytometry for individual conventional CSC-related markers (ALDH+, CD44+/CD24- or CD133+), or by the side-population assay, each of which recognize distinct cell populations as opposed to the entire population of CSCs. Thus, we need to uncover and understand the common functional properties of CSCs to identify them more reliably in different tumors and discover new therapeutic targets.
Our study investigates whether CSCs can be identified through differences in glucose and protein metabolism. We propose that only non-CSCs exhibit the Warburg effect of aerobic glycolysis and thereby require higher levels of glucose than CSCs, which we propose exhibit less glycolysis and more oxidative phosphorylation. CSCs may also produce and degrade proteins more slowly than non-CSCs. We are focusing on developing assays for metabolism using FACS to allow identification, quantitation, and additional phenotypic analyses of purified cell populations. Preliminary data using novel flow cytometry assays demonstrate that the glucose transporter, GLUT-1, glucose metabolizing enzymes, mitochondrial activity, and protein synthesis/degradation rates are heterogeneous within clonal cancer cells. Importantly, we have discovered that altered protein and/or mitochondrial activities are associated with CSC phenotypes.
Hence, these newly developed innovative assays improve CSC identification and characterization and imply that combined targeting of CSC metabolic alterations with conventional radio- or chemotherapy will be a useful approach to selectively eliminate these cells.

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SINGLE CELL PROTEIN PROFILING DEFINES CELL POPULATIONS ASSOCIATED WITH TRIPLE-NEGATIVE BREAST CANCER AGGRESSIVENESS

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Triple-negative breast cancer (TNBC) is an aggressive and profoundly heterogeneous subtype of breast cancer that lacks targeted therapy. Such TNBC heterogeneity influences disease progression and drug response. Apart from tumor cells, solid TNBC tissue is composed of diverse populations of immune cells, endothelial cells, and stromal cells which together with the extracellular matrix form the tumor microenvironment. In addition, both tumor and non-tumor cells can exist in various phenotypic and signaling states, resulting in different biological functions. To understand how selected subpopulations and cell states shape tumor biology and influence the clinical outcome of TNBC patients we developed a single-cell proteomics pipeline allowing complex analysis of TNBC tumor and microenvironmental heterogeneity using mass cytometry. We designed and constructed antibody-based detection protocol for 26 phenotypic markers and 13 signaling molecules targeting cancer, stromal and immune cells. The multiparametric panel was measured in 26 treatment-naive primary TNBC specimens. Obtained data were analyzed with advanced supervised and unsupervised algorithms, including dimensionality reduction, clustering, and visualization.
The TNBC tumors contained phenotypically distinct subpopulations of cancer and stromal cells that were associated with the patient clinical status. Furthermore, we classified epithelial-mesenchymal status of tumor cells and described phenotypically diverse populations of tumor-associated stroma. Expression of molecules of interest identified by mass cytometry was then validated in retrospective tissue microarray cohort and their levels were examined for stratifying potential. Finally, we identified level of cancer cell CD97 as a predictor of worse clinical outcome.

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BIOMATERIAL SELECTION DOES NOT AFFECT AML ENGRAFTMENT IN HUMANIZED OSSICLES

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Objectives: Humanized ossicles can improve engraftment in patient-derived xenografts. Different biomaterials were reported for ossicle creation although their direct comparison is lacking. Here we aimed to test engraftment of primary acute myeloid leukemia (AML) samples in ossicles created from 4 different biomaterials.
Methods: Ossicles were created by seeding human bone marrow mesenchymal stromal cells on β-TCP granules (Kasios® TCP Dental HP, Kasios, France), extracellular matrix gel (ECM gel, Sigma-Aldrich, USA); collagen with hydroxyapatite in form of a sponge and loose fiber (CEITEC BUT, CR), directly before implantation. The scaffolds were implanted into NOD SCID gamma mice. After 8 weeks, the ossicles were either analyzed for formation of bone and bone marrow niche or the mice were intravenously injected with AML cells and further followed.
Results: The materials were first tested for bone- and bone marrow-niche formation. Ossification was successful in 75 – 100% of scaffolds (9/12 ECM gel, 9/10 collagen fiber, and 8/8 collagen sponge and 22/22 β-TCP granules) and infiltration with murine hematopoiesis in more than 64% scaffolds (8/12 ECM gel, 14/22 β-TCP, 9/10 collagen fiber, and 8/8 collagen sponge). The AML engraftment was then tested using 2 primary samples – 4 ossicles per material, 2 ossicles per mouse, plus 2 control non-implanted mice per AML. The engraftment in ossicles (median 99%) generally outperformed murine bone marrow (BM, median 7%) and peripheral blood (PB, median 2%), in the same mice. Between the individual biomaterials, no difference was observed in engraftment, the number of the extracted hu-cells, or in the percentage of primitive CD34+CD38- cells, for any of the AMLs. In AML #1, the mice with ossicles showed generally better conservation of the CD34+CD38- population compared to non-implanted mice, (implanted mice: median 0.72% in PB and 0.02% in BM; non-implanted mice: 0% in PB and <0.01% in BM). The ossicles harbored less CD34+CD38- cells (0.03%) than PB, indicating a migration of the primitive cells outside the human niche. Similar comparison in AML #2 was prohibited by low engraftment in murine tissues.
Conclusions: Biomaterial selection did not affect quantity or quality of the AML cells engrafted in humanized ossicles. The ossicles in general produced faster engraftment and better conservation of primitive AML phenotype than non-implanted mice.

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PD1 AND TIM3 EXPRESSION AS PREDICTOR OF EARLY HCC RECURRENCE AFTER PERCUTANEOUS THERMAL ABLATION

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Percutaneous thermal ablation is a cornerstone in the management of early hepatocellular carcinoma (HCC), but intrahepatic distant recurrence occurs up to 80% at 5 years. Combined approaches with immunotherapies are being studied to prevent recurrence. We evaluated the expression of immune checkpoint molecules (ICM) on intrahepatic and peripheral lymphocytes and investigated the association with early intrahepatic distant recurrence after local ablation.
This monocentric study was conducted between 2018 and 2023. After percutaneous thermal ablation for HCC, patients were followed and later divided into two groups, an "very early recurrence" group in case of intrahepatic distant recurrence within 12 months after percutaneous thermal ablation, and a "prolonged recurrence free" group in case of no recurrence before 12 months of follow-up. Freshly harvested intratumoral and non-tumoral liver tissue biopsies and circulating blood samples were obtained before percutaneous thermal ablation and were explored with multiparametric flow cytometry.
We observed that the frequency of $PD1^+CD4^+$ T cells was higher in the very early recurrence group compared with the prolonged recurrence free in circulating blood and in the non-tumoral liver, but not in the tumor. Similarly, the frequency of $Tim3^+CD8^+$ T cells was significantly higher in the early recurrence group in both peripheral blood and non-tumoral liver.
This study highlights the importance of the baseline systemic immunosuppressive status for tumor recurrence. The expression of immune checkpoint molecules on T cells, such as PD1 and TIM3 (in blood and in non-tumoral liver) identifies HCC patients at risk of early intrahepatic distant recurrence after percutaneous thermal ablation and gives a rationale for evaluating anti-PD-1 and/or anti-TIM3 to prevent tumor recurrence in pre-selected HCC patients.

THE ROLE OF CASEIN KINASE 1 INHIBITION IN TREATMENT OF CHRONIC LYMPHOCYTIC LEUKEMIA

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Chronic lymphocytic leukemia (CLL) is the most common leukemia in the western world and due to refractory behavior of the disease, CLL still remains incurable. Casein kinase 1 (CK1) is an important player in Wnt signaling but it also regulates multiple intracellular processes including autophagy, circadian rhythm or apoptosis-related signaling. In previous study, our research group has shown that CK1 is a potential therapeutic target in CLL, as its inhibition *in vivo* outperformed the effects of ibrutinib, the best therapeutic approach in CLL treatment so far. Therefore, we focused on the mechanism by which CK1 inhibition slows CLL progression, inspecting leukemic B cells and several subsets of T cells in CLL microenvironment. For that, we used approach combining *in vivo* treatment of Eμ-TCL1 mice with flow cytometric measurements using 13-color panel aimed at phenotyping of distinct B and T cell subsets in peripheral blood, lymph nodes, spleen and bone marrow, and subsequent multiparametric data analysis using FlowSOM. Moreover, we supplemented the analysis with RNA sequencing data of splenic leukemic B cells, which we used for differential expression analysis and for inference of the activity of distinct transcription factors and signaling pathways.
Preliminary results of our study have not only confirmed that CK1 inhibition leads to deceleration of CLL progression in Eμ-TCL1 mouse model, but also helped us elucidate multiple alterations in gene expression profile of leukemic B cells linked to potential cell cycle arrest of the leukemic B cells present in splenic microenvironment. Besides, our results have shown several alterations in abundances of specific T cell subsets, namely the cytotoxic T cells and regulatory T cells which might be potentially contributing to deceleration of CLL progression.

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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 the primary 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. A tumor cell spiking assay is frequently used to evaluate the sensitivity, 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. These data demonstrate the high sensitivity, specificity, and accuracy of CTC detection by flow cytometry.

THE ROLE OF ENDOPLASMIC RETICULUM STRESS ON IMMUNE SURVEILLANCE AND THE EFFECTIVENESS OF IMMUNOTHERAPY IN TREATMENT OF OVARIAN AND RENAL CELL CARCINOMA

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Advanced ovarian cancer (OC) or renal cancer (RCC) patients have poor prognosis, with a 5-year survival rate of less than 20%. Recently, immunotherapy has emerged as a promising novel approach for cancer treatment. However, most patients develop resistance. Many factors in the tumor microenvironment (TME), such as low pH and hypoxia, cause cellular stress, including Endoplasmic Reticulum stress (ERS), which is a crucial inducer of inflammation. This leads to under expression of MHC1 in tumor cells, increased angiogenesis, resistance to chemo and radiotherapy, and hypoxic survival. Immune cells exposed to ERS lack antigen presentation, exhibit a suppressor phenotype, and can initiate apoptosis. Thus, ERS can trigger immunosuppression in chronic inflammatory states. Modulation of ERS may be beneficial for immune surveillance and response to immunotherapy. Here, we co-cultured OC and RCC cells with peripheral blood mononuclear cells (PBMC). We modulated ERS in these co-cultures using tunicamycin (TM) and the chemical chaperone TUDCA. The alleviation of ERS by TUDCA increased the viability of cancer cells when cultured by themselves, whereas in co-culture with PBMC, their viability decreased, suggesting the increased effectiveness of the immune cell reaction. Moreover, TUDCA decreased the expression of the ERS markers HSPA5/BiP, DDIT3/CHOP, and EIF2AK3/PERK in these co-cultures. Next, we employed fluorescent IRE and PERK-YFP reporter systems and found that both PERK and IRE1 activity were induced by TM and abolished by TUDCA. TUDCA increases the capacity of immune cells to target cancer cells by inducing PERK promoter activity in OC cells and reducing the negative effects of TM on the viability of PBMC. These results were related to the molecular background of ERS in patient samples and showed a positive correlation between good clinical outcomes and higher lymphocyte infiltration at the tumor site and elevated expression of the ERS markers BiP and CHOP in tumor cells. Based on these results we expect that alleviating ER stress could improve the survival and effectiveness of the immune cells in hostile tumor-induced microenvironment and represent suitable approach for improving immunotherapy treatment.

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DIRECT CO-CULTURE MODEL OF CHRONIC LYMPHOCYTIC LEUKAEMIA: CK1 INHIBITION COUNTERACTS THE PROTECTIVE EFFECT OF BONE MARROW STROMAL CELLS

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We have previously shown in Eμ-TCL1 mouse model of chronic lymphocytic leukaemia (CLL) that inhibition of casein kinase (CK) 1 by a small molecule inhibitor PF-670462 can improve overall survival. CK1 is a key component of Wnt signalling pathway, which regulates CLL chemotaxis and communication of CLL cells with their microenvironment. Communication with microenvironment is essential for survival of CLL cells.
We mimicked the CLL natural microenvironment by direct co-culture of B cells from 9 CLL patients with murine bone-marrow stromal cell line M2-10B4. Monocultured and co-cultured cells were also treated by CK1 inhibitor (PF-670462) and submitted to RNA sequencing. Human reads were isolated in silico and their differential expression was evaluated by the limma-voom method. Activity changes of oncology-related pathways was further analysed by Progeny R package.
The most prominent change was found in activity of NFkB pathway, which was upregulated in co-cultured CLL cells (compared to mono-cultured) and downregulated in co-cultured CLL cells treated with CK1 inhibitor (compared to co-cultured CLL cells). Deregulated NFkB pathway activity was confirmed using intracellular flow-cytometry by upregulated IkBa in co-cultured CLL cells and downregulated IkBa in co-cultured CLL cells treated with CK1 inhibitor. Downregulation of key NFkB components (p65 and IkBa) by CK1 inhibition was further confirmed in a CLL cell line (HG3) stimulated by a NFkB activator (TNFa) using western blot.
This study uncovers the key role of CK1 in mediating the pro-survival signalling provided by the CLL microenvironment.

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TRANSCRIPTIONAL REGULATION OF SURFACE FINGERPRINT DURING EPITHELIAL-TO-MESENCHYMAL TRANSITION IN BREAST CANCER CELLS

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Epithelial-to-mesenchymal transition (EMT) and its reversed counterpart mesenchymal-to-epithelial transition (MET) are two sides of dynamic cellular process engaged during cancer dissemination. Transcriptional program of epithelial cells rewires during EMT to remodel established cell-to-cell contacts and to enable dynamic shape-shifting and invasion of cancer cells through various tissue niches. *Vice versa*, transcriptional program of mesenchymal cells regains back epithelial characteristics once cancer cells colonise new organ site. EMT/MET-related phenotypic plasticity is reflected on cellular surface as we´ve previously reported. In this study we asked a question: Are surface signatures observed in cells of epithelial (EPCAM, TROP2, CD9) or mesenchymal (CD29, CD49c, ITGB5, GD2) phenotype regulated at transcriptional level and if yes, can we identify specific transcriptional regulator? To address this, transcription factors (TF) interacting with the regulatory regions of genes encoding components of EMT surface signatures were identified, their expression profiles were analysed in breast tissue-related *in vitro* EMT models and final TF candidates (EBF1, FOXA1/ELF3, ZEB1) were chosen for further experiments to investigate impact of their deregulation on EMT-related surface signature ("6+ fingerprint"), migration capability and/or cellular morphology. Preliminary data indicate alterations of migratory features in cells with ectopically expressed TFs ZEB1 and ELF3, however, 6+ fingerprint reflects these changes only mildly. This is consistent with our observation that the expression of genes encoding epithelial markers is suppressed in cells with a mesenchymal phenotype, whereas gene expression of mesenchymal surface markers is not significantly different between epithelial and mesenchymal cells. Our data suggest the existence of a complex mechanism that involves both transcriptional and non-transcriptional regulation of surface marker expression during EMT with a limited ability of selected TF candidates to remodel the surface fingerprint.

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PROFILING OF GLYCOSPHINGOLIPIDS AND THEIR RELATED SURFACE EPITOPES IN EPITHELIAL AND MESENCHYMAL/STROMAL-LIKE CELLS OF BREAST NON-TUMOR AND TUMOR ORIGIN

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In this study, we asked a question if cellular profiles of glycosphingolipids (GSLs) alter with epithelial-to-mesenchymal transition (EMT) status and if so, can flow cytometric single-cell profiling of GSL-related epitopes present in non-tumor/tumor breast tissue serve as a tool for reproducible description of BCa-related heterogeneity and phenotypic plasticity driven, among others, by EMT?
Total levels of GSLs were analyzed in *in vitro* EMT model derived from breast tissue to reveal association of GSLs with the phenotypic status of cells (epithelial vs. mesenchymal). These associations were then investigated in clinical samples of breast non-tumor and tumor tissue using set of commercial antibodies recognizing GSL-related epitopes, lineage and EMT surface markers. Our data indicate that in comparison with stromal-like/mesenchymal cells, breast epithelial cells appeared more positive for surface SSEA1 staining and have higher levels of Gb3. In contrast, promising enhancement of Gb3 positivity was observed in stromal-like cells of tumor origin when compared with their paired non-tumor counterparts. Therefore, further analyses are essential to validate more precisely the unique associations we observed between the surface presence of specific GSL-related epitope (e.g., Gb3, SSEA1) and distinct cellular phenotype (e.g., non-tumor myoepithelial vs. tumor stromal-like cells).
In conclusion, single-cell profiling of surface GSL-related epitopes provides a more detailed description of heterogeneity present in subpopulations of epithelial and stromal-like cells residing in breast tissue microenvironment. However, except for Gb3 and SSEA1, it fails to recapitulate the EMT-specific *in vitro* pattern of GSL levels in clinical samples of breast tissue.

Acknowledgement
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DROPLET DIGITAL PCR ANALYSIS OF THE ANDROGEN RECEPTOR GENE AMPLIFICATION IN LIQUID BIOPSY SAMPLES FOR MONITORING OF PROSTATE CANCER PROGRESION

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Prostate cancer (PC) is the most common cancer as well as the second leading cause of cancer-related deaths in men in Western countries. Androgen deprivation therapy in combination with androgen receptor targeted therapy (ARTA) represents a current standard of therapy in patients with metastatic hormone-sensitive (mHSPC) or castration-resistant PC (mCRPC). However, despite therapeutical advancement, all patients eventually become resistant to therapy regardless of the low androgen environment. Our goal is to monitor changes that may indicate therapy failure, disease progression, and metastatic activity of PC. Therefore, we collected liquid biopsy samples of advanced PC patients before, during, and after ARTA therapy treatment. We designed and validated the assay for droplet digital PCR analysis of amplification of the androgen receptor (AR) gene using VCaP and LNCaP cell lines. Plasma samples of the selected patients with advanced PC (n=77) were then analysed and the amplification of the AR gene was found in 15 % of them. The patients with AR CNV were significantly older (p=0.0072) and had a shorter time between castration and CRPC occurrence (p=0.0122) compared with patients without amplification. The patients had also significantly increased levels of PSA, LDH, and ALP (p=0.0006, 0.0073, and 0.0130, respectively) at the start of ARTA, which indicated more aggressive PC. These results are currently being validated in a larger cohort (more than 460 samples from 150 patients). The analysis of AR gene amplification could be a useful tool for PC progression monitoring and could help clinicians with a difficult treatment decision-making process.

THE IMPACT OF INHIBITION OF GLYCOSPHINGOLIPID SYNTHESIS ON COLON CANCER CELL PROLIFERATION AND DEATH

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The remodeling of lipid metabolism and lipid-mediated signaling often contributes to cancer development. Glycosphingolipids (GSLs) represent an important group of bioactive lipid molecules shown to be specifically deregulated in colon cancer cells, which may affect the progression of the disease. We aimed to investigate the impact of inhibition of specific step in GSL synthesis, synthesis of lactosylceramides (via selective targeting of relevant GSL metabolism enzymes, in particular lactosylceramide synthases, using pharmacological inhibition with the D-PDMP inhibitor, siRNA gene silencing, and CRISPR/Cas9 gene knockout) on control of cell proliferation and death in DLD-1 human colon adenocarcinoma cells. The efficacy of chemical inhibition of selected enzymes or their downregulation was verified by several approaches including the detection of decrease in levels of related GSL products (LC-MS/MS), or changes of expression at the level of mRNA (RT-qPCR) and protein (western blotting). We found that down-regulation of lactosylceramide synthase B4GALT6 in DLD-1 cells did not substantially affect their proliferation and cell cycle progression (flow cytometry); however, it enhanced their sensitivity to the cytotoxic effects of oxaliplatin, a drug frequently used in the colorectal cancer treatment. We continue to evaluate functional roles of individual GSL enzymes in modulation of colon cancer cell chemosensitivity/chemoresistance and colon cancer cell phenotype/behavior.

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QUALITATIVE AND QUANTITATIVE EVALUATION OF THE TISSUE MICRO-ENVIRONMENT BY HIGH-RESOLUTION 17-PLEX IMMUNOFLUORESCENCE REVEALS DISTINCT CELL POPULATIONS

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Inflammatory tumor micro-environments contain cells of various types and sub-types. The composition and spatial location of the cell populations reflects the host reaction to the inflammatory stimulus and increasingly is understood to influence responsiveness to tumor immunotherapies. Multiplexed imaging technologies allow identification of cell types and states within the spatial context of tissue architecture. We present here a prototype workflow that combines rapid high-resolution, whole-slide highly multiplexed immunofluorescence imaging with advanced image analysis tools for 1) segmenting tissues, cells, and quantifying cellular phenotypes based on multiple markers and 2) determining regional densities and proximity between cells. We apply the workflow to comparative assessment of three lymphoid tissues: tonsil (follicular hyperplasia); lymph node (quiescence); lymphoma (architectural effacement). Regional masks that were defined by predominance of B-cells (CD20) or T-cells (CD3d) matched known lymphoid micro-anatomy of follicles and inter-follicular cortex respectively. Within the regions, populations and sub-populations of B-cells, T-cells, macrophages and vessels were measured, and their densities calculated and compared between tissues. Rare cell types of potential importance in immuno-oncology were investigated. The results demonstrate differences between the tissues at a phenotypic level that correspond to the morphologic differences seen by light microscopy. Orion imaging combined with HALO image analysis provides a powerful and intuitive workflow for visualization and quantification of distinct microenvironment populations for use in translational and clinical research.

FIBROBLAST ACTIVATION PROTEIN EXPRESSING MESENCHYMAL CELLS INFLUENCE T CELL ABUNDANCE AND FUNCTION IN GLIOBLASTOMA

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Background: Glioblastomas (GBMs) are aggressive brain tumors with strong immunosuppressive properties. In epithelial cancers, mesenchymal cells expressing fibroblast activation protein (FAP) have been described to affect T cell response. Recently we have detected the presence of FAP+ mesenchymal cells in GBMs. The aim of this study was to determine whether the presence of FAP+ mesenchymal cells affect T cell abundance and function in the GBM microenvironment.
Method: Immunohistochemistry (IHC) was used to detect T cells in paraffin-embedded tissue sections. Co-localization of FAP+ mesenchymal cells and T cells was evaluated by double labeling IHC in frozen sections. FAP+ mesenchymal cells were derived from human GBMs and characterized by immunocytochemistry. T cells were isolated from healthy donors´ buffy coats. T cell proliferation and expression of activation markers CD69 and CD25 were analyzed using flow cytometry in activated T cells exposed or not exposed to conditioned media from FAP+ mesenchymal cells. The production of TGF-β1 by FAP+ mesenchymal cells was quantified by ELISA.
Results: T cells were more abundant in GMBs with a higher proportion of FAP+ mesenchymal cells. T cells were frequently in the proximity of FAP+ mesenchymal cells. T cell proliferation and expression of activation marker CD69 were decreased after exposure to conditioned media from FAP+ mesenchymal cells. TGF-β1 was secreted by six out of ten FAP+ mesenchymal cell cultures.
Conclusions: FAP+ mesenchymal cells may impair T cell functions in GBMs by limiting their activation and proliferation.

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UNRAVELING THE TROP-2 INTERACTOME: INSIGHTS INTO THE ROLE OF TROP-2 AND DESMOGLEIN 2 IN BREAST CANCER METASTASIS

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The majority of cancer-related deaths result from metastasis, and epithelial-mesenchymal plasticity (EMP) is one of the crucial mechanisms during dissemination. Epithelial marker Trop-2 is considered to be linked with worse outcome of patients with breast and other carcinomas. Despite efforts to develop Trop-2 antibody-based cancer therapy, its role during metastatic cascade is still unclear. As Trop-2 is involved in organization of epithelium, we investigated the relationship between Trop-2 loss and mesenchymal phenotype of cancer cells. We concentrated on identifying novel interactions of Trop-2 that influence the epithelial phenotype of tumor cells by strengthening cell-cell adhesion. For this purpose, we performed Trop-2 interactome screen using proximity-dependent biotin identification assay (BioID).
We detected interaction of Trop-2 and desmogleins in breast non-tumor and tumor epithelial cells and confirmed this interaction in epithelial cell in general. Interaction is affecting DSG2 function as the loss of Trop-2 is accompanied by DSG2 protein level downregulation and change in its localization on the membrane of breast cancer cell model. Trop-2 KO cells show decreased DSG2 level and altered subcellular localization of DSG2. Trop-2 loss is associated with decreased intercellular adhesion manifested as reduced epithelial monolayer integrity and this phenotype is reflected after DSG2 loss in breast cancer cells.
In conclusion, we first describe the Trop-2 interactome using the BioID assay. One of the identified interactions – DSG2 – plays a significant role alongside Trop-2 during EMP as both proteins enhance the integrity of epithelial cell-cell connections. Remodeling of surface molecules is a crucial step during metastasis, and impact of EMP on both Trop-2 and DSG2 level fits in the formulated hypothesis.

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CELL THERAPY

FLOW CYTOMETRIC ANALYSIS OF NORMAL AND OSTEOARTHRITIC CHONDROCYTES

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Osteoarthritis (OA) is the most common degenerative joint disease characterized by increased degradation of cartilage tissue in the joint due to overexpression of proteolytic enzymes degrading extracellular matrix (ECM). OA is characterized by cartilage degradation, osteophyte formation, and stiffening of joints. Due to the inability of resident chondrocytes to regenerate the ECM with the same properties as it was formed during development, the probability of spontaneous regeneration is very low. Treatment is aimed at alleviating symptoms such as pain and swelling. The last option is a total replacement of the joint. Regenerative medicine using cells as therapeutics offers a large hope in treating OA. The main problem and challenge of advanced therapy medicinal product (ATMP) based on cultured human articular chondrocytes (HACs) is that chondrocytes transferred into monolayer culture, lose their typical properties – round morphology changes to fibroblast-like spindle shape, and cartilaginous protein synthesis decreases. This phenomenon is termed dedifferentiation. Our working hypothesis was that modification of growth media can modify chondrocytes' properties, ideally increasing the synthesis of ECM, mainly collagen type II (COL2). We needed to identify surface markers correlated with the differentiation status of monolayer expanded HACs and to define the degree of dedifferentiation. We selected 3 surface markers that should determine the state of the cell (CD44, CD54, CD90). Our results indicate that increased expression of CD54 is therefore associated with a response to inflammatory stimulation. In the absence of an inflammatory reaction, the cellular distribution of this marker is reduced, in healthy cartilage the expression of this marker was not detected at all. The high level of expression of the markers CD44 and CD90 is directly connected with the good chondrogenic capacity of the cells and the ability to produce a matrix with a high content of COL2.

ISOLATING INDUCED PLURIPOTENT STEM CELLS USING THE WOLF G2 GENTLE CELL SORTER

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The culture and sorting of induced pluripotent stem cells (iPSCs) require special care that can be both time consuming and laborious in order to achieve reliable results. Although maintaining a homogeneous stem cell culture is possible, researchers greatly benefit from other tools to succeed and reduce laborious maintenance. Microfluidic cell sorting is essential to the success of gently sorting homogeneous stem cells and eliminating unwanted cells. Here we demonstrate how the dual laser WOLF G2 Cell Sorter was imperative to identify and sort naive stem cell populations. The WOLF G2 accurately identified and enriched hiPSCs that were labeled with two cell surface markers SSEA-4 (Stage Specific Embryo Antigen 4) and TRA-1-60-R (Tumor-related Antigen-1-60 [R]), that are widely used to label undifferentiated stem cells. High purity and viability of the stem cells was also affirmed by successful seeding and adherence of the sorted iPSCs into a new 6- well plate. NanoCellect's microfluidic sorting technology enables researchers to generate consistent high-quality results.

POTENTIAL OF INVARIANT NKT CELLS IN ALLOGENEIC STEM CELL TRANSPLANTATION

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Allogeneic stem cell transplantation (HSCT) is an only curative treatment for severe hematological malignancies. Despite the progress in the management of post-transplant events, serious complications requiring new treatment options remain. Invariant NKT cells (iNKTs) represent a lymphocyte subpopulation with the ability to regulate immune reactions including anti-tumor response. Therefore, iNKTs might be promising treatment for post-transplant complications.
The study aimed to set and test manufacturing protocol for iNKTs.
We measured circulating iNKTs in healthy donors (HD; n=16) and patients 1 months after HSCT (n=24). Then, we cryopreserved mononuclear cells (MNC) from which iNKTs were isolated. The purified iNKTs were 2x activated with irradiated MNC, IL-15 and α-galactosylceramide. After 4-week culture, the cells were used for the evaluation of CD4+/CD4- cells, cytotoxic potential (again tumor cell lines) and immunomodulation (level of CD25 on activated T cells).
The number of circulating iNKTs was 16.8x lower in patients than in HD (median in HD for 1L of blood was $0{,}6x10^6$). Using these data, the minimal applied iNKT number to get the level of HD, was estimated at 10^6. The yield of iNKTs from apheresis product containing $7x10^9$ MNC, was between 0.5-$1.2x10^7$ (median $0.9x10^7$). The purity was always higher than 90%. Subsets distribution was very heterogeneous (CD4+ range 1-76, median 43.6%; CD4- range 12.2-99, median 21,5) except in one donor, all subsets were always present. Addition of iNKTs to stimulated T cells, led to decrease of CD25 expression about 50% (range 42-63%). The killing ability was very low (proportion of dead cells was up to 15%) except CD1d positive cell line with α-galactosylceramide where 90% of cells were dead after 12hrs.
Our protocol gives us sufficient number of iNKTs with the strong immunomodulatory function. More optimization is needed to increase the cytotoxic potential.

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DENDRITIC CELLS MATURED WITH RELEASED COMPONENTS OF ACTIVATED LAD2 HUMAN MAST CELLS HAVE IMPAIRED INFLAMMATORY PHENOTYPE BUT ENHANCED CAPACITY TO INDUCE PROLIFERATION OF AUTOLOGOUS T CELLS

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Background: *Ex vivo*-produced DCs are attempted to be used for active cellular immunotherapy of multiple diseases, including cancer and autoimmunity. One of the critical steps during their production is their maturation which shapes their functional phenotype, including the DC capability to induce T cell proliferation and production of inflammatory cytokines. We previously found that coculture of immature DCs with activated LAD2 human mast cells (MCs) induces DC maturation. However, the mechanism of the maturation and the DC functional phenotype was not known.
Methods: LAD2 human MCs were stimulated with an inhibitor of sarco/endoplasmic reticulum Ca2+-ATPases, thapsigargin, for 30 min. The cells were extensively rinsed and then cultured overnight. The conditioned supernatant was collected and used to overnight mature *ex vivo*-produced monocyte-derived immature DCs. The matured DCs were then used to stimulate the proliferation of autologous lymphocytes. DC maturation was determined by the surface expression of CD80, CD83, CD86, and HLA-DR, and the inflammatory phenotype by the intracellular production of TNFα. Ki-67 expression in DC-stimulated T cells was used to determine their proliferation.
Results: We found that LAD2 human MC-mediated DC maturation is caused by the released components of the activated MCs and does not require MC-DC coculture. Despite the maturation, the inflammatory phenotype was yet still comparable with the immature DCs. However, MC-matured DCs induced a similar proliferation of allogeneic T cells as the one induced by R-848(TLR7/8 agonist)-matured DCs.
Conclusions: MC release products could be used for the production of mature DC with an inflammatory pattern of immature DCs.

11th HLDA WORKSHOP: PRELIMINARY EVALUATION OF ANTIBODY CLONES FOR NEW CD MARKER NOMENCLATURE

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Human Leukocyte Differentiation Antigens (HLDA) workshops have been run since the 1980´s. So far, almost 400 cluster of differentiation (CD) markers were defined; some of which include multiple isoforms. Currently, the 11th workshop is underway with the goal to validate new clones for previously assigned CD markers or for defining new CD markers.
The experimental work is shared across three laboratories in Prague, Barcelona and Melbourne with the aim to evaluate antibody binding capacities of all submitted clones to blood leukocyte subsets of 12 unique donors. We employed 12-color flow cytometry and optimized two panels covering innate (n=12) and adaptive (n=15) cell subsets present in peripheral blood. The PE channel was dedicated to the new clones; the antibody binding capacity was recalculated using BD Biosciences PE Quantitation kit. All the new clones were pre-titrated using a mixture of barcoded cell lines and peripheral blood leukocytes.
In total, 111 new antibody clones were submitted by industry and academia. 21 clones were excluded due to the lack of a second clone. So far, we have evaluated 90 new antibody clones targeting 41 markers on 4 donors. For 3/41 markers all clones were completely undetectable on all evaluated cell subsets. 2 markers were excluded from the analysis due to technical issues. For 36 markers we have performed a direct comparison between (at least) two clones. Out of those, for 10 markers the antibody clones showed same pattern of staining. For the remaining 26 markers the pattern of surface expression differs between the clones. The differences between clones were caused either by high background or low affinity of one clone. For the clones showing reproducible staining pattern but lacking a reliable counterpart a validation with induced expression of its target gene will be needed.

IPSCS DERIVED NK CELLS AS CANDIDATE OFF-THE-SHELF IMMUNOTHERAPY

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Human induced pluripotent stem cells (hiPSCs) are an important tool in the search for various cellular therapies. Here we explore one aspect of their unique capacity for self-renewal and potential to differentiate into any cell type. Natural killer (NK) cells possess efficient anti-leukemic properties and have been used in many experimental protocols to treat hemato-oncologic disorders. iPSCs show a particular promise as a source for an off-the-shelf immunotherapeutic product because they represent a consistent source of cells capable of differentiating into NK cells.
In this project we have demonstrated the capacity of iPSCs to differentiate into hematopoietic progenitors and subsequently into NK cells whose cytotoxicity can target acute myeloid leukemia.
We chose to optimize an existing differentiation protocol for iPSC-derived NK cells and verified that the correct transfection and formation of iPSCs was carried out via the immunocytochemical measurement of pluripotent markers using a confocal microscope. We furthermore constructed a cytometric panel that allowed us to continuously monitor the expression of surface markers at different stages of ongoing differentiation. We were thus able to track the changing phenotype of NK cells throughout their differentiation. Another aim of this project will be to assess the different roles of the cytokines involved in NK cell expansion and differentiation; namely, IL-2, IL-12, IL-15, IL-18 and IL-21. Some of them with newly define roles in NK cell differentiation.
Most importantly, our goal is to determine whether iPSC-derived cells are capable of ADCC/ other cytotoxic activity and to assess their efficacy as an AML treatment in comparison to NK cells derived from other sources. Finally, the project aims to define the main parameters of the therapeutic capacity of iPSC-derived NK cells, as well as to delineate the main obstacles we face in our further development of iPSC-derived NK cells as a finished product of adoptive transfer therapy for AML.

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

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Collecting and using a patients' own immune cells is a rapidly emerging immunotherapy approach. Genetically reprograming T cells to express a chimeric antigen receptor (CAR) has already paved the way for successful immunotherapies to fight against leukemia and lymphoma, and research into solid-tumor CAR-T cells is also underway. Alot is still unknown in terms of exactly how these re-engineered cells will behave once reinfused into a patient, including efficacy and potential side-effects. We review single-cell polyfunctional profiling results from several different sets of pre-infusion CAR-T samples, including anti-CD19; CAR-T samples from both Kite Pharma (Gilead) [1] and Novartis Pharmaceuticals [2], GOCAR-T cell products from Bellicum Pharmaceuticals [3], and Bispecific CD19/22 CAR-T cells from the NIH [4]. In each case, CD4+ and CD8+ CAR-T cells were stimulated (details in [1]. [2], and [3]) and subsequently analyzed at a single-cell level using IsoPlexis' IsoCode chip. A 17-plex cytokine panel was used in study [2], while studies [1] and [3] used a 32-plex cytokine panel. Stimulated CAR-T cells were loaded onto Isoflexis IsoCode chips containing ~12000 microchambers pre-patterned with a complete, 17-plex, antibody array. Cells on the IsoCode chips were imaged to identify single-cell locations and incubated for 16 hours at 37°C. 5% CO2: single-cell cytokine signals were then captured and digitized with a microarray scanner. The polyfunctional expression of single CAR-T cells was evaluated using IsoPlexis' software across the panel of profiled cytokines comprising effector, stimulatory, regulatory, inflammatory, and chemo attractive functional groups.

ANALYSIS OF VIRAL PARTICLES ON AMNIS® FLOW CYTOMETERS

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Until recently, the analysis of viruses and viral particles by flow cytometry was limited due to the range of detection (300-500 nm)1 and the low signal-to-noise ratio of traditional flow cytometers in the lower size range. However, the development of camera-based systems in flow cytometry now allows for the analysis of viruses and viral particles (VPs). Due to the high sensitivity of the time-delay integration (TDI) and CCD-camera technology that is unique to Amnis® systems, the Amnis® CellStream® and ImageStream® flow cytometers can be used to study viruses and VPs. These flow cytometers also offer a low
signal-to-noise ratio, which further increases their ability to resolve particles 100 nm or smaller. The recent coronavirus pandemic has demonstrated that flow cytometry can be used as a high-throughput tool for studying viruses and their effects on cells. The research presented here demonstrates the ability of two Amnis systems to analyze VPs. The VPs (MV-M-sfGFP) used in these studies were developed by ViroFlow Technologies, Inc.2 MV-M-sfGFP are inactivated murine retroviruses produced in mouse cells that express superfolder green fluorescent protein (sfGFP) on the outer surface of the viral envelope and have a size of ~120 nm. First, we analyzed the viral particles on the CellStream Flow Cytometer, which is a compact, camera-based benchtop system that uses TDI-CCD technology to deliver highly sensitive small particle resolution with low background noise. Then, we analyzed the viral particles on the ImageStream®X Mk II Imaging Flow Cytometer, which obtains image data in addition to flow cytometric data. The new High Gain mode for ImageStream was used for these experiments in conjunction with a high-powered 400 mW 488 nm laser for increased detection of viral particle signal. In conclusion, both the Amnis CellStream Flow Cytometer and the Amnis ImageStreamX Mk II Imaging Flow Cytometer are excellent choices for flow virometry.

IDENTIFICATION OF CD318, TSPAN8 AND CD66C AS TARGET CANDIDATES FOR CAR T CELL BASED IMMUNOTHERAPY OF PANCREATIC ADENOCARCINOMA

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Spatial biology bridges the gap between cell phenotyping and tissue imaging by high-plex approaches to unlock exciting possibilities for advances in immuno-oncology and beyond. This includes amongst others: deep phenotyping; biomedical research and discovery; and drug target and validation. In this webinar, we will show you how we identified and evaluated novel target candidates for the cellular immunotherapy of pancreatic ductal adenocarcinoma. Our spatial biology solution, the MACSima™ Platform, helped to dissect the expression of more than 100 markers on tumor cells at single-cell resolution. It was also used to investigate CAR T cell infiltration into tumors. We will also present the MACSima Platform in detail. This spatial biology platform provides the perfectly orchestrated combination of a fully automated instrument, specifically designed sample carriers, a broad portfolio of pre-tested antibodies, and a tailor-made and powerful image analysis software. This reduces the time needed to obtain ready-to-publish data to a minimum.

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COMBINATION THERAPY USING INVARIANT NATURAL KILLER T CELLS AND IMMUNOMODULATORY DRUGS IN MULTIPLE MYELOMA

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Invariant natural killer T cells (iNKTs) are lymphocytes connecting innate and adaptive immunity. iNKTs interact with B cells in all maturation stages through the invariant TCR and CD1d presenting lipid antigens. In progressive multiple myeloma (MM), a cancer of plasma cells, the expression of CD1d is downregulated and iNKT function is affected. However, CD1d can be upregulated by all-trans retinoic acid (ATRA). Lenalidomide (LND) and pomalidomide (PMD) are therapeutic agents enhancing the activity of natural killer (NK) cells in MM patients. Therefore, they might also promote iNKT function.
This work aimed to monitor the effect of LND and PMD on iNKTs and CD1d expression after ATRA application.
iNKTs isolated from 7 healthy donors (magnetic selection), were 3x activated using irradiated C1R-CD1d cells, α-galactosylceramide and IL-15. During the last activation, LND and PMD were added to iNKTs in concentrations 0.25–5 μM. After 96hrs, the cell number, metabolic activity (measured by AlamarBlue) and the main subsets (measured by flow cytometry using CD45, CD8, CD4) were evaluated. Myeloma cell lines were cultured 48hrs with 0.25 – 10μM ATRA and CD1d expression was measured using FACS.
No positive effect on the metabolic activity was observed after LND/PMD application. In addition, the number of iNKTs was 1.2–1.6 higher in the control group suggesting the decrease in proliferation rate in treated cells. The subpopulations of iNKTs remained unchanged. The expression of CD1d after ATRA application increased in myeloma cell lines. The highest increase without any loss in viability, was seen at 1 μM, higher concentrations caused a significant decrease in viability.
LND/PMD have no significant positive impact on iNKTs. The iNKTs function was improved by ATRA-related increase of CD1d on myeloma cells. These data need to be further investigated in co-culture system.

Acknowledgement
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ANALYSIS OF UHKT CAR19 COMPARED TO TISAGENLECLEUCEL CELLULAR PRODUCT

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Anti-CD19 CAR T cells are becoming a well-established option for treatment of B-cell malignancies. For example, tisagenlecleucel (Kymriah, Novartis) has been on the market for 6 years. However, it is still a very expensive therapy, mainly due to the manufacturing costs. To make it more available, in-house CAR T cell therapies are being developed. It has been reported that early memory phenotype of T cells is linked to better outcome of the treatment. Therefore, there is need for manufacturing conditions producing cells of those phenotypes.
Here, we present analysis of UHKT CAR19 product (N=7), which has been approved for phase 1 clinical trial. Manufacturing costs have been cut down by omitting retroviral vectors and using transposon system instead. In addition, cultivating CAR Ts in the presence of IL-21 maintains early memory phenotype of CAR T cells which should enhance antitumor response.
Moreover, we compare the clinical trial data with products of patients treated with tisagenlecleucel (N=15). Detailed immunophenotype analysis by flow cytometry revealed increased percentage of early memory phenotype and decreased amount of inhibitory receptor PD-1 in UHKT CAR19 cells.

CLINICAL CYTOMETRY

THE ROLE OF FLOW CYTOMETRY IN DIAGNOSTICS OF MYCOSIS FUNGOIDES AND SÉZARY SYNDROME

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Mycosis fungoides (MF) is the most common primary cutaneous T-cell lymphoma (CTCL), making up about 60% of all CTCLs. It typically manifests an indolent course but may progress to a leukemic form Sézary syndrome. The diagnosis is difficult and requires a comprehensive approach including clinical, pathological, and haematological examination. The prognosis of patients closely depends on the clinical stage. The staging system, which also includes flow cytometry (FCM), is used to assess this. FCM can detect an increased proportion of CD4+7- or CD4+26- T cells in peripheral blood (PB) accompanied by possible clonality.

Since 2016, we have performed more than 1,000 examinations of patients with a diagnosis or suspicion of CTCL at our institution. Since 2022, we have been using a 7-color tube including anti-TRBC1 to determine clonality. The testing also includes a B rating, which assesses the absolute number of MF/SS cells per µL of PB. Finding an immunoregulatory index >10 and a B rating of B2 correlates with a diagnosis of SS and is usually accompanied by clonality of the pathological population. However, these are rare cases. The majority of findings are normal, with pathological T lymphocytes predominantly inhabiting the skin and not found in increased numbers in the PB. Nevertheless, it is the monitoring of MF/SS cells in the periphery of CTCL patients at diagnosis or during treatment that provides a useful diagnostic role for FCM.

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FULL SPECTRUM 3-LASER CLINICAL CYTOMETRY: 24-COLOR HUMAN IMMUNOPHENOTYPING PANEL

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The detection of some fluorochrome combinations by conventional flow cytometry presents a challenge due to high amounts of peak emission spectral overlap (Figure 1). The Cytek Northern Lights™ systems (CE-IVD spectral flow cytometer) 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. Whole blood from a healthy donor was stained, lysed, washed, and analyzed on a 3-laser Northern Lights system. Subsets of NK and NK T cells that co-express CD56 Alexa Fluor 647 and CD8 APC were easily identified. For comparison, blood from the same donor was stained with CD56 PE and CD8 APC and yielded similar percentages of NK and NK T cells, demonstrating that the use of these two highly overlapping dyes did not impact results.

IDENTIFICATION OF POLARIZED HUMAN ADIPOSE TISSUE MACROPHAGE POPULATIONS IN RELATIONSHIP TO PLASMA CHOLESTEROL LEVELS

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Adipose tissue macrophages are important in inflammation development within the adipose tissue and contribute to inflammation within the whole body. That low-grade chronic inflammation plays an important role in various diseases such as atherosclerosis development. In the project, we focused on adipose tissue macrophages in relationship to blood cholesterol and lipoprotein fractions in relationship to atherosclerosis development.
Macrophages were measured in stromal vascular fractions isolated from visceral and perivascular human adipose tissue obtained from living kidney donors. Samples were analysed by flow cytometry using a set of antibodies (CD45, CD14, CD16, CD36, CD163, and CD206). Although tissue macrophages do not represent easily defined populations based on a specific set of markers, we defined pro-inflammatory and anti-inflammatory macrophage subpopulations. Subpopulation CD14+CD16+CD36high we defined as pro-inflammatory and Subpopulation CD14+CD16-CD163+ we defined as an anti-inflammatory subpopulation.
At such defined macrophage phenotypes we found a strong positive correlation between the proportion of pro-inflammatory macrophages with plasma cholesterol level, LDL concentration, remnant cholesterol, and triglycerides concentration and on the contrary negative correlation with the proportion of HDL of total cholesterol. On the contrary, the proportion of anti-inflammatory macrophages showed reverse trends. Similarly, those results were found in both visceral and perivascular adipose tissue.
We established adipose tissue macrophage phenotypes based on the expression of surface markers measured by flow cytometry. The present study proves a strong relationship between the proportion of pro-inflammatory and anti-inflammatory macrophages and lipoprotein particles. Finally, the higher plasma cholesterol, LDL, remnant cholesterol, and triglycerides levels also contribute to cardiovascular disease development also through increased chronic inflammation due to a higher proportion of pro-inflammatory adipose tissue macrophage polarisation.

COMPARISON OF LINEAR AND NON-LINEAR UNMIXING METHODS IN SPECTRAL FLOW CYTOMETRY

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Spectral flow cytometry is a technique that enables quantitative high-dimensional analysis of multiple biological targets within cells. However, spectral unmixing, the process of separating the mixed spectra into individual fluorophores, can be challenging and prone to errors. This work compares both established and novel unmixing methods in spectral flow cytometry, with a focus on unmixing methods that can account for non-linear interactions between endmember spectra. We explore both regression based and machine learning based methods evaluating their performance on simulated and experimental data. We discuss the potential of non-linear methods to improve the accuracy and robustness of spectral unmixing, especially for complex panels with highly overlapping fluorophores. We also discuss the advantages and limitations of the compared methods and make appropriate recommendations providing practical guidelines for data preparation and analysis.

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A PREDICTIVE MODEL FOR PROGRESSION TO CLINICAL ARTHRITIS IN AT-RISK INDIVIDUALS WITH ARTHRALGIA BASED ON LYMPHOCYTE SUBSETS AND ACPA AUTOANTIBODIES

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Background: The presence of antibodies against citrullinated proteins (ACPA) in serum significantly increases the risk of developing rheumatoid arthritis (RA). Dysregulation of lymphocyte subpopulations was previously described in early and established RA.
Objective: To propose the predictive model for progression to arthritis based on peripheral lymphocyte subsets and ACPA in at-risk individuals with arthralgia.
Methods: Individuals from the At Risk of RA (ARRA) prospective observational cohort were defined as having arthralgia without arthritis and being either ACPA+ and/or meeting the EULAR definition of clinically suspect arthralgia (CSA). The percentage of CD19+, CD3+, CD3+CD4+, CD3+CD8+, and CD3-CD16/56+ (B, T, Th, Tc, and NK) cells and non-conventional CD3+CD16/56+ and CD3bright (NK-T and γδ-T) cells were analyzed in peripheral blood samples by flow cytometry.
Results: Out of 191 at-risk individuals, 36 developed clinical arthritis (progressors) within a median of 9 months of follow-up. For the construction of predictive models, 81 individuals who have not yet progressed to arthritis (non-progressors) with symptom duration longer than 12 months were included. The resulting predictive model for arthritis development performed in all ARRA individuals based on the percentage of B cells, NK cells, and anti-CCP IgG U/ml showed AUC=0.732 ($p<0.001$). According to this model, a high percentage of B cells, high anti-CCP IgG serum levels, and a low percentage of NK cells increase the probability of developing clinical arthritis. AUC was even higher when the model with the same set of predictors was applied to ACPA+ individuals.
Conclusion: We propose a predictive model to distinguish individuals with arthralgia who progress to clinical arthritis from non-progressors, especially applicable in ACPA+ individuals. The final model includes B cells and NK cells, which are involved in the pathogenesis of RA.

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DATA ANALYSIS

AUTOFLUORESCENCE EXTRACTION WITH CYTEK AURORA AND NORTHERN LIGHTS

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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.

CHOLESTEROL'S SURPRISING ROLE IN THE REGULATION OF ADENYLYL CYCLASE 7: REVEALING THE BINDING THROUGH CARC AND CRAC MOTIFS IN THE CYTOSOLIC DOMAINS

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Adenylyl cyclases (AC) are enzymes synthesizing cAMP, which plays a crucial role in intracellular signaling and metabolism. Since the cAMP signaling pathway is involved in various physiological functions, the number of evidence between the change of AC activity and specific pathologies is increasing. Since the AC allosteric activator forskolin (FSK) is absent *in vivo*, the question of the structurally similar native regulatory molecule has arisen. The regulation of various transmembrane proteins by cholesterol was already described, so we speculate that cholesterol molecule can be an undescribed native regulator of AC7 activity. Using the multiple sequence alignment and literature, we observed that the human AC7 isoform possesses many cholesterol-binding motifs (CARC and CRAC). In this study, we identified the most promising CARC and CRAC motifs in the AC7 primary sequence and studied them using a robust bioinformatic analysis that utilized multiple sequence alignment (MSA), binding site prediction, *AlphaFold2* structure modeling, and docking. The experimental approach uses targeted mutations in the CARC and CRAC motifs near the allosteric and Gαs binding site to reveal how cholesterol may modulate AC7 enzymatic activity. Our findings provide a new avenue for understanding the connection between AC7 activity and cholesterol-related pathologies, such as depression. Further, given the low conservancy of transmembrane parts across tmAC proteins (AC1-AC9), identifying CARC and CRAC motifs in the cytosolic parts is especially promising for understanding the conserved regulatory mechanisms.

RAMAN BASE – A NEW MULTIPURPOSE ONLINE PLATFORM FOR RAMAN SPECTROSCOPIC ANALYSIS

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Raman spectroscopy provides analysis of chemical structure of a wide range of samples in a label-free, non-destructive, in-situ, and real-time manner. Moreover, Raman microspectroscopy provides spatially resolved chemical information on a microscopic scale, e.g., in individual cells or cellular components, for mapping biochemical changes that occur during various cellular processes. All this makes Raman spectroscopy an extremely powerful research tool. However, the effective use of any modern analytical technique is strongly conditioned by the availability of reference data and the tools to share, search, compare and interpret them. In certain cases, the knowledge-dissemination format of scientific article is not effective enough anymore. This is especially true, when the techniques for generation of large data-sets come to play. In such cases, it takes a paradigm-shift for the progress to accelerate, as we witnessed during the molecular revolution. Raman spectroscopy urgently needs such a paradigm-shift from its outdated culture of data-handling. Pictures of spectra and tables of peaks within an article are way too ineffective. We are developing Raman Base, a specialized online platform for the global Raman-spectroscopic community, which allows fast, simple, direct means to share, search, compare and interpret Raman-spectroscopic data. Raman Base provides a free personalized space to store the data, access them worldwide and to optionally share them with the Raman-community. It involves full-text spectra-searching engine as well as multiple algorithms to search spectra based on their similarity. Additionally, it provides AI-based capacity to predict the identity of certain spectra, with a training set of more than 100 000 spectra. We plan to gradually extend and improve the capabilities of Raman Base with software for filtering noise and background, ability to analyze complex mixtures, to determine and predict the character of individual molecular vibrations of unknown molecules, by including links to chemical-structure databases, proteomic databases, and many more functionalities. We hope Raman Base will become the paradigm-shifting global platform for Raman spectroscopy.

DISSECTING THE GENOMES OF *AEGILOPS BIUNCIALIS* AND *AE. GENICULATA* BY CHROMOSOME FLOW SORTING

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Breeding of wheat adapted to new climatic conditions and resistant to diseases and pests is hindered by a limited gene pool due to domestication and thousands of years of human selection. Annual goatgrasses (*Aegilops* spp.) with M and U genomes are potential sources of new gene variants. Development of wheat-*Aegilops* introgression lines can be facilitated by the knowledge of DNA sequences of U and M chromosomes. *Aegilops* chromosomes purified by flow cytometric sorting provide an attractive opportunity to sequence the big and complex *Aegilops* genomes. The present study extends the potential of chromosome genomics to allotetraploid *Ae. biuncialis* and *Ae. geniculata* by dissecting their M and U genomes into individual chromosomes. Hybridization of FITC-conjugated GAA oligonucleotide probe to chromosomes suspensions of the two species allowed the application of bivariate flow karyotyping and sorting some chromosomes. Bivariate flow karyotype FITC vs. DAPI of *Ae. biuncialis* consisted of nine chromosome-populations, but their chromosome content determined by microscopic analysis of flow sorted chromosomes indicated that only $7M^b$ and $1U^b$ could be sorted at high purity. In case of *Ae. geniculata*, fourteen chromosome-populations were discriminated allowing the separation of nine chromosomes ($1M^g$, $3M^g$, $5M^g$, $6M^g$, $7M^g$, $1U^g$, $3U^g$, $6U^g$ and $7U^g$) out of the 14. To sort the remaining chromosomes, a partial set of wheat-*Ae. biuncialis* and a whole set of wheat-*Ae. geniculata* chromosome addition lines were also flow karyotyped revealing clear separation of the GAA-rich *Aegilops* chromosomes from GAA-poor A- and D-genome chromosomes of wheat. All of the alien chromosomes represented by individual addition lines could be isolated at purities ranging from 74.5 to 96.6% and from 87.8 to 97.7%, respectively. Chromosome-specific genomic resources will facilitate gene cloning and development of molecular tools to support alien introgression breeding of wheat.

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PERTURBANCE OF B CELL SUBSETS IN MBL AND CLL PATIENTS

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Monoclonal B lymphocytosis (MBL) is defined as presence of clonal B-cell populations in the peripheral blood with fewer than 5–10^9/l cells and no other signs of lymphoproliferative disorder. It can progress to chronic lymphocytic leukemia (CLL), which is the most common leukemia in Western countries with high counts of clonal CD5+ malignant B cells in the peripheral blood. Infectious complications have been known to be major cause of morbidity and mortality among CLL patients. Susceptibility to infectious diseases can be caused by therapy or by malfunction of immune system induced by depletion of specific types of immune cells.
We aim to compare presence and percentage of main B cell subsets in healthy donors vs. MBL and CLL patients, including MBL cases in early disease stage, defined by presence of a minor clone/multiple clones of CD5+ B cells. These rare samples have been so far understudied due to the indolent nature of MBL, which is commonly diagnosed at later stages when the clonal populations already form majority of the B cell populations. For this reason, the early changes of B cell subsets connected to MBL/CLL development are poorly documented.
PBMCs from peripheral blood of healthy donors, MBL and CLL patients were collected. In those samples, we detected 15 surface lymphocytic markers by spectral flow cytometer. B cell markers were selected to facilitate identification of healthy B cell subsets and malignant cells. Data were transformed and subjected to clustering algorithm (FlowSOM) and dimensional reduction (tSNE, UMAP). Resulting clusters were mapped on tSNE, annotated, and verified by manual gating in FlowJo. Comparison of B cell subsets in healthy and MBL/CLL samples revealed major differences, mainly in count of naïve cells. Correlation of B cells subsets perturbances and disease progression in MBL/CLL samples was analyzed.

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HEMATOLOGY

SEMI AND FULLY AUTOMATED IMMUNOSTAINING SAMPLE PREPARATION PLATFORMS IMPROVE LIVE LEUKOCYTE RECOVERY, REPRODUCIBILITY, AND FLOW CYTOMETRY DATA QUALITY

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Limited innovation in automated cell and organelle sample preparation methodology limits the effectiveness of modem analytical methods, such as single-cell 'omics, flow and mass cytometry. These techniques traditionally rely on manual centrifugation-based protocols for cell washing and suspension preparation, hampering researchers' access to the reproducibility and scalability benefits of automation. We have developed a suite of cell suspension preparation systems that enable semi and full automation of cell washing protocols. These Laminar Wash™ technologies robustly, gently, and efficiently remove debris, dead cells, and unbound reagent using laminar flow and liquid handling robotics, rather than turbulent and harsh pelleting-plus-pipetting methods. Murine and humanized mouse peripheral blood mononuclear cells (PBMCs) and tumor infiltrating lymphocytes (TILs) were prepared and immunostained for flow cytometry analysis. Workflow improvements were assessed, as well as data quality by flow cytometry gating strategies isolating live cells and various lymphocyte subpopulations. Adaptation of standard protocols to Laminar Wash automation typically improves repetitive immunostaining processes and workflows, in terms of reduced hands-on time and inter- and intra-operator variability. We demonstrate the superior live cell retention and reproducibility of Laminar Wash over centrifugation in processing murine and humanized mouse PBMCs and TILs for flow cytometry. Furthermore, we show how Laminar Wash improves flow cytometry data quality, in terms of debris removal and separation of immune cell subsets for both PBMCs and TILS. Overall, these results show how Laminar Wash methodology assists in standardizing sample preparation for cytometric analysis, an important and unmet need in cancer immunotherapy discovery and manufacturing workflows.

FLOW CYTOMETRY ASSESMENT OF REGULATORY LYMPHOCYTES IN MYELOPROLIFERATIVE DISORDERS

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Background: Myeloproliferative neoplasms (MPNs) represent a group of hematological malignancies where the inflammation probably plays a crucial role in promoting MPN initiation and affecting disease evolution. The role of regulatory T cell (Treg) and especially B cell (Breg) in MPNs is not fully elucidated yet.
Aims: 1) Development of highly sensitive 10-color flow cytometric protocol for detection of immomodulatory lymphocytes. 2) Tregs and Bregs assessment and enumeration in MPNs and their comparison with healthy controls (HC).
Method: Peripheral blood of MPN patients (n = 180) and healthy blood donors (n = 30) was incubated with appropriate MoAbs. Analysis was done by 4-lasers Omnicyt (Cytognos) and data reanalysis by Infinicyt (Cytognos). Relative (and absolute, not shown) numbers of Tregs and subpopulations of Bregs were evaluated together with other lymphocyte subpopulations (not shown).
Results: MPD patients were divided into diagnostic groups: ET (n = 79), PV (n = 70) and PMF (n = 31) and compared together with HC. Relative numbers of $CD4^+$ Tregs were 4.7 vs 4.2 vs 4.7 vs 4.5 % in ET vs PV vs PMF vs HC, respectively. Similarly, Breg subpopulations were: transitional 3.6 vs 2.4 vs 1.9 vs 3.6 %; naïve 61.1 vs 63.0 vs 51.6 vs 64.4 %; activated 8.8 vs 7.8 vs 10.2 vs 7.2 %; memory 19.0 vs 18.5 vs 26.6 vs 22.9 % and plasmablasts 0.4 vs 0.4 vs 0.4 vs 0.4 %) in ET vs PV vs PMF vs HC, respectively.
Conclusion: There were found small differences in relative and/or absolute counts of Tregs and Bregs when compared MPDs vs. HC. Further analysis of patients regarding to clinical parameters and obtained treatment will be done together with statistics.

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IMPACT OF CYTOKINES AND SOLUBLE PLASMA PROTEINS ON THE IMMUNE PROFILE OF ACUTE MYELOID LEUKEMIA PATIENTS

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Acute myeloid leukemia (AML) is a malignant hematological disorder characterized by aberrant expansion of myeloid progenitor cells called myeloblasts in the bone marrow and peripheral blood. It is well established that the immune system protects the human body from malignant disease by eliminating transformed cells. However, the ability of the immune system to control AML is influenced by the direct and indirect crosstalk between myeloblasts and immune cells. Soluble molecules present in the leukemic environment, i.e., cytokines, chemokines, soluble receptors, etc., create intricate networks that shape the immune profile of the patients.
For example, pro-inflammatory and anti-inflammatory cytokines can shape immune reactions as well as influence the growth and chemotherapy resistance of myeloblasts. Soluble isoforms of various receptor molecules are produced in the process of alternative splicing or are shed from the cell surface. These soluble isoforms subsequently bind to their counterparts and can mimic cell-to-cell interactions.
In the presented work, we are combining deep functional phenotyping using a 36 fluorescent parameter spectral flow cytometry panel and the measurement of soluble plasma protein levels using the cytometry-based Legendplex assay to expand the knowledge of how cytokines and other soluble proteins affect the immune system's function in AML patients.

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ROLE OF FLOW CYTOMETRY IN DIAGNOSTICS OF AHUS

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Background: Atypical hemolytic uremic syndrome (aHUS) is an ultra-rare disease characterized by microangiopathic hemolysis, thrombocytopenia, and renal impairment and is associated with dysregulation of the alternative complement pathway on the microvascular endothelium. Although membrane cofactor protein (MCF, CD46) deficiency may not be enough to induce aHUS, mutation in CD46 are present in 5–9 % of primary aHUS. Thus, flow cytometry may serve as useful and quick method to reveal such mutations and aHUS confirmation.
Aim: Flow cytometry analysis of CD46 expression on blood cells of healthy controls (HC) to cover normal range of expression through different ages and their comparison with patients with suspected aHUS.
Method: Peripheral blood of healthy controls (n = 48) and patients (n = 7) was incubated with CD16/CD45/CD46. Two clones of CD46 were used (E4.3 from BD Biosciences and MEM258 from Exbio) in different tubes. Analysis was done by 3-laser BD FACSCanto II (BD Biosciences) and 4-laser Omnicyt (Cytognos) where MFI of CD46 expression on neutrophils, monocytes and lymphocytes was detected.
Results: HC were divided into 9 groups according to age and MFI of CD46 (mean ± 2SD) was analysed separately for every group. Some groups are still not sufficiently covered with HC thus their acquisition will continue. CD46 MFI of patient should be lower and exceed 2SD of appropriate HC group mean to confirm aHUS. The ratio of CD46 MFI of patient/CD46 MFI of HC group median was analysed as well, cut-off for aHUS positivity will be tested and should be <0.8. There was a case of previously confirmed aHUS used as a positive control in this study which fulfilled both criteria (mean+ratio).
Conclusion: Although further analyses are needed, flow cytometry seems as an optimal screening method for CD46 mutation.

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INTRACELLULAR CD3 AS A T LINEAGE MARKER IN LEUKEMIA: ROLE OF INTENSITY

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Arbitrary criteria are needed in diagnostics in order to distinguish various diseases and their variants. Most challenging are borderline cases, who frequently possess features that may resemble canonical findings but their biological meaning may be different in the context of borderline characteristics.
We participate in several international task forces, which aim at identifying the optimal type of treatment for children with borderline types of leukemia. Within this, we appraise the arbitrary markers used in subtype definition. One of the crucial cytometric markers is intracellular CD3 (iCD3), a strong indicator of T lineage differentiation. In rare cases, its expression intensity is lower, possibly leading to misinterpretation and diagnostic ambiguities.
From 1997 to 2022, we diagnosed acute leukemia or its relapse in over 1000 patients. We are currently re-analyzing FCS files of approximately 900 of patients aiming at iCD3 expression. We set up reference values in each sample – iCD3 negative cells according to CD45++19+ cells (non-malignant B lymphocytes) and iCD3++ (non-malignant surface CD3+ T lymphocytes). The intensity between iCD3neg and iCD3++ is described as "iCD3 dim". At the time of this abstract submission, we have completed the reanalysis of 396 Czech children with ALL, AML or ALAL.
Within the cohort containing all subtypes of acute leukemia, we found iCD3dim above 30% in 8 patients. Surprisingly, canonical T ALL cases so far analyzed never expressed dim level of iCD3 (0 cases of 31 primary T-ALL and 4 T-ALL relapses).
The on-going reanalysis of diagnostic and relapsed specimens show that dim intracellular positivity of CD3 is an almost exclusive feature of ambiguous lineage leukemias. In the next step, we plan to assess the biological meaning of iCD3dim cells in normal T lineage development and to evaluate response of iCD3dim leukemias to various types of treatment.

Acknowledgements
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CYTOMORPHOLOGY AND QUANTITATIVE IMMUNOPHENOTYPING USING ARTIFICIAL INTELLIGENCE IN MATURE CD5+ B-CELL NEOPLASMS

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Background: The diagnostics of mature CD5+ B-cell neoplasms is multidisciplinary, data on quantitative immunophenotyping is limited. The study aimed at evaluation of cytomorphology and quantitative immunophenotyping in 233 specimens of mature CD5+ B-cell neoplasms.
Methods: The cytomorphology was assessed by light microscopy using the microscope Nikon Eclipse. The BD FACS Canto II flow cytometer and the FACS Diva clinical software were used to analyse the positivity/negativity and MFI of surface expression in the panel of 18 markers. Advanced data mining methods including construction of data networks and the diagnostic algorithm based on artificial intelligence were used to define the key differential diagnostic features and most informative markers of CLL/SLL, MCL and CD5+ MZL.
Results: The occurrence of cytomorphological types was: in CLL – typical form and atypical CLL (CLL/PL, pleomorphic CLL); in SLL – small cell type; in MCL – classical type, small cell, pleomorphic, blastoid and MZL-like variant, in CD5+ MZL – small cell type, medium/large lymphocyte type, pleomorphic type and type with villous lymphocytes. Most informative markers for the distinction of CLL/SLL, MCL, and CD5+ MZL, including atypical cases, were MFI values of CD79b, CD20, CD23, CD43, CD38, CD11c, FMC7, CD200, kappa LC, and their combination. CD23+CD200 were the most discriminant between CLL/SLL and MCL; CD23+CD79b between CLL/SLL and CD5+ MZL. Although quantitative immunophenotyping failed to accurately distinguish MCL and CD5+ MZL, it improved their distinction as compared with the qualitative assessment. The combination of cytomorphology and quantitative immunophenotyping increases the diagnostic effect.
Conclusions: The quantification of expression of informative markers increases the diagnostic value of immunophenotyping as compared with qualitative/semiquantitative assessments. The routine use of quantitative immunophenotyping requires the development of sophisticated mathematical models, minimizing the subjectivity of expert-based assessment.

UNCOVERING THE ORIGINS OF EMBRYONIC HEMATOPOIESIS

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The emergence of blood cells during mammalian embryonic development occurs in three independent waves. Importantly, the very first blood cells emerge in the extra embryonic yolk sac (YS). YS derived hematopoiesis provides the embryo with primitive erythrocytes (EryPs, wave 1) and erythro-myeloid progenitors (EMPs, wave 2) and is essential for embryonic development as well as early life. Later on, hematopoietic stem cells (HSCs, wave 3) emerge in the aorta-gonado-mesonephros region of the embryo proper. After birth the HSCs gradually take over for the hematopoiesis, however, they are dispensable during embryogenesis.
Due to high similarity of above-mentioned hematopoietic progenitors and spatiotemporal overlap of individual waves during embryonic development, it is not fully understood how individual waves emerge, differentiate and cease, and most importantly how is this dynamic process balanced to provide the embryo with sufficient amount of blood cells and at the same time generate a stable pool of quiescent HSCs.
The overall goal of this project is to reveal the molecular mechanism behind specification of YS derived hematopoietic lineages. More specifically, using scRNA-Seq approach combined with full spectrum flow cytometry, *in vitro* assays and development of new lineage-tracing mouse models for labelling of cells simultaneously expressing two genes, we aim to reveal the contribution of EMPs to the first embryonic myeloid cells and megakaryocytes.
Based on the analysis of scRNA-Seq data of YS-derived progenitors and their progeny, we propose, that the first embryonic macrophages derive from the EMP wave and that EryPs emerge from hemato-endothelial cells and not directly from mesoderm as proposed previously.
In summary, we will generate a novel genetic tracing system for precise labelling of EMPs and their progeny to reveal their fates in developing embryos and to tackle the theorized existence of myeloid cells derived from the primitive wave.

MAPPING THE SURFACE MARKERS OF CHILDHOOD T-CELL ACUTE LYMPHOBLASTIC LEUKEMIA CELLS

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T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive malignant disease with very heterogenous genetic and immunophenotypic background arising from T-lymphoblasts. While T-ALL accounts for only 15% of childhood ALL and at diagnosis the prognosis is comparable to other ALL subtypes, around 30% of patients undergo relapse with a dismal prognosis. Level of minimal residual disease (MRD) during induction therapy remains one of the crucial prognostic markers, however due to partial immunophenotypic overlap with non-malignant T-lymphocytes its precise evaluation is still challenging. Therefore, development of new approaches leading to possible discovery of potentially targetable molecules and improvement of flow cytometry-based MRD monitoring represents a hope for a better therapy outcome.

In this study, using conventional flow and full-spectrum cytometry we mapped surface markers (n=307) on T-lymphoblasts obtained from patients at diagnosis (n=18) and compared its expression to healthy donor T-cells. To reduce technical variability, we barcoded patient samples and healthy donors using anti-CD45 antibodies directly conjugated to various level of Dy396XL and Dy647P1 or Pacific Blue. Additionally, we applied a simplified panel for identification of T-lymphoblasts and non-malignant T-cells. First, we identified aberrantly expressed markers of T-lymphoblasts (over- or under-expressed compared to healthy donor T-cells) for each patient individually, ranging from 8 to 19. A set of markers was shared across patients: CD38 was over-expressed in 18/18 of T-ALL patients; CD48 was under-expressed in 18/18 of T-ALLs. Also CD147, CD82, CD71, CD11a and CD44 showed differential expression in at least 16/18 of investigated samples. Although we did not find any novel characteristic set of markers reflecting maturation stages (T-II, T-III or T-IV immunophenotype), we identified CD31 as a marker solely over-expressed in T-II subtype.

In conclusion, we demonstrated the feasibility of surface mapping of childhood T-ALL cells offering new approaches for targeted therapy. We identified 7 potential markers for MRD monitoring.

Acknowledgement

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INSIGHT INTO THE MECHANISM OF IMPAIRED CD34+ CELL MOBILIZATION IN MULTIPLE MYELOMA PATIENTS TREATED WITH ANTI-CD38 THERAPY

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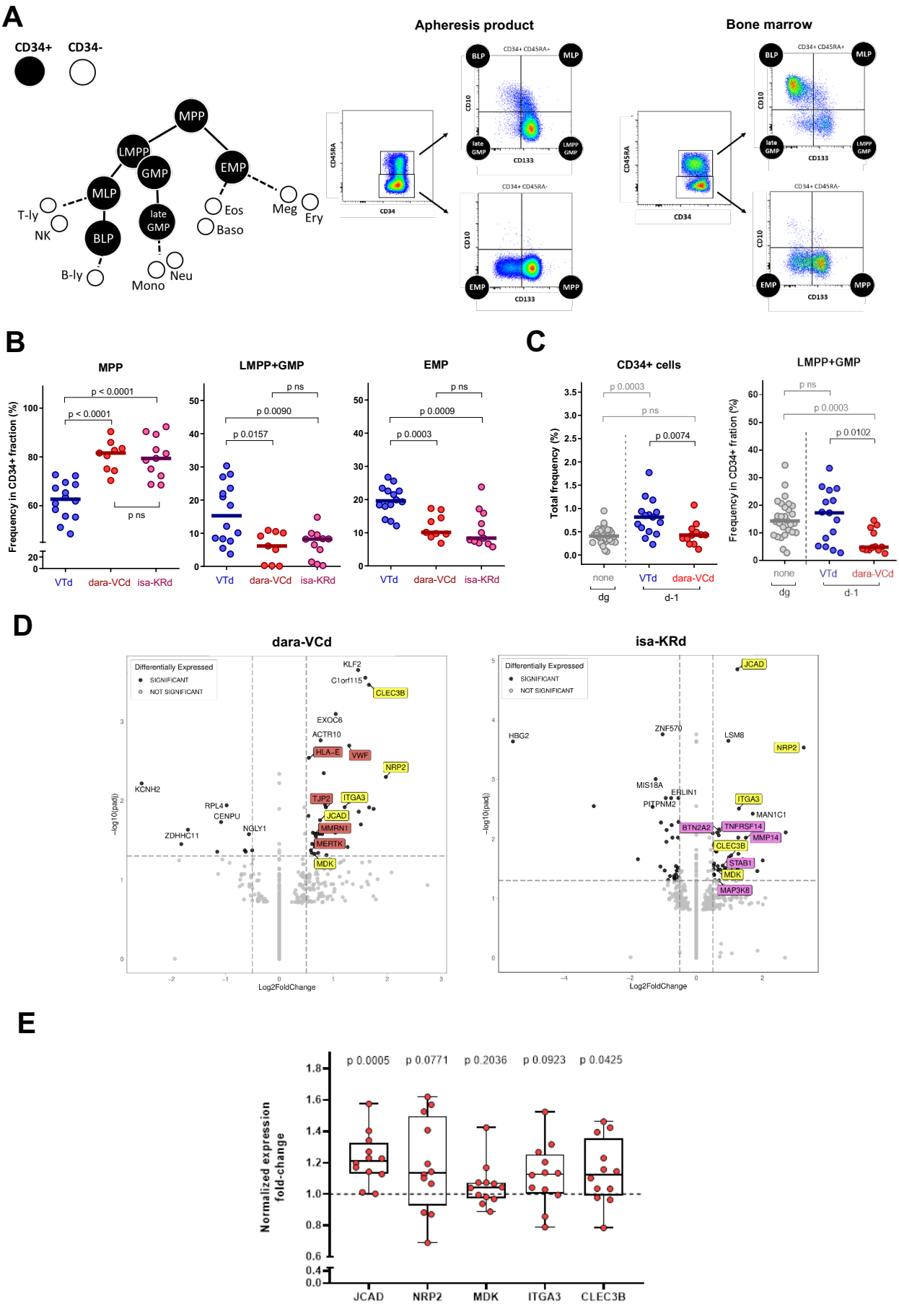
Background: High-dose induction followed by CD34+ hematopoietic stem/progenitor cell (HSPC) mobilization, apheretic collection, and autologous transplantation is standard of care in multiple myeloma (MM) patients. However, daratumumab (dara) and isatuximab (isa), anti-CD38 monoclonal antibodies used in modern myeloma regimens, impair mobilization and post-ASCT hematopoietic recovery (1).
Aims/methods: To elucidate the effect of dara and isa on CD34+ cells, phenotyping of CD34+ subsets was assessed in apheresis product (AP) and bone marrow (BM) samples of MM patients treated with conventional (VTd) vs anti-CD38-based induction (dara-VCd and/or isa-KRd). Transcriptomic analysis was also performed in mobilized AP CD34+ cells.
Results: Fundamentals of the study were built by mobilization efficacy and engraftment analysis in patients treated with VTd (n=14) vs dara-VCd (n=9) and isa-KRd (n=11). Decrease in mobilized HSPCs, prolonged AP collection, and delayed neutrophil/platelet recovery reflected already reported data.
AP CD34+ subset phenotyping (2,3) (fig. 1A) revealed significant ($p < 0.05$) changes in mobilized HSPC pool with decreased frequency of erythro-myeloid (EMP) and lympho-myeloid (LMPP+GMP) progenitors (fig. 1B). Analysis of BMs (VTd n=15, dara-VCd n=12) aspired on day before transplantation showed reduction of CD34+ cells in dara-VCd compared to VTd, mainly because of LMPP+GMP decrease (fig. 1C). RNAseq analysis performed on mobilized AP CD34+ cells sorted with FACS (VTd n=7, dara-VCd n=6, isa-KRd n=8), revealed only small differences, but cell adhesion was among the top gene-ontology terms upregulated after dara-VCd and isa-KRd. *JCAD, NRP2, MDK, ITGA3,* and *CLEC3B* represented common adhesion targets in dara-VCd/isa-KRd, while other adhesion genes were also identified in each group respectively (fig 1D). Direct effect of anti-CD38 therapy to upregulate *JCAD* and *CLEC3B* significantly was demonstrated by qPCR performed on cDNA of CD34+ cells isolated with MACS from AP bags (n=12), cultured for 20 hours with 10μg/ml isa *in vitro* (fig 1E).
Summary: The study provides first insights into the impact of anti-CD38 therapy on CD34+ cells and suggest upregulated adhesive interactions as a main cause of mobilization and hematopoietic recovery impairment.

Keywords: anti-CD38 therapy, daratumumab, isatuximab, monoclonal antibodies, mobilization, multiple myeloma, flow cytometry, CD34+ cells, hematopoietic stem cells, transcriptomics, RNA sequencing

Figure 1


A
CD34+
CD34-
MPP
LMPP
GMP
EMP
MLP
late GMP
BLP
T-ly
NK
B-ly
Mono
Neu
Eos
Baso
Meg
Ery
Apheresis product
Bone marrow
CD34+ CD45RA+
CD34+ CD45RA-
CD45RA
CD34
CD10
CD133
B
MPP
LMPP+GMP
EMP
Frequency in CD34+ fraction (%)
p < 0.0001
p < 0.0001
p ns
p ns
p 0.0090
p 0.0157
p ns
p 0.0009
p 0.0003
VTd
dara-VCd
isa-KRd
C
CD34+ cells
LMPP+GMP
Total frequency (%)
p 0.0003
p ns
p 0.0074
p 0.0102
none
dg
d-1
D
dara-VCd
isa-KRd
Differentially Expressed
SIGNIFICANT
NOT SIGNIFICANT
-log10(padj)
Log2FoldChange
KLF2
C1orf115
CLEC3B
EXOC6
ACTR10
HLA-E
VWF
NRP2
KCNH2
TJP2
ITGA3
RPL4
CENPU
JCAD
NGLY1
ZDHHC11
MMRN1
MERTK
MDK
HBG2
ZNF570
LSM8
MIS18A
PIPNM2
ERLIN1
MAN1C1
BTN2A2
TNFRSF14
MMP14
STAB1
MAP3K8
E
Normalized expression fold-change
p 0.0005
p 0.0771
p 0.2036
p 0.0923
p 0.0425
JCAD
NRP2
MDK
ITGA3
CLEC3B

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IMMUNOLOGY

IMMUNOPHENOTYPING AND EXPRESSION OF AGING-MARKERS IN ELDERLY COHORTS WITH CHRONIC HEALTH CONDITION

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The age-related decline of many functional systems including immune system is associated with chronic low-grade inflammation (CLGI), that has been proposed to be a driver of many aging-related diseases development. While CLGI and immunosenescence can potentiate each other, the prevalence of immunosenescence has not yet been associated with ageing-related diseases development or progression.
In order to determine whether the chronic conditions in elderly may promote immunosenescent phenotype and expression of aging-associated markers, we analysed immune signature of peripheral blood mononuclear cells and measured expression of activation/exhaustion/differentiation/ senescent plasma markers in cohorts of elderly with high BMI and/or with chronic ischemic heart disease.
We detected differences in expression of aging- and inflammation- associated plasma markers and we observed different frequencies of immune cells populations between these cohorts.
Understanding the link between immunosenescence and age-related disease development followed by identification of aging- and immunity- associated markers together with traditional clinical screening may improve patient stratification for targeted preventive interventions.

SPATIAL IMMUNOPROFILING OF THE TUMOR TISSUE IN PATIENTS WITH SOFT TISSUE SARCOMAS

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Soft tissue sarcomas (STS) are an extremely heterogeneous and rare group of tumors. The incidence of STS is increasing while the mortality rate remains the same over time. This underlines the need for a detailed analysis of these tumors and development of novel therapeutic approaches. The current standard of care is surgery with or without radiotherapy. Nevertheless, many patients are diagnosed with metastases, and effective treatment is very limited. The benefits of chemotherapy are disputable. The introduction of immune checkpoint inhibitors (ICIs) has delivered novel encouraging era for many cancer types in metastatic setting. Besides displaying unprecedented response rates, ICIs also provide new opportunities to explore multimodal strategies in managing solid tumors. The role of ICIs in the treatment of STS remains controversial. However, the presence of tumor-infiltrating immune cells and the expression of immune checkpoint molecules provide a rationale for further investigating and testing of ICIs in these tumors. We have investigated by flow cytometry and immunohistochemistry the tumor-infiltrating lymphocytes (TILs) with a particular focus on the immune checkpoint molecules (LAG-3, TIM-3, PD-1, PD-L1) at multiple sites within one tumor of selected histology. Interestingly, the infiltration of immune cells and expression of inhibitory molecules varied substantially within one tumor. This observation highlights that multiple biopsies are superior to a single biopsy in detecting the expression of immune checkpoint molecules. Thus, taking additional biopsies should become the standard practice of STS biopsy as it may possibly broaden indications for therapy with ICIs.

CELLULAR AND MOLECULAR INTERACTION OF MAIT CELLS IN MUCOSAL TISSUE AND THEIR ROLE IN INFLAMMATORY BOWEL DISEASE

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Inflammatory bowel disease (IBD) manifests as chronic inflammation and is characterized by a deregulated immune response. The identification of the cellular immune players involved represents a major approach to unravelling the pathogenesis of IBD and may suggest new therapeutic strategies. Recently, MAIT cells have been identified as a possible key player in IBD. Activated MAIT cells produce cytokines including IL-26, a newly discovered cytokine involved in the pathology of IBD. IL-26 is not expressed in mice and therefore its effect on the course of inflammation has not been yet sufficiently investigated.
Our laboratory described a model of intestinal inflammation based on human iPSCs-derived intestinal organoids (IOs). Using this model, we aim to help to understand the role of MAIT cells and IL-26 in the pathology of IBD. We have described the relevancy of IOs to model IBD using various methods such as immunofluorescent labelling, flow cytometry and RNAseq. We have shown that MAIT cells can be isolated from human blood and intestinal tissue. Upon *in vitro* activation, they produce cytokines and effector molecules including IL-26 and their response differs depending on the origin of the stimuli. RNAseq and subsequent analysis have allowed us to describe the crucial role of IL-26 in healthy and inflamed tissue.

DATA REPRODUCIBILITY AND SIMPLE ASSAY TRANSFER BETWEEN CYTEK AURORA SPECTRAL ANALYZER AND AURORA CS SPECTRAL SORTER

Jaimes M.[1], Brewinska-Olchowik M.[2]

Cytek Biosciences, Fremont, CA, USA

The Cytek® Aurora CS Spectral sorter provides advanced cell sorting capabilities while incorporating the same Full Spectrum Profiling (FSP™) technology as the Aurora and Northern Lights™ systems. Cytek's built-in instrument setup standardization allows for seamless assay transferability between the Aurora and Aurora CS platforms which results in similar assay resolution and allows the user to employ the same gating strategy from analysis to sorting.

In this presentation, we will discuss:
- The Cytek® Aurora CS concept and features
- How we achieve assay reproducibility through instrument standardization
- Data demonstrating Aurora and Aurora CS assay reproducibility
- Workflow to transfer assays from analysis to sorting
- Sorting with Cytek®'s 25-Color Immunoprofiling Assay

TURNING ASPIRATIONS INTO REALIZATIONS: 40 COLORS, ONE TUBE WITH CYTEK SPECTRAL TECHNOLOGY

Jaimes M.[1], Brewinska-Olchowik M.[2]

Cytek Biosciences, Fremont, CA, USA

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 performer 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.

SARS-CoV-2 SPECIFIC CELLULAR IMMUNE RESPONSES AFTER BNT162b2 VACCINATION IN HOSPITAL HEALTHCARE WORKERS

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The COVID-19 pandemic has undeniably posed the most serious healthcare crisis of our century so far, with millions death and widespread social and economic repercussions worldwide. Vaccination has provided the most important tool for prevention and control of COVID-19 and proven to be crucial in the management of the pandemic. Novel mRNA vaccines, chiefly BNT162b2 (Comirnaty; developed by BioNTech and Pfizer), have been particularly important due to the speed and flexibility of development and widespread of use. While the induction of humoral immunity, i.e., robust production of neutralizing antibodies, has been the primary endpoint of vaccination efforts, T-cell based cellular immunity, in particular the induction of Th1 polarization, is likely equally or even more important for the successful tackling of a viral disease like COVID-19. Healthcare workers represent one of the earliest groups vaccinated, as well as one of the groups most at risk from COVID-19 exposure. In our study we used flow cytometry to measure the production of IFN-gamma by CD4+ and CD8+ T-cells upon SARS-CoV-2 Spike protein stimulation in a cohort of 173 healthcare workers vaccinated by BNT162b2. The data was also correlated with the production of IgG and IgM anti-SARS-CoV-2 antibodies in sera in order to compare the induction of humoral and cellular immunity by the vaccine.

Acknowledgements
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BIOLOGICALLY ACTIVE SUBSTANCES AND THEIR INFLUENCE ON THE REDUCTION OF STRESS AND RADIATION BURDEN IN THE ORGANISM

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The importance of radioprotective research stems from the deteriorating security situation worldwide and the growing risk of exposing soldiers and the civilian population to the effects of weapons of mass destruction. The most destructive of these are nuclear weapons, which cause extensive human and material losses. Radioprotective substances work on various biological principles, but their common feature is that they mitigate the side effects of ionizing radiation, thereby increasing the combat capability of troops, reducing the loss of life and improving the quality of life of all affected.
Juvenil® containing biologically active substances (a natural complex of dietary nucleotides, peptides, amino acids and minerals) that have a confirmed positive biological effect on a living organism. The radioprotective effects of the product was measured in combination with the stress load.
We used female BALB/c mice divided into eight groups: C group (control), J group (Juvenil), S group (stress), JS group (Juvenil + stress), I group (Irradiated), IJ group (Irradiated + Juvenil), IS group (Irradiated + stress) and IJS group (Irradiated + Juvenil + stress). The mice in I, IJ, IS, and IJS group received a dose of 5.4 Gy from a 60Co gamma source. The mice in S, JS, IS and IJS groups were stressed by social isolation. Half number of the mice in each group were euthanized 14 days after time point 0 (irradiation and/or the start of stress induction), and the second half of mice were euthanized 30 days after time point 0. Blood from heart, thymus and spleen were analyzed by an automatic hematological veterinary analyzer and/or by a flow cytometer.

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A FORSKOLIN-MEDIATED INCREASE IN cAMP PROMOTES T HELPER CELL DIFFERENTIATION INTO THE TH1 AND TH2 SUBSETS RATHER THAN INTO THE TH17 SUBSET

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The cyclic adenosine monophosphate (cAMP) signaling pathway is involved in various physiological and pathophysiological processes. Forskolin (FSK), a labdane diterpene well known as an activator of cAMP production, is suggested to possess significant immunomodulatory potential. However, the specific effects of elevated cAMP levels caused by the FSK-mediated activation of adenylate cyclase (AC) on T helper (Th) cell differentiation and functions are still unclear. We speculated that the increased levels of cAMP in Th cells affect the differentiation program of distinct Th populations differently, in particular the development of Th1, Th2, and Th17 subsets. Only minor changes in the expressions of isoforms of ACs and phosphodiesterases (PDE), enzymes responsible for the degradation of cAMP, were observed in differentiating human Th cells with prevailing *ADCY1, ADCY3, ADCY7*, and *ADCY9 and PDE3B, PDE4A/B/D, PDE7A/B*, and *PDE8A* isoforms. FSK mediated elevation in Th1-specific markers reinforcing the Th1 cell phenotype. The differentiation of Th2 was supported by FSK, though cell metabolism was affected. In contrast, the Th17 immunophenotype was severely suppressed leading to the highly specific upregulation of the level of CXCL13. The causality between FSK-elicited cAMP production and the observed modulation of Th2 differentiation was proven by using cAMP inhibitor 2',5'-dideoxyadenosine that reverted the FSK effects. Overall, an FSK-mediated cAMP increase has an effect on Th1, Th2 and Th17 differentiation and can contribute to the identification of novel therapeutic targets for the treatment of Th cell-related pathological processes.

HUMAN CELL LINE ACTIVATION TEST (h-CLAT) WITH ALTERNATIVE FLUOROPHORES AS A CONTRIBUTION TO THE STANDARD PROTOCOL DESIGNED TO ASSESS THE SKIN SENSITISATION POTENTIAL OF CHEMICALS

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The human Cell Line Activation Test (h-CLAT, OECD 442E) represents one of the methodologies (NAMs) leading to human-relevant non-animal testing of skin sensitization potential. The method is based on overexpression of cell surface markers associated with the activation of monocytes and dendritic cells (CD86 and CD54 membrane proteins in THP-1 human monocytic leukemia cell line). The expression levels of CD86 and CD54 are quantified following 24h exposure to serial dilutions of chemicals selected on the basis of the CV75 (i.e., the concentration that allows 75% of cell viability). Chemicals are classified as sensitisers if the relative fluorescence intensity (RFI) of either CD86 and/or CD54 exceeds a defined threshold (i.e., RFI CD86≥150 and RFI CD54≥200) compared to the vehicle control. RFI values are considered for the prediction if the cell viability is above 50%. The method can be used in combination with previously implemented methods (i.e., Direct Peptide Reactivity Assay – DPRA, LuSens assay). Proficiency chemicals listed in TG442E were tested in three independent runs. CD86 (B7-2)-APC-Alexa Fluor 750 and CD54-PE labeled antibodies were used as alternative fluorophores to FITC-labelled antibodies (described in the standard protocol), in order to prevent the autofluorescence background interferences, the fluorescence spill-over, and the spectral overlapping at the FITC-specific wavelengths. DAPI was used as an alternative for the determination of viability instead of Propidium Iodide staining. Troubleshooting options have been suggested to prevent inconclusive or false negative predictions, including the description of representative cell morphology, dot-plot patterns, recommended gating strategy, 96-wells plate design, etc. Further research is planned in order to investigate also the role of recently suggested relevant markers, e.g., CD109, CD181, CD183, CD354 (Karhanis et al., 2021).

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INTERACTION OF LYVE-1 WITH HYALURONAN OF DIFFERENT MOLECULAR WEIGHT

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Hyaluronan (HA) is a large glycosaminoglycan composed of D-glucuronic acid and *N*-acetyl-D-glucosamine, which is found in the extracellular matrix (ECM). This glycosamiglycan is a key substrate in cell migration during inflammation, wound healing, and neoplasia. Recently, research suggested that HA is important in lymphatic trafficking, whereby it interacts with cells via receptors. The most widely known HA receptor is CD44; however, others have been discovered. One of them is a lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1), a protein belonging to Link protein superfamily. LYVE-1 is mostly expressed on lymphatic endothelial cells, but its expression can also be found on liver sinusoidal endothelial cells, lung alveolar epithelial cells, and some macrophages. HA binds to LYVE-1 through the HA-binding domain. This domain contains specific amino acid residues that form hydrogen bonds and other non-covalent bonds with HA. Some studies suggest that LYVE-1 has a higher affinity towards high molecular weight HA than low molecular weight HA. In our study we aim to investigate the interaction of LYVE-1 with hyaluronan of different molecular weight, and to see which HA has higher affinity towards LYVE-1. Furthermore, TSG-6, a HA-binding protein, also contains a Link domain through which it interacts with HA. Binding of HA to TSG-6 creates a complex that may influence HA binding to LYVE-1. However, some studies suggest that if there is a higher occupancy of HA with TSG-6, then this may inhibit the interaction of HA with LYVE-1. Therefore, the interaction of HA and LYVE-1 in the presence of TSG-6 is investigated as well.

Keywords: Hyaluronan, LYVE-1, TSG-6, lymphatic endothelial, complex

IMMUNOMETABOLIC PROFILING OF MYELOID CELLS IN PEDIATRIC PATIENTS REPRESENTS A TOOL HOW TO DEPICT THE DYNAMICS OF SEPSIS PROGRESSION

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Immunometabolism represents the main governing force for immunity and its defensive functions. The exposure of myeloid cells to environmental stimuli such as invading pathogens or pathogen-originated ligands is followed by their activation leading to a metabolic switch, represented by increased glycolysis linked with anabolic processes. This immunometabolic switch ensures the gain of required amount of energy and intermediate metabolites for defensive processes, vital for host protection. Metabolic processes are altered in many pathologies including sepsis progression.
Sepsis is heterogeneous syndrome with dynamic progression, in late phases associated with immunosuppression, which can persist many months after sepsis onset. Changes in immunometabolic profile occur within minutes and represent the main source of intermediate molecules for defensive processes and also for long-lasting epigenetic changes. We hypothesize, that metabolic changes can characterize the sepsis dynamics and can reflect the functional ability of cells during sepsis progression.
We performed the flow cytometry profiling of single-cell energetic metabolism by SCENITH completed with immunophenotypic analysis of myeloid cells. Using metabolic inhibitors 2-deoxy glucose, oligomycin ad harringtonine we investigated glucose dependence or fatty acid oxidation capacity. We also focused on detailed analysis of myeloid cells, their subsets and activation status by CD86, CD163, CD354 or HLA-DR, together with cytokine production. All markers were correlated to the severity of sepsis (SOFA score).
In summary, we showed a detailed immunometabolic analysis of myeloid cells of pediatric septic patients together with a characterization of sepsis-altered cells' subset frequency and their ability to perform defensive processes.

THE IMPACT OF CASEIN KINASE 1 ACTIVITY ON T CELL FUNCTION AND RESPONSE

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T helper (Th) cells play a critical role in maintaining homeostasis, and their dysregulation has been implicated in the development and progression of various diseases, such as autoimmune diseases, allergic reactions, and transplant rejection. The casein kinase 1 (CK1) family members belonging to serine/threonine kinases have been shown to phosphorylate key regulatory molecules involved in T cell signaling pathways. Inhibition of CK1 has been demonstrated to slow down the development of chronic leukemic-like disease, pancreatic tumors, and cardiovascular diseases, but its effect on T cells remains unclear. Our objective was to investigate the impact of CK1 inhibition on T helper cell function and response. Human $CD4^+$ Th naïve cells were isolated from peripheral blood and stimulated with anti-CD3 plus anti-CD28 monoclonal antibodies. A part of the samples was treated with selective CK1 inhibitors of isoforms α, δ, and ε. We analyzed the expression of CD25 and CD69 markers, production of IFN-γ, cell cycle, proliferation and viability of the cells. Our findings demonstrated that various inhibitors of CK1 decrease T helper cell activation by reducing the expression of CD25 molecule and production of effector cytokine IFN-γ. The inhibitors, particularly the isoform α inhibitor, were found to arrest cells in the G1 phase and decrease cell proliferation. These findings provide new insights into the role of CK1 in T cell function and response and highlight its potential as a therapeutic target.

MODELLING INFLAMMATION-DRIVEN FIBROSIS USING HUMAN LUNG ORGANOIDS

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Fibrotic tissue formation is a crucial mechanism of physiological tissue repair. Dysregulated, pathological fibrosis is characterized by uncontrolled growth and activation of fibroblasts, increased production of extracellular matrix, and stiffening of the surrounding tissue. This can lead to tissue disruption, loss of function, and even organ failure. Fibrosis is associated with inflammation and immune response, with several cytokines such as TGF-β, TNFα, IL-6, and IL-1β playing a key role in its development. Here we show 3D human lung organoids (LOs) as a tool to model how fibrosis is induced and progresses in an environment mimicking real human tissue. We show that LOs comprise both epithelial and mesenchymal cells. As such we describe LOs as a model for studying the development and progression of fibrotic changes in response to external stimulation, including epithelial-mesenchymal crosstalk. Our laboratory has previously shown that human lung organoids are able to form an immunocompetent environment in response to pathogen stimulation, as seen by upregulation of inflammatory markers. We show LOs response to a panel of inflammation inductors (e.g., cytokines, DAMPs, PAMPs, …), and to conditions linked to fibrosis development assessed as structural changes, changes in fibroblast activation (fibroblast activation protein expression), proliferation/differentiation (α-smooth muscle actin expression), function (collagen expression), and ECM composition (collagen/fibronectin deposition). A single cell approach using flow cytometry shows insight into the role of mesenchymal ($CD73^+$, $CD90^+$) and epithelial ($EpCAM^+$) cell subpopulations in fibrosis progression. Our results describe the fibrotic processes induced in LOs upon inflammatory signaling.

SPECTRAL CYTOMETRY ASSESSMENT OF T CELLS IN PATIENTS WITH INBORN ERRORS OF THE IMMUNITY – INTERFERONOPATHY AND EXHAUSTION

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Primary immunodeficiencies are rare diseases manifesting with increased susceptibility to infections, higher prevalence of autoimmunities and dysregulation of the immune response. Depending on the underlying genetic cause, patients may have both numeric or functional deficiencies of various immune cells, frequently including T cells. Here we used regular and spectral cytometry, transcriptomics and functional assays to explore T cell function and phenotype in patients with 22q11.2 deletion syndrome (22q11.2DS, DiGeorge syndrome) with congenital dysgenesis of the thymus, and compared them to cells of healthy children, patients with activated PI3-kinase delta syndrome (APDS) who have normal thymi but suffer from cell-intrinsic acceleration of T cell maturation, and cells of patients with cystic fibrosis (CF) who suffer from large load of infectious pathogens.

We show that 22q11.2DS leads to accelerated T cell maturation and skew towards follicular helper (Tfh) phenotype. 22q11.2DS T cells displayed increased production of IL-21, IFN-g and IL-10 and corresponding transcriptional enrichment of interferon pathways.

Interestingly, cells from CF patients remained largely normal in phenotype and function despite the *in vivo* bacterial load, however, i.v. ATB treatment was able to augment their cytokine production to supra-normal levels.

We then further interrogated the subpopulations, function and resilience against exhaustion of patient T cells with deep phenotyping using a 30-color spectral cytometry panel, the analysis of which is currently ongoing and will be presented at the conference.

POSTSURGICAL CHANGES IN THE ACTIVATION OF NETOSIS IN NEUTROPHIL SUBPOPULATIONS

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Each surgery and trauma are associated to activation of the immune system which can be deregulated in some cases and can lead to the development of serious states resulting to systemic inflammatory response syndrome (SIRS) and sepsis. The early step of inflammation is related to changes in neutrophil activation. Activation of neutrophils is connected to phagocytosis and degranulation but also to formation of neutrophil extracellular traps (NETs) which are composed of chromatin and released extracellularly to paralyze pathogens during process of NETosis. Low density neutrophils (LDNs) are a suggested subpopulation of neutrophils, which have a similar density to peripheral blood mononuclear cells (PBMCs). Pro-inflammatory LDNs have been described in autoimmunity and infection while LDNs with inducible immunosuppressive activity have been identified in septic shock. The ability of LDNs to generate neutrophil extracellular traps (NETs) also varies between diseases.
In this study, we described the changes in neutrophils and state of NETosis in the group of 40 patients undergoing hip/knee joint replacement. Blood samples were collected before surgery, 1 and 4 days after surgery. The experiments were performed in whole blood cells and in LDNs from isolated PBMC fraction.
Elevated numbers of neutrophils were observed in whole blood after surgery, similar results were gained also for low density neutrophils from PBMC fraction. The ability to form NETs decreased over time in unstimulated and ionomycin-stimulated cells in both neutrophil populations studied.
Our data suggest that the potential of neutrophils to be activated is probably successfully attenuated by postsurgical medication. However, individuals who respond very distinctly from the group have been observed and they could be potentially at risk of excessive inflammatory responses, which could play an important role in the development of SIRS or sepsis.

THE ROLE OF TSG-6 PROTEIN IN THE REGULATION OF T HELPER CELLS

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Tumor necrosis factor-stimulated gene 6 (TSG-6) is a multifunctional protein that is secreted mainly at sites of inflammation where it has been found to play a crucial role in immune regulation. Recent research has suggested that TSG-6 can modulate the immune response at the cellular level but also at the cytokine level. However, the mechanisms by which this protein modulates the adaptive immune system have not been investigated in almost any way. Understanding this problem may provide insight into the development of novel therapies for Th-mediated diseases. Therefore, our objective was to study the effects of TSG-6 on the viability and proliferation of activated T helper (Th) cells, as well as on the production of pro-inflammatory cytokine interferon gamma using ELISA. For that human Th naïve cells were sorted from buffy coat and stimulated with anti-CD3 plus anti-CD28 monoclonal antibodies. Subsequently, samples were treated at different time points with various concentrations of TSG-6. To further investigate the possible change in cell phenotype, the surface expression of early (CD69) and late (CD25) activation markers was examined by flow cytometry. Overall, the data show that certain concentrations of TSG-6 negatively affect Th cell activation and contribute to a suggestion of the potential of TSG-6 in therapeutic application in Th cell-related pathological processes.

THE ROLE OF HA IN ENDOMETRIAL RECEPTIVITY STUDIED IN 3D IN VITRO MODEL OF HUMAN ENDOMETRIUM

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Despite long-term research, the percentage of successfully implanted embryos during in vitro fertilization (IVF) process stays low (ca. 30%) creating a significant number of patients with so called non-receptive endometrium pathological condition. For the contact between the embryo and endometrial epithelial cells play a key role, glycosaminoglycans on the surface of both binding partners. Among them, hyaluronic acid (HA) comprises a major component. Our main hypothesis is that formation of cross-linked multimolecular assemblages and their interaction with HA binding proteins (TSG6, heavy chains (HC) of Inter-α-inhibitor (IαI)) on the surface of endometrium is necessary for successful implantation of embryo during WOI, and that insufficient formation of these complexes on uterus surface leads to non-receptive endometrium. Owing to ethical considerations, the collection of clinical human samples, especially at the implantation site, is challenging. Since the experimental results of the animal studies could not be fully adapted to humans due to the physiological and anatomical specificity of the human endometrium, there is an increasing interest in the use of in vitro 3D models to simulate the physiological conditions.
3D model of endometrium was established using endometrial stromal cells T HESCs and two endometrial epithelial cell lines: RL95-2 ($CD44^+$, receptive endometrium) and AN3CA ($CD44^-$, non-receptive endometrium). Molecular and cytometric approaches were used to study the differences between receptive and non-receptive endometrium. Expression of CD44, HA synthases and hyaluronidases in epithelial cells and TSG6 in stromal cells were analyzed using RT-PCR. The presence of TSG6 in culture medium was determined by ELISA. HA, TSG6, and HC of IαI on the surface of epithelial cells were detected by immunocytochemistry.

HIGH-RISK NEUROBLASTOMA THERAPEUTICS, TOPOTECAN AND 13-*cis* RETINOIC ACID, MODULATE AUTOPHAGY IN CORD BLOOD HEMATOPOIETIC STEM AND PROGENITOR CELLS, WHILE 13-*cis*-RETINOIC ACID POTENTIATES MONOCYTE ACTIVITY

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The therapy of neuroblastoma, the most frequent extra-cranial solid tumor in early childhood, targets important functions of tumor cells. While induction of their replication arrest and/or further differentiation are the ultimate therapeutic goals, the damage of other cell types forms an eventual risk in long-term perspective. Senescence of immune cells is characterised by decline of both innate and adaptive immune functions. Immunosenescence is associated with a number of pathologies linked to aging, such as higher susceptibility to infections and cardiovascular diseases, and has been described in childhood cancer survivors. Therefore, we hypothesize that intensive therapy including treatment with topotecan, induction treatment before stem cell harvest, and 13-cisRA, serving as maintenance therapy after hematopoietic stem cell transplantation, serve as inducers of accelerated aging of immune system.
In this study, we focused on effects of topotecan and 13-cisRA on cord blood hematopoietic stem and progenitor cells (HSPCs) and on monocytes from healthy donors. 13-cisRA treatment potentiated cytokine production and decreased the amount of ROS by monocytes. We show that topotecan and 13-cisRA change the HSPCs' differentiation profiles and the expression of cyclin-dependent kinases' genes related to senescence was ongoing after *in vitro* expansion. Moreover, both, topotecan and 13-cisRA, manipulate metabolism through autophagy and sirtuins 1 and 3 expression. Altered differentiation/expression profiles found in HSPCs *in vitro* may represent long-lasting effects of topotecan and 13-cisRA that involve epigenetic regulation in HSPCs and thus, these therapeutics might potentially alter the immune system function in survivors later in life.

ACUTE IMMUNE RESPONSE TO SURFACES FUNCTIONALIZED BY PLASMA-ENHANCED CHEMICAL VAPOR DEPOSITION

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Immune response to foreign bodies is a complex process that involves intricate communication between immune cells, mainly macrophage and T-lymphocytes. Neutrophils as first responders also play a crucial role in these communications by controlling other cell types' responses and phenotypes. However, they are quite overlooked. This study demonstrates the neutrophil complex role in reaction to foreign bodies.
The main property of foreign bodies dictating immune response is surface. Therefore, in this project, we assessed immune response to surfaces deposited by cyclopropylamine (CPA). The layer of CPA was deposited by the plasma-enhanced method (PE-CVD) which has its benefits, mainly lower working temperature, sterility, and increase in hydrophilicity. On the surfaces, CPA forms thin films generally called plasma polymers. The composition of these polymers varies and primarily is dependent on the working conditions of PE-CVD. These plasma polymers tend to have a decent impact on overall cytocompatibility as was described previously by our group[1,2].
We used HL-60 cell line and primary cells isolated from healthy donors as models to evaluate neutrophil response. We mainly focused on unique cell death, NETosis, and cytokine production. At first, we established HL-60 cell line as a neutrophil model thanks to their ability to differentiate into a neutrophil-like phenotype. Differentiation was controlled by increased expression of CD11b and their ability to phagocytose particles. Neutrophil response was characterized as proinflammatory if they underwent NETosis and secreted proinflammatory cytokines, such as TNF-α. Their ability to moderate inflammation was recognized by apoptosis and by the production of pro-macrophage chemokines, IL-8 and CCL2. We found substantial differences. The presence of NETs is dependent on the composition and hydrophilicity of plasma polymers.
This study's findings provide further insight into neutrophil response to surfaces of foreign bodies and highlight the significance of controlling their phenotype to promote overall biocompatibility.

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INFLUENCE OF SYNOVIAL FLUID ON OSTEOARTHRITIC JOINT-DERIVED CELLS

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Introduction: Although synovial fluid (SF) plays a key role in the pathophysiology of osteoarthritis (OA), its contribution to inflammation and healing processes has not been fully elucidated. We were interested in whether SF from OA patients differing in immune cell populations alter the immunophenotype of synovial cells.
Aim: To investigate the effect of different SFs from OA joints on fibroblasts-like synoviocytes (FLS) and chondrocytes.
Material and methods: Commercially available FLS and chondrocytes (Sigma-Aldrich) derived from the knee joint of OA patient were cultured in the presence of 10% SF from OA patients (n=21) in the culture medium for 24 hours. Subgroups were formed according to the prevalence of immune cell populations in the SF as follows: lymphocyte-predominant SF, neutrophil-predominant SF and monocyte/macrophage-predominant SF. Expression of surface markers and proliferative ability were measured by flow cytometry, the wound healing assay and the dynamics of cell migration in the presence or absence of SF was detected by time-lapsed microscopy.
Results: The SFs of patients suffering from OA show high inter-individual heterogeneity in immune cell composition and those were reflected by different effects on expression of studied surface markers on FLS and chondrocytes. Particularly, changes in expression of cadherin11, CD11c, CD55, and CD90 on FLS, and CD49c, CD62L, CD44, CD151, and CD90 on chondrocytes were detected after cultivation with different types of SF. A trend towards the highest proliferative capacity was observed after culturing cells with lymphocyte-predominant SFs. Differences between migration in the presence or absence of different SFs were not detected.
Conclusion: Our study revealed differences in the effect of SF with different immune subpopulations on the studied articular cells. Knowledge of the complex microenvironment is essential to understand the pathological processes ongoing in the OA joint.

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THE INFLUENCE OF SILYBIN ON THE METABOLIC ACTIVITY, CELL CYCLE, MITOCHONDRIAL MEMBRANE POTENTIAL, APOPTOSIS, AND THE GENE EXPRESSION OF CYTOKINES IN THE HEALTHY AND CARCINOGENIC GUT CELLS

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In addition to the hepatoprotective effect, the silymarin complex also has a confirmed neuro-, nephro- and cardio-protective effect, experimentally proven in animals and humans. Another effect of silymarin is immunomodulation, which can be oriented towards stimulation, but also suppression of the immune system, depending on its concentration.
Moreover, the anticancer potential of silymarin is well known, including its anti-inflammatory as well as antiproliferative effect mediated by influencing the cell cycle, suppression of apoptosis, and inhibition of cell-survival kinases. However, less is known about silybin, the main component of the silymarin complex. This study is focused on the concentration-dependent effect of silybin on the healthy porcine gut epithelial cells (IPEC-1) and the human colorectal adenocarcinoma cells (CaCo-2). The metabolic activity, cell cycle, mitochondrial membrane potential, apoptosis, and the relative gene expression for pro- and anti-inflammatory cytokines were studied in cells treated with silybin. Silybin showed a cytoprotective effect in IPEC-1 cells by stimulating their metabolic activity and proliferation and protecting mitochondrial membranes, while having only a minimal effect on the gene expression of pro-inflammatory cytokines, but significantly stimulating the gene expression of anti-inflammatory TGF-β. On the other hand, silybin inhibited metabolic activity of carcinogenic gut CaCo-2 cells, exerted an antiproliferative effect by increasing apoptosis, and significantly reduced the gene expression of pro-inflammatory interleukins as well as TGF-β. The antiproliferative and anti-inflammatory effect of silybin on carcinogenic cells without a negative effect on healthy gut cells is a prerequisite for its potential use in the adjuvant therapy of colon cancer; however, further studies are necessary.

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ROLE OF FAM83H IN IMMUNE SYSTEM HOMEOSTASIS

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FAM83H is expressed mainly in epithelial cells and has been suggested to be responsible for intracellular transport, regulation of cytoskeletal networks, and enamel formation. Deficiency of *FAM83H* was reported to be the cause of amelogenesis imperfecta (AI), a soft enamel disease. Interestingly, two AI patients from one family in Czechia with confirmed *FAM83H* mutation developed a juvenile rheumatoid arthritis. To understand the function of FAM83H in immune system homeostasis, we generated Fam83h knock-out (KO) and mutant mouse (*Fam83h*$^{tg/tg}$). Both mutant and KO animals exhibit decreased body size, sparse and scruffy coat, scaly skin, weakness and hypoactivity. While we have not observed dentin-related phenotype, mutant and KO pups show severe swelling of their forepaws accompanied by bone deformation at as early as 3 weeks. However, the soft tissue lesions are being resolved and peripheral blood leukocytes return back to normal levels at 7 weeks of age.
Moreover, mutant juvenile animals have increased neutrophils as well as G-CSF and inflammatory cytokine levels in their peripheral blood. Additionally, the development of leukocytes including T, B and NK cells is severely impaired in *Fam83h* mutants. Specifically, T cell development is arrested at the double-negative stage, B cells at the pro- and pre-B cell stages, and NK cells at the immature NK cell stage. Altogether, our findings advocating for the importance of FAM83H role in the hematopoietic niches and leukocyte development will contribute to the unravelling of the role of *Fam83h* in the onset of arthritic lesions and, in general regulation of the immune system.

MECHANISM OF CYANOBACTERIAL LIPOPOLYSACCHARIDES PRO-INFLAMMATORY EFFECT

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Cyanobacteria are photosynthetic procaryotic organisms. They can produce various toxins and bioactive molecules. Their cell wall contains lipopolysaccharides (LPS), similarly to G- bacteria. It is known, that bacterial LPS has a pro-inflammatory effect on immune cells, but also fibroblasts or epithelial cells. In recent years several studies have shown pro-inflammatory activation of eucaryotic cells after exposure to cyanobacterial LPS (cyanoLPS) *Microcystis aeruginosa* and *Geitlerinema sp* or cyanoLPS isolated from harmful algal blooms containing a mixture of several cyanobacterial species. However, not all LPS and cyanoLPS have pro-inflammatory properties. The model receptor for LPS is toll-like receptor (TLR) 4. Nevertheless, other receptors such as TLR2 or different saccharide receptors could be involved.
The aim of our study was to elucidate the mechanism of pro-inflammatory activation of eucaryotic cells after cyanoLPS exposure. We isolated cyanoLPS from axenic laboratory cultures of *Aphanizomenon flos-aquae* (Ap), *Planktothrix agardhii* (Pl), *Dolichospermum curvum* (Do) and *M. aeruginosa* (Mi). All used cyanoLPS were able to induce expression of IFNβ RNA in mouse macrophage cell line RAW 264.7. However, not all of them were able to induce production of TNFα, IL-6 or NO into the culture medium. Furthermore, Ap, Pl and Mi but not Do activated NFκB signalling pathway and none of them were able to activate p38 and SAPK/JNK signalling pathway. Positive control *Escherichia coli* LPS was able to activate all measured parameters.
To study if cyanoLPS signals via TLR4 or TLR2 inhibitor of TLR4 and neutralising antibody of TLR2 were used. Moreover, TLR4 and TLR2 knock-in HEK293 and knock-out THP-1 cell lines were employed. All our data suggest that cyanoLPS does not use the TLR4 receptor, but TLR2 receptor.

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INFLUENCE OF EARLY POST-HATCH ADMINISTRATION *ENTEROCOCCUS FAECIUM* AL41 ON INTESTINAL AND SYSTEMIC IMMUNITY IN CHICKENS

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The microflora of the GIT plays a key role in the anatomical, physiological and immunological development of the organism. Early post-hatch administration of *Enterococcus faecium* (EF) stimulates innate and acquired immune responses, protects intestinal epithelial barrier, promotes small intestine development, and its higher absorptive capacity for nutrients contributes to the positive effects on growth performance. Our study (Albrecht et al. 2022) showed that 7 days *per os* EF AL1 supplementation improves pectoralis major muscle growth and health due to positive effects on bioavailability and fusion capacity of satellite cells progeny and better tissue perfusion. Simultaneously with examination of the muscles, we monitored the immune parameters by evaluating the white blood cells count haematologically, phagocytosis, metabolic activity, subpopulations of T and B lymphocytes in the peripheral blood and jejunum by flow cytometry. Heterophils were responsible for the insignificant increase in leukocytes in the EF group after 4 days, and lymphocytes and monocytes after 7 days. The increase in the metabolic activity of phagocytes occurred earlier than the rise in phagocytosis. In the peripheral blood, the most consistent effect in EF group was shown by subpopulation of CD8 T lymphocytes, with a permanent increase during the monitored period in three samples on day 4, 8 and 11. Post 3 days of EF probiotic administration, the EF group also presented an increase in CD3 and IgA subpopulations. Jejunal mucosal immunity was monitored by IEL and LPL isolation and cytometric measurement of CD3, CD4, CD8, IgM and IgA lymphocytes. Administration of the probiotic EFAL41 showed an increase in all determined subpopulations, but with significance only in CD3 and CD45 LPL at day 11. The targeted transfer of antigens by Peyer's patches is important in the formation of a local secretory immune response. The intestinal epithelium with high number of lymphocytes that produce a wide range of cytokines thus influencing the local immunoregulatory microenvironment. Cytokines affect a number of defence mechanisms of the macroorganism through signalling across cytokine network. The strain-specific immunomodulatory effect of tested bacteria *Enterococcus faecium* AL41 was confirmed.

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THERAPEUTIC ACTIVITY AND BIODISTRIBUTION OF A NANO-SIZED POLYMER-DEXAMETHASONE CONJUGATE INTENDED FOR THE TARGETED TREATMENT OF RHEUMATOID ARTHRITIS

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Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease caused by the alteration of the immune system. Current therapy includes various approaches; however, it is often associated with various side effects. The conjugation of the drug to a nanosized delivery system improves its therapeutic outcome. Therefore, our objective was to evaluate the biological effects of nanopolymer drug conjugates based on biocompatible N-(2-hydroxypropyl) methacrylamide carrying glucocorticoid dexamethasone (DEX). The drug was derivatized prior the attachment to polymer with the aim to introduce suitable functional group for the attachment via stimuli sensitive spacer. The biological effects of polymer conjugates were evaluated *in vivo* using the adjuvant-induced arthritis mouse model. The biodistribution of fluorescently labelled conjugates was evaluated by *in vivo* imaging and fluorescence analysis in organs. Therapeutic efficacy was evaluated by observation of the clinical severity of arthritis in mouse paws and measurement of the level of RANKL in homogenates of paw tissue. We observed an increased accumulation of nanopolymer conjugate in the arthritic mouse paw using *in vivo* imaging. This was also confirmed by analysis of fluorescence in organ homogenates. The DEX copolymer conjugate showed a dose-dependent healing effect based on the evaluation of the clinical severity of RA. The dose of 0.5 mg/kg was shown to be the best dose/effect option. The conjugation improved the therapeutic outcome compared to free DEX. Overall, our data suggest that the conjugation of DEX to a nanopolymer carrier by a stimuli-sensitive spacer is a suitable strategy for improving RA therapy.

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ENTEROCOCCUS FAECIUM-DERIVED EXTRACELLULAR VESICLES AND THEIR INTERACTION WITH IMMUNE CELLS

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Inflammatory bowel disease (IBD) is a chronic disease leading to serious health problems. It is often accompanied by dysbiosis and enrichment in certain bacterial species (such as *Enterococcus* spp.). In consequence, presumably, also the production and composition of bacterial membrane-derived particles, enabling intercellular communication, called extracellular vesicles (EVs), change. We hypothesize that *Enterococcus faecium (Ef)*-derived EVs may contribute to IBD. In this work, we aim to characterize these EVs and describe their interaction with immune cells.
The EVs were isolated from *Ef* (CCM 7167T) culture, using multiple centrifugation, filtration, and ultracentrifugation steps. The EVs were then purified by sucrose cushion and visualized by Cryo-EM. Further analyses included particle concentration assessment by MADLS, protein characterization by SDS-PAGE with Coomassie staining and LTA detection by western blotting. To detect the supposed EV uptake by innate immune cells, the EVs were stained with Rhodamine B and their interaction with RAW264.7 cells was visualized by confocal microscopy. To describe also the effect of the EVs on the inflammatory response, pro- and anti-inflammatory cytokines were measured by ELISA and reactive species of nitrogen (RNS) production was determined by Griess reaction. To further specify the exact EV-receptor interaction, transfected HEK293 cells were used.
Ef-derived EVs size ranges between 100-150 nm in diameter and they were shown to contain LTA. The first results have shown a rather immunostimulatory action of these EVs, inducing formation of RNS, as well as production of pro-inflammatory cytokine TNFalpha and immunoregulatory IL-10 by RAW264.7 macrophages. Further, the cultivation of transfected HEK293 cells with *Ef*-derived EVs suggested that the TLR2/6 heterodimer might be an important receptor for these EVs' entrance to target cells.
Our results provide a starting point for further functional studies of *Enterococcus*-derived EVs. These EVs seem to induce a pro-inflammatory reaction in macrophages, supposedly by interaction with the TLR2/6 receptor.

CHARACTERISATION OF MACROPHAGES FROM SYNOVIAL TISSUE AND FLUID BASED ON IMMUNOPHENOTYPING

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Introduction: Osteoarthritis (OA) is a degenerative joint disease that is most common in the elderly population. Although synovial macrophages play an important role in the modulation of joint inflammation and cartilage repair and regeneration in OA through various secreted mediators and interactions with synovial fibroblasts and other immune cells, there is limited data on the differences between synovial tissue (ST)- and synovial fluid (SF)-derived macrophages.
Aim: We aimed to investigate the differences in immunophenotypes of macrophages derived from ST and SF obtained from patients with late stages of knee OA.
Material and Methods: STs were obtained during total knee arthroplasty from 13 patients with late stages of knee OA, in 6 patients SFs were obtained by needle aspiration. STs were enzymatically digested to release cells using collagenase IV and incubated for 90 minutes in 37°C. 14-color flow cytometry analysis was used for immunophenotyping of macrophages and other cell populations present in STs and SFs.
Results: In all examined samples, myeloid cells described as CD45+HLA-DR+CD11b+ were present as the main predominant population, being slightly more represented in ST than in SF (medians 75.8% vs. 61.5% of all immune cells; P=0.236). Within this population, population of macrophages detected as CD1c-CD14+ was more abundant in ST (97.1% in ST vs. 62.2% in SF; P<0.001) and divided into CD16+ and CD16- subpopulations. Macrophage subpopulations derived from ST and SF were further characterized by CCR2, CD206, CX3CR1, CD86, CD36 expression. Moreover, myeloid dendritic cells described as CD1c+CD14- population was less abundant in ST (0.9% in ST vs. 23.5% in SF; P<0.001).
Conclusion: Our pilot study revealed differences between the composition and immunophenotypes of ST- and SF-derived macrophages. Knowledge of the immune microenvironment may contribute to the understanding of the pathophysiological processes ongoing in the OA joint.

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IMMUNE RESPONSE OF KERATINOCYTES AND INTESTINAL CO-CULTURE MODEL TO WATER BLOOM LIPOPOLYSACCHARIDES

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Cyanobacterial water blooms represent a global ecological problem that we are also facing in the Czech Republic. Water blooms are a source of a wide range of toxins, including lipopolysaccharides (LPS) derived from the cell wall of cyanobacteria and associated heterotrophic bacteria. Humans can be exposed to LPS during water recreational activities (e.g., swimming) in contaminated water bodies. In this case, skin and intestinal epithelium can get in direct contact with contaminated water. We used HaCaT cells as a model of keratinocytes and Caco-2/PBMC (peripheral blood mononuclear cells) co-culture as a simplified model of the intestine. These models were exposed to LPS isolated from water blooms and the possible pro-inflammatory response was studied. The water bloom samples were collected from recreational water bodies in the Czech Republic. As a marker of inflammatory response, levels of pro-inflammatory cytokines (IL-6, IL-8, CCL2, CCL20) were measured by ELISA. We also determined the migration potential of the cells by automatized wound-healing assay in the keratinocyte model. In the co-culture model, the changes in monocyte population were monitored by flow cytometry and the Caco-2 monolayer integrity was assessed by TEER measurement and presence of tight junction proteins. We observed that several LPS samples showed pro-inflammatory activity, with three samples highly active in the keratinocytes and one in the co-culture model. These LPS caused substantial increase in pro-inflammatory cytokines level. In the co-culture model, we detected the elevation of monocyte activation marker CD16, while the integrity of the monolayer was maintained. We conclude that these water bloom LPS are capable of inducing an inflammatory response that, alone or in combination with other cyanobacterial toxins present in contaminated water, may pose a risk to human health.

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DEXAMETHASONE-BASED POLYMER THERAPEUTICS FOR THE TREATMENT OF RHEUMATOID ARTHRITIS

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Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease caused by alteration of immune system. Current therapy includes various approaches; however it is often connected with various side effects. The conjugation of the drug to nano-sized delivery system improves its therapeutic outcome. Therefore, we aimed to evaluate the biological effects of nanopolymer drug conjugates based on biocompatible N-(2-hydroxypropyl) methacrylamide carrying glucocorticoid dexamethasone (DEX). The drug was derivatized prior the attachment to polymer with the aim to introduce suitable functional groups for the attachment via stimuli sensitive spacer. The biological effects of polymer conjugates were evaluated both *in vitro* and *in vivo*. Acute toxicity and anti-inflammatory properties were tested using murine peritoneal macrophages RAW 264.7 activated by lipopolysaccharide. Adjuvant induced arthritis mouse model was used for evaluation of pharmacokinetics and therapeutic efficacy of nanopolymer conjugates. Acute toxicity was not observed for any tested compound. All tested compounds retained the anti-inflammatory potential of DEX as they decreased production of pro-inflammatory mediators (NO and IL-6) by stimulated RAW 264.7. We observed the increased accumulation of nanopolymer conjugate in arthritic mouse paw using *in vivo* imaging. All tested polymer conjugates showed better therapeutic efficacy in comparison with free DEX. The data suggest that the conjugation of DEX to polymer carrier did not affect its biological activity and the therapeutic potential was retained and even higher than for free DEX. Therefore, the conjugation of DEX to nanopolymer carrier by stimuli sensitive spacer is a suitable strategy for improvement of RA therapy.

Animal experiments were approved by the institutional Animal Care and Use Committee (protocol n.52/2020 from 15th June 2020).

CYANOBACTERIAL TOXINS AND THEIR EFFECT ON IMMUNE SYSTEM CELLS

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Cyanobacterial water blooms (WBs) are an important contaminant of water bodies, among others because they produce a wide range of toxins. Among the best known are microcystin LR (MC-LR) and cylindrospermopsin (CYN). Although both have been intensively studied, their effect on the immune system has long been overlooked. Another important toxin is lipopolysaccharide (LPS), a component of the cyanobacterial cell wall. As a result, it is present in every locality occupied by WBs. The aim of our work was to study effects of these toxins on immune cells. MC-LR and CYN are commercially available, LPS were isolated at RECETOX from WB biomasses from Czech Republic water bodies. These biomasses were dominated by various cyanobacterial species, including those with documented MC-LR and CYN production. Both primary cells from peripheral blood of healthy donors and the murine macrophage cell line RAW 267.4 were used for the study. Flow cytometry, ELISA and western blotting were mainly used to detect pro-inflammatory effects of non-toxic concentrations. MC-LR and CYN experiments showed that after exposure, activation of signalling pathways, phosphorylation of NFκB, induction of TNFα and IL-6 production, activation of inducible NO synthase and, in the case of CYN, production of reactive oxygen species occurred. The pro-inflammatory effect of LPS of *Escherichia coli* or LPS isolated from *Aphanizomenon flos-aquae*-dominated WB was potentiated by exposure to CYN. LPS from *Microcystis aeruginosa*-dominated WB induced the production of IL-6, TNFα and IL-1β in whole blood as well as in isolated monocytes and polymorphonuclear leukocytes (PMNLs). PMNLs activation was further confirmed by increased expression of CD11b and CD66b on the cell surface as well as activation of p38, ERK 1/2 and NFκB signalling pathways. Our results suggest that both secondary metabolites and LPS produced by WBs have pro-inflammatory effects and their effects in environmental mixtures may potentiate each other.

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FLOW CYTOMETRY AS A REPLACEMENT FOR THE E-ROSETTE TEST IN AN IMMUNOSUPPRESSED GUINEA PIG MODEL? NOT YET

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The animal model of immunosuppressed guinea pigs with azathioprine is standardly used to determine the effectiveness of immunomodulating preparations. The disadvantage of the current model is that the main monitored parameter is the result of the E-rosette test, which is time and technically demanding historical method. The aim of this study was to compare the established E-rosette test with the detection of T lymphocytes using flow cytometry. In the case of guinea pigs, very limited number of commercial antibodies is available. Therefore, only the ratio of B and T lymphocytes and the subpopulations of T lymphocytes differentiated based on the expression of CD4 and CD8 markers were monitored in the study. Obtain data showed unchanged T/B ratio. Percentage of CD4+ and CD8+ T cells were also without significant changes after immunosuppression. Only CD4-CD8- T cell population showed significant decrease after azathioprine administration. It could be population of γδ T lymphocytes, but this claim is currently unverified. While the representation of total number of T lymphocytes measured by flow cytometry was without significant change, the E-rosette test showed a strong impact of azathioprine on the reduction of the number of rosette cells. Flow cytometry in guinea pigs still requires considerable development, especially in the search for new antibodies, to be a competitive method to the historical E-rosette test.

NEUTROPHIL AND EOSINOPHIL GRANULOCYTE IMMUNOPHENOTYPING AND BURST TEST

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Neutrophils and eosinophils are among the largest granulocytes observed in clinical flow cytometry. They are commonly gated from the basic FSC vs SSC dot plot based on their size and granularity, or by using the pan-leukocyte antigen CD45 and SSC. Routine laboratories usually measure only the relative numbers of neutrophils or eosinophils. In terms of functional tests, these include the test of oxidative burst in neutrophils to rule out the diagnosis of chronic granulomatosis, or the test for the presence of myeloperoxidase (MPO) in case of suspected immunodeficiency of MPO.
Inflammation or bone marrow disorders are the most common causes of increased neutrophil representation and an increased presence of immature neutrophils in peripheral blood (PB). Eosinophil granulocytes are commonly increased in PB of various diseases including allergic eosinophilic asthma, allergic eczema, parasite infections or immunodeficiencies like Omenn syndrome and hyper IgE syndrome.
Our study aimed to perform a functional test of the oxidative burst of neutrophils and eosinophils with their simultaneous phenotyping in patients with eosinophilic asthma and patients after hip surgery. The results showed that immature neutrophils and eosinophils have a reduced oxidative burst capacity compared to their mature forms.
Immunophenotyping of neutrophils and eosinophils allows the recognition of their mature and immature forms, which differ in their functional properties. Our findings could help in the interpretation of findings in patients with partially reduced oxidative burst.

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IMMUNOMODULATORY ENVIRONMENT OF BONE MARROW NICHE OF MYELOID LEUKEMIA

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In recent years, cellular immunotherapy has been an attractive new candidate to treat different types of leukemia along with new inhibitors and transplantation of hematopoietic cells. Despite preliminary success, there are still several limitations. Different types of leukemia are able to modulate the bone marrow (BM) niche and, as such, affect the immune response. Leukemic cells produce wide spectrum of modulatory molecules and communicate with other cells via direct cell-to-cell interaction. In this manner, leukemic cells are able to reshape the BM niche and establish protective environment providing a shelter to particular leukemic clones. This project is focused on describing the immunomodulatory microenvironment of myeloid leukemia BM environment and its potential impact on therapeutically administered effector immune cells. First, we aim to address the influence of transforming growth factor β (TGF-β) on cells within the BM niche as well as on incoming effector immune cells. Furthermore, the co-regulatory role of cytokine network including IL-2 and IL-15 will be explored, along with other major factors affecting the niche. Aim of this study is to develop a realistic *in vitro* model of leukemic niche using available cell lines and primary cells in order to describe the cytokine profile and general conditions resembling the situation *in vivo*. This project benefits from established complex BM model cultures (consisting of leukemic cells, mesenchymal stem cells and other cell types found in BM) combined with flow cytometry and advanced imaging methods. So far, utilizing intracellular staining, we were able to show that the presence of leukemic cells in a co-culture induces changes in TGF-β production in mesenchymal stem cells. We intend to further employ this method to analyse how the establishment of the leukemic niche affects production of other immunomodulatory molecules. Another aim of this project is to use surface marker phenotyping to describe in detail how the presence of leukemic cells reshapes its surroundings. In the end, these models should then help us understand the complex dynamics in the leukemic niche in context of cell-based therapies. In conclusion, this project aims to deepen the knowledge of leukemic environment and to translate these findings into improvement of efficiency of immunotherapy.

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THE INFLUENCE OF NEUROTROPHINS ON THP-1 CELL LINE POLARIZATION

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Introduction: Neurotrophins (NTs), a family of structurally similar growth factors (BDNF, NGF, NT-3 and NT-4), have been shown to contribute to the joint pain and stimulation of the proliferation of osteoarthritic fibroblast-like synoviocytes in ostearthritis (OA). Although NTs have been found to contribute to monocyte/macrophage dysfunction in animal studies, their role on monocytes/macrophages in OA is unknown.
Aim: To study the influence of BDNF, NGF and NT-3 on polarization of THP-1 cell line, a model to study monocyte and macrophage activities.
Material and methods: THP-1 cells in M0 state were treated with BDNF/NGF/NT-3 at three different concentrations for 24 hours. Five markers of macrophage polarization: CD64 (%), CD86 (%) and CCR7 (%) for M1 and CD163 (MFI) and CD206 (%) for M2 state; were measured by flow cytometry on BD FACSCanto II. Expression of these markers was compared with the untreated control and between different concentrations of the tested proteins.
Results: Treatment of THP-1 cells with BDNF, NGF and NT-3 resulted in a significant decrease in M1 markers, particularly CD86 and CCR7 expression, but this decrease was not concentration-dependent. BDNF and NGF did not influence on CD64 expression as opposed to NT-3, which decreased the counts of CD64 positive cells at all studied concentrations. Regarding M2 markers, expression of CD163 on THP-1 cells increased significantly under the highest concentration of tested proteins, as well as percentage of CD206-expressing cells, which also showed concentration-dependent trend. The measurements of soluble mediators produced during the treatment with studied NTs is ongoing.
Conclusion: Treatment of THP-1 cells with BDNF, NGF and NT-3 resulted in the shift to M2 polarization state. This pilot study may contribute to a deeper understanding of the role of NTs in OA joints.

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IMMUNOMODULATORY POTENTIAL OF POLYSACCHARIDES ISOLATED FROM MEDICINAL PLANTS

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Polysaccharide-rich fungi and plants have been employed for centuries worldwide for their dietary and medicinal benefits. Often thought to merely support normal bowel function and blood glucose and lipid levels, certain polysaccharides have attracted growing scientific interest for their ability to exert marked effects on immune system function, inflammation and cancers [1]. When PSs are administered orally, they are poorly absorbed through the gut, but interact with the mucosal immune system in the small intestine by epithelial cells, Peyer's patches and complement, thus positively modulate immune system and the activity of circulating leukocytes [2–4]. Aim of the present study was to compare the chemical features of water-extractable PSCs from leaves and roots of *G. sanguineum*, as well as their immunomodulatory activities on leukocytes in response to inflammation. Additionally, their effects on the growth of probiotic and pathogenic bacteria were evaluated.
PSs were isolated by aqueous extraction and their structural properties were studied by 2D NMR spectroscopy. The stimulatory effects of PS on human leukocytes were analyzed by flow cytometry. Their effect on the oxidative burst in blood and isolated neutrophils and the functional response of macrophages was investigated. The prebiotic and antibiotic activity of PS was also evaluated.
The total carbohydrate content of the samples was significant (73.6–76.8%). PS contained pectins that were rich in homogalacturonan, rhamnogalacturonan type I with 1,5-α-L-Araf, 1,4- and 1,6-β-D-Galp, 1→3,5-α-L-arabinans and 1→3,6-β-D-galactans side chains. PS stimulated the oxidative burst and functional response (NO and cytokines) of professional phagocytes. On the other hand, they were able to attenuate the inflammatory response of neutrophils activated by OZP. PS stimulated probiotic cultures of *Clostridium beijerinckii* and *Lactobacillus* sp. ZK9 strains and inhibited the growth and biofilm formation of *Escherichia coli*, *Streptococcus mutans* and *Salmonella enterica*.
Our results show that isolated PS have immunomodulatory effects and in case of inflammation anti-inflammatory effects (especially PS from the root). Based on the overall results, these PS could be part of dietary supplements to support the treatment of inflammation and infection in the gut.

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NEUTROPHILS – IMMUNITY INFLUENCERS INFLUENCED BY NFAT

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Calcineurin (CN) – Nuclear factor of activated T-cells (NFAT) inhibitors are commonly used immunosuppressants inhibiting adaptive immunity but leaving patients vulnerable to opportunistic infections. CN-NFAT pathway is also active in myeloid cells, where inhibition of CN-NFAT affects anti-infectious responses. We and others showed the importance of CN-NFAT in monocytes and dendritic cells, while the impact on neutrophils' function after NFAT inhibition remains elusive.
We used neutrophils (CD11b+, CD16+, CD66b+) isolated from the peripheral blood of healthy donors and evaluated the effects of CN-NFAT inhibitors on response to heat-killed *Candida albicans*, *Aspergillus fumigatus*, and other pattern recognition receptors (PRRs) ligands. We performed RNAseq to see global changes in expression and qPCR analysis of selected genes and used the ELISA method for analysis of released chemokines.
We showed that after PRRs activation, neutrophils massively change gene expression patterns and CN-NFAT inhibitors inhibit the increase of expression of genes involved in inflammation regulation. In the presence of CN-NFAT inhibitors, human neutrophils significantly reduce the expression of chemotactic cytokines CLL-2, CCL-3, and CCL-4; an expression of inflammation-mediating molecule COX-2, early growth response transcription factors EGR1, EGR2, and EGR3, and hydroxycarboxylic acid receptors HCAR1, HCAR2, and HCAR3 is also affected. By chemotaxis assay using trans wells, we showed the impaired neutrophil ability to chemo-attract other immune cells in the presence of CN-NFAT inhibitors. NFAT inhibition in human neutrophils impairs their response to pathogens and dysregulates the inflammatory environment.
Presented negative effects of CN-NFAT inhibitors on human neutrophils complete our information about the impacts of NFAT inhibition in immune cells. Together with the already published adverse effects of CN-NFAT inhibitors on other myeloid cells, these findings can help us explain the increased vulnerability of CN-NFAT inhibitors-treated patients.

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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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. The ideal universal structure for potent CAR T cells is undergoing continuous improvements, therefore novel CAR T cells require in-depth studies of their potency to ensure efficacy and identification of any nonspecific behaviors such as antigen-independent signaling. 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). CART19 cell cytolysis of CD19 expressing HEK-293T cells with different E:T (Effector to Target) ratios were monitored by the xCELLigence RTCA system. Concurrently, assessment of T cell differentiation and activation as well as cytokine production was measured on the NovoCyte Quanteon. This workflow provides a thorough examination of CAR T cell function, RTCA data demonstrates that CART19 killing is highly potent, specific and dose-dependent. In conjunction, cytokine increased with higher numbers of effector CART19 cells. CART19 cells express stem memory cell (TSCM) markers and upregulate activation and exhaustion markers after expansion. Coculture with HEK-293-CD19 resulted in upregulation of CD25, a decrease in CD127 expression and increase in exhaustion markers, PD-1, TIM-3, and LAG-3. In summary, this study demonstrates the combination of RTCA and flow cytometry cell analysis is a powerful workflow for immunotherapy research.

MICROFLUIDICS

PLASMA SURFACE TREATMENT OF CLOSED CYLINDRICAL MICROCHANNEL TO CREATE ON-CHIP VASCULAR ENDOTHELIUM

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On-chip vascular microfluidic models provide a great tool to study aspects of cardiovascular diseases *in vitro*. To produce such models, polydimethylsiloxane (PDMS) has been the most widely used material. For biological applications, its hydrophobic surface needs to be modified. The major approach has been plasma-based surface oxidation, which has been very challenging in the case of channels enclosed within a microfluidic chip.
The preparation of the chip combined 3D printed mould with soft lithography and commonly available materials. We have introduced the high-frequency low-pressure air plasma surface modification of seamless channels enclosed within a PDMS microfluidic chip. The plasma treatment modified the luminal surface more uniformly than in previous works. This setup enabled a higher degree of design freedom and a possibility of rapid prototyping. Further, plasma treatment in combination with collagen IV coating created a biomimetic surface for efficient adhesion of vascular endothelial cells as well as promoted long-term cell culture stability under flow. The cells within the channels were highly viable and showed physiological behaviour confirming the benefit of the presented surface modification.

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We would like to kindly acknowledge the funding from Czech Science Foundation (grant No. 21-01057S) and research support of IBP CAS (project No. 68081707).

ARTIFICIAL INTELLIGENCE-AIDED SINGLE-MOLECULE BIOAFFINITY ASSAYS WITH PHOTON-UPCONVERSION LABELS FOR MICROFLUIDIC APPLICATIONS

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Microfluidics and femtoliter arrays became essential for single-molecule enzyme-linked immunosorbent assays with femtomolar limits of detection.[1] Although successful commercially, a key impediment is shared with other heterogeneous assays – the separation of immunochemical complexes on solid surfaces.[1,2] In contrast, homogeneous bioaffinity assays perform all assay steps in a free dispersion – very useful for automated microfluidic analysis. Here we present a new approach for homogeneous assays, which utilizes background-free luminescence of photon-upconversion nanoparticles (Figure 1),[2] customized optical instrumentation[2,3], and artificial intelligence.[3] As a model system, the formation of bioaffinity complexes between nanoparticle-conjugated streptavidin and biotin is investigated, and a homogeneous single-molecule competitive assay for biotin is developed. The instrumentation utilizes a laboratory-built epiphoton-upconversion microscope. High-intensity near-infrared excitation (976 nm, 14 kW cm^{-2}) enables the imaging of freely diffusing photon-upconversion labels as diffraction-limited spots (5 ms exposition time). The detection of bioaffinity interactions from micrographs is automated by using convolutional neural networks.

Figure 1. Developing bioaffinity assays with photon-upconversion in hand (λ_{ex} 976 nm).

Acknowledgement

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MICROFLUIDIC RAMAN MICROSPECTROSCOPY WITH HIGH-POWER LASER FOR ULTRA-SENSITIVE MONITORING OF ENZYMATIC DEHALOGENATION

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Microfluidic systems combined with Raman microspectroscopy are useful for quasi-continuous flow-through analysis of liquid or suspended samples, such as microorganisms, micro- and nano-plastics, and chemical pollutants. We used as a model system a highly toxic environmental pollutant 1,2,3-trichloropropane (TCP) and studied its dehalogenation by a 3-enzyme cascade [1]. We employed microfluidic Raman microspectroscopy to monitor the gradual decomposition of TCP to harmless glycerol. In order to optimize the efficiency of the reactions catalyzed by these enzymes, it is necessary to analyze the dynamics of their action in various conditions. To this end, it is essential to monitor low concentrations of glycerol. Here, we report using microfluidic Raman spectroscopy with a high-power laser (up to 6 W) for fast periodic quantification of glycerol in concentrations undetectable by conventional Raman systems. We tested the effect of the laser power on the limits of glycerol quantification by Raman spectroscopy in a simulated reaction environment within a microfluidic system. The developed system can quasi-continuously quantify glycerol in sub-millimolar concentrations, which makes this setup ideal for the analysis of the devised enzymatic reactions.

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DIRECTED EVOLUTION OF ENZYMES ON MICROFLUIDIC CHIP

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Fluorescence–Activated Droplet Sorting (FADS) is a cutting-edge technology that opens up new possibilities for the directed evolution of enzymes. A FADS system was constructed by integrating several optical, electrical, and computational modules, and validated in terms of high-throughput detection of microfluidic droplets. Haloalkane dehalogenases (HLDs) were used as a target model enzyme. HLDs are α/β−hydrolases that cleave the bond between the carbon and the halogen in organic substrates and have significant potential in bioremediation.
For the validation of the newly assembled FADS system, the benchmark experiment was performed with two different populations of HLDs enzymes over-expressed in *Escherichia coli* cells (AncHLD-RLuc, DhaA31) and one population of cells containing empty vectors (gene–free pIDR9).
Specifically, each analysed droplet represents a different mutant from the mixture of cells with over-expressed enzyme co-encapsulated with a fluorescent assay for halide detection. The experiment employs two different microfluidic chips: one for droplet generation and another for droplet sorting. This setup enables a lengthy off-chip incubation period to be completed for the enzymatic assay. An application to support FADS analysis was programmed in LabVIEW and bundled with Python. This application allows initial analysis and prediction of a threshold voltage, validation and graphical interpretation of all measured data, and final reporting of all processes. The developed platform was subsequently used to sort a library of HLDs (circa 60 000 variants) at a rate of 1,000 events (droplets) per second. We will present the results of the directed evolution experiment, assess the strengths and weaknesses of the method, and identify areas of further development.

CELL LYSIS IN THE MICROFLUIDIC CHIP: TOWARDS SINGLE-CELL ANALYSIS IN A FREE SOLUTION

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There is a growing interest in single-cell studies as well as in developing new methodologies for single-cell analysis. Especially, automated handling of single-cell contents in a free solution is still challenging.[1] We propose a microfluidic platform that integrates: a) passive cell encapsulation; b) mixing the microdroplet content; and c) the cell lysis (Figure 1A, B). Microdroplets are produced continuously at high rates by pumping fluids (Fluorinert oil and assay buffer containing 0.01% (v/v) of detergent Triton-X) from external pressure-driven reservoirs. Hela cells in assay buffer stained with Propidium iodine (a fluorescent stain that differentiates between live and dead cells) are passively encapsulated into the droplets (Figure 1C). Further, the droplet content is mixed (Figure 1D) and the cells are lysed down in the incubation part of the chip (Figure 1E, F). We take the advantage of the proposed microfluidic platform and the single-molecule immunochemical method based on photon-upconversion labeling is discussed.

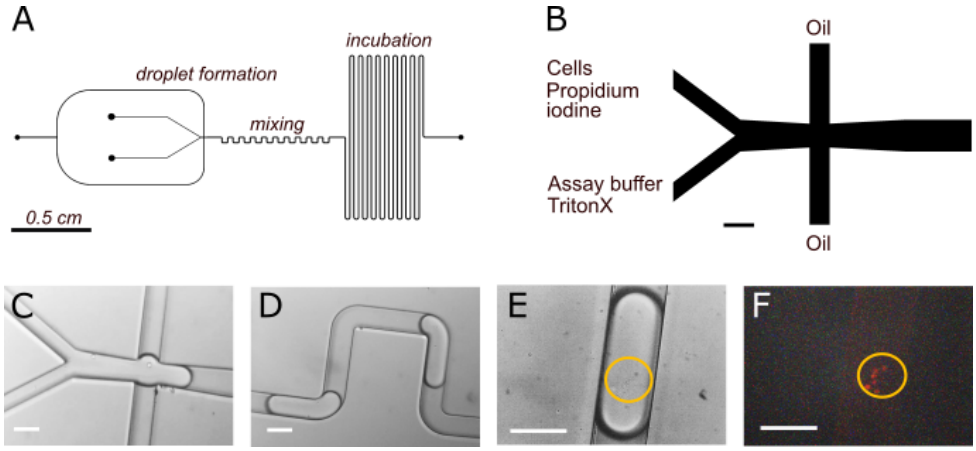

Figure 1. Microfluidic chip for cell lysis. Scheme of the chip (A). Scheme of the chip inputs (B). Micrographs of: (C) cell encapsulation; (D) mixing of droplet content; (E) lysed cells (bright-field mode); (F) lysed cells (epifluorescence mode). Scale bar: 100 mm.

Acknowledgement

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EXPLORING AMYLOID β SELF-ASSEMBLY IN NATIVE-LIKE MEMBRANES

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As the global population is ageing, dementia becomes a cause of growing worldwide epidemiological concerns. Alzheimer's disease (AD) accounts for 60% to 70% of all cases of dementia. AD is characterised by the presence of senile plaques of Amyloid β (Aβ) peptides and neurofibrillary tangles of hyperphosphorylated Tau protein. Aβ is cut-out from the larger, plasma membrane-spanning Amyloid Precursor Protein (APP), by the action of β- and γ-secretases in the brain. APP processing produces Aβ peptides of varying length (between 37 and 44 residues), with the most common being Aβ40, and the most up-regulated during AD being Aβ42. The latter may either aggregate into amyloid fibrils or pore-forming oligomers at the plasma membrane. Controlling this process is a promising therapeutic concept, but it requires a thorough understanding of Aβ42's aggregation pathway in its native membrane environment. We developed a microfluidic platform to generate highly homogeneous giant unilamellar vesicles (GUVs) whose composition mimics that of neuron plasma membranes. We use these vesicles to monitor Aβ aggregation and liposome destruction by fluorometry. Tweaking the lipid composition of our GUVs, we reveal interactions between specific lipids and Aβ42, and assess structural changes using infrared spectroscopy, and NMR. Finally, we build a kinetic model of Aβ42 aggregation and membrane toxicity in native-like environment, which could guide future drug discovery in the field of AD.

FIRST SINGLE-CHROMOSOME SEQUENCE ANALYSIS USING MICROFLUIDIC PLATFORM

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Individual chromosomes of important grass crops cannot be identified and purified using DNA-based flow-sorting because the chromosomes usually cluster together within a flow karyotype, forming composite peaks. However, two options exist towards true single-chromosome sequencing: i) flow-sorting into individual wells of a PCR plate (or into tubes); and ii) chromosome enrichment using flow-sorting with subsequent microfluidic processing. The later approach enables high-throughput analysis, statistically improves sequence coverage, and reduces price and bias. Here, we describe a method for single-chromosome sequencing that uses a microfluidic platform to capture, amplify, and barcode the individual chromosomes. Chromosome encapsulation in "gel microbeads" allows lysis of chromosomes and tagmentation of DNA, while a "microfluidic double-merger" efficiently pairs each chromosome with a unique oligonucleotide barcode, allowing together single-chromosome sequencing. Prior to down-stream analyses, the sequencing data are de-multiplexed by barcode, resulting in subsets of reads originating from a single chromosome. This high-throughput and low-bias method will enable a wide range of (cyto)genomic studies, such as allocating of genes or scaffolds to individual chromosomes, precisely delimiting translocation events, and studying recombination. In the future, the method is foreseen to be utilized in organisms with large genomes (e.g., *Triticeae* crops) as well as organisms with numerous small chromosomes (e.g., sugarcane).

USING RAMAN SPECTROSCOPY IN THE DIFFERENTIATION BETWEEN LEUKOCYTES FROM HEALTHY DONORS AND TUMOR CELL LINE THP1

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Raman spectroscopy analyses changes in the vibrations of chemical bonds caused by interaction with photons, the source of which is a laser beam. Its involvement in detecting and evaluating the chemical composition of cells or even tissues is currently the domain of research. It is a non-destructive, label-free, spectroscopic technique capable of revealing specific biochemical information about the examined sample. Our work aimed to measure the spectra of physiological neutrophils, the spectra of the THP1 tumor line cells, and their unequivocal differentiation using multivariate statistical analysis. The experiment used blood samples from healthy donors. For this experiment, standard blood smears were made in panoptic staining. Neutrophils recognized based on their typical morphology were measured in the blood smear using an immersion objective and a microscope connected to a Raman spectrometer. The second group consisted of cells of the tumor line THP1, which were cultured in a standard way. Then, a modified protocol for the manual preparation of a blood smear was used for their preparation with subsequent measurement on a Raman spectroscope. The final control sample was created by combining all used chemicals, i.e., methanol, May-Grünwald and Giemsa-Romanowski dyes, phosphate buffer, and immersion oil. The mentioned substances are a part of the resulting spectrum. After measuring all the mentioned spectra, a model for discriminant analysis was created, with the help of which the individual groups can be clearly distinguished. The neutrophil group showed the greatest variability of the spectra, as inter-individual variability also plays a certain role here. On the contrary, both the measured population of THP1 cells, as they are cells of the tumor line, and the individual measurements of the control sample were relatively homogeneous.

SYNTHESIS OF PHOTON-UPCONVERSION NANOPARTICLES FOR MICROFLUIDIC SINGLE-MOLECULE ASSAYS

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Photon-upconversion nanoparticles (UCNPs) are luminescent nanocrystals, which are doped with lanthanide ions. UCNPs are excited by near-infrared light (976 nm) and emit light of shorter wavelengths in both visible and near-infrared ranges. Photon-upconversion luminescence is detected without a background autofluorescence, which makes UCNPs extremely suitable for single-molecule assays.[1]

Here, we describe the development and optimization of UCNPs for microfluidic single-molecule bioaffinity assays. The size, composition, and internal architecture of UCNPs are carefully tuned to provide bright emission (Figure 1). The core-shell structure was optimal for Er^{3+} doped nanoparticles (emission wavelengths 554 nm and 660 nm), and the core-only structure was used in Tm^{3+} doped nanoparticles (emission wavelengths 450 nm and 802 nm).[1] Two types of surface modification were tested. The first type utilized silanization, and the second one encapsulated the UCNPs into a hydrophilic acrylic polymer. The monodispersity of all samples is characterized by optical microscopy and single-nanoparticle counting is used for absolute quantification.[2]

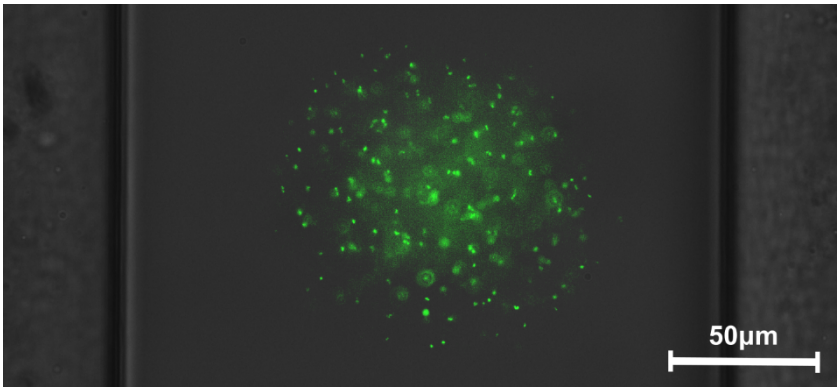

Figure 1. Microscopy of freely diffusing UCNPs in a microfluidic channel; green channel: epiphoton-upconversion (λ_{ex} 976 nm, λ_{em} 800 ± 25 nm), gray channel: dark-field (λ 800 ± 25 nm).

Acknowledgement

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

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NEW METHODS

GOING BEYOND VISUAL INSPECTION: MASTERING CELLULAR MORPHOLOGY ANALYSIS

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Cellular morphology is a crucial aspect in the study of biological systems, and accurate quantification of cellular morphology is essential for gaining insights into cellular function. Here, we present an automated assay for the assessment and quantification of cellular morphology based on standard principles of confocal microscopy. Our procedure employs the combination of 3 cellular compartments staining, including the nucleus, cytoplasm, and membrane, to obtain a comprehensive analysis of cellular characteristics.
The image processing and analysis in our assay are fully automated using R script programming, enabling accurate and efficient quantification of cellular shape and morphology parameters. In addition, our strategy determines the functional aspects of cell metabolism/viability and the cell cycle phase on a single-cell level.
The method supports the statistical comparison of numerous cell lines or established cell clones' morphology, facilitating the identification of unique cellular characteristics across different populations. Importantly, our assay is designed for researchers without any knowledge of programming, and the outputs are provided in a PDF report, including images of publication quality. Additionally, the open data format of the results enables subsequent analysis in third-party software for further data exploration. Overall, our automated assay represents a significant simplifying of cellular morphology analysis, providing accurate quantification of cellular characteristics to aid in the understanding of cellular function.

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PULSE SHAPE DETECTION WITH ANGULAR RESOLUTION FOR FLOW-CYTOMETRIC CELL SORTING

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Currently, cell sorting in flow cytometry is heavily reliant on fluorescence intensity signals from respective stainings of cell subsets of interest. Over decades, this approach has been improving and became a well established technique in biomedical research. The use of fluorescent staining provides a deep insight for immunological questions but it also has disadvantages. The staining procedure can affect cell functionality or even require fixation. Clearly, this impedes, e.g., culturing of sorted cells.
In addition to fluorescence signals, flow cytometers also acquire scattered light intensities for each measured object. When cells pass the laser beam, they generate time-dependent intensity signals at the detectors. In standard instruments, the resulting signal pulses are characterized by three read-out values: height, area, and width. For a profound analysis of scattered light signals, the complete pulse shapes and angular resolution of the light scatter are required.
For this purpose, we set up a flow cytometer with custom-made signal acquisition electronics, modified optics, a fiber array in forward scatter direction, and a mechanical sorting unit. The decisions for cell sorting are made by assigning the events to clusters. The clusters are generated in the space of wavelet transform coefficients that are calculated from the pulse shapes in real time.
With this custom-built setup, sorting based on comprehensive information from scattered light is possible. As a proof-of-principle, we showed specificity of the scattered light signals for the cell cycle phases. Fluorescent staining was used to select appropriate clusters and for validating the results of the sort. Ongoing and future work is targeted to sorting purely based on scattered light. Additionally, further applications in, e.g., bacteria analysis and sorting are under investigation.

BIOLOGICAL EVALUATION OF UPCONVERSION NANOPARTICLES

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In the last decade, luminescent lanthanide-doped upconverting nanoparticles (UCNPs) have gained considerable attention as a promising theranostic agent in biomedicine. In this study, particles, ca. 25 nm and 112 nm in diameter, were prepared by a high-temperature coprecipitation of lanthanide chlorides. To ensure optimal dispersion of UCNPs in aqueous milieu, they were coated with three different polymers containing reactive groups, i.e., poly(ethylene glycol)-alendronate (PEG-Ale), poly(N,N-dimethylacrylamide-co-2-aminoethylacrylamide)-alendronate (PDMA-Ale), and poly(methylvinyl ether-co-maleic acid) (PMVEMA). For cytotoxicity tests we used C6 cancer cell line (a rat glioma cell line); as a model of healthy cells, human mesenchymal cells (hMSC) were utilized. All prepared UCMPs were tested for their cytotoxicity by Alamar Blue assay and comet assay. In general, large nanoparticles (112 nm) were less toxic than small nanoparticles (25 nm). UCNP-PMVEMA particles were the most toxic due to their high solubility in artificial lysosomal fluid. The highest uptake was seen with neat UCNPs, followed by UCNP@Ale-PDMA and UCNP@PMVEMA. UCNP@Ale-PEG particles were the least abundant in the cells because PEG is bioinert to a number of biological components found in the human body including proteins and does not exhibit cell-adhesive properties. PDMA was identified as the most promising coating for the UCNPs to be used in cell experiments because it provided reasonably high cellular uptake and relatively low cytotoxicity by protecting the particles from dissolution in PBS; in addition, it provided superior colloidal stability in water. The amino groups also made PDMA suitable for subsequent conjugation with biomolecules. This study emphasized the importance of combined cytotoxicity and degradation testing to fully evaluate the safety of the UCNPs intended for in vitro and in vivo applications.

COMPARISON OF COOH-RICH SURFACES WITH HMDSO POLYMERS USING HOLOGRAPHIC MICROSCOPE

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This study aimed to compare two types of films prepared by plasma assisted polymerization on standard polystyrene cultivation dishes (TPP). First, the carboxyl-rich films (COOH1 and COOH2), which are of high interest for bio-applications thanks to their negative surface charge, high hydrophility and reactivity, allowing the formation of covalent linkages between biomolecules and a surface. Second, the films based on hexamethyldisiloxane (HMDSO and HMDSO+O2), that are naturally hydrophobic, but the condition of the polymer deposition and presence of oxygen molecules may change it to more hydrophilic surface. We studied viability of HaCaT (human keratinocytes) and VSMC (rat vascular smooth muscle cells) cells growing on these surfaces, their ability to resist the trypsin treatment and their attachment and migration rate using holographic microscope (Telight). HMDSO based polymers do not allow attachment of cells in the same time scale as standard cultivation surface or COOH dishes – it takes much longer for cells to start to spread. This delay might be the reason for cells to achieve lower numbers in 3 days-long cultivation compared to both COOH dishes and untreated control (assessed by ATP and resazurin assays). In our previous study we confirmed association of the resistance to TRYPSIN treatment and the strength of cell adherence (Buchtelova 2023). We can than conclude that cells growing on HMDSO layer have lower adherence to this surface than to the control surface and COOH surfaces, which is also reason for their higher migration rate.

Acknowledgement
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Reference
Buchtelová, M. et al., 2023. "Insight into peculiar adhesion of cells to plasma chemically prepared multifunctional “amino-glue” surfaces." Plasma Processes and Polymers; doi:10.1002/ppap.202200157.

RAMAN MICROSPECTROSCOPY OF STREPTOMYCES AND THEIR SECONDARY METABOLITES

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Streptomyces is a genus of bacteria known for its filamentous growth and ability to produce a wide range of bioactive compounds. Streptomycetes are used in the production of many antibiotics, antifungals, and other drugs, and have shown potential as a source of novel natural products with pharmaceutical and biotechnological applications.
Raman microspectroscopy is a non-destructive analytical technique that can provide information about the chemical composition and molecular structure of a sample. It involves illuminating a sample with a laser and measuring the scattered light, which is shifted in frequency due to the Raman effect, to obtain a vibrational spectrum.
We analysed several strains of *Streptomyces* cultivated under different growth conditions and reference samples of *Streptomyces*-derived antibiotics and antifungals with Raman microspectroscopy. We found spectral signatures of the secondary metabolites in the living mycelia of the *Streptomyces* sp. samples. This research could be useful for the identification of potential new antibiotics and their producers, analysis of strains producing known antibiotics, optimization of fermentation conditions and times for maximization of antibiotic production, as well as selection of the most effective producers of antibiotics.

ENDOPLASMIC RETICULUM STRESS RESPONSE IN EXPANDABLE LUNG-LIKE EPITHELIAL CELLS

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Lung cells are constantly exposed to internal or environmental stress cues that can evoke various pathologies. Endoplasmic reticulum (ER) is primarily involved in proteosynthesis of secreted and membrane proteins, but also provides a cellular signaling hub integrating various stress factors. Accumulation of misfolded and/or underglycosylated proteins then evokes a general cell stress response driven by unfolded protein stress (UPR) signaling. Understanding how the UPR affects proliferation and differentiation of lung alveolar cells and regeneration of pulmonary tissues is so far unclear.
To study the stress signaling in lung cells, the primary human lung cells or lung cancer cell lines are used. Such models are however difficult to propagate, they quickly acquire senescent phenotype or they represent a pathological model. Here we established the human embryonic stem cells (hESCs)-derived expandable lung-like epithelial (ELEP) cells with high proliferation rate and differentiation capacity to all principal lung cell types. Using ELEPs we addressed the ER-driven cell response to pharmacologically induced proteosynthetic stress.
We show the high sensitivity of ELEP cells to disruption of ER homeostasis that is followed by changes in cell morphology and molecular phenotype. These changes can be modulated by chemical chaperons or small molecule inhibitors of UPR and translation machinery. ER signaling is therefore an important molecular circuitry affecting the phenotype of lung cells and tissue homeostasis.

Acknowledgement
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PITFALLS OF MEASUREMENTS CYANOBACTERIAL VIABILITY AFTER STRESS EXPOSURE VIA FLOW CYTOMETRY

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Cyanobacteria are ecologically important phototrophic bacteria capable of oxygenic photosynthesis. Cyanobacteria played a key role in planet evolution and are responsible for the oxygenic atmosphere. Cyanobacteria synthesize many interesting metabolites such as glycogen, lipids, various pigments or polyhydroxyalkanoates (PHA). PHAs are biodegradable biopolymers with similar properties as petrochemical plastics and they occur as intracellular granules. Numerous prokaryotes including cyanobacteria use PHA primarily as a storage of carbon and energy in the form of intracellular granules, secondary PHB raises the fitness of bacteria because it helps the bacteria to survive stress conditions e.g., osmotic stress, UV radiation or freezing. As PHAs are economically interesting metabolites PHA production employing cyanobacteria seems to be the promising way of CO_2 valorisation. Nevertheless, a reliable technique for measurement of the viability of employed cultures is an important tool to monitor the biotechnological process. In this study, we sought a reliable and suitable probe for our cyanobacterial cultures capable of PHA accumulation. The biggest difference from heterotrophic bacteria is that cyanobacteria contain a wide spectrum of various pigments, from chlorophylls, through carotenoids to phycocyanines or phycobiliproteins and the pigmentation and even resulting autofluorescence changes during cultivation. Therefore, numerous commonly used probes are due to the presence of pigments inapplicable. Moreover, cyanobacterial strain *Synechocystis* possess an extra layer on the surface, called S-layer, which could make staining of cells difficult. For that purpose, we were looking for a probe(s) that does fit with cyanobacteria, stains dead cells, is suitable for flow cytometry, and does fit with our cyanobacteria cells at various physiological states and flow cytometer. Together with the probe, also troubleshooting of sample preparation was necessary.

HYALURONIC ACID IN RADIOBIOLOGY

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Hyaluronic acid is a polysaccharide that is the main component of the extracellular matrix. It is involved in the regulation of various biological and damage-response processes in the organism. This interesting molecule can find application in many biomedical fields, including the field of radiation protection or for the therapy of damaged tissues after the harmful effects of ionizing radiation. Common feature of all organ damage is a significant change in the composition and structure of extra cellular matter, which is dependent on the received radiation dose and cellular composition of the organs. It has been proved that exogenous administration of hyaluronic acid reduces liver and lung tissue damage through the mechanism of reducing the production of pro-inflammatory cytokines induced by T lymphocytes. The administration of hyaluronic acid particles is rising many questions regarding their production, size, stability, biodegradability and their viability of cell populations. Another question is their stability after the application of ionizing radiation.
Our research aim is the treatment of radiation lung damage, which is lung condition leads to irreversible fibrotization of the lung tissue especially in oncology patients. Therefore, an extensive study was conducted to address the safety of hyaluronic acid nanoparticles, which could effectively prevent this disease after application before planned radiotherapy.
The study was carried out in an in vitro system on cell lines derived from lung cell progenitors without and with the application of ionizing radiation and verified higher biocompatibility using a cytotoxic test. The viability of the tested lines was in favor of the lines that were given hyaluronic acid nanoparticles.

MODELING DRUG DELIVERY TO BRAIN IN VITRO

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Glioblastoma (GB) is the most common primary brain tumor in humans that is uniformly fatal, since tumor resistance to conventional therapy which is associated with low permeation capacity of the drugs through the blood brain barrier (BBB). Brain microvascular endothelial cells, pericytes, astrocytes, tight junctions and the basement membrane together form a physical barrier to maintain a intracranial environment. Although in GBM the BBB is partly disrupted, it still not enough permeable for the effective drug delivery.
Our aim to design a bioengineered multicellular blood brain barrier in vitro model to improve permeation ability of nanopolymers by functionalization with adenosin 2A receptor agonists or peptide BBB shuttles.
Transwell studies are widely used and have provided a great array; however, its static nature limits the extent to which results can accurately model its physiology.
Polymers carrying trans-BBB peptides AGILKRW and SGTQEEY and labelled with fluorescent ATTO488 were tested on static model of blood-brain-barrier. After 24 h, green fluorescence was detected in the lower compartment representing the brain matrix. Also, the polymer based on HPMA linked to adenosine agonist lexiscan and polymer with fluorescent label only were used. No fluorescence from lexiscan-binded polymer was recorded.
The dynamic model consists of a 3D layered system (similar to a Transwell ensemble) on an enclosed ibidi slide and employing flow of cell media to better resemble the physiological environment of BBB. This allows the recreation of BBB barrier in vitro with the added feature of establishing fluidic circuits to perfuse modeled system.
The shape, density and orientation of cell culture under flow is changed as cells react to shear stress by reorganization of whole cell layer. Cultivation of cells under flow mimic more physiological, in vivo cell behaviour.
Peptide BBB shuttles will be further tested in dynamic model of BBB.

SINGLE CELL BIOLOGY AND SIGNALING

NEW ROLES OF PATTERN RECOGNITION RECEPTOR-INDUCED TRANSCRIPTION FACTOR SIGNALLING IN MONOCYTE RESPONSES

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Background: Tight regulation of signalling cascades, that are activated through pattern recognition receptors (PRRs) is crucial for proper function of innate immune cells, including monocytes. Efficient immune response and clearance of microbial pathogens requires strictly controlled cooperation of several transcription factors (TFs), such as NFkB and NFAT. While the role of Calcineurin/NFAT pathway in terminally differentiated myeloid cell responses is well established, potential impact of treatment with Calcineurin inhibitors on monocyte responses is unknown. Moreover, rapid analysis of TF activation at the single cell level remains important challenge.
Methods: Using commercially available kits for flow cytometry, we have established intracellular flow cytometry (IC-FC) protocol as a tool for rapid evaluation of TF activity in primary monocytes. We analyzed nuclear translocation of NFAT in human monocytes upon PRR activation using confocal microscopy. Similarly, we analyzed the transcripts regulated by NFkB and NFAT in stimulated human monocytes.
Results: Confocal microscopy analysis revealed that both LPS and Zymosan induce NFAT nuclear translocation in primary monocytes. Using IC-FC method, we identified non-classical monocytes as the major NFAT-expressing subset. Preliminary results obtained from TF activity assessment indicate that this high-throughput method could be a valuable tool for studying TF pathway activation in the cell subset of interest. Based on the mRNA expression analysis in stimulated $CD16^+$ monocytes, we hypothesized that NFAT co-regulates some cytokines and chemokines including *CSF2, CCL2* and *CXCL1,* but not *CXCL2*.
Conclusion: In summary, our data suggest that NFAT plays an important role in regulating anti-microbial immune responses in human monocytes. Further studies will aim to identify NFAT regulated genes and interaction partners. Collectively, these results could contribute to development of targeted therapies for acute and chronic inflammatory diseases.

SPATIALLY RESOLVED SINGLE-CELL TRANSCRIPTOMIC PROFILING IN FORMALIN-FIXED PARAFFIN-EMBEDDED (FFPE) TISSUES

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Formalin-fixed paraffin-embedded (FFPE) tissues are the most widely used clinical sample types in histology and molecular diagnosis, but these samples are often challenging for single-cell transcriptomic analysis due to RNA degradation and protein crosslinking. A spatial transcriptomics technique with high detection efficiency and single molecule resolution is required to accurately profile the gene expression in FFPE samples in situ. Vizgen's MERSCOPE® Pplatform, built on multiplexed error robust in situ hybridization (MERFISH) technology, directly profiles intact tissue's transcriptome with subcellular spatial resolution. Here, we demonstrate the FFPE MERSCOPE workflow in tissues from 10 mouse and human samples, including archival clinical samples. In each sample, hundreds of thousands of cells were captured with >100 million transcript counts, generating detailed spatial transcriptomic data for the profiled genes in each sample. A comparison of FFPE and matched fresh frozen samples indicated that the FFPE workflow performs similarly in detection efficiency as compared to the fresh frozen protocol. We further demonstrated the MERSCOPE FFPE workflow is compatible with protein imaging by performing simultaneous protein-based cell boundary staining with MERFISH to accurately profile gene expression and map cell types in archival clinical human samples. Finally, we constructed a spatially resolved single-cell atlas across eight major tumor types, mapped and cataloged different cell types within the tumor microenvironment and systematically characterized the gene expression among cells. This study demonstrates the potential for spatially resolved transcriptomic profiling of FFPE samples at single- cell level with MERSCOPE to contribute to a wide range of biomedical research areas, including many applications to study human diseases.

THE EFFECT OF TSG-6 IN COMPLEX WITH HYALURONAN ON CELL THROUGH SURFACE RECEPTOR LAYILIN

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Tumor necrosis factor stimulated gene 6 (TSG-6) is a small soluble protein expressed in various cell types during proinflammatory conditions and acts as an anti-inflammatory cytokine. Despite its size, TSG-6 can interact with a broad range of molecules. Among these is hyaluronan (HA), which is one of the most abundant glycosaminoglycans in the human body. HA can interact with cells through surface receptors and act as both an extracellular matrix scaffold and a signaling molecule. The molecular weight (MW) and structural organization of HA are crucial for its effects on the surrounding environment. TSG-6 can modify the structure of HA, leading us to hypothesize that it can indirectly alter the cell response to inflammation.
Different cell types have various surface receptors for HA, and one such receptor is LAYN, a transmembrane protein with a link module that is a domain typical for HA binding proteins. LAYN is believed to play a role in cell adhesion and motility, but its behavior in the context of various MW HAs under proinflammatory conditions, where HA structure is modified by TSG-6, is unknown.
To investigate this, we generated MCF-7 clones that overexpress LAYN and examined the effects of TSG-6 and HA of varying MW on these cells. We used different biochemical and cytometric approaches to study the molecular interactions of these molecules and subsequently monitored the expression of different protein markers to observe their effects on the cell response.

MULTIOMIC PROFILING OF IMMUNE DIVERSITY, CELL BY CELL

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The immune system is incredibly dynamic, comprising many complex cell types and innumerable specificities to respond to infection, injury, and inflammation. While single cell transcriptional profiling is a powerful tool for studying the immune system, to truly understand immune complexity is also helpful to use multiomic approaches that can measure protein expression, antigen interaction, and clonal diversity from the same cell. With novel single cell multiomics techniques, was showed, that you can perform multiomic phenotyping in hundreds to hundreds of thousands of cells by accessing cell surface proteins, T-and B-cell repertoire diversity, antigen specificity, and gene expression analysis. There will be demonstrated an option to discover new cell types and states using whole transcriptome analysis even within ultra-rare cell populations as well as development of advanced cell therapies using CAR-T and TCR-T cells.

HIGH EFFICIENCY GENTLE SORTING IMPROVES OUTGROWTH FOR SINGLE-CELL CLONING OF MAMMALIAN CELLS

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Single-cell cloning is required for biological workflows such as production of antibodies, stem cell therapy, and gene editing. Colonies that do not arise from a single cell can result in discrepancies in product quality, instability of recombinant proteins, and issues with cellular growth rates. Efficient cloning methods should be able to discriminate cells of interest from a heterogenous population and ensure that it can deposit one cell per well to establish cell lines with single cell origin. In addition to selecting a cloning method, cells require optimized cloning medium to ensure survival at such low densities by using formulations that mimic a greater number of cells to support cell outgrowth. The WOLF and WOLF G2 Cell Sorters alongside the N1 Single Cell Dispenser rely on microfluidics technology to gently sort at a pressure of <2 psi, reduce shear stress, and maintain a close system for favorable biosafety. Furthermore, 50 mL of customized sheath buffer can be used to prevent increase cell death during sorting. The WOLF Cell Sorter with the N1 Single Cell Dispenser was used to sort suspension (CHO ES, Expi293F, and Jurkat) and adherent (HEK293, MCF-7, A549, CHO-K1) cell lines at 1 cell/well and were grown up to day 14 in suggested optimal cloning medium. Dispensing with the WOLF was compared with limiting dilution to demonstrate increased efficiency when using this platform for cell line development.

AUTOMATED PROCESSING OF SOLID TISSUES INTO SINGLE CELLS OR NUCLEI FOR SEQUENCING

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Single cell sequencing is transforming our knowledge of cells and tissues, revealing new cell types and states, and increasing our understanding of what cells are present, what they are doing, and how cells and tissues function in normal and diseased states. Single-cell sequencing technology, including targeted sequencing, single cell or nuclei RNA-Seq, ATAC-Seq and other sequencing application, is now routinely providing un-paralleled insights into the genomics of tens of thousands or more single cells and nuclei per experiment. The single cell workflow after the generation of single-cell suspensions is well established with multiple commercial systems available to prepare single cell libraries. 35,000 30,000 25,000 20,000 15,000 10,000 5,000 The development of standardized solid tissue dissociation processes into single cells is critical to the development of single cell biology. Automated systems remove user variability and deskill the production of high-quality single cell and nuclel suspensions, helping democratize single cell biology. Reproducible methods with simple to use instrumentation will be required for future clinical applications. S2 Genomics has developed the patent-pending Singulator System to automatically dissociate solid tissues into single cell or nuclei suspensions. The system quickly processes solid mouse, rat, human, or patient-derived xenograph tumors (or other) solid tissues in disposable cartridges into single cell suspensions using tissue-specific enzymatic, or into single nuclei suspensions using almost universal chemical formulations. The system was used process a wide range of fresh mouse, rat, or human solid tissues, e.g., 3 to >600 mg, into highly viable single-cell suspensions in 20 to 60 min with high yields or to process fresh, frozen, or OCT tissue samples into nuclei in seven min. Ten pre-production prototype instruments were developed, and six pre-production prototype systems have been deployed and tested in an Early Technology Access program.

A NOVEL PLATFORM PROVIDING AUTOMATED, CONSISTENT AND GENTLE CELL ISOLATION FROM TISSUES FOR DOWNSTREAM APPLICATIONS

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Tumor-infiltrating lymphocytes (TILs) are lymphocytic cells that have invaded the tumor tissue. Adoptive cell therapy (ACT) of TILs is a strategy to modify the immune system to recognize tumor cells and carry out an anti-tumor effector function. Today, TIL therapies consist of ex vivo expansion of TIL from resected tumor material and adoptive transfer into the cancer patient. Although the treatments have shown promising results in various tumor types, the production and reactivity of TIL products from many solid tumor types is variable and requires further research. Therefore, an efficient way to isolate TILs is crucial for basic and clinical research applications. Conventional cell separation technologies such as Ficoll gradient centrifugation, column-based magnetic separation, and FACS single cell sorting have issues in low cell recovery, compromised cell viability, and are time-consuming. Here we present an easy and efficient immune cells separation method from a lung tumor biopsy sample using the novel cell separation platform -MARS®. MARS® platform employs two cell separation technologies: active-microfluidics acoustics as well as magnetic separation. Acoustic separation is based on the difference in physical parameters (size, etc.) and allows for the removal of lysed cell debris, dead cells, and other small particles. the MARS® magnetic cell separation technology performs a specific selection of tumor cells or immune cells. Both processes produce high purity and high recovery of target cells. The high-speed separation is controlled at low pressure and maintains TIL cell viability and functionality, so the isolated cells are ready for expansion.

T-CELL SIGNATURES CHARACTERIZING PEDIATRIC TREATMENT NAIVE TYPE 1 DIABETES MELLITUS

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Type 1 Diabetes Mellitus (T1DM) is a chronic autoimmune disease characterized by the destruction of pancreatic beta cells by autoreactive T cells. In this study, we aimed to identify T-cell signatures characterizing pediatric treatment naive T1DM using single-cell RNA sequencing (scRNAseq) phenotyping, single-cell immune profiling, and T-cell receptor (TCR) repertoire analysis. We performed a large prospective study that included patients with T1DM at diagnosis and one-year post-diagnosis, healthy controls, and healthy relatives with detectable pre-diabetic autoantibodies. Our analysis revealed clonal expansion of effector and memory CD8 T cells and CD4 T cells in patients with T1DM, indicating a dysregulation of T-cell homeostasis. Using established scRNAseq analytical methods, we identified specific gene expression patterns that were associated with T1DM progression. We discovered gene signatures characterizing CD4 and CD8 T cells that correlated with disease progression at one year. The integration of scRNAseq, single-cell immune profiling, and TCR repertoire analysis allowed us to obtain a comprehensive understanding of the cellular and molecular mechanisms underlying T1DM progression. Our findings provide valuable insights into the pathogenesis of T1DM and have important implications for the development of novel therapeutic targets.

ANALYSIS OF CELLULAR RESPONSE TO SELECTIVE INHIBITORS OF HASPIN KINASE WITH FUCCI CELL CYCLE REPORTER

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The atypical kinase Haspin is required for normal mitosis progression in mammalian cells, and has a specific function in chromosome alignment, centromeric cohesion, and spindle stability. Haspin is expressed throughout the entire cell cycle, with the highest expression in non-dividing haploid spermatids. However, it is still not sufficiently explained why Haspin-null embryos develop normally and show no anatomical defects except testicular anomalies. Several studies elucidating the biological role of Haspin in the cell cycle use available but insufficiently selective small-molecule inhibitors. Therefore, we used novel (proprietary) Haspin inhibitors that fulfil the requirements for a selective chemical probe and allow us to address mechanistic and phenotypic questions regarding Haspin-regulated processes in cell-based assays.
We and others have demonstrated the great potential of the Fucci (= Fluorescent Ubiquitination-based Cell Cycle Indicator) reporter system in the quantitative analysis of the response of cell populations to drugs. Here we used the Fucci4 system, which uses 4 cell cycle sensors to label the entire cell cycle and can reveal detailed dynamics of cell behaviour in the presence of Haspin inhibitors. Moreover, we developed pipeline for automatic cell tracking and analysis of the cells in time lapse images. The results presented herein demonstrate that the new highly selective Haspin inhibitors induce a different effect on cell cycle progression, in contrast to less selective commercially available inhibitors.

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DISRUPTION OF PROTEIN SYNTHESIS INDUCED BY PAHs IN HUMAN HEPATIC CELL LINES

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Polycyclic aromatic hydrocarbons (PAHs) and related pollutants are a prominent group of environmental contaminants. Human exposure can occur primarily through ingestion, inhalation and skin contact. PAHs are primarily metabolized by an enzymatic pathway regulated by the aryl hydrocarbon receptor (AhR), including cytochrome P450 family I enzymes, which may contribute both to detoxification of PAHs and to production of their reactive toxic metabolites. Both parent PAHs and their metabolites have been shown to exhibit multiple types of toxicity in exposed cells, including their known genotoxic, carcinogenic, or teratogenic effects. Current studies indicate that they may also contribute to disruption of endocrine signaling and cell metabolism. In the present study, we evaluated their potential impact on proteosynthesis disruption in HCC-derived and immortalized hepatocyte cell lines (HepaRG, MIHA), using model PAHs, which were identified as either potent human AhR ligands or low affinity AhR agonists. We monitored translation using surface sensing of translation (SUnSET) flow cytometric assay, as well as by Western blotting detection of puromycin incorporation into newly synthesized proteins. Following 72-h exposure to PAHs, we found that a strong AhR ligand decreased the rate of total protein synthesis in both liver cell lines, in a concentration-dependent manner. Our results suggest that PAHs may, likely in AhR-dependent manner, decrease proteosynthesis in target cells and that their AhR-dependent metabolism could contribute to these effects.

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Keywords: AhR; liver cells; PAHs; translation; SUnSET

ESSENTIAL ROLE OF FLOW CYTOMETRY IN SCREENING OF BACTERIAL CELLS AFTER EXPOSURE TO STRESS CONDITIONS

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Microorganisms belong to a very important part of the environment because they are near the bottom of the food chain. They serve as markers of changes in the environment due to close physical and chemical interactions and influence the quality of soil and to face adverse conditions through the protective mechanisms (pigments, storage compounds, cryoprotectants, etc.). The understanding of the principle of robustness of bacteria with respect to various stress conditions could be used in wide applications in biotechnology, the food industry and bioremediation. Therefore, it is necessary to define the viability of bacterial cells. For this purpose, flow cytometry provides valuable information about the cells such as the impact of stressors via viability parameters utilizing specific fluorescent probes.

In our study, we focused on the impact of stressors such as high temperature (50, 80°C), low temperature (–20°C), osmotic stress (0, 50, 150, 200 g/L NaCl) and cycles of repetitive freezing and thawing on cell viability. The experimental conditions were adjusted to individual microorganisms that we selected based on the production of polyhydroxyalkanoates which are known as intracellular storage and stress protectants. Within stress experiments, we used a mesophilic purple bacterial strain *Rhodospirillum rubrum* (DSM 467) and a thermophilic bacterial strain *Caldimonas thermodepolymerans* (DSM 15344). As well as duration and the intensity of stresses, the fluorescent probes (propidium iodide, SYTOX™ Blue and fluorescein diacetate) were optimized to determine the viability of cells regarding the optimal temperature of cultivation, stressor, and presence of pigment. To confirm our results, we have also characterized samples by electron microscopy, in particular cryo-SEM and TEM.

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CRISPR-CAS9 ENGINEERED TETRASPANIN-DEFICIENT CELL LINES FOR THE ANALYSIS OF EXTRACELLULAR VESICLES

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Tetraspanins are essential proteins that play a crucial role in the biogenesis, secretion and uptake of extracellular vesicles (EVs). These transmembrane proteins can form tetraspanin-enriched microdomains (TEMs) on the plasma membrane by interacting with each other and with additional membrane proteins and lipids. These TEMs are critical for sorting proteins and lipids into EVs, regulating EV biogenesis and release, and facilitating EV uptake by target cells.
Our study focuses on understanding the roles of individual tetraspanins in EV biology. Specifically, we are analysing EVs from four cell lines, including the wild type cell line DU145 and three tetraspanin-deficient cell lines prepared using CRISPR-Cas9 gene editing. After producing of conditioned media with EVs, we isolate them using ultracentrifugation coupled to sucrose cushion. We confirm the tetraspanin deficiency by western blotting and analyze the size and concentration of EVs using dynamic light scattering and cryo-electron microscopy. Next, we quantify their protein composition using tandem mass spectrometry.
Each tetraspanin has a unique function in the process of EV biogenesis and secretion. CD9, for example, engages in the biogenesis and secretion of EVs in various cell types, while CD63 is implicated in the sorting of proteins and lipids into EVs. CD81 plays a role in the fusion of EVs with target cells, leading to the uptake of EVs by target cells. However, comprehensive analysis of the major tetraspanin knock-out (KO) cells, *i.e.,* CD9 KO, CD63 KO and CD81 KO, in the same background has not been performed.
Understanding the specific roles of individual tetraspanins in EV biology is essential for revealing the mechanisms underlying EV biogenesis, secretion and uptake. Our experimental approach will provide a comprehensive understanding of the roles of tetraspanins in EV biology, therefore contributing to the broader field of EV research.

TRYPTAMINE-BASED COMPOUNDS PREVENT INFLAMMATORY DAMAGE IN A MODEL OF INTESTINAL EPITHELIAL CELLS

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Chronic inflammation in gut can lead to the development of several disorders, including inflammatory bowel disease, Crohn's disease, or inflammation-associated cancer. Pregnane X receptor (PXR) is a ligand-activated nuclear transcription factor expressed in gastric tissues with a major role in drug transport and metabolism processes. In addition, PXR can regulate the inflammatory process and maintains the intestinal epithelium function. Human gut microbiota produces tryptophan metabolites, which can activate PXR, and other receptors involved in the maintenance of gut homeostasis, the aryl hydrocarbon receptor (AhR). Here we used chemically modified tryptamines targeting the PXR and/or AhR and assessed their anti-inflammatory and epithelial barrier function protective activity in human colon cancer epithelial Caco-2 cells. We showed that selected tryptamine derivatives activating PXR prevented the upregulation of pro-inflammatory chemokines (CXCL-8 and MCP-1) in cells exposed to a mixture of inflammatory cytokines. In line with this, tryptamine derivative activating both AhR and PXR prevented the downregulation of tight junction constituents, occluding and TJP-1/ZO-1, under inflammatory conditions. Finally, we evaluated the epithelial barrier-protective activity of tryptamine derivatives by detecting transepithelial resistance and permeability of differentiated Caco-2 cell monolayers. In conclusion, we show that specific tryptamine derivatives may decrease inflammatory damage elicited by inflammatory cytokines.

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Keywords: PXR; AhR; inflammation; microbiota; tryptamine