10th International Conference Analytical Cytometry

21. – 24. 9. 2019

Hotel Jezerka, Seč-Ústupky, Czech Republic

CSAC

Czech Society for Analytical Cytometry

BOOK OF ABSTRACTS

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CONTENTS

CELL BIOLOGY AND SIGNALLING

(P9) The impact of loss of the aryl hydrocarbon receptor (AHR) on metabolism and functional properties of human breast carcinoma MCF-7 cells
Karasová M., Fedr R., Ciganek M., Machala M., Matthews J., Vondráček J.

(P10) Functional role of AHR in dioxin-mediated disruption of apoptosis control in a human model of adult liver progenitors?
Krkoška M., Svobodová J., Kabátková M., Hyršlová Vaculová A., Vondráček J.

(P11) CHK1 inhibition facilitates the cooperative anticancer action of platinum-based drugs and trail
Krkoška M., Herůdková J., Paruch K., Nevědělová K., Sova P., Hyršlová Vaculová A.

(P12) Evaluation of membrane bound isforms of adenylate cyclases
Kubala L., Daďová P., Litvinchuck A., Dobler L., Jaroušek R.. Šindelář M., Švenda J., Parůch K.

(P13) Modulation of radiation-induced injury by hyaluronic acid nanoparticles
Lierová A., Pejchal J., Jeličová M., Kašparová J., Korecka L., Bílkova Z., Šinkorová Z.

(P14) Treatment of glioblastoma using superparamagnetic nanoparticles with modified surface
Mareková D., Turnovcová K., Horák D., Kaiser R., Jendelová P.

(P15) Evaluation of targeting and efficacy of nanoparticles-mediated combined cancer treatment
Buociková V., Miklíková S., Ploth K., Tyčiaková S., Majerová K., Smolková B., Matúšková M.

(P16) Resistance to Trypsin/Edta treatment is caused by increased cell adhesion on CPA layers
Medalová J., Černochová P., Bartošíková J., Bláhová L., Vondálová Blanářová O., Zajíčková L.

(P17) Toll-like receptors in prostate cancer carcinogenesis and therapy-resistance
Muresan X.M., Drápela S., Slabáková E., Remšik J., Fedr R., Culig Z., Souček K.

(P18) Characterization of anoikis resistance in mammary cancer clones derived from long lived CTCS
Pícková M., Dvořák V., Fedr R., Souček K.

(P19) KDM5D is associated with neuroblastoma chemoresistance to ellipticine
Podhorská N., Hraběta J., Eckschlager T.

(P20) Non-telomeric function of telomerase complex in pattern recognition receptors signalling driven inflammation of human tissue organoids
Jose S.S., De Zuani M., Buřilová P., Tidu F., Frič J.

(P21) Electrostimulation of murine embryonic stem cells enhanced cardiomyogenesis
Šafaříková E., Stříteský S., Ehlich J., Omasta L., Salyk O., Vala M., Weiter M., Kubala L., Víteček J.

(P22) NF-kB and NFAT pathways shape mesenchymal stem cell response to PAMPs
Tidu F., De Zuani M., Jose S. S., Bendickova K., Fric J.

(P23) Live-cell analysis of 3D spheroids: label-free & fluorescent cell health reporters
Alcantara S. L., Lovell G., Oliver M., Patel K., Dale T., Holtz N., Endsley E., Trezise D.

(P24) Glioblastoma multiforme-derived cells and their contribution to neovascularization
Turnovcová K., Mareková D., Kaiser R., Herynek V., Jendelová P.

(P25) Role of TROP-2 expression in beta catenin expression and activity in mammary cancer cells
Vacek O., Kryštofová K., Remšík J., Binó L., Beneš P., Souček K.

(P26) Comparison of ALDH expression in huvecs and rabbit endothelial progenitor cells using flow cytometry
Vašíček J., Baláži A., Svoradová A., Tomková M., Chrenek P.

(P27) Time-lapse microscopy of primary chronic lymphocytic leukaemia cells locomotion in response to microenvironmental stimuli
Vondálová Blanářová O., Čada Š., Janovská P., Poppová L., Kotašková J., Pavlová Š., Vališová M., Bryja V.

(P28) Role of CD9 tetraspanin in breast cancer
Kvokačková B., Fedr R., Remšík J., Souček K.

(P29) Development of 3D cultivation model of immortalized human MIHA hepatocytes for evaluation of endobiotic and xenobiotic metabolism
Nevědělová K., Krkoška M., Kabátková M., Nekvindová J., Hyršlová Vaculová A., Vondráček J.

(P30) Revival of chemosensitivity and resistance assay in solid tumors – a single-cell significance evaluation
Šímová M., Drbal K.

(P31) Urban dust and diesel exhaust particles elicit estrogen-like effects on er-dependent gene expression and cell cycle progression
Vázquez-Gomez G., Pivnička J., Machala M., Ciganek M., Hýžďalová M., Karasová M., Vondráček J.

CLINICAL CYTOMETRY

LECTURES:

Reproducibility of flow cytometry through standardization: Opportunities and challenges
Tomáš Kalina

The effect of early postnatal supplementation of newborns by probiotic strain escherichia coli on regulatory T cells
Jiří Hrdý

Granulocytes in CVID patients are activated and possess suppressory function
Marcela Vlková

POSTERS:

(P32) Does characteristic phenotype for plasma cell leukemia exist?
Bezdekova R., Kralova R., Vsianska P., Penka M., Stork M., Pour L., Rihova L.

(P33) Usefulness of 6-colour multiparameter flow cytometry in canine lymphoma phenotyping
Jeklová E., Levá L., Faldyna M.

(P34) The dysregulation of NK cells and monocyte subpopulations in in the preclinical phase of rheumatoid arthritis
Prajzlerová K., Kryštůfková O., Hánová P., Hulejová H., Gregová M., Pavelka K., Vencovský J., Šenolt L., Filková M.

(P35) Flow cytometric characterization of lymphoma in dogs from the Chech Republic – A retrospective study
Levá L., Jeklová E., Pichlová B., Faldyna M.

(P36) Analysis of gene expression in CD26+ CML leukemic stem cell population
Smitalová D., Loja T., Čulen M., Herudková Z., Budinská E., Mayer J., Ráčil Z., Romžová M.

(P37) A simple no-wash assay for the detection of tumor nuclei ploidy, phenotype and function followed by unsupervised analysis
Ogan B.M., Drbal K.

IMMUNOLOGY

LECTURES:

Recent thymic emigrants and activation status in diagnostic immunophenotyping of severe primary immunodeficiencies: An euroflow
Marina Bakardjieva

Metaboprofiling of human monocytes by flow cytometry
Marcela Hortova Kohoutkova

The human circulating hematopoietic stem cell compartment is impaired during sepsis
Marco De Zuani

POSTERS:

(P43) Role of transcription factors in peripheral blood monocyte responses to sepsis
Andrejčinová I., Hortová Kohoutková M., Tidu F., De Zuani M., Tomášková V., Helán M., Šrámek V., Frič J

(P44) The effects of cytokines on development of suppressive B lymphocytes
Boháčová P., Heřmánková B., Hájková M., Holáň V.

(P45) Accelerated aging of immune cells in childhood and adolescent cancer survivors
Burilova P., Bendickova K., Kohoutkova M.H., Jose S.S., Frankova B., Kepak T., Krenova Z., Fric J.

(P46) Regulation of CD163 expression in human blood monocytes by IL-10
Čurnová L., Fialová M., Kotschwarová K., Švachová V., Stříž I.

(P47) Importance of adenylate cyclase isoforms in regulation of T lymphocytes differentiation
Daďová P., Kubala L., Fedr R.

(P48) Functional flow cytometry-based approaches for validation of novel mutations causing inborn errors of immunity with autoinflammatory phenotype
Fejtková M., Bakardjieva M., Racková M., Svatoň M., Froňková E., Hložková K., Borna Š., Králová J., Klocperk A., Škvárová Kramarzová K., Kalina T., Šedivá A., Hrušák O., Brdička T., Kanderová V.

(P49) Characterization of neutrophils in synovial fluid from patients with knee osteoarthritis
Gabcova G., Gallo J., Manukyan G., Mikulkova Z., Savara J., Dihel M., Kriegova E.

(P50) Anti-tumorigenic activity of probiotic mixture: crosstalk between tumour environment, microbiota and immune system
Hradicka P., Demeckova V., Zakutanska P., Kassayova M.

(P51) Effect of electric field on human polymorphonuclear leukocytes
Chorvátová M., Kocurková A., Šafaříková E., Ehlich, J., Víteček J., Kubala L.

(P52) Effect of mesenchymal stem cells in combination with cyclosporin A on an inflammatory response in mouse model
Jaburek F., Hajkova M., Vasek D., Porubska B., Bohacova P., Krulova M.

(P53) Effect of low, middle and high molecular weight hyaluronan on immune response in vivo
Kocurková A., Ambrožová G., Nešporová K., Šimek M., Hermannová M., Velebný V., Kubala L.

(P54) Immunophenotyping extracellular vesicles using the amnis CellStream flow cytometer
Pugsley H.R., Davidson B.R., Morrissey P.

(P55) Could be CD39+ regulatory T lymphocytes distinguishing marker of SIRS and SEPSIS diasnostics?
Mrázová V., Zuzulová M., Jarčušková J., Záhorec R.

(P56) Sertoli cells as a potential therapeutic tool for acute myocardial infarction
Porubská B., Hájková M., Krulová M.

(P57) Effects of pseurotin alkaloids on selected immune cell functions
Rubanova D., Daďová P., Skoroplyas S., Fedr R., Vasicek O., Kubala L.

(P58) Natural pseurotins affect human B-lymphoma cells in vitro
Skoroplyas S., Mosejova E., Bosnjakovic R., Kubala L., Vašíček O.

(P59) LDNs in CVID patients
Slanina P., Štíchová J., Litzman J., Hel Z., Vlková M.

(P60) Characterization of unique urothelial carcinoma cell lines from BBN-induced murine bladder carcinoma
Stepanek I., Kerzeli I., Lord M., Mangsbo S.

(P61) NK cells in reproductive immunology
Szaboova K., Haramiova K., Tibenska E.

(P62) Quantifying immune cell subsets in living cultures over time using IncuCyte® live-cell analysis system
Szybut C.

(P63) Immophenotyping and cytokines profile after total body irradiation
Šinkorová Z., Lierová A., Pejchal J., Jeličová M., Andrejsová L., Kašparová J., Korecka L., Bílkova Z., Němcová M.

(P64) Low molecular weight leukocyte extract (transfer factor) activates porcine T-lymphocytes
Štěpánová H., Hlavová K., Gebauer J., Hodkovicova N., Krejci J.

(P65) Optimization of a methodology for the analysis of immune cells in glioblastoma microenvironment by flow cytometry
Ternerova N., Houdova Megova M., Stollinova Sromova L., Busek P., Sedo A.

(P66) The effect of cold adaptation on the immune systeme
Vašek D., Žurmanová J., Jabůrek F., Hájková M., Krulová M.

(P67) Evaluation of probiotic candidates for the re-programming of macrophage subset-driven pro-inflammatory responses: rebuilding the gut ecosystem via faecal microbial transplantation in inflammatory bowel disease (IBD)
Zakutanska P., Hradicka P., Demeckova V.

CYTOMETRY METHODS FOR CELL BIOLOGY

LECTURES:

Spectral Cytometry advances in high dimensional data acquisition – The possibilities of a 24 colour panel on a 3 laser platform using the Cytek Aurora
Yacine Kharraz

HAEMATOLOGY

LECTURES:

Peripheral blood of CLL patients is repopulated with complement resistant malignant cells one day after rituximab infusion
Marek Borský

Development of multiparameter flow cytometry combination for myeloid maturation
Michaela Nováková

NK cells for the prevention and treatment of relapse after allogeneic haematopoietic stem cells transplantation
Monika Holubová

Extramedullary expansion of myeloma plasma cell into CNS
Lucie Říhová

Dynamic changes of inhibitory KIR receptors after allogeneic HSCT
Tereza Dekojová

Single cell profi ling of signaltransduction pathways in pediatric t-cell acute lymphoblastic leukemia
Daniela Kužílková

POSTERS:

(P39) High quality multiparametric flow cytometry: seeing the full picture through full spectrum cytometry
Kharraz Y., Gu H., Zhong A., Jaimes M.

(P40) KIRnome analysis for improved transplantation efficiency
Pitule P., Houdova L., Fatka J., Dolejsova M., Kralovcova E., Holubova M., Jindra P.

(P41) The development of erythrocytes populations of hereditary spherocytosis patient identified by flow cytometry in time
Kralova R., Bezdekova R., Sebelova B., Blatny J., Penka M., Rihova L.

MICROFLUIDICS

LECTURES:

Detection of photon-upconversion luminescence from microdroplets in microfluidic chips
Antonín Hlaváček

High-throughput characterization of haloalkane dehalogenases by using capillary microfluidics
Michal Vašina

Detection of chloroalkanes by surface-enhanced Raman spectroscopy in microfluidic chips
Martin Kizovský

Fabrication of a microfluidic chip to study the biology of vascular endothelium under flow conditions
Marek Černík

Optical micromanipulation and raman spectroscopy of cells in microfluidic systems
Tereza Klementová

DATA ANALYSIS

POSTERS:

(P38) Multidimensional data analysis and breast cancer heterogeneity
Fedr R., Remšík J., Navrátil J., Binó L., Slabáková E., Fabian P., Svoboda M., Souček K.

BOTANY

POSTERS:

(P1) DNA replication timing program in barley (hordeum vulgare)
Čížková J., Němečková A., Vrána J., Doležel J., Hřibová E.

(P2) Characterization and dissecting the complex nuclear genome of crested wheatgrass by chromosome flow sorting
Said M., Kubaláková M., Karafiátová M., Molnár I., Čížková J., Cabrera A., Vrána J., Doležel J.

NEW METHODS

POSTERS:

(P68) Probiotic effect on mucosal imunity to campylobacter jejuni in chickens
Revajová V., Karaffová V., Herich R., Ševčíková Z., Lenhardt L., Gancarčíková S., Žitňan R., Levkut M. Jn., Šefcová M., Levkut M.

(P69) Quantitative, live-cell kinetic analysis of microglial function and morphology
Overland A.C., Rauch J.N., Oupicka L., Bowe M.L., Alcantara S.L., Lovell G., Dále T., Appledorn D.M.

(P70) High parameter flow cytometry – setup and optimalization
Hnilicová Š., Dvořák P., Musil J.

(P71) 3D bioprinting of stem cells for creating tissue models
Jaroš J., Chochola V., Spustová K., Kandra M., Pospisil J., Proks V., Hampl A.

(P72) Isolation of sterlet (acipencer ruthenus) type a spermatogonia using fluorescence-activated cell sorting
Kislik G., Xie X., Pšenička M.

(P73) Maldi-tof biotyping in quality control of clinically relevant cell types
Porokh V., Moráň L., Hampl A., Vaňhara P.

(P74) Automated scoring of the in vitro micronucleus assay for genetic toxicology testing using imaging flow cytometry
Rodrigues M.A.

(P75) Test of metalloproteinases production by mesenchymal stromal cells after combination of different biophysical stimuli
Průcha J., Skopalík J., Švík K., Slavík J.

(P76) Screening for production of intracellular benzo[a]pyrene metabolites by spectral flow cytometry
Tylichová Z., Fedr R., Souček K., Vondráček J.

(P77) Cell separation of CD3+ and CD19+ cells from samples of leukemic patients
Rejlová K., Semerák P., Thürner D., Luknárová P., Mecerodová M., Kalina T., Vášková M.

(P78) CTHRC1: A novel prognostic marker in chronic lymphocytic leukaemia
Zezula N., Janovská P., Popová L., Bryja V.

(P79) High quality multicolor flow cytometry - seeing the whole picture through full spectrum cytometry on the 5 laser aurora
Kharraz Y., Gu H., Zhong A., Jaimes M.

CELL BIOLOGY AND SIGNALLING

BIDIRECTIONAL INTERACTIONS OF CANCER CELLS WITH THEIR MICROENVIRONMENT: BIOLOGICAL EFFECTS OF CASEIN KINASE 1 INHIBITION

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Our recent work has shown that Wnt signaling pathways and specifically the Wnt5a→CK1δ/ε→DVL signalling axis are key driving forces of the chronic lymphocytic leukemic (CLL) progression. Casein kinase (CK) 1 controls CLL chemotaxis and communication of CLL cells with their microenvironment that further re-inforces disease development. By using Eμ-TCL1 mouse model of CLL, we have shown that CK1δ/ε inhibitor PF-670462 can improve overall survival and demonstrated that CK1 inhibitors can serve as a new class of drugs in CLL therapy, targeting Wnt signaling in CLL cells and potentially also in other CK1-driven malignancies.
In this study, we aim at identification and better understanding of biological processes that are under control of CK1 in context of CLL and define how they change upon CK1 inhibition. We focus mainly on the context-dependent (=microenvironment-induced) biological processes that were shown to have a crucial role in CLL pathogenesis and treatment. Taking advantage of direct co-culture experimental systems, we analyze the effects of isoform-specific CK1 inhibitors. We analyze the consequences of CK1 inhibition both in an unbiased way at the global scale using (dual species) RNA Seq as well as using focused analysis of selected important biological features of CLL-microenvironment system.
This complex, multi cell type, co-culture approach has great potential to identify not only CK1-induced changes in the leukemic CLL cells but also to assess how CK1 inhibition affects behaviour and signalling of cells that constitute their microenvironment.
This work was supported by the following grants: GX19-28347X (Czech Science Foundation), FNBr. 65269705 (Ministry of Health, Czech Republic).

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ADAPTATION OF CANCER CELLS TO EXTREME METABOLIC STRESS

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Cancer cells inside fast-growing tumor mass are often subjected to periods of transient lack of oxygen and vital nutrients. To survive, they need to rewire their energy metabolism as well as cellular signaling pathways regulating cell growth and programmed cell death. In this study, we analyzed the response of human malignant melanoma cell lines to the inhibition of main glucose metabolism pathways *in vitro*. We found a remarkable ability of human cancer cells to survive extended periods of extreme metabolic stress. Our findings will be discussed in the context of cell energy metabolism and signaling pathways sensing intracellular levels of nutrients.

This work was supported by Masaryk University (MUNI/A/1087/2018) and the Ministry of Education, Youth and Sports of the Czech Republic (Translational Medicine; LQ1605).

WNT SIGNALING-INDUCING ACTIVITY OF ASCITES PREDICTS POOR OUTCOME IN OVARIAN CANCER PATIENTS

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High grade serous carcinoma of the ovary, fallopian tube and peritoneum (HGSC) is the deadliest gynecological disease with five-year survival rate below 30 %. HGSC is characterized by early and rapid development of metastases and frequent presence of ascites, a pathological accumulation of fluid within peritoneum. Here we show that ascites is capable to induce Wnt signaling in primary HGSC cells and cell line Kuramochi (a novel clinically relevant *in vitro* model of HGSC). Importantly, a fraction of patients whose ascites cannot activate Wnt pathway has less aggressive disease and considerably better outcome, especially in terms of overall survival. Functionally, the activation of non-canonical Wnt/planar cell polarity (PCP) signaling by Wnt5a (and not the activation of Wnt/β-catenin signaling by Wnt3a) promoted self-renewal, migration and invasion of HGSC cells. Accordingly, casein kinase 1 inhibitors and knock-out of DVL3 abolished the effect of Wnt5a. Additionally, Wnt/PCP pathway components were differentially expressed between healthy and tumor tissue as well as between the primary tumor and metastases and consistently only ascites activating Wnt/PCP signaling contained typical Wnt/PCP ligands Wnt5a and Wnt11. Together, our results suggest the existence of positive feedback loop between tumor cells, producing Wnt ligands, and ascites that distribute Wnt activity to cancer cells in the peritoneum in order to promote their pro-metastatic features and drive HGSC progression. Furthermore, our data highlight role of Wnt/PCP signaling in ovarian cancerogenesis and imply that analysis of Wnt pathway activity in the ascites has a prognostic value and casein kinase 1 inhibitors have a therapeutic potential in HGSC.

INTERACTION OF SKP2 AND SLUG PROTEINS IN AGGRESSIVE PROSTATE CANCER

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BACKGROUND: Skp2 is a substrate recruiting component of E3 ubiquitin-ligase complex, while Slug is a transcriptional repressor involved in epithelial-mesenchymal transition. Skp2 plays an important role in prostate cancer progression, e.g. via recently reported stabilization of EZH2 or Twist1, however, relationship with Slug needs further elucidation. METHODS: Prostate cancer patients cohort (N=101) was analysed by immunohistochemistry for the following proteins (Skp2, Slug, AR, Ki-67 and E-cadherin). Colocalization analysis was performed using Perkin Elmer Opal Multiplex kit, Vectra 3.0 imaging system and confocal microscope Carl Zeiss LSM 780. Prostate cancer PC3 cells were treated with a SCF^{Skp2} E3 ligase inhibitor MLN4924 and analysed by western blot. RESULTS: High Gleason score was significantly associated with higher Skp2 and lower E-cadherin expression ($p<0.001$ and 0.011, respectively). Skp2 slightly correlated with Slug and AR in the whole cohort (Rs 0.32 and 0.37, respectively), which was enhanced in patients with high Gleason score (Rs 0.56 and 0.53, respectively) or with metastasis to lymph nodes (Rs 0.56 and 0.37, respectively). Confocal microscopy revealed colocalization of Skp2 and Slug in prostate cancer cells. Chemical inhibition of Skp2 by MLN4924 upregulated p27 and decreased Slug expression which supports a possible link between Skp2 and Slug proteins. CONCLUSION: Immunohistochemistry, colocalization studies and in-vitro experiments support association between Skp2 and Slug in aggressive prostate cancer. Acknowledgement: The study was supported by grants NV15-28628A and DRO: FNOL00098892 from the Czech Ministry of Health.

CHK1 INHIBITION AS A HIGHLY PROMISING THERAPEUTIC APPROACH TO BYPASS CHEMORESISTANCE IN PRECLINICAL PROSTATE CANCER MODELS

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Prostate cancer (PCa) represents one of the most heterogeneous and clinically common malignancies in men. Despite a high initial effectivity of androgen deprivation, nearly 20% of patient experience progression to the lethal metastatic castration-resistant prostate cancers (mCRPC). Given the limited effectivity of chemotherapeutics, identification of a novel actionable target in mCRPC is crucial for more successful therapy. Checkpoint kinase 1 (CHK1) refers to the highly conserved protein kinase activated by single-stranded DNA that controls cell cycle progression and prevents accumulation of DNA damage. In the respect of PCa, enhanced activation of CHK1 correlates with castration resistance and reduced overall survival of mCRPC patients. Based on these considerations, synergistic modulation of DNA damage and inhibition of CHK1 might represent a novel particularly intriguing and promising strategy to eradicate chemoresistant mCRPC. Here we comprehensively investigate the effectivity of the novel CHK1 inhibitor MU380 alone or in combination with antimetabolite gemcitabine in docetaxel-resistant PCa cell lines DU145 and PC3 and clinically relevant PC346C and PC339 patient-derived xenografts (PDX). We showed that CHK1 inhibition, significantly sensitized all PCa models to gemcitabine. Flow cytometric analysis revealed robust effect of MU380 related to significantly increased phosphorylation of DNA damage sensor g-H2AX and activation of premature mitotic entry. Moreover, treatment by MU380 alone resulted in significantly prolonged S-phase duration and reduced spheroid size of docetaxel-resistant PDX cell cultures. Most importantly, we observed significant inhibition of tumor growth in both PC339 DOC and PC346C DOC PDXs after the combination of gemcitabine and MU380. Collectively, these data revealing unique features of MU380 suggest that inhibition of CHK1 significantly potentiate gemcitabine cytotoxicity and hence might represent promising therapy option for the patients with incurable mCRPC.

This work was supported by funds from the Ministry of Health of the Czech Republic, grant nr. 15-33999A, 15-28628A, 17-28518A, NV18-08-00245 all rights reserved (KS), projects no. LQ1605 from the National Program of Sustainability II (MEYS CR), and by project HistoPARK (CZ.1.07/2.3.00/20.0185; KS).

MOLECULAR MECHANISMS OF DIFFUSE PERITONEAL ADHESIONS DEVELOPMENT

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Peritoneal adhesions represent one of the major complications following intraabdominal and pelvic surgery leading to symptoms such as abdominal pain, bowel obstruction and infertility. Adhesions are mostly initiated by mechanical disruption of the mesothelial layer and its underlying tissues in the peritoneal membrane. Moreover, diffuse adhesions are formed randomly in areas of peritoneal cavity distant from the site of the mechanical disruption. This phenomenon is not sufficiently understood, however, hypoxia, coagulation, fibrinolysis and inflammation probably play a role in diffuse peritoneal adhesions incidence and development. In our study, we focused on hypoxia as a crucial factor triggering diffuse peritoneal adhesions development.

Within our research, an *in vivo* model of diffuse peritoneal adhesions was implemented in C57Bl/6J mice by desiccation of intestines and application of acidic HBSS and $CoCl_2$ (inducers of hypoxia) to peritoneum. Score of adhesion-creation (grade, localization) was observed, adhesive tissue was sampled for IHC staining and further analyses. Moreover, mesothelial cells and peritoneal fibroblasts were isolated from inert peritoneal membrane of C57Bl/6J mice. Using TGF-β1 (pro-fibrotic cytokine) and DMOG (stabiliser of HIF-1α), these primary cultures were transformed to pro-fibrotic phenotype. In both, extracted samples of adhesive tissues and primary cultures, expression of pro-fibrotic markers (FSP-1, αSMA), markers of proliferation (cyclins, Ki-67) and HIF-1α stabilisation were studied using WB and PCR. Since inflammation is participating in adhesion development, leukocytes were isolated from peritoneal lavage and analysed using clinical haemocytometer and flow cytometer. Systemic pro-fibrotic and inflammatory markers (including TGF-β1, SDF-1 and IL-6) were measured in plasma by ELISA.

Our results confirmed that hypoxia in combination with tissue desiccation is crucial for diffuse peritoneal adhesions development. Understanding underlying molecular mechanisms is necessary for implementation of effective strategies (including anti-adhesive membranes, gels and fluids) for prevention of this pathological process.

This work was supported by MEYS CR (project OP VVV INBIO CZ.02.1.01/0.0/0.0/16_026/0008451).

HYPERICIN IN HYPOXIA: FOCUS ON CANCER STEM-LIKE CELLS AND THERAPY RESISTANCE

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Hypoxia, the important part of the tumour microenvironment, is being linked with the promotion of the malignant progression, therapy resistance and maintenance of the cancer stem-like cell (CSC) phenotype. The ability of CSCs to face the effect of chemotherapeutic agents is due to the increased expression of transport proteins. CSCs can be detected by "side population" (SP) technique based on the enhanced efflux activity (mainly via BCRP protein). As several chemotherapeutics used in clinical practice are among the BCRP substrates, we have focused on the effect of hypoxia on the action of hypericin (HY), the metabolite of St. John's wort (*Hypericum perforatum* L.), since it is predicted competitive inhibitor, substrate and modulator of BCRP. HY is known photosensitizer for photodynamic therapy (PDT), however, above mentioned biological activities of this natural compound happen without its light-activation.

The aim of our study was to determine, if HY under hypoxia, similarly to *in vivo* tumour microenvironment, could decrease the activity of BCRP by competitive inhibition, thus representing an alternative natural inhibitor of simultaneously administered drugs or, conversely, hypoxia will stimulate HY efflux and decrease its effect.

We demonstrated that hypoxia differentially affected the level of ABC transporters, altered the intracellular HY accumulation to a various extent among different cancer cell lines and increased resistance of cells to HY-PDT. Hypoxia and HY synergistically decreased the size of the SP population and also affected the clonogenicity of SP and nonSP cells, as well as the size of the colonies, as was established by automatized single cell cloning.

Altogether, our results suggest that hypoxia can modify resistant phenotype of cancer cells and their ability to eliminate hypericin, however, the exact mechanism needs to be further elucidated.

This work was supported by the Scientific Grant Agency of the Ministry of Education of the Slovak Republic under the contract No. VEGA 1/0022/19 and by the Slovak Cancer Research Foundation.

A NOVEL LONG NON-CODING RNA MIAT IS ASSOCIATED WITH NMYC NEUROBLASTOMA STATUS

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Long non-coding RNAs (lncRNA) are a type of RNA with lengths exceeding 200 nucleotides that are not translated into protein. Expression levels of lncRNAs were deregulated in various diseases, including malignant tumors. Neuroblastoma (NBL) is the most common solid, extracranial malignant tumor of children. Amplification of the oncogene NMYC is a well-established poor prognostic marker in NBL, and it strongly correlates with higher tumor aggressivity and resistance to treatment. lncRNA MIAT was found to demonstrate aberrant expression in various diseases (myocardial infarction, schizophrenia, ischemic stroke, diabetic complications, age-related cataract, and cancers).
We examined the expression of lncRNA MIAT in the panel of NBL cell lines with and without NMYC amplification. We found that MIAT expression strongly correlates with NMYC amplification. Non-malignant human neonatal fibroblasts had higher lncRNA MIAT compared to NBL cells without NMYC amplification. Knock-down of MIAT by siRNA resulted in a significant arrest of cells at G_0 phase and a significant decrease in S phase in both UKF-NB-4 (MYCN ampl.) and SH-SY5Y (MYCN nonampl.) cells. Moreover, MIAT downregulation decreases UKF-NB-4 and SH-SY5Y cells migration. Interestingly, silencing MIAT in human neuroblastoma cell lines resulted in the induction of apoptosis in lines with NMYC amplification compared to cell line without NMYC amplification, where we found only growth inhibition. Mitochondrial oxidative phosphorylation, as measured by oxygen consumption rate, and also glycolysis function, as measured by extracellular acidification rate were reduced in the MIAT-siRNA group.
These results demonstrate that MIAT levels are potentially associated with neuroblastoma and with NMYC status and seem to be important for NBL proliferation, particularly for those with MYCN amplification. One may speculate that lncRNA MIAT could be used as a therapeutic target in the future.

Supported by GAUK 2120247.

LIPOPOLYSACCHARIDE FROM *MICROCYSTIS AERUGINOSA* DOMINATED WATER BLOOM ACTIVATES DIFFERENT CELL TYPES *IN VITRO* AND *EX VIVO*

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Cyanobacterial water blooms are a worldwide problem of freshwater reservoirs where they pose a significant risk to human health. In addition to highly toxic cyanotoxins, they also produce mixture of cyanobacterial and also bacterial lipopolysaccharides (LPS) with not much studied biological activity. *Microcystis aeruginosa* (MA) is a globally widespread species abundant also in the Czech Republic, therefore water bloom with its predominance was chosen.
Intestinal epithelium represents the first contact of human internal environment and LPS. Therefore, differentiated Caco-2 and confluent HT-29 cell lines were used for subsequent analyses. Increased production of pro-inflammatory cytokines interleukin-12 (IL-12) and IL-1β was detected by the cytokine array. Significantly higher secretion of IL-8 assessed by ELISA was also observed. Endotoxin activity of the MA in Pyrogene assay was shown to be two times greater of compared to Escherichia coli (EC).
Via activated intestinal epithelium, the LPS can pass into the blood stream. In whole blood, the major phagocytes are polymorphonuclear leukocytes (PMNL) and monocytes. Their activation was confirmed by flow-cytometric detection of CD11b and CD66b surface markers expression. This was complemented by analysis of signalling pathways from isolated PMNLs for p38, NF-KB and p38-MAPK, which were also shown to be significantly elevated.
For further understanding of activation mechanism of LPS, macrophage cell line RAW 264.7 was employed. The production of tumour necrosis factor-α, IL-6 and nitric oxide (NO) was monitored. However, IL-6 and NO have not been activated, therefore, it could be concluded that LPS of MA possibly uses different mechanisms for activating macrophages compared to EC. This fact was supported by the experiment with Toll-like receptor 4, a model receptor for LPS, where its inhibition did not attenuate the effects of cyanobacterial LPS.
Taken together, LPS from MA-dominated water bloom exerts high endotoxin activity and pro-inflammatory effects in intestinal epithelial cells as well as in immune cells *in vitro* and *ex vivo*.

Acknowledgements: This study was supported by Czech Science Foundation (19-09980S).

PERSPECTIVES OF MESENCHYMAL STEM CELL THERAPY FOR RETINAL DEGENERATIVE DISORDERS

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Retinal degenerative disorders represent a group of diseases affecting millions of people worldwide. So far, there is still no effective treatment protocol available for many of retinal disorders. A perspective approach for these patients represents stem cell-based therapy. The promising candidate are mesenchymal stem cells (MSCs) which can be obtained from patient's tissues in sufficient amount. In this study we analyzed the immunomodulatory properties of mouse bone marrow-derived MSCs after their intravitreal delivery to inflammatory environment in the eye caused by the application of pro-inflammatory cytokines. We found that MSCs decrease the number of immune cells in the retina and inhibited production of pro-inflammatory molecules by retinal cells in the eyes with injected cytokines. The same effect was detected *in vitro* after cultivation of MSCs with retinal explants in inflammatory environment. In addition to these properties, MSCs are also able to produce a number of growth and trophic factors that have important role in retinal regeneration. We conclude that all of these properties make MSCs a perspective cell candidate for treatment of retinal degenerative disorders.

This study was supported by project LO1508 from the MEYS of the Czech Republic.

DETECTION OF DISRUPTION OF FATTY ACID SYNTHESIS AND LIPID ACCUMULATION IN HEPATOCYTE-LIKE CELL MODELS

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Dysregulation of liver functions can lead to accumulation of lipids in the liver resulting in the development of non-alcoholic fatty liver disease (NAFLD) and subsequent progression to non-alcoholic steatohepatitis (NASH), liver fibrosis, cirrhosis and hepatocellular carcinoma. Although a large part of fatty liver can be attributed to alcohol consumption, the global prevalence of NAFLD is increasing. Disruption of lipid metabolism leading to NAFLD has been suggested to affect over 25% of the global population, and both occupational and environmental chemical exposures have been suggested to contribute to the development of NAFLD. The compounds disrupting metabolism have been also linked with increased incidence of metabolic syndrome, obesity and type 2 diabetes. Therefore, there is a growing need for human *in vitro* cellular models allowing to evaluate effects of both endogenous and exogenous compounds on disruption of lipid metabolism. In the present study, we evaluated effects of model lipid metabolism disruptors (AhR ligands, amiodarone) on alterations of fatty acid (FA) synthesis and accumulation of lipids in liver cell models (immortalized human MIHA hepatocytes, differentiated liver HepaRG cells). We also compared suitability of various dyes specific for phospholipids or neutral lipids (such as LipidTOX™ Neutral Lipid Stain, LipidTOX™ Phospholipidosis Stain, BODIPY™ 493/503, Nile Red, etc.), using flow cytometry and/or confocal microscopy, with a perspective to apply them to routine screening of effects of various chemicals. We found anti-arrhythmic drug amiodarone, causing phospholipidosis and/or vacuolar sequestration, to be a suitable inducer of lipid accumulation and FA metabolism disruption in our cell models. Amiodarone increased mRNA levels of a set of genes involved in lipid metabolism/transport (such as ACC, ACLY, CD36, FASN and SCD1), in our selected cell models, and it was found to promote in particular phospholipidosis. A model AhR ligand, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, was somewhat less effective. Our ongoing investigation currently focuses on evaluation of the impact of model chemicals on regulatory factors controlling lipid metabolism. [Supported by the Czech Science Foundation (project No. 19-00236S) and the Czech Academy of Sciences.]

THE IMPACT OF LOSS OF THE ARYL HYDROCARBON RECEPTOR (AHR) ON METABOLISM AND FUNCTIONAL PROPERTIES OF HUMAN BREAST CARCINOMA MCF-7 CELLS

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The aryl hydrocarbon receptor (AhR) is a transcription factor that has been shown, apart from its essential role in adaptive response to exposure to many toxic compounds (such as polychlorinated dibenzo-*p*-dioxins or polycyclic aromatic hydrocarbons), to modulate activities of several intracellular signaling pathways involved in tumor development. The AhR is frequently overexpressed in tumor cells, and its target pathways include those controlling genes linked with regulation of cancer cell growth, survival, migration, invasivity and others. Notably, several recent studies have indicated its potential role in regulation of endogenous metabolism, particular fatty acid/lipid metabolism. AhR has been recently also shown to decrease the tumorigenic properties of the highly metastatic breast cancer cell lines. Therefore, in the present work, we focused on evaluation of the role of the AhR in human breast carcinoma MCF-7 cell model. We employed a combination of flow cytometric, microscopic and biochemical approaches to characterize the metabolic and phenotypical alterations of MCF-7 AhR knockout (AhR KO) cells. The loss of AhR resulted in reduced proliferation and alteration of cell cycle progression in AhR KO cells, as compared with wild-type cells. Laser scanning confocal microscopy revealed significant morphological alterations, including altered texture, intensity and distribution of endoplasmic reticulum, altered nuclear texture or distribution of mitochondria. Perhaps the most profound changes have been observed in metabolism of AhR KO cells, where their oxidative metabolism was significantly inhibited, which was accompanied also with altered glucose consumption and reduced proteosynthesis. Notably, in AhR KO cells, we observed a reduced expression of genes involved in fatty acid synthesis, such as *ACC*, *ACLY*, *FASN* and *SCD*, as well as their corresponding regulatory factors. This was associated with reduced oleic-to-stearic acid ratio (saturation index). Together, the results indicate that the loss of AhR (here in a model of human breast cancer cells) may lead to reduced growth and fitness of tumor cells. [Supported by the Czech Science Foundation, project no. 19-00236S, and the Czech Academy of Sciences.]

FUNCTIONAL ROLE OF AHR IN DIOXIN-MEDIATED DISRUPTION OF APOPTOSIS CONTROL IN A HUMAN MODEL OF ADULT LIVER PROGENITORS?

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We have previously shown that the aryl hydrocarbon receptor (AhR) activation may alter proliferation, apoptosis or differentiation of adult rat liver progenitors. Here, we investigated the impact of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-mediated AhR activation in a human model of bipotent liver progenitors. As a model, we used undifferentiated human liver HepaRG cells representing human liver progenitor-like cells. We employed both intact cells and those with silenced Hippo pathway effectors, YAP1 and TAZ, which are known to play key role(s) in progenitor cell self-renewal and expansion, e.g. in liver, cardiac or respiratory tissues. We have previously shown that TCDD induces cell proliferation in confluent undifferentiated HepaRG cells. By contrast, following the siRNA-mediated downregulation of YAP1 and TAZ, we observed induction of apoptosis, which was further promoted by TCDD. Using specific caspase inhibitors, we then evaluated the role of caspases, in particular that of caspase-8 and -9 in YAP1/TAZ siRNA-mediated apoptosis. Our results seem to suggest that, unlike in mature hepatocytes or hepatocyte-like cells, activation of the AhR by TCDD may sensitize HepaRG cells to apoptotic stimuli. TCDD also promoted induction of apoptosis by a death ligand, FasL, in undifferentiated HepaRG cells. Finally, siRNA-mediated AhR knockdown again seemed to enhance the FasL-induced apoptosis. Collectively, our results suggest that the AhR, and/or its deregulation by its persistent toxic ligands such as TCDD, may contribute to the control of programmed cell death pathways in human liver progenitor-like cells.

Supported by the Czech Health Research Council (grant No 17-28231A), by the Czech Science Foundation (project No. 18-00145S), by the Czech Academy of Sciences, and The Brno City Municipality (Brno Ph.D. Talent).

CHK1 INHIBITION FACILITATES THE COOPERATIVE ANTICANCER ACTION OF PLATINUM-BASED DRUGS AND TRAIL

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Checkpoint kinase 1 (Chk1) plays an essential role in the cell signaling processes involved in regulation of the cell cycle, DNA damage response and cell death. Chk1 has been recognized as an attractive target for pharmacological inhibition in cancer treatment. Small-molecule inhibitors of Chk1 kinase have been identified and intensively investigated as single agents or in combination with various chemotherapeutic drugs, and showed to significantly enhance the chemosensitivity of numerous tumor types. However, the molecular mechanisms involved in these effects still remain not fully explained.
We have found that pharmacological inhibition of Chk1 using the potent and selective inhibitor SCH900776, currently profiled in phase II clinical trials, or specific siRNA-mediated Chk1 silencing, significantly potentiate the cooperative cytotoxic effects of platinum(II) (cisplatin) or platinum(IV) (LA-12) complexes and TRAIL cytokine in human prostate cancer cells. Our results demonstrate that SCH900776 enhances cisplatin/LA-12 and TRAIL combination-induced DNA damage and cell death, associated with stimulation of mitochondrial apoptotic pathway. We have further investigated a specific role of several Bcl-2 family proteins in the observed effects, and selected the most important players in the Chk1 inhibition-mediated potentiation of anticancer effects of platinum drugs and TRAIL, which will be presented within our contribution.

This work was supported by Czech Science Foundation (15-06650S), HistoPARK (CZ.1.07/2.3.00/20.0185), and The Brno City Municipality (Brno Ph.D. Talent). Institutional support was provided by the Czech Academy of Sciences.

EVALUATION OF MEMBRANE BOUND ISFORMS OF ADENYLATE CYCLASES

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Adenylate cyclases (ACs) produce cyclic AMP (cAMP) which is an important intracellular signalling messenger. Ten mammalian AC isoforms were identified up to date that differ in their tissue distribution and mechanisms of regulation. Despite ACs are involved in many physiological functions, little is known about alternations in their function due to their gene polymorphism. A loss or a gain of function of these enzymes is reported to be related various pathological phenotype. Further, interestingly, most of membrane AC isoforms (AC1-8) are activated by forskolin (FSK), a labdane diterpene containing a tetrahydropyran-derived heterocyclic ring produced by plant Plectranthus barbatus. Recently, a 24-step synthesis of FSK was developed that opened a potential for the preparation of new FSK analogues (Hylse, 2017).
New screening methods suitable to monitor specific modulation of the enzymatic activity of different isoforms of human ACs were developed. We prepared various clones of HEK293 cells overexpressing distinct isoforms of ACs. These methods enable profiling of new, fully synthetic derivatives of FSK. Further, information about known missense mutations in selected AC7 and AC8 were collected and predicted functional impact and their possible correlation with a certain pathology was analysed. The functional impact of selected mutations was further evaluated by preparing mutated variants of AC isoforms and comparing them to their wild type forms *in vitro*.
We anticipate that the results will help to define regulatory effects of FSK analogues on specific isoform AC and help to identify potential compounds for therapeutic use in wide range of diseases related to pathogenic cAMP signalling alternations including chronic inflammation, autoimmune diseases and other.
This work was supported by MEYS CR (project number LQ1605 from the National Sustainability Program II).

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MODULATION OF RADIATION-INDUCED INJURY BY HYALURONIC ACID NANOPARTICLES

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Radiation-induced lung injury (RIPI) is a dose-limiting side effect of thorax or total body irradiation. The thoracic irradiation is performing frequently, especially in cases of lung, breast, mediastinal, and certain rare forms of heart cancer. Clinically, pathologically and radiologically, two successive phases of RIPI can be recognize – acute radiation pneumonitis (RP) and chronic radiation fibrosis (RF). Understanding the temporal relationship between molecular and cellular changes during individual phases may help to prevent misdiagnosis and possibly improve patient prognosis.

The purpose of our study was to determine whether treatment of C57Bl/6J mice with hyaluronic acid nanoparticles (HA-NPs) could mitigate the acute or chronic radiation effects. Hyaluronic acid has excellent biological properties such as biocompatibility, biodegradability and non-toxicity. Additionally, the molecule can be self-assemble into HA-NPs. These nanoparticles keep their biocompatibility and receptor-binding properties. In our study, female C57BL/6J mice were whole thorax irradiated by 17 Gy. Nanoparticles of two different sizes (84.5 and 124 nm) were delivered by intratracheal instillation directly into lung 1 hour before irradiation. Samples of blood and lung tissue were collected 113 (phase of RP), 155 (intermediate phase) and 180 day (phase of RF) after irradiation. Blood count, immunophenotypisation and cytokine profile were measured in blood. In lung, immunophenotypisation, cytokine profile and histopathology were evaluated.

The results suggest that HA-NPs in our experimental model contribute to the mitigation of radiation induced changes in blood and lung tissue. The most significant effects were observed in B-lymphocytes in blood and on T helper population in lung during intermediate and fibrotic phases. According to our findings HA-NPs application seems promising to attenuate RIPI.

TREATMENT OF GLIOBLASTOMA USING SUPERPARAMAGNETIC NANOPARTICLES WITH MODIFIED SURFACE

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Glioblastoma multiform (GBM) is frequent brain tumor, representing 80% of malignant primary central nervous system tumors. The lifespan of patients with treatment is about 12 months. Aim of the study was to develop multifunctional superparamagnetic iron oxide nanoparticles (SPION) suitable for magnetic resonance imaging (MRI), hyperthermia and drug delivery (carrying HPMA polymer with bound doxorubicin). We assumed that SPION, when placed into the high frequency alternating magnetic field will increase their temperature. Therefore cancer cells incubated with SPION may in high frequency magnetic field go into apoptosis (thermotherapy).

We used glioblastoma lines (GAMG, A172) and primary cultures isolated from solid patient tumors; as a model of healthy cells, human mesenchymal cells (hMSC) were utilized. For thermotherapy experiment, the cells were divided into three parts. The first part of the cells was incubated with SPION 48 h; the second part of the cells was mixed with SPION just before thermotherapy and the third part of the cells stayed intact as control.

The therapeutic effect of SPION-HPMA-dox on glioblastoma and healthy cells was analysed using real time proliferation assay (RTCA-DP xCELLigence).

Transmission electron microscopy confirmed uptake of SPION into the cell cytoplasm, MRI visualized SPION-labeled cells. SPION-HPMA-dox reduced proliferation in all tumor cells in 10 nM concentration, while free dox was effective only up to 50 nM. Moreover, SPION-HPMA-dox did not affect growth of hMSC. Adhesion and proliferation were affected by hyperthermia only in cells mixed with SPION in concentration of 16-32 mM, in which temperature reached 50° C. On contrary, the exposure to high frequency magnetic field did not affect cells that contained nanoparticles only in the cytoplasm. The temperature of these samples did not exceed 30° C. In conclusion, SPION-HPMA-dox can offer enhanced cell permeation, increased cell death and reduced nonspecific toxicity.

Acknowledgments: GAČR 17-04918S

EVALUATION OF TARGETING AND EFFICACY OF NANOPARTICLES-MEDIATED COMBINED CANCER TREATMENT

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Chemoresistance and metastasis cause the majority of cancer-related death, and innovative therapeutic approaches are needed to break this unfavourable situation.
Therapy that combines two or more therapeutic agents targeting more key pathways can lead to additive or synergistic effect. Nanomedicine offers a new frontier for targeted drug delivery, enabling the development of alternative strategies to improve the treatment efficacy.
Multifunctional nanocarriers are able of targeted co-delivery of more therapeutic molecules. These multifunctional platforms allow conjugation of drugs, therapeutic genes, targeting molecules and fluorescence dye on a single molecule.
Many types of nanocarriers have been developed. In this study we used liposomes and PAMAM (polyamidoamine) dendrimers. SiR dye was used for visualisation of nanoparticles (NPs). Interaction of NPs with tumour cells and cells derived from healthy tissues was determined by flow cytometry using FASC Canto II and ImageStream devices.
In order to improve tumour cells targeting we analysed expression of surface molecules such as folate receptor, cMet and Axl. On the panel of model cell lines we demonstrated different expression of above mentioned antigens which enables us to analyse the targeted delivery of NPS conjugated with appropriate ligand or antibody. In order to evaluate combinational effect, cells were treated with chemotherapy (Doxorubicine or 5-Fluorouracil), epigenetic drug (Decitabine) and gene therapy (Deoxycitidine kinase or TNF-related apoptosis-inducing ligand coding plasmid) and with combinations of these molecules. Apoptosis was determined by Annexin V assay. CellTiterGlo luminescence assay was used for detection of cytotoxic effect. Subsequently combinational effects (synergy, additive effect of antagonism) were calculated by CalcuSyn software.

Financial support
JRP SAS – TÜBITAK SBAG (project code: 118S126, TAcTiCAI); the 7FP platform ERA-NET EuroNanoMed II Innocent; VEGA 02/0050/19 and 1/0271/17; APVV-16-0178 and APVV-0052-12; HISENTS (H2020-NMP-2015- 685817); UVP BIOMED-ITMS 26240220087. The experiments mentioned in this study were enabled with the kind help and the financial support from the Cancer Research Foundation and League against Cancer of Slovak Republic.

RESISTANCE TO TRYPSIN/EDTA TREATMENT IS CAUSED BY INCREASED CELL ADHESION ON CPA LAYERS

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Cell cytometry is commonly used for assessment of cytokinetic parameters - proliferation, differentiation and cell death. However, from the point of view of biomaterial science, there is another, primal parameter – cell adhesion. Proper adhesion of anchorage-dependent cells in fact affects all the other parameters, i. e. failed attachment results in anoikis, too strong attachment can affect proliferation or differentiation (Huang et al. 1999). We aim to study cytokinetic parameters of multiple cell lines growing on cyclopropylamine (CPA) plasma-functionalized surfaces, resulting in amine-rich positively charged layers. Our study was compromised by extreme resistance to TRYPSIN/EDTA treatment of cells growing on these surfaces, thus we focused on this phenomenon. With use of time-lapse microscopy we realized that cells can attach on functionalized surface in only few minutes, their spontaneous migration is slower and they are more resistant also to the cell scratching technique (AFM microscopy based technique). However, we did not observed any changes in expression of main adhesion molecules. We can thus conclude, that amine-rich surface results in increased adhesion of cells, which is also accompanied by decreased proliferation.

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Acknowledgement:

This project was supported by GACR grant 18-12774s and grant for specific research MUNI/A/1380/2018.

TOLL-LIKE RECEPTORS IN PROSTATE CANCER CARCINOGENESIS AND THERAPY-RESISTANCE

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Prostate cancer (PCa) is the most frequent non-skin cancer affecting men in developed countries. One common curative strategy aimed to maintain disease under control is the androgen-deprivation therapy, which consists of lowering testosterone circulating levels by the use of anti-androgen molecules, such as Enzalutamide and Abiraterone. Despite initial effectivity, patients might eventually develop resistance, progressing into a condition of castration-resistant PCa (CRPC) associated with further development of the tumor. Thus, curative strategy shifts to the use of chemotherapeutic agents, like Docetaxel, which ultimately may also lead to treatment-resistance, with disease progress into metastatic condition. Toll-like receptors (TLRs) are key proteins in immune response and have been reported to be expressed also in epithelial cells. Moreover, studies showed that TLRs affect prognosis in patients affected by PCa, by the activation or inhibition of pathways involved in carcinogenesis. The aim of our study was to evaluate the expression of TLRs in PCa and to assess a possible connection between TLRs and therapy-resistant PCa. For this purpose, we screened 38 TLR- and oncogene-associated molecules in normal and tumor patient sample, in clinically relevant patient-derived xenografts (PDX) and in four PCa cell lines with different resistance status. Regardless the heterogeneous expression of the assayed markers in our models, we found that TLR3, TLR4 and MUC1 genes were up-regulated in all resistant models and their modulation was observed also at a protein level, by methods including flow cytometry. Given that TLRs exert their action through the activation of the corresponding pathways, we evaluated two main downstream transcription factors, NF-kB and IRF3. Although no striking difference was observed as baseline expression, we detected a defect in TLRs activation ability in therapy-resistant models. Taken together, our results show a potential implication of TLRs in PCa and therapy-resistance.
This work was supported by funds from the Ministry of Health of the Czech Republic, grant no. 17-28518A and 15-28628A, project no. LQ1605 from the National Program of Sustainability II (MEYS CR) and by project HistoPARK (CZ.1.07/2.3.00/20.0185).

CHARACTERIZATION OF ANOIKIS RESISTANCE IN MAMMARY CANCER CLONES DERIVED FROM LONG LIVED CTCS.

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The cancer dissemination into distant organs is mediated by circulating tumor cells (CTCs), which are released from the primary tumor into the blood circulation. One of the key steps in metastatic dissemination is survival of cancer cells in the blood and successful colonization of the tissue. An important feature of CTCs leading to their enhanced survival in blood, followed by increased chance of metastatic founding, is the acquired resistance to *anoikis*. We hypothesized that cancer cell clones derived from long-lived CTCs will be suitable for modeling of anoikis resistance *in vivo*. Therefore we employed previously described 4T1 12B luc mCherry model of CTCs [Dvorak et al., 2017] and established single CTC-derived clones which were isolated from blood circulation of experimental animals after 12h from intracardial injection into Balb/c mice and expanded *in vitro*.
The aim of this work was to characterize CTC-derived clones with focus on their metastatic potential and number of CTCs in context of *anoikis* resistant phenotype. Therefore we performed *in vitro* and *in vivo* characterization, including 3D *in vitro* cultivation assays from a single cell suspension o*f in vitro* cultivated cell lines, *in vivo* experiments investigating dissemination capability using immunodeficient SCID mice as well as syngeneic Balb/c mice, and clonogenic assay of CTCs isolated from blood. From the current data, 3D *in vitro* cultivation assays did not reveal any differences in resistance to anoikis, between these cell lines which was reflected by an equal number of formed spheroids but interestingly, there were significant differences in size of spheroids from the CTCs-derived cell line. Further, *in vivo* experiments also did not show differences in the resistance to anoikis, metastatic potential neither number of CTCs between clones derived from long-lived CTCs and parental cell line. However, there were significant differences in size of colonies formed from the isolated CTCs and expanded ex vivo. All together, these data can be related to altered proliferation properties of CTC-derived clones.

Acknowledgement
This work was supported by funds from the Ministry of Health of the Czech Republic, grant nr. 15-28518A all rights reserved (KS), The Czech Science Foundation, grant nr. 15-11707S, projects no. LQ1605 from the National Program of Sustainability II (MEYS CR), and by project HistoPARK (CZ.1.07/2.3.00/20.0185; KS).

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KDM5D IS ASSOCIATED WITH NEUROBLASTOMA CHEMORESISTANCE TO ELLIPTICINE

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Histone lysine-demethylases KDM5s are enzymes, which are capable of removing tri- and di- methyl marks from lysine 4 on histone H3 (H3K4) and depending on the methylation site, their effect on transcription can be activating or repressing. Therefore, they could play an important role in the downregulation of tumor suppressors or repressing of oncogenes and in appearance of drug tolerance. Neuroblastoma (NBL) is the most common pediatric extracranial solid tumor and there exist needs for new biomarkers for developing more effective therapeutic strategies that improve treatment outcome.

We investigated expression of KDM5A-D in sensitive NBL cell line (UKF-NB4) and its derived chemoresistant (to cisplatin –CDDP / doxorubicin –DOXO / vincristine –VCR / ellipticine - ELLI) cell lines. We found that expression of KDM5B-D were significantly increased in all chemoresistant NBL cell lines compared to sensitive NBL cells. Expression of of KDM5B-D in sensitive NBL cells after treatment with cytostatics, were reduced or remain unchanged. KDM5D wasn't expressed in VCR chemoresistant cells while in all other chemoresistant cells was KDM5D highly over-expressed, the highest was expression in NBL cells resistant to ELLI.

To investigate whether KDM5D demethylase is involved in ELLI chemoresistance in NBL, we inhibited KDM5D by JIB-04, a multi-KDM inhibitor. We showed that this molecule can efficiently inhibit NBL cell growth in native and chemoresistant NBL cells. We have shown that JIB-04 demethylates H3K4 and activates the expression of genes which are sufficient to activate death mechanisms.

We observed that inhibition of KDM5D in NBL cell lines lead to inhibition of cell growth followed by induction of apoptosis in chemoresistant NBL cells compared with sensitive NBL cell lines where we found only inhibition of cell growth. These results demonstrate that KDM5D expression levels are potentially associated with NBL chemoresistance to ELLI.

Supported by GAUK 822219 and *GACR 17-12816S.*

NON-TELOMERIC FUNCTION OF TELOMERASE COMPLEX IN PATTERN RECOGNITION RECEPTORS SIGNALLING DRIVEN INFLAMMATION OF HUMAN TISSUE ORGANOIDS

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Non-telomeric roles of telomerase complex have been reported recently, suggesting the involvement of telomerase in many more cellular processes than merely telomere maintainance. We have indicated that chronic inflammation is closely correlated with telomerase activity in a range of disease associated pathologies (Jose *et al.* Front. Genet. 2018; Jose *et al.* Front. Immunol. 2017). However, the mechanism of how pattern recognition receptors (PRRs) mediated inflammatory signalling drives tissue senescence remains largely unknown. Here we report how telomerase influence PRR mediated signalling in 3D lung and intestinal organoids differentiated from human induced pluripotent stem cells. Previous studies have shown links between mucosal infection, and inflammation with impaired telomerase activity. Our organoids resemble human tissues, expressing important organ specific markers such as NKX2.1, CDX2, EpCAM-1, E-Cad and Mucin, and furthermore exhibit tissue polarity and lumen formation. Interestingly, both lung and intestinal organoids express TLR2, TLR4 and TLR9 as shown by FACS analysis. Stimulation of organoids with PRR-ligands including LPS, Poly I:C and heat-inactivated-fungal conidia results in expression of pro-inflammatory cytokines IL-1β, IL-6, IL-8, IL-10, IL-12, MCP-1 and TNF-α at both mRNA and protein level. PRR stimulated organoids also express inflammatory markers COX-2 and MMP-9 as well as tissue injury associated DAMPs such as S100A 8/9 and HMGB-1. Strikingly, the expressions of IL-1β, IL-6, IL-8, TNF-α and MMP-9 were significantly impaired upon treatment with telomerase component TERT inhibitors (BIBR1532). Furthermore we show that activity of NF-κB pathway is downregulated upon inhibition of telomerase activity using the luciferase reporter organoids. Taken together our results suggest the crosstalk of of telomerase complex with inflammatory signalling driven by NFkB transcription factor. These findings might have potential in broarder understanding of mechanims leading to accelerated aging in different chronic inflammatory dissorders.

ELECTROSTIMULATION OF MURINE EMBRYONIC STEM CELLS ENHANCED CARDIOMYOGENESIS

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Heart diseases are of serious socioeconomic impact. Potentially, the heart damages could be treated by using cardiomyocytes differentiated out of embryonic stem cells (ES). For stem cells application it is necessary to understand principles of their differentiation into cardiac cells [1]. Recent studies have indicated that ES differentiation can be manipulated also by exogenous electric field.
To apply exogenous electric field a unique platform based on organic electronics developed in cooperation with Brno University of Technology was used [2]. Embryonic bodies prepared out of murine ES were subjected to electric pulses from 65 to 200mV/mm at frequency of 1Hz [3].
It was found, that electric stimulation induced cell membrane depolarization and increase of cytosolic calcium level. Both events were positively dependent on the total stimulation time. Further, the longer electrostimulation enhanced cardiomyocyte beating during maturation. In order to provide support for the above-mentioned the data analysis of gene expression of two marker genes (Nkx2.5 – cardiomyogenesis marker, Oct4 – pluripotency marker) was estimated.

The work was supported by Czech Science Foundation grant No. 17-24707S
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NF-KB AND NFAT PATHWAYS SHAPE MESENCHYMAL STEM CELL RESPONSE TO PAMPS

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Mesenchymal stem cells (MSCs) are multipotent cells present in the stromal fraction of many tissues. It is now clear that they play an important role in immune response homeostasis during both steady state and inflammation. Their ability to migrate to inflamed site and secrete immunoregulatory factors makes them an interesting candidate for cell therapies.
MSCs-expressed pattern recognition receptors (PRRs) allow direct sensing of pathogen associated molecular patterns (PAMPs) or damage in tissue microenvironment. So far most of the studies pointed at TLR-NF-κB axis as the main pathway controlling MSCs inflammatory profile. However a clear insight on PRRs activation patterns and cross-talks between NF-κB and other inflammatory pathways has not sufficiently been described, thus hampering MSC's potential in therapy.
The aim of this work is to investigate calcineurin-NFAT axis in MSCs response to PRR ligands and compare its activity to NF-κB. Here we show that zymosan, a fungal antigen recognized by PRRs, leads to NF-kB activation and production of MCP-1, IL-6 and IL-8. Together with expression of inflammatory cytokines, we observe nuclear translocation of NFAT transcription factor, which culminates in regulation of genes associated to extracellular matrix composition.
The presented work suggests a dual mechanism in MSCs response to PAMPs: on one side the cells produce inflammatory cytokines, mainly under the control of NF-kB pathway. On the other side NFAT pathway regulates the expression of genes associated to extracellular matrix, which eventually shapes the crosstalk with the immune cells.

LIVE-CELL ANALYSIS OF 3D SPHEROIDS: LABEL-FREE & FLUORESCENT CELL HEALTH REPORTERS

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A growing body of evidence suggests that 3D cell models yield more translational biological insight than 2D monolayers. Here we describe a simple kinetic live-cell imaging approach based on brightfield, in combination with fluorescent image analysis of spheroids. Brightfield analysis enables the monitoring of spheroid size (proliferation) and when combined with cell health markers (cytotoxicity or apoptosis) mechanisms of cell death may be elucidated. Expression of fluorescent proteins provides a surrogate for cell viability, where the fluorescence increases during proliferation and decreases following treatment with cytotoxic agents.These assays are flexible, simple and provide automated and direct measures of tumour size and health in real-time.

GLIOBLASTOMA MULTIFORME-DERIVED CELLS AND THEIR CONTRIBUTION TO NEOVASCULARIZATION

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Glioblastoma multiforme is a human brain tumor with bad prognosis. The only hope for patients is radical surgery with subsequent chemotherapy with temozolomide, but less then 10 % of patient survive 5 years. Glioblastomas are characterized by necrotized areas and microvascular proliferation, but antiangiogenic therapy is not effective as expected. As shown in sporadic reports, glioblastomas are able to derive blood vessels de novo from their tumor cells independently on VEGF. Thus, we focused on tumor-derived stem cells and tumor neoangiogenesis in vitro.

Samples from human glioblastoma multiforme were obtained from perioperative biopsies from neurosurgery unit, Military Hospital, Prague. These samples were minced, enzymatically digested and the cells were seeded in cultivation flasks in order to obtain tumor cell culture. Cell suspension before seeding and after reaching passage 3 was analyzed using flow cytometry and deeply frozen before subsequent testing. To analyze their angiogenic potential, in vitro tube formation test was performed.

We found that while freshly isolated cells from humans specimens were positive for astrocyte markers (GLAST1, A2B5), only few cells (< 5%) were positive for endothelial markers like CD31 or CD146. We did not find distinct stem cell populations positive for CD133 or CD15, single positive cells disappeared during first passage. Cells in culture did not express any determining markers specific for particular cell population (glial or neuronal), but were positive for pluripotent markers as sox2. We found remarkable and variable expression of growth factor receptors (CD271, EGFR, PDGFRB, but not PDGFRA or VEGFR). At least, after initiation and stimulation with VEGF in tube formation assay, the human glioblastoma specimens gave mostly positive results in induction media as well as in basal media without VEGF (control), whereas other formed capillary-like nets only in control media and minority remained unresponsiveness in both.

In conclusion, we confirmed strong plasticity in glioblastoma mutiforme-derived cells and their ability to form their own vasculature and to contribute to progression based on VEGF-independent pathways.

Supported by GACR 17-04918S

ROLE OF TROP-2 EXPRESSION IN BETA CATENIN EXPRESSION AND ACTIVITY IN MAMMARY CANCER CELLS.

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The cell surface glycoprotein and stem cell marker Tumor-Associated Calcium Signal TransDucer 2 (Trop-2, TACSTD2) is known to be overexpressed in carcinomas and its deregulation is associated with cancer progression and poor prognosis. Although Trop-2 may represent promising target for therapy, biological function of Trop2 in tissue maintenance and tumorigenesis remains unclear. As we described previously intratumoral heterogeneity in Trop-2 expression may significantly contribute to response and resistance to therapies targeting Trop-2-expressing cells [1]. We suppose that revealing more details about Trop-2 role and biological role in tumor development and metastasis is essential to evaluate an advantage of its targeting. One of already described Trop-2 role is associated with the Wnt signaling pathway. This pathway controls the balance between stem/progenitor self-renewal and differentiation and is frequently dysregulated during tumorigenesis. Proteolysis of Trop-2 on membrane leads to activation of β-catenin and thus contributing to oncogenic signaling. Aim of this study was to analyze *in vivo* phenotype of Trop-2 CRISPR knock-outed clones prepared from murine breast cancer cell line 4T1 12B luc2 and, in case of differences, reveal if Wnt is signaling pathway deregulated. We tested link between β-catenin activation in the cells with deregulated Trop-2 expression and nature of Trop-2 expression in this cells. Using western blot, flow cytometry and confocal microscopy, we characterized expression of total and active beta catenin as well as presence of Trop-2 with antibodies specific to different domains of Trop-2. Based on our data we can conclude that deregulation of Trop-2 expression is linked with modulation of beta-catenin expression and activity.

This work was supported by funds from the Ministry of Health of the Czech Republic, grant no. 17-28518A and 15-28628A, project no. LQ1605 from the National Program of Sustainability II (MEYS CR) and by project HistoPARK (CZ.1.07/2.3.00/20.0185).

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COMPARISON OF ALDH EXPRESSION IN HUVECS AND RABBIT ENDOTHELIAL PROGENITOR CELLS USING FLOW CYTOMETRY

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Aldehyde dehydrogenase (ALDH) seems to be a promising stemness marker of adult rabbit stem cells. We have already observed ALDH expression in rabbit endothelial progenitor cells (rEPCs) and adipose-derived mesenchymal stem cells (AD-MSCs) using confocal microscopy and also by flow cytometry in rabbit AD-MSCs. The aim of this preliminary study was to compare the expression of ALDH in human umbilical vein endothelial cells (HUVECs) and rabbit EPCs via flow cytometry. Briefly, rEPCs were isolated and cultured as described in our previous studies. The adherent cells were cultured until the confluency about 90% for at least 2-3 weeks till passage 3 as described previously. Frozen HUVECs culture (PCS-100-010, ATCC) were thawed and cultured the same way as rEPCs till the passage 3. Then cells were harvested and stained using Aldefluor kit (STEMCELL Technologies, USA) as follows: cells were washed and incubated with Aldefluor substrate for 15 min at 37°C, with or without the ALDH inhibitor diethylamino-benzaldehyde (DEAB) according to the manufacturer's protocol. Samples were analysed using a FACSCalibur flow cytometer (BD Biosciences, USA) according to the manufacturer's recommendations. At least 10,000 cells were analysed in each sample. Obtained data were statistically evaluated using SigmaPlot software and expressed as mean ± SEM. According to the flow cytometry analysis we observed higher ALDH expression in rEPCs (92.9±4.1%) in comparison to HUVECs (78.8±1.9%), although the difference was not statistically significant. In conclusion, the stemness of rabbit EPCs could be assessed on the basis of ALDH expression detected by flow cytometry.

This work was supported by the grants: APVV-14-0348, APVV-18-0146 and VEGA 1/0160/18.

TIME-LAPSE MICROSCOPY OF PRIMARY CHRONIC LYMPHOCYTIC LEUKAEMIA CELLS LOCOMOTION IN RESPONSE TO MICROENVIRONMENTAL STIMULI

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Motility is one of the crucial characteristics of B lymphocytes. It enables them to perform immune surveillance by circulating between lymph nodes and periphery, and across tissues, responding to the plethora of various chemotactic signals. This capacity is to some extent retained also in malignant CLL cells. It can influence efficacy of CLL therapy and contribute to the chronic nature of the disease.

Wnt/planar cell polarity (PCP) pathway controls cellular polarity, migration, and invasion, and is implicated in the pathogenesis of several diseases, including CLL. Expression of several components of this signaling pathway is altered in CLL, some of them, for instance WNT5A and WNT5B, are connected to more aggressive phenotypes, high migratory capacity, and poor prognosis (Janovská et al., 2016). Employing live cell imaging, we demonstrated that polarization of the core PCP protein VANGL2 is dependent on CK1 activity (Kaucká et al., 2015). Furthermore, inhibition of casein kinase 1δ/ε significantly blocked microenvironmental interactions of CLL cells and slowed down development of CLL-like disease in mouse model (Janovska et al., 2018). Taken together, we concluded that Wnt/PCP pathway provided additional regulatory signals, modulate interactions of CLL cells with their environment and affected their fate.

Time lapse microscopy is a versatile tool for studies of locomotion of CLL cells and for exploration of mechanisms and signaling pathways governing both chaotic and chemotactic movements and interactions with surrounding extracellular matrix and other immune and stromal cells. We test 2D as well 3D systems that provide various support and signals with or without gradients of chemotactic substances. We aim to dissect type or modes of movement and signaling pathways employed by CLL cells facing various microenvironmental challenges. Knowledge of these mechanisms can be utilized in optimizing and designing new strategies for CLL therapy.

This work was funded by the Czech Science Foundation (GA17-16680S) and the University Hospital Brno (6529705).

ROLE OF CD9 TETRASPANIN IN BREAST CANCER

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CD9 molecule (TSPAN-29) is a member of tetraspanin family – transmembrane proteins, which function in variety of cellular processes either in physiological conditions or in disease. CD9 protein is widely distributed in nearly all tissues, including variety of tumors and plays role in proliferation, invasiveness and adhesion, and dysregulation of later is often associated with epithelial-to-mesenchymal (EMT) transition and metastatic process. Previously we showed that CD9 associates with epithelial phenotype and favorable prognosis in breast cancer patients [1]. Hence, we aimed to investigate complex role of CD9 molecule in cancer plasticity in the context of breast cancer. To study the impact of CD9 on breast cancer biology, we transfected luminal breast cancer cell line MCF-7 with plasmids encoding shRNA targeting CD9. We hypothesized that abrogation of CD9 expression in generated MCF-7 CD9shRNA clones will lead to suppressed proliferation, adhesion capacity and more mesenchymal like phenotype compared to control clones with maintained levels of CD9. We found out that CD9 knockdown did not affect the proliferation of cancer cells in 2D. Using multicolor protocol for identification of cell phenotype, we analyzed the EMT signature in MCF-7 CD9-silenced clones. Decreased epithelial marker CD9 in CD9-silenced clones was associated with low levels of CD49f (integrin alpha-6) protein. As CD49f integrin protein (integrin alpha6)/integrin beta1 (CD29) as well as integrin alpha6/integrin beta4 (CD104) are receptors for laminin, we next investigated if decreased expression of CD49f affects capability of the CD9-silenced cells to bind this compound of ECM. In conclusion we examined the impact of CD9 on biological properties of proliferation, adhesion and EMT phenotype in MCF-7 cell line. Because tetraspanins specifically interact with other tetraspanins, integrins, and cell-specific receptors, and through these interactions might influence the cellular functions, further studies of CD9 associated proteins are needed to elucidate the complex role of this molecule in breast cancer progression.

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This work was supported by funds from Ministry of Health of the Czech Republic, grant nr. 15-28628A, NV18-08-00245, projects no. LQ1605 from the National Program of Sustainability II (MEYS CR), and by project HistoPARK (CZ.1.07/2.3.00/20.0185; KS). We would like to thank M. Huličiak for generation of MCF-7 CD9shRNA clones.

DEVELOPMENT OF 3D CULTIVATION MODEL OF IMMORTALIZED HUMAN MIHA HEPATOCYTES FOR EVALUATION OF ENDOBIOTIC AND XENOBIOTIC METABOLISM

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Currently, there are numerous problems with obtaining functional *in vitro* liver cells models. The isolated primary hepatocytes often lose their metabolic capacity, when cultivated as conventional *in vitro* cultures, while liver-derived cancer cell lines miss it almost completely. A number of approaches have been proposed to overcome these limitations. The cultivation of three-dimensional (3D) liver cell spheroids seems to be a suitable approach to improve features of hepatoma-derived cells, as well as to stabilize hepatocyte functional properties in primary hepatocyte cultures. Notably, this experimental approach may contribute to promote expression of receptors and enzymes/transporters involved in both phase I and II biotransformation, which play key roles in metabolism of drugs and other xenobiotics, as well as other, endogenous, compounds. In the present study, we attempted to introduce 3D-cultivation spheroid model of MIHA cell line representing immortalized human hepatocytes. Following 3D cultivation, MIHA cells were found to express higher levels of constitutive androstane receptor (CAR), a principal regulator of drug metabolism, as compared with their 2D counterparts. Importantly, this was accompanied with enhanced expression of cytochrome P450 (CYP) enzymes that are typically highly expressed in mature hepatocytes, such as CYP1A2 and CYP3A4, as well as with an increased production of albumin. Overall, both 2D- and 3D-cultivated MIHA cells showed a good response to the model ligand of the aryl hydrocarbon receptor (AhR); however, 3D-cultivated cells showed an improved response to ligands of CAR and pregnane X receptor (PXR), such as rifampicin, CITCO or amiodarone. In conclusion, 3D cultivation of MIHA cell spheroids might serve as a promising model for evaluation of mechanisms regulating expression/activity of enzymes contributing to metabolic activities of liver cells. In particular, cultivation of spheroids of MIHA cells could be in future also optimized for increased throughput methods suitable for screening of enzymatic activities. [Supported by the Czech Health Research Council (grant No 17-28231A), by the Czech Science Foundation (project No. 19-00236S), by the Czech Academy of Sciences, and The Brno City Municipality (Brno Ph.D. Talent).]

REVIVAL OF CHEMOSENSITIVITY AND RESISTANCE ASSAY IN SOLID TUMORS – A SINGLE-CELL SIGNIFICANCE EVALUATION

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More than a 50-year failure in the adaptation of experimental chemosensitivity and resistance assay (CSRA) in the clinics is caused by ignorance of the cellular heterogeneity in a sample and tumor heterogeneity in a patient. Rapid visualization and statistical single-cell evaluation of a patient-specific tumor cell response to chemotherapy (CT) would make selection of the optimal therapeutic modality possible. In the era of immunotherapy, a fast 3-day turnover of functional data will facilitate the clinical decision-making process.

Therefore, we test an *in vitro* outcome of a combinatorial CT treatment in two ways – viability/proliferation of each cancer cell and its alteration in the surface expression profile. CT-induced surface exposure of "danger-associated molecular patterns" (DAMP) during diverse cell death processes initiates antitumor response by activating innate immune cells. We focus on complex detection of so-called immunogenic cell death (ICD) markers, hence improving the uniparametric retrospective studies performed nowadays in the clinics. ICD has been defined by sequential externalization of three crucial DAMP molecules: endoplasmic reticulum-resident calreticulin (CALR), cytoplasmic ATP and nuclear HMGB1. The effect of DAMP and other positive regulators is counterbalanced by increased surface expression of innate immune cell inhibitors. The immunogenic properties of dying tumor cells are therefore determined by the final balance of these regulatory signals, which change over the course of CT.

An 8-color panel was designed for the evaluation of CSRA and ICD. Comparative single-cell data from flow cytometry and microscopy analysis describe kinetic changes in the bladder carcinoma followed by a single-cell analysis using a novel unsupervised dimensionality reduction algorithm EmbedSOM. Visual significance testing in 2D-embedded plot reveals a unique fingerprint for each cell population and perturbation combination. The final assay output shows tumor cell heterogeneity with respect to the functional and expression profile. Minor resistant cell populations will be characterized and the outcome of statistically significant changes during a 3-day CT treatment will be discussed in detail.

URBAN DUST AND DIESEL EXHAUST PARTICLES ELICIT ESTROGEN-LIKE EFFECTS ON ER-DEPENDENT GENE EXPRESSION AND CELL CYCLE PROGRESSION

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Polycyclic aromatic hydrocarbons (PAHs) are persistent environmental contaminants interacting with the aryl hydrocarbon receptor (AhR). Increasing attention has been paid to the impact of PAHs on estrogen receptors (ER). However, their mechanisms of action are not clear. Here, we analyzed the effects of extracts derived from urban dust (SRM 1649b) and diesel exhaust particles (SRM1650b) reference standards, using both total extracts (CE) and their polar aromatic compounds fraction (F3). First, we evaluated the effect on ER-dependent reporter gene induction in ovarian cancer cells, MCF-7 and BG-1, respectively. We observed a high ER-dependent reporter gene induction in MCF-7 cells exposed to CE and F3 of both SRMs. In contrast, the ER-dependent reporter gene induction was only minor in BG-1 cells. Neither CE nor F3 fractions inhibited the 17β-estradiol (E2) induced reporter gene activity in MCF-7 cells. Using wild type and AhR knock-out (AhR KO) variant of MCF-7 cells, we then analyzed the impact of AhR on the ER-dependent reporter gene expression, cell cycle progression (flow cytometry), expression of endogenous ERα-regulated genes (qRT-PCR), cell proliferation, as well as induction of retinoblastoma tumor suppressor protein (pRb) phosphorylation (Western blotting). We found that, although higher doses of CE and F3 could be cytotoxic and genotoxic in MCF-7 cells, CE and F3 also stimulated accumulation of cells in S-phase of cell cycle, induction of pRb phosphorylation (Ser 807/811) and that they partly induced the expression of the ERα-regulated endogenous gene, *PGR*. The absence of AhR in AhR KO cells altered estrogen-like effects of CE and F3 fractions, in particular those derived from urban dust particle sample. In conclusion, our results suggest that both urban dust and diesel exhaust particles could display estrogenic-like effect in a human model of estrogen-sensitive cells, and that the AhR, as well as the AhR-dependent metabolism of polyaromatic compounds may, perhaps in a tissue-dependent manner, modulate their impact on estrogen-dependent gene expression and cell proliferation. [G. V.-G. is supported by the National Council of Science and Technology. Mexico City, Mexico. The Czech Science Foundation support (project no. 16-17085S) and support of the Czech Academy of Sciences are acknowledged.]

CLINICAL CYTOMETRY

REPRODUCIBILITY OF FLOW CYTOMETRY THROUGH STANDARDIZATION: OPPORTUNITIES AND CHALLENGES

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Standardization is both a promising and controversial issue in current clinical and translational flow cytometry. Standardization can provide a platform for improved reproducibility of cytometry results over prolonged periods of time, across different sites and across different instruments. For the purpose of structured discussion, we describe several standardization aims: common interpretation of the immunophenotype definition of a target subset, accurate quantification, standardized multicolor and standardized intensity of all measured parameters. We provide an overview of how standardization was approached by several large consortia: EuroFlow, The ONE Study, Human Immunology Project Consortium (HIPC) and several other groups. We note their particular aims and the tools adopted to reach those aims. We review how those standardization efforts were adopted in the field and how the resulting outcome was evaluated. Multiple challenges in instrument hardware design, instrument setup tools, reagent design and quality features need to be addressed to achieve optimal standardization. Furthermore, the aims of different studies vary, and thus, the reasonable requirements for standardization differ. We offer a framework of reference for the reasonable outcomes of different approaches. Finally, we argue that complete standardization is important not only for the reproducibility of measurements but also for education, for quality assessment and for algorithmic data analysis. The different standardized approaches can and in fact should serve as benchmarking reference tools for the development of future flow cytometry studies.

THE EFFECT OF EARLY POSTNATAL SUPPLEMENTATION OF NEWBORNS BY PROBIOTIC STRAIN ESCHERICHIA COLI ON REGULATORY T CELLS

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Increasing incidence of allergic diseases during last couple of decades together with steep raise of financial and sociological demands brought up the need to stop this trend or even reverse it. Due to the growing knowledge of the key role of microbiota in the maturation of neonatal immune system it seems that manipulation of microbiota could be exploited in hampering allergy development. In our study, *Escherichia coli* O83:K24:H31 (EcO83) was administered to newborns within 48 hrs after delivery and children were followed prospectively. Several immunological characteristics (cytokines, specific IgE, total T regulatory cells (Treg) and subpopulation of natural Treg (nTreg) and induced Treg (iTreg)) were tested in peripheral blood of eight year old children. Incidence of allergic disease was decreased in EcO83 supplemented children and significantly elevated levels of IL-10 and IFN-ɣ were detected in serum of EcO83 supplemented children. Probiotic supplementation did not influence the numbers of the total Treg population but their functional capacity (intracellular expression of IL-10) was significantly increased in children supplemented with EcO83 in comparison to non-supplemented children. Moreover, decreased proportion of iTreg was present in peripheral blood of non-supplemented in comparison to EcO83 supplemented children. Interestingly, children suffering from allergy have lower numbers of iTregs and intracellular presence of IL-10 in distinct subpopulations of Tregs. Stimulation of cord blood cells with EcO83 *in vitro* promoted both gene expression and secretion of IL-10 and IFN-ɣ without induction of Treg. After coculture of EcO83 primed dendritic cells with naive CD4+ T cells, increased levels of IL-10 were detected by flow cytometry. We can conclude that beneficial effect of EcO83 in prevention of allergy development could be mediated by promotion of regulatory responses (by IL-10) and Th1 immune response (IFN-γ). This work was supported by AZV15-26877A and Progres Q25/LF1.

GRANULOCYTES IN CVID PATIENTS ARE ACTIVATED AND POSSESS SUPPRESSORY FUNCTION

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Background: Common variable immunodeficiency disorders (CVID) is a group of primary immunodeficiency diseases characterized by hypogammaglobulinemia, disturbed specific antibody production and recurrent infections due to dysfunctional immune response to invading pathogens. Our previous data suggested that altered cytokine profile observed in patients with CVID may be attributed to activation of cells of the neutrophile lineage. Methods: A detailed phenotypic and functional multicolor cytometric characterization of neutrophils and IFN-gamma intracellular T cells production, measurement of proliferation was performed in 46 CVID patients and 44 healthy donors. Results: Neutrophils from CVID patients exhibited elevated surface levels of CD11b and PD-L1 and decreased levels of CD62L, CD16, and CD80, consistent with a phenotype of activated neutrophils with suppressive properties. CVID neutrophils actively suppressed T cell activation, proliferation, and production of IFN-gamma. Furthermore, CVID was associated with increased frequency of low-density neutrophils (LDNs)/granulocytic myeloid-derived suppressor cells (G-MDSCs), which suppressed T-cell activation and production of IFN-gamma but not proliferation of PBMCs. Conclusion: We present evidence that neutrophils in CVID patients acquire a specific activated phenotype and exert potent T cell suppressive activity, a previously unrecognized mechanism of immune suppression in CVID patients. This observation may be of a high potential importance as it provides a qualitatively new view on the mechanisms of immune suppression in CVID and potentially other immunodeficiencies and inflammatory conditions. This work was supported by grant 15-28732A from the Czech Ministry of Health.

DOES CHARACTERISTIC PHENOTYPE FOR PLASMA CELL LEUKEMIA EXIST?

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Background: Plasma cell leukemia (PCL) is characterized by a presence of circulating plasma cells (cPCs) in peripheral blood. Primary PCL (pPCL) occurs in patients *de novo* while secondary PCL (sPCL) is a leukemic transformation of multiple myeloma (MM). Detection of cPCs by flow cytometry (FC) is important for PCL diagnostics and its discrimination from reactive plasmacytosis.

Aim: Analyses of pPCL and sPCL cases to identify their phenotype profile in comparison with MM.

Methods: Peripheral blood (PB) and/or bone marrow (BM) PCs of 12 patients with pPCL, 10 patients with sPCL and 64 MM patients were analysed. Expression of CD19, CD20, CD27, CD28, CD44, CD56, and CD117 together with nestin was studied by FC.

Results: There were found similar % of cPCs in both PCL types. Infiltration of BM was the highest in sPCL, compared to pPCL and MM. No CD19+ and/or CD20+ PCs was found in sPCL, slightly increased number of positive cases was found in pPCL when compared to MM. Number of CD56+ cases was higher in BM of MM then in pPCL and sPCL; in PB slightly higher for pPCL compared to sPCL. Number of CD27 positive cases was the highest in BM of MM compared to PCLs. Expression of CD44 was decreased in MM compared to PCLs, where CD44 was expressed in 100% of BM samples; PB expression was lower in pPCL than in sPCL. CD117 was mostly expressed in BM of sPCL and MM when compared to pPCL. Nestin was highly expressed in both PCLs and decreased in MM.
Summary: Phenotype profile of pPCL and sPCL is similar, except for a disappearance of CD19 and CD20 in sPCL and decrease of CD117 in pPCL. Lower expression of CD56, CD27 and overexpression of CD44 with nestin was characteristic for both PCLs when compared to MM.

Supported by AZV NV18-03-00203
Keywords: Flow cytometry, Plasma cell leukemia, Phenotype

USEFULNESS OF 6-COLOUR MULTIPARAMETER FLOW CYTOMETRY IN CANINE LYMPHOMA PHENOTYPING

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Flow cytometry (FC) is a critical tool in the objective diagnosis and further characterization of lymphoma. Features such as expression of cell surface markers or cell size can provide important prognostic information. In many laboratories, immunophenotyping of canine leukocytes is usually performed using 2-colour or 3-colour FC. The aim of this study was to introduce in praxis a compatible 6–colour multiparameter FC protocol for routine lymphoma phenotypisation using commercially available monoclonal antibodies. For 6-colour FC, directly conjugated (CD3-FITC, CD8-AF700, CD25-eFluor660, CD21-PE) as wells as unconjugated (CD4 and γδTCR) anti-dog monoclonal antibodies and appropriate conjugates (PeCy7 and BV421) in optimal dilution were used. Furthermore, fluorescent minus one controls were prepared for compensation set-up. Fifteen samples of peripheral blood or samples of fine-needle aspirates of peripheral lymph nodes from dogs with suspected lymphoproliferative disease were stained using 2-colour and 6-colour protocol and analysed with a flow cytometer LSRFortessa operated with BD FACSDiva software v. 6.2 (BD Biosciences) to compare the results. From all tested samples, 9 were diagnosed as B-cell and 6 as T-cell lymphoma (including 3 T-zonal and 2 γδ-TCR lymphoma). No spectral overlaps or nonspecific binding of used fluorochromes were detected. Six-colour FC is powerful method to increase the accuracy of lymphoma phenotyping by simultaneous staining of lymphocyte markers with six specific antibodies in one test tube. When compare to 2-colour FC, 6-colour approach proves to be helpful mainly in case of aberrant phenotypes and in small cell types of lymphoma, where neoplastic cells are hardly differentiable from other lymphocytes. Moreover, lesser total amount of samples and reagents are used, since less duplications of the reagents among multiple tubes is required.

This study was supported by the Ministry of Agriculture of the Czech Republic (MZE-RO0518).

THE DYSREGULATION OF NK CELLS AND MONOCYTE SUBPOPULATIONS IN IN THE PRECLINICAL PHASE OF RHEUMATOID ARTHRITIS.

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The anti-citrullinated proteins antibodies (ACPA) are present months before the rheumatoid arthritis (RA) manifestation with 8-10x higher risk in ACPA+ individuals. The individuals with arthralgia suspicious for RA progression are characterised with clinical features (clinically suspect arthralgia, CSA). The alteration of natural killer (NK) cells and monocyte subpopulations was reported in patients with established RA. We present their analysis in the preclinical phase of RA.

Methods: The peripheral blood lymphocyte subpopulations; defined as B (CD19), T (CD4 and CD8) and NK (CD16/56) cells and monocyte subsets; classified to classical, intermediate and non-classical (based on CD14 and CD16 expression) were evaluated by flow cytometry in 49 individuals with arthralgia and 80 age and gender matched healthy controls (HC). Data were analysed with Mann Whitney test ($p<0.05$ considered significant) and median values of proportion [%] or absolute counts [G/l] compared to HC are expressed.

Results: The 36 individuals with arthralgia were ACPA+, 28 met CSA definition and 10 developed RA within a median 4 months follow up.

Individuals with arthralgia had higher %CD3+ T cells (78.10 vs. 75.04) and lower %NK (11.20 vs. 12.89) as well as absolute count of NK cells (0.18 vs. 0.24). In ACPA+ individuals higher %CD3+ T cells (77.95 vs. 75.04), lower %NK (10.85 vs. 12.89) and absolute NK cells (0.20 vs. 0.24) were confirmed. Patients who developed RA, had lower baseline absolute NK cells (0.14 vs. 0.19).

The higher % of non-classical (5.99 vs. 4.03) and intermediate (5.82 vs. 4.31) monocytes and lower classical monocytes (86.06 vs. 89.41) in individuals with arthralgia were present. ACPA+ individuals had higher % of non-classical (6.07 vs. 4.03) and intermediate (5.86 vs. 4.31) and lower % of classical (85.43 vs. 89.41) monocytes.

Conclusions: The lower numbers of NK cells and classical monocytes and expansion of intermediate and non-classical monocytes in the preclinical phase of RA, especially in ACPA+ individuals may be a prognostic marker. Such disproportion or pro-inflammatory potential of these cells was previously shown in the pathogenesis of established RA. We hypothesize that the dysregulation observed in at-risk individuals may predispose to further development of RA.

Acknowledgement: AZV-17-32612A and MHCR 023728.

FLOW CYTOMETRIC CHARACTERIZATION OF LYMPHOMA IN DOGS FROM THE CHECH REPUBLIC – A RETROSPECTIVE STUDY

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Lymphoma is the most common hematologic malignancy in dogs. The diagnosis of lymphoma in dogs is based mainly on cytopathology, immunohistochemistry, flow cytometry and PCR for antigen receptor rearrangements (PARR). Flow cytometry is a very precise and accurate method to determine the immunophenotype of neoplastic cells and can provide important information regarding prognosis and response to therapy. The aim of this retrospective study was to determine the relative frequencies of various lymphoma subtypes in lymph node aspirates from 112 dogs with suspected lymphoproliferative disease using flow cytometry. Results of flow cytometry examination performed between January 2012 and December 2018 at the VRI were included. Following monoclonal antibodies were used for two or one colour flow cytometry: CD45, CD3, CD4, CD8, γδ-TCR, CD79α, CD21, MHCII, CD45RA and CD90. B-cell lymphoma was diagnosed in 70% (n=78) and T-cell lymphoma in 30% (n=34) of the cases. All cases of B-cell lymphoma were positive for CD79α and CD45 and all except 4 samples were positive for CD21. Of the CD79α+/CD45+/CD21+ tumors 48 were MHCII+, 6 were MHCII- and 20 lymph node aspirates expressed various levels of MHC II. Of the T-cell lymphoma 4 samples each were CD4+ or CD8+, and 3 samples expressed the γδ-TCR phenotype. Four aspirates were immunophenotypically consistent with T-zone lymphoma (CD45-, CD3+, CD21+). Co-expression of CD3 with CD79α was detected in 11 cases. In the remaining 8 samples predominance of CD3+ cells with different expression of other T-cells markers were found. In accordance with other studies, we confirmed a higher frequency of B-cell vs. T-cell lymphoma in the dog and frequent occurrence of different phenotypes in T-cell lymphomas.

This study was supported by the Ministry of Agriculture of the Czech Republic (MZE-RO0518).

ANALYSIS OF GENE EXPRESSION IN CD26+ CML LEUKEMIC STEM CELL POPULATION

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Background: Nowadays, chronic myeloid leukemia (CML) has become a well manageable disease and majority of the patients achieve remission on tyrosine kinase inhibitor (TKI) treatment. However, the disease usually shows a low-level persistence during therapy that arises from putative leukemic stem cells (LSC), which are, despite *BCR-ABL1* positivity, resistant to TKI treatment. LSC can be identified and isolated based on a surface expression of specific surface markers of which CD26 is perhaps the best described with high positive correlation with the occurrence of *BCR-ABL1*.

Aims:

Our aim was to compare gene expression (GE) profiles of CD26+ LSC, CD26- HSC and CD38+ progenitor cells (PC) from CML patients and identify potential novel CML LSC markers or disrupted pathways.

Methods:

Bone marrow (BM) cells were obtained from 18 CML patients and stained for the following surface markers – CD34, CD38 and CD26. Cells were FACS sorted into 3 populations – LSC (CD34+CD38-CD26+), HSC (CD34+CD38-CD26-) and PC (CD34+CD38+) using BD FACSAria Fusion cell sorter. cDNA samples from 5 patients were pre-amplified with Ovation Pico WTA System V2 whole transcriptome amplification kit (NuGEN) and analysed on SurePrint G3 human GE 8x60k microarray (Agilent). Gene expression data validation was performed on the same cell populations collected from a novel extended cohort of 13 CML patients on SmartChip real-time PCR system (Takara Bio).

Results:

Analysis of GE microarray data identified over 272 differentially expressed genes (160 upregulated and 112 downregulated) between CD26- HSC and CD26+ LSC, and over 1330 differentially expressed genes (531 upregulated and 799 downregulated) between CD26- HSC and CD38+ progenitors. ($P \leq 0.05$; fold change ≥ 1). The subset of the most deregulated genes between populations was validated on an extended cohort of different CML patients using SmartChip real-time PCR, showing high correlation between both systems (R^2_{LSC} = 0.808; R^2_{PC} = 0.913) and supporting the results of GE microarray analysis. Validated candidate genes (n = 15), consisting of those involved in important pathways (e.g. IL1, mTOR signalling - TAB2, PELI2, MYCBP2) or associated with oncogenic transformation (e.g. MYB, RAD51), will be further studied in functional assays. Of highest interest are the top

deregulated genes histidine decarboxylase (HDC) and immunoglobulin J chain (IGJ), whose expression was previously reported deregulated in *BCR-ABL1* positive cells or CML stem cells. Both HDC and IGJ exhibited opposite expression pattern in CD26+ LSC and CD38+ PC subpopulations compared to CD26- HSC (FC_{HDC} = 2.8 vs 4.3; FC_{IGJ}= 2.8 vs -2.8; FC - fold diference in expression). Further, we identified CD69 (Early lymphocyte activation antigen) as a gene specifically upregulated in CD26 + LSC, whose expression was also shown to be elevated in cell lines expressing *BCR-ABL1*. Currently, we are analysing its potential as a specific surface marker.

Conclusions:

In summary, we identified consistent changes of gene expression in CD26+ LSC as well as CD38+ progenitor populations, with only 3% gene overlap showing specificity of these patterns for selected populations. These results were successfully verified using high-throughput real-time PCR. The involvement of identified candidate genes and pathways, which could provide important insights into the CML stem cell biology, are currently evaluated.

Supported by MUNI/A/1105/2018, by Ministry of Health of the Czech Republic, grant nr. 17-30397A, and by institutional support of Faculty of Medicine, MU.

We acknowledge the CF Genomics CEITEC MU supported by the NCMG research infrastructure (LM2015091 funded by MEYS CR) for their support with obtaining scientific data presented in this paper

A SIMPLE NO-WASH ASSAY FOR THE DETECTION OF TUMOR NUCLEI PLOIDY, PHENOTYPE AND FUNCTION FOLLOWED BY UNSUPERVISED ANALYSIS

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Tumor cell heterogeneity is still poorly understood at a single-cell level and its correlation to the disease outcome has long remained unclear. Diverse differentiation states of carcinomas (here detected by KRT5/KRT14/KRT20) contains also heterologous cell populations originating from different germ layers (here detected by VIM/KRT18). Moreover, the expression switch in these intermediate filaments is a hallmark of epithelial-mesenchymal transition (EMT) during metastasis. Currently, flow cytometry starts to become the method of choice also for the analysis of solid tumors composed mainly of cell aggregates in the analyzed samples. Here, we present a combined DNA/antibody staining no-wash method of isolated nuclei and a novel unsupervised analytical pipeline. **The aim of our work is to provide clinicians a one-day feedback on the quality of surgical tumor removal and the respective rare tumor cell quantification.**
For the detection of early stage tumors, we have selected bladder cancer (BCa) as one of the top heterogeneous malignancies with a very high mutation burden. From urine and three washouts (pre/mid/post) during tumor resection (TURBT), cell nuclei were prepared using a modified Vindeløv method. We have selected 4 small cohorts of control, non/recurrent and progressing BCa patients from over 200 patients analyzed. A standard 8-color cytometer setup was used for a panel design to be compatible with a typical clinical lab equipment. Cell cycle/aneuploidy was detected in the isolated nuclei together with antibody staining of nucleus-attached KRT/VIM and a nuclear proliferation marker Ki67. Altogether, we have been able to classify normal and tumor profiles in each sample into three main subtypes: basal, intermediate and luminal or squamous, respectively.
Tumor and other populations of nuclei have been classified by manual gating (FlowJo) as well as unsupervised analysis using a novel dimensionality reduction tool EmbedSOM/DiffSOM. Both results were compared in detail and a significance testing allowed us to define tumor cell populations correlating with the disease recurrence and/or progression. Eventually, a patient tumor fingerprint will be compared to the complex TNM disease classification, including grading (G) and standard urine cytology assessment in BCa patients.

IMMUNOLOGY

RECENT THYMIC EMIGRANTS AND ACTIVATION STATUS IN DIAGNOSTIC IMMUNOPHENOTYPING OF SEVERE PRIMARY IMMUNODEFICIENCIES: AN EUROFLOW STANDARDIZED APPROACH

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The EuroFlow PID consortium developed a set of flow cytometry tests for evaluation of patients with suspicion of primary immunodeficiency (PID). In this technical report we evaluate the performance of the SCID-RTE tube that explores the presence of recent thymic emigrants (RTE) together with T-cell activation status and maturation stages.
We have analyzed peripheral blood cells of 26 patients diagnosed between birth and 2 years of age with genetically defined primary immunodeficiency disorder: 15 SCID patients had disease-causing mutations in *RAG1 or RAG2* (n=4, two of them presented with Omenn syndrome), *IL2RG* (n=4, one of them with confirmed maternal engraftment), *NHEJ1* (n=1), *CD3E* (n=1), *ADA* (n=1), *JAK3* (n=3, two of them with maternal engraftment) and *DCLRE1C* (n=1) and 11 other PID patients had diverse molecular defects (*ZAP70* (n=1), *WAS* (n=2), *PNP* (n=1), *FOXP3* (n=1) del22q11.2 (DiGeorge n=2; complete DiGeorge n=2), *CDC42* (n=1) and *FAS* (n=1). In addition, 44 healthy controls in the same age group using the SCID-RTE tube in 4 EuroFlow laboratories using standardized 8-color approach.
RTE were defined as CD62L+CD45RO-HLA-DR-CD31+ and the activation status was assessed by the expression of HLA-DR+. Naïve CD8+ lymphocytes and naïve CD4+ lymphocytes were defined as CD62L+CD45RO-HLA-DR-.

With the SCID-RTE tube we identified patients with PID by low levels or absence of RTE in comparison to controls as well as low levels of naïve CD4+ and naïve CD8+ lymphocytes (100% sensitivity in SCID). All SCID patients had absence of RTE, even the patients with confirmed maternal engraftment or oligoclonally expanded cells characteristic for Omenn syndrome having normal T-cell counts. Another dominant finding were the increased numbers of activated CD4+HLA-DR+ and CD8+HLA-DR+ lymphocytes in these patients.

Therefore, the EuroFlow SCID-RTE tube together with the previously published PIDOT PID tube forms a sensitive and complete cytometry diagnostic test suitable for patients suspected of severe PID (SCID or CID) and for children identified via newborn screening programs for SCID with low or absent T-cell receptor excision circles (TRECs).

METABOPROFILING OF HUMAN MONOCYTES BY FLOW CYTOMETRY

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Energy metabolism of monocytes in steady-state is represented by low-levels of fatty acid oxidation (FAO). While, their exposure to environmental stimuli is accompanied by changes in energy metabolism. Monocyte activation leads to the metabolic switch, represented by increased glycolysis. The initiation of the inflammatory response is linked with anabolic processes and requires glucose as the primary source of energy. In contrast, the late phases of inflammation are mainly catabolic, fueled by oxidation of fatty acids.
All metabolic pathways, which can be used for generation of ATP in the cell, are unique and their parts can be determined by flow cytometry. We observed changes in the expression of metabolic markers CPT-1, CD36, GLS, GLUT-1 and ATP5A which represent components of different metabolic pathways responsible for the energy generation.
The development of metabolic changes is regulated by various signaling pathways. The amplified glucose fueling after monocyte activation is accompanied by disrupted mitochondrial pyruvate oxidation. These modifications, leading to changes in expression levels of glycolysis controlling genes, are under HIF-1α control. Whereas late phase of activation leading back to FAO and OXPHOS is under PGC-1 supervision. Calcineurin (CN)-NFAT has been shown to play an essential role in myeloid cells, and its inhibition leads to various impairments, including increased susceptibility to infections, but there is lacking evidence about the role of CN-NFAT in monocyte metabolism.
In summary, we suggest the new role of CN-NFAT pathway in the co-regulation of glycolysis state and fatty acid metabolism, suggesting CN as a vital candidate participating in the control of metabolic processes upon monocyte stimulation and the resolution of inflammation.

THE HUMAN CIRCULATING HEMATOPOIETIC STEM CELL COMPARTMENT IS IMPAIRED DURING SEPSIS.

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Sepsis is a complex clinical syndrome affecting ~30 million people worldwide every year, 6 million of whom do not survive. This pathology is generally characterised by an early hyper-inflammatory phase followed by a latter immunosuppressive phase. Although being one of the leading causes of death in ICU, the immunological bases of this pathology remain poorly understood.
During infection, pattern recognition receptors (PRRs) bind microbial ligands and activate signalling cascades culminating in immune cells activation and pathogen clearance. Hematopoietic stem and progenitor cells (HSPCs) can directly sense infection by PRR-triggering, which promotes their proliferation and differentiation towards the myeloid lineage, in order to replenish the pool of cells lost during the clearance. Different PRR agonist can affect HSPCs differentiation, cell fate and phenotype of terminally differentiated cells. However, a massive and continuous triggering by bacterial products can induce a state of unresponsiveness of myeloid cells known as "endotoxin tolerance".

Here we show that human HSPCs are expanded and myeloid skewed during the early phases of sepsis. As a possible result of impaired HSPCs development, patients affected by sepsis show also an expansion of different subsets of myeloid-derived suppressor cells (MDSCs), which likely participate in the latter immunosuppressive stages of the pathology. Moreover, we demonstrate that HSPCs from septic patients can give rise to a myeloid progeny with impaired immunological functions.

Understanding the derailments that can occur during hematopoiesis will help identify novel pathologic hallmarks of sepsis and facilitate the design of new therapeutic targets.

The research is supported by European Social Fund and European Regional Development Fund—Project MAGNET (No. CZ.02.1.01/0.0/0.0/15_003/0000492).

ROLE OF TRANSCRIPTION FACTORS IN PERIPHERAL BLOOD MONOCYTE RESPONSES TO SEPSIS

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Sepsis is characterized by dysregulation of innate immune responses to infection. An immunosuppressive state commonly occurring in sepsis survivors leaves them vulnerable to secondary exposure to pathogens. The circulating peripheral blood monocytes are an important first line of host defence against pathogenic organisms. As sepsis progress, monocytes are subjected to functional reprogramming, which was reported to be driven by transcription factor HIF-1α. However, engagement of other transcription factors, such as NFAT and NF-κB, as well as their eventual crosstalk in monocyte responses during sepsis is poorly described. In order to analyse the activity of transcription factor in monocytes, we established HIF-1α and NF-κB luciferase reporters derived from the human monocytic cell line and challenged them with fungal and bacterial stimuli in the presence or absence of their respective inhibitor. In order to address the potential crosstalk, we also treated the reporter cell lines with inhibitors specific for other transcription factors. Similarly, we examined the crosstalk in peripheral blood monocytes obtained from healthy donors by analysing the transcripts regulated by specific transcription factor. Along with these experiments, we collected peripheral blood monocytes obtained from septic patients for assessment of nuclear translocation of HIF-1α, NFAT and NF-κB within defined timepoints of sepsis progression using confocal microscopy. We hypothesize that cellular localization of said transcription factors during sepsis progression changes accordingly to severity of a disease. In conclusion, we found that specific inhibitors of HIF-1α, NFAT and NF-κB are partially affecting activation of unrelated pathways as well. The importance of reported transcription factor crosstalk in the progress and for eventual changes in therapy of sepsis is yet to be concluded.

THE EFFECTS OF CYTOKINES ON DEVELOPMENT OF SUPPRESSIVE B LYMPHOCYTES

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Cytokines represent a broad group of small molecules that are indispensable for the communication among cells of the immune system. Cytokine environment strictly influences the development of individual subpopulations of immune cells. We analysed the effects of different cytokines on the development of IL-10-producing B cells (suppressive B cells) *in vitro*. It was shown that the percentage of IL-10-producing B cells is significantly enhanced by IL-12 and IFN-γ and negatively regulated by IL-21 and transforming growth factor-β. Moreover, macrophages, which were cocultivated with B cells stimulated with LPS and IFN-γ, showed decreased expression of costimulatory molecule CD86 and reduced production of IL-6. These macrophages displayed also decreased ability to stimulate a proliferation of activated $CD8^+$ T cells. Furthermore, we studied the role of transcription factors GATA3 and Foxp3 in IL-10 production by LPS-stimulated B cells. It was found that IL-10 production by B cells is independent of GATA3 and Foxp3 expression. These findings are in a sharp contrast with the observation in T cells, where IL-10 production correlates with GATA3 or Foxp3 expression. The results have shown that IFN-γ enhances activation of suppressive functions of B cells which have the ability to suppress immune response through their effect on macrophages. Nevertheless, the production of IL-10 by B cells is not dependent of the presence of GATA3 or Foxp3.

This study was supported by project LO1508 from the MEYS of the Czech Republic.

ACCELERATED AGING OF IMMUNE CELLS IN CHILDHOOD AND ADOLESCENT CANCER SURVIVORS

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The therapy of neuroblastoma, the most frequent extra-cranial solid tumor in early childhood, is targeting important functions of tumor cells. While replication arrest and induced differentiation of these cells are the therapeutic goals, the damages of other cell types form an eventual risk in long-term perspective. By-products of cancer therapy and severe tissue damage, called damage-associated molecular patterns (DAMPs), can spread within the blood stream where they can engage with myeloid cells and their progenitors, leading to their activation and initiation of sterile inflammation.
Senescence of immune cells is characterised by the decline of a plethora of immune cell functions including adaptive as well as innate responses and is associated with a number of pathologies linked to aging, such as higher susceptibility to infections and cardiovascular diseases. Deterioration of immune responses in context of immunosenescence has been described in adults (>40 years) and frailty, a condition linked to chronic inflammation and accelerated aging, has been already described in young adult childhood cancer survivors. Therefore, we hypothesise that intensive therapy including treatment with 13-cis-retinoic acid and topotecan and/or inflammatory burden caused by acquired comorbidities, serve as inducers of accelerated aging of immune system. In this study we focused on the molecular mechanism of how neuroblastoma therapy, namely 13-cis-retinoic acid and topotecan, or the presence of DAMPs affect the immune cells by addressing the changes in telomere control, hematopoietic differentiation and ability to induce senescence. Furthermore, analysis of immunosenescent phenotype and other signs of accelerated aging in high-risk neuroblastoma patients are also included in this study.

REGULATION OF CD163 EXPRESSION IN HUMAN BLOOD MONOCYTES BY IL-10

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CD163 represents well-conserved myeloid membrane antigen serving as a scavenger molecule for hemoglobin-haptoglobin complexes and receptor for cytokine TWEAK. In tissue macrophages derived from blood monocytes, CD163 expression seems to be a marker of subpopulation downregulating immune responses known as M2 macrophages. The aim of our study was to evaluate regulation of two different epitopes of CD163 in peripheral blood monocytes in response to IL-10, an anti-inflammatory cytokine associated with M2 differentiation. Peripheral blood mononuclear cells from healthy donors or monocytes from transfusion unit buffy coats were cultured for 6 days and CD163 expression measured by flow cytometry (Navios, Beckman Coulter) with monoclonal antibody clones GHI/61 (BioLegend) and RM3/1 (BioLegend). Different subpopulations of monocytes ($CD14^{++}CD16^{-}$; $CD14^{+}CD16^{+}$; $CD14^{low}CD16^{+}$) were evaluated separately.

The basal expression of GHI/61 epitope on blood monocytes was higher as compared to RM3/1 epitope. The expression of both CD163 epitopes spontaneously increased until day 3 and was markedly augmented by IL-10 at 20 ng/ml. The induction of CD163 on non-classical monocytes ($CD14^{low}CD16^{+}$) was relatively limited. In monocytes from buffy coats, spontaneous induction of GHI/61 epitope reaches the maximum at 24h.

We conclude from our data that monocyte subpopulations differ in their capacity to express CD163 with pro-inflammatory subset of non-classical monocytes ($CD14^{low}CD16^{+}$) being the less sensitive one. Buffy coat separation at the transfusion unit seems to facilitate the spontaneous upregulation of GHI/61 epitope of CD163.

Supported by Ministry of Health of the Czech Republic, grant nr. 15-26883A.

IMPORTANCE OF ADENYLATE CYCLASE ISOFORMS IN REGULATION OF T LYMPHOCYTES DIFFERENTIATION

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Cyclic AMP (cAMP) is an important intracellular second messenger, which is produced by adenylate cyclases (ACs). Ten mammalian AC isoforms identified up to date differ in their tissue distribution and biochemical regulation. The only known specific activator of membrane AC isoforms 1 to 8 is labdane diterpene forskolin. cAMP regulates both innate and adaptive immune cell activities as a potent immunosupressor. In leukocytes, three dominant AC isoforms (AC3, AC7 and AC9) are suggested.
Our study is aimed to clarify importance of different AC isoforms in regulation of function of selected subpopulation of T-lymphocytes and to elucidate possibilities how to specifically modulate production of cAMP in these cell types. T helper cells (CD4+) cells were sorted and stimulated by selected activators. Formation of cAMP was measured by homogenous time-resolved fluorescence resonance energy transfer (TR-FRET) together with cell proliferation and immunophenotypic characterization of lymphocyte activation state. Further, analogues of forskolin were screened using unique assay covering all membrane AC isoforms. Obtained data suggest changes in AC isoform expressions during activation of T-lymphocytes and potential of some forskolin analogues to specifically modulate particular AC isoforms. AC isoform selective modulation may represent a new therapeutic approach for the treatment of T-lymphocytes related pathological processes.

FUNCTIONAL FLOW CYTOMETRY-BASED APPROACHES FOR VALIDATION OF NOVEL MUTATIONS CAUSING INBORN ERRORS OF IMMUNITY WITH AUTOINFLAMMATORY PHENOTYPE

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Introduction

Next-generation sequencing reveals new mutations in immune-related genes, leading to Inborn Errors of Immunity (IEI). Since many mutations haven't been previously described, it is necessary to devise methods to properly characterize their biological impact at the cellular level.

Methods and Results

We developed and optimized series of cytometric tests for detailed characterization of novel mutations in *Hematopoietic cell kinase* and *Toll-like receptor 8* which were found in patients with IEI and autoinflammatory complications. These genes are expressed primarily in myeloid cells and control their inflammatory properties. Both primary cells from patients and lentivirally-transformed cell lines were used in the study.

Mutant form of Hematopoietic cell kinase enhanced expression of beta 2 integrins (CD11a, CD11b, CD11c, and CD18) which was accompanied by increased affinity to their ligand Intercellular Adhesion Molecule-1 (ICAM-1) measured by Ligand-complex-based Adhesion Assay. Moreover, increased intracellular level of inflammatory cytokines (IL-6 and TNFα) was found in mutant cells which were stimulated with recombinant ICAM-1. Altogether these results could partly explain patient's vasculitis.

The cells with mutant form of Toll-like receptor 8 presented with enhanced basal NFκB (p65) (Ser536) phosphorylation measured by single-cell phospho flow, increased intracellular expression of inflammatory cytokine IL-1β, and increased expression of activation markers (e.g. CD40, CD80, CD83, CD69, ICOS-1). Upon TLR ligands stimulation, we found abnormalities in activation of mutant cells, however the question whether the mutation is either activating or inhibiting still needs to be solved in other experiments.

Discussion

Proving the causality of novel gene mutations is crucial for diagnostics and subsequent therapeutic decision in patients with IEI. In that regard, single-cell flow cytometry serves as extremely important platform allowing a deep insight into a functional behavior of the cells which are affected by these mutations.

Supported by: GAUK 251089, NV19-05-00332, NV18-08-00245, Primus/MED/28, UNCE 204012, LO1604

CHARACTERIZATION OF NEUTROPHILS IN SYNOVIAL FLUID FROM PATIENTS WITH KNEE OSTEOARTHRITIS

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Background:
There is growing body of evidence that neutrophils may be involved in initiation and perpetuation of aberrant immune responses and tissue damage in osteoarthritis (OA). To date, little is known about the immunophenotype of neutrophils in synovial fluid (SF) in knee OA (KOA).
Aim:
To characterize neutrophils in SF from KOA patients and to determine their reactive oxygen species (ROS) production.
Methods:
Neutrophils in SFs from 39 patients with KOA were immunophenotyped using 8-colour flow cytometry (FACSCanto II, BD). Patient subgroups were formed based on the neutrophil counts in SFs: low neutrophils (<30 %, n=10), moderate (31-70 %, n=13), infection-like KOA with high neutrophils (>71 %, n=16). Basal and fMLP-stimulated ROS generation by neutrophils was investigated using DHR-123 *ex vivo*.
Results:
High variability in the distribution of neutrophils in SFs in KOA (min-max 0.59–68 %), with highest counts in infection-like KOA (71–97%) was observed. Low neutrophilic group displayed higher percentage and expression (MFI) of ROS at basal state comparing to moderate (P=0.09, P=0.01) and high neutrophilic (P=0.01, P<0.01) groups, respectively. After fMLP stimulation, trend to higher ROS production was observed in low comparing to moderate and high neutrophilic groups (P=0.07, P=0.09), but it did not reach significance. ROS production at both basal (r_s=0.6, P=0.02) and fMLP stimulated cells (r_s=0.7, P=0.05) positively correlated with the expression level of activation marker CD62L (L-selectin) on neutrophils. When calculating absolute number of neutrophils, the formation of ROS was markedly higher in infection-like KOA in both basal (P<0.01) and stimulated (P<0.01) conditions compared with moderate neutrophilic group. Of studied groups, the lowest oxidative burst was detected in patients with low neutrophils (P=0.03, P<0.01).
Conclusions:

Our study revealed high variability in neutrophil distribution in SFs from KOA patients. Highest oxidative burst was observed in infection-like KOA cases, the lowest in patients with less than 30% neutrophils. Further studies are ongoing.

Acknowledgements:
MZ ČR (VES16-31852A, RVO FNOl, 00098892), IGA UP_2019_014

Disclosure of Interest:
None declared

ANTI-TUMORIGENIC ACTIVITY OF PROBIOTIC MIXTURE: CROSSTALK BETWEEN TUMOUR ENVIRONMENT, MICROBIOTA AND IMMUNE SYSTEM

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Colorectal cancer (CRC) is one of the most common forms of cancer worldwide. Nowadays studies suggest that alteration in microbiota can play a pro-tumorigenic role, therefore bacterial shift towards "good" bacteria is essential in prevention and potential management of CRC. It is well-established, that especially probiotics can serve as a promising tool via their ability to enhance activity of immune system in different diseases, notably CRC. Therefore, the aim of this study was to test anti-cancer activity of patented mixture of six probiotic strains (University of Plymouth, UK) from *Lactobacillus salivarius* and *L. plantarum* species in chemically induced CRC rodent model.

Male Wistar rats were s.c injected with 1, 2 dimethylhydrazine once a week for 15 weeks while received 10^9 cfu/dose of probiotic mixture p.o. daily. After another 12 weeks, rats were euthanised, colon and tumour tissues were longitudinally dissected and one half was snap frozen and tested for cytokine presence by Bio-Plex® Multiplex Immunoassay (Bioclarma Sr.l., Torino, Italy). Other halves of the tissues were homogenized and stained for flow-cytometric analyses (panel I: CD45, CD161, Mϕ factor; panel II: CD45, CD3, CD4, CD8). Samples were measured using BD FACSVerse and analysed using KALUZA 2.1 software.

CD4/CD8 ratio did not differ between experimental groups in tumour tissue. However, CD4/CD8 ratio in colon of probiotic cancer group was lower ($P<0.01$) compared to all experimental groups suggesting higher abundance of anti-tumorigenic $CD8^+$ cells. Presence of NK cells and macrophages was also higher in tissues of probiotic-treated cancer group compared to cancer group. These results are in agreement with cytokine profiling of tissues, where IL-18 level (chemokine for NK cells) had increasing tendency after probiotic treatment. Tumour concentration of VEGF was significantly lower ($P<0.001$) and IL-6 level showed decreasing trend in probiotic-treated cancer group indicating switch towards anti-tumorigenic M1 subset.

Altogether, our results suggest that oral administration of selected probiotic mixture had positive effect and was able to alter gut immune response towards favourable anti-tumour activity proved also by better clinical performance of probiotic-treated animals.

This work was supported by the project VEGA 1/0476/17.

EFFECT OF ELECTRIC FIELD ON HUMAN POLYMORPHONUCLEAR LEUKOCYTES

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The phagocytic cells circulating in the bloodstream play a key role in both the defence of the body and the pathology of inflammatory diseases. Thus, targeting their functions has a potential utility for modulating the adverse immune response. Interestingly, an application of electric field is a therapeutic biophysical method that is employed in clinical practice for treatment of various chronic inflammatory conditions. [1] However, effect of electric field stimulation on immune cells and particularly on polymorphonuclear leukocytes, the most abundant phagocyte population in human peripheral blood, is not well understood.
In this study, an effect of electric stimulation on human polymorphonuclear leukocytes was tested using a unique platform with a network of gold electrodes. The activation of reactive oxygen species production, degranulation of metalloproteinases, surface expression of selected receptors and citrullination of histones was determined after application of electrical pulses in a range of 10 mV to 1V at frequency of 1 Hz and pulse duration 100 ms. [2]
Overall, we have observed activation of neutrophil granulocytes by electrical pulses, however, a significant effect was observed only for 1 V electrical pulses in the case of ROS production and for 100 mV and 1 V in the case of MMP8 collagenase degranulation. Furthermore, it was found that electrical pulses induced citrullination of histone H3 and increased expression of surface receptors CD11b and CD15 but without significant differences. The limited significance of our observation could be due to unwanted activation of neutrophils caused by platform adherence.

This work was supported by GACR (17-24707S).

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EFFECT OF MESENCHYMAL STEM CELLS IN COMBINATION WITH CYCLOSPORIN A ON AN INFLAMMATORY RESPONSE IN MOUSE MODEL

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Mesenchymal stem cells (MSCs) are known for their immunomodulatory, protective and anti-apoptotic properties. Several studies have shown that combined therapy of graft recipients with MSCs along with various immunosuppressive drugs increases the graft survival. However, the exact mechanism of such synergy is yet not known. In our study we confirmed therapeutic effect of MSCs in the combination with cyclosporine A (CsA) in mouse model of allogeneic cell transplantation. Combined therapy significantly increased the survival of transplanted allogeneic cells and prevented the apoptosis of T cells caused by application of CsA. Analysis of distinct T cell subpopulations revealed switch in the balance between effector Th cell populations into anti-inflammatory. We detected significant decrease in inflammatory Th1 (CD4+Tbet+) and Th17 (CD4+RORγt+) cells, while the proportion of Th2 (CD4+GATA3+) cells significantly increased and Treg (CD4+FoxP3+) population remained unchanged in comparison to CsA monotherapy. In accordance with this, the production of Th1/Th17 related cytokines was attenuated with a simultaneous increase in anti-inflammatory cytokine IL-10. Moreover our study revealed that CsA altered migration and survival of transplanted MSCs, which further improved their therapeutic effect.
We propose that combined therapy of MSCs and CsA has significant clinical relevance and enable to reduce dosage of immunosuppressive drugs while maintain the outcome of therapy.

This work is supported by grant 1154217 from Grant Agency of Charles University and by project LO1508 from the MEYS of the Czech Republic.

EFFECT OF LOW, MIDDLE AND HIGH MOLECULAR WEIGHT HYALURONAN ON IMMUNE RESPONSE IN VIVO

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Hyaluronan (HA) is a glycosaminoglycan naturally produced in the body as one of the components of extracellular matrix. Due to its biocompatibility, HA and its derivatives are used in many pharmaceuticals and therapeutical devices. It is supposed that ability of HA to activate immune response and to induce an adverse reaction in the body is based on its molecular weight. There are studies which promote that low molecular weight HA has a pro-inflammatory potential compared to high molecular weight HA. In this case degradation of high molecular HA-based devices into smaller HA fragments could activate an immune response. However, current information on this subject from different authors is contradictory. Importantly, mechanism of degradation of pharmaceuticals and therapeutical devices seems to be affected by HA molecular weight However, pharmacokinetic of HA of different molecular weight is still poorly understood.
In this study, we examine potential of HA of different molecular weight sizes (12; 197; 460 and 1640 kDa) to activate neutrophil granulocytes and induce an inflammatory response *in vivo*. HA was applied intravenously to the tail vein of C57BL/6 mice (50 mg/kg). After different time intervals blood was collected. Activation of neutrophil granulocytes was analysed from whole blood based on levels of expression of selected surface markers (e.g. CD11b, CD62L) by multispectral flow cytometer SONY SP6800. Pro-inflammatory cytokines (including e.g. TNF-α, IL-6, IL-1β) were determined in plasma by ELISA. Pharmacokinetics of selected molecular weight HA was determined based on tracking of HA and its metabolites by LC-MS/MS of 13C-labeled HA. Obtained data show differences in activation of neutrophil granulocytes and innate immune response in mice after application of HA of different molecular weight. Similarly, significant differences were observed in pharmacokinetics of HA of different molecular weight. Overall, these observations significantly improve our understanding of an impact of different HA sizes on immune response that can help to properly employ HA and its derivatives in the development of HA-based pharmaceuticals and devices.
This work was supported by MEYS CR project OP VVV INBIO (CZ.02.1.01/0.0/0.0/16_026/0008451).

IMMUNOPHENOTYPING EXTRACELLULAR VESICLES USING THE AMNIS CELLSTREAM FLOW CYTOMETER

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Extracellular Vesicles (EVs) are membrane derived structures that include exosomes, microvesicles, and apoptotic bodies. The importance of EVs as key mediators of intercellular communication is not well understood. Exosomes have been shown to transfer molecules between cells, potentially transmitting signals. Exosomes are released under normal physiological conditions; however, they are also believed to serve as mediators in the pathogenesis of neurological, vascular, hematological, and autoimmune diseases, as well as cancer. Quantifying and characterizing extracellular vesicles in a reproducible and reliable manner is challenging due to their small size (exosomes range from 30 to 100 nm in diameter). EV analysis can be done using high magnification microscopy; however, this technique has a very low throughput. Attempts to analyze EVs using traditional PMT based flow cytometers has been hampered by the limit of detection of such small particles and their low refractive index. To overcome these limitations, we have employed the CellStream ® System, which has the advantage of high throughput flow cytometry, with higher sensitivity to small particles due to the CCD based, time delay integration, image capturing system. Data will be presented using the CellStream Flow Cytometer to immunophenotype EVs derived from red blood cells and platelets.

COULD BE CD39+ REGULATORY T LYMPHOCYTES DISTINGUISHING MARKER OF SIRS AND SEPSIS DIASNOSTICS?

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Sepsis is a disease defined as a systemic inflammatory response of an organism to infection associated with high mortality. The initial inflammatory stage is quickly counterbalanced by an anti-inflammatory response, which compromises the immune system, leading to immune suppression. Septic states often lead to multiorgan failure. For early therapy it is important to assess which organ is primarily affected, the degree of disability or phase when organ failures occur. Therefore it is important to field the transition of SIRS (systemic inflammatory response syndrome) to sepsis by selecting appropriate markers as well as combination of diagnostic-specific markers, which could leads to quickly intervention and prevention of sepsis progression.

The inflammatory process leads to depletion of the immune system and subsequent immunosuppression. Regulatory T cells (CD4 + CD25 +) are involved in the pathogenesis of sepsis by inducing the immunosupression. CD39+ Treg cells have more significant suppression ability compared with CD39- Treg cells. An increase in CD39 expression on Tregs has been observed in patients with HIV infection and is strongly associated with disease progression. Furthermore, an increase in CD39 expression on Tregs has also been identified in tumor and autoimmune diseases. Recent studies suggested that in the case of septic patients, increased expression of CD39 on regulatory T cells is associated with poor disease prognosis and increased mortality.

We monitored CD39 + regulatory T cells from peripheral blood of SIRS/SEPSA solid tumor patients. Present results suggested that CD39 + Tregs may serve as a predictive survival marker of cancer patients

SERTOLI CELLS AS A POTENTIAL THERAPEUTIC TOOL FOR ACUTE MYOCARDIAL INFARCTION

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Sertoli cells (SCs) are increasingly studied lately, with the focus on their immunomodulatory properties. SCs, localized in seminiferous tubules in testes, nourish differentiating germ cells and create immunologically privileged site by modulating the immune system. SCs promote Th2 immune response and induce production of Th2 cytokines by immune cells. These cytokines along with other molecules induce M2 phenotype in macrophages, which are known as alternatively activated or wound healing. Activity of SCs in the body after transplantation is broadly similar to that observed for mesenchymal stem cells (MSCs). Because of this similarity, a theory that SCs are a kind of MSCs, has been proposed. Among others, MSCs are studied as a possible therapeutic tool for myocardial infarction (MI). In the organism MSCs are responsible for maintaining tissue homeostasis, whereas one of the main SCs function in testes is the induction of anti-inflammatory environment. Therefore, we hypothesized that SCs may be more suitable candidate for MI treatment. In our study, we established a mouse model of Isoprenaline-induced acute MI, followed by intravenous administration of 1 million cells (MSCs/SCs). Using flow cytometry, we monitored immune cells infiltration and production of pro-inflammatory or anti-inflammatory cytokines (TNFα and IL-10 respectively) in heart after MI. The SCs application resulted in decrease in TNFα level accompanied with an increase in IL-10 production compared to control (MI only) and MSCs treated mice. Also infiltration of various immune cell populations in the heart was reduced after SCs administration. Our study confirmed that actions of SCs after MI are overall beneficial and lead to myocardial tissue healing in a function-preserving manner, but further study is needed to reveal molecular mechanisms of this phenomena.

EFFECTS OF PSEUROTIN ALKALOIDS ON SELECTED IMMUNE CELL FUNCTIONS

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Pseurotins A and D are secondary metabolites that are synthetized by filamentous fungi. Interestingly, pseurotins show significant effects on functions of different body cell types including immune system cells. However, effects of pseurotins on function of most type of leukocytes is still unclear.

In this study we tested effects of pseurotins on function of two different leukocyte types, murine macrophages and human lymphocytes. Results obtained with murine macrophages RAW 264.7 reveals that pseurotins attenuate response of macrophages to bacterial lipopolysaccharide. Pseurotins also slow down proliferation of this murine macrophage cell line. Analysis of selected signaling pathways revealed that pseurotins interfere with STAT signaling pathway in these cells. Next, the effect of pseurotins on human T- and B-lymphocytes isolated from blood of healthy donors was tested. T-lymphocytes were activated by anti-α CD3 and anti-α CD28. B-lymphocytes were activated by IL-4. Interestingly, significant inhibitory effects of pseurotins on expression of surface markers CD69 and CD25 was observed. Similarly, an inhibition of lymphocyte proliferation was observed. These effects were accompanied by inhibitory effects of pseurotins on JAK/STAT signaling pathway.

Overall, it can be concluded that natural pseurotins shown inhibitory effects on leukocytes of both myeloid and lymphoid origin that is connected with inhibition of JAK/STAT signaling pathway inhibition.

The study was supported by the GACR of the Czech Republic (17-18858S).

NATURAL PSEUROTINS AFFECT HUMAN B-LYMPHOMA CELLS *IN VITRO*

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Pseurotin A is a secondary metabolite produced by many species of fungi, mainly by Aspergillus sp. and Penicillium sp. During the pseurotin A biosynthesis, a large number of closely related bioactive compounds, such a pseurotin D or synerazol are also formed. Natural pseurotins and structurally related small organic molecules have documented antimicrobial and antiparasitic activity. Interestingly, our unpublished data also suggest that pseurotins can significantly inhibit activation and proliferation of primary lymphocytes. However, their potential to affect malignant lymphocytes is not clear.
In this study, we focused on effects of natural pseurotins A and D on human chronic B-lymphoma cell line MEC-1. We analyze a modulation of cell proliferation and cell cycle mediated by pseurotins. Effects of pseurotins on lymphoma cell metabolism and cell viability was also analyzed. Our current results show that pseurotins have a potential to decrease the mitochondrial activity and cell proliferation together with induction of cell apoptosis. It can be concluded that pseurotins are able to affect proliferation of lymphoma cells.
The study was supported by the GACR of the Czech Republic (17-18858S) and the research support of IBP CAS (No.888811).

LDNS IN CVID PATIENTS

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Common variable immunodeficiency disorder (CVID) represents a group of primary immunodeficiency diseases characterized by hypogammaglobulinemia and dysfunctional immune response to invading pathogens. Previous studies have indicated that CVID is associated with microbial translocation and systemic neutrophil activation. Neutrophils were traditionally considered as a homogenous population with inflammatory phenotype. However, recent studies suggest two different subpopulations of neutrophils based on density gradient centrifugation of peripheral blood. The first subpopulation is included in sediment together with erythrocytes after classic density gradient centrifugation and is known as a classic or normal density neutrophils (NDNs), whereas the second subpopulation remains in a layer of mononuclear cells and is known as a low density neutrophils (LDNs). LDNs have been originally identified in patients with several types of solid tumours or haematological malignancies, in acute and chronic inflammatory disease conditions or sepsis. LDNs suppress T cell responses such as proliferation and interferon-γ production. Last year we described increased incidence of LDNs in peripheral blood of CVID patients. The goal of this study was to determine whether patients with CVID display elevated production of LDNs after stimulation with LPS and fMLP compared to healthy controls and whether the levels of LDNs are further influenced by intravenous immunoglobulin (IVIg) infusions. After stimulation of heparinized peripheral blood and isolation of PBMC the percentage of LDNs contained in PBMC was determined by using multicolour flow cytometry in CVID patients and healthy controls. All CVID patients were in a stable state with no apparent acute infection. The percentage of LDNs in PBMC was significantly higher in CVID patients compared to healthy controls in non-stimulated peripheral blood. The percentage of LDNs was further increased following stimulation by LPS and fMLP in both groups, but the growth of a newly emerging LDNs was significantly higher in CVID patients compared to healthy controls. Moreover, after in vitro stimulation of CVID patients whole blood using IVIg in a therapeutically relevant dose for 2h resulted in a significant increase of LDNs compared to unstimulated blood. This data presented here indicate, that CVID is associated with chronic neutrophil activation, which is further exacerbated by administering IVIg. Increased LDNs may contribute to comorbidities associated with CVID patients.

CHARACTERIZATION OF UNIQUE UROTHELIAL CARCINOMA CELL LINES FROM BBN-INDUCED MURINE BLADDER CARCINOMA

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Set of novel murine, carcinogen (BBN)-induced urothelial bladder carcinoma cell lines were established using the Hgf-Cdk4^{R24C} mouse strain, by treating animals for 10 weeks with BBN in drinking water. We compared our model to the widely used MB49 orthologous bladder cancer model. At week 10 the animals developed low grade urothelial bladder tumors of an early stage and BBN was removed from the drinking water. Depending on the length of follow-up period mice developed tumors of different size and grade. One, five and ten weeks after the follow-up period, bladders were dissected, mechanically/enzymatically disrupted and cultured for at least 16 weeks. Cell lines from bladder carcinomas of different grade/size were established from single cell colonies and further characterized. Tumor cell lines grew as the mosaic sheet monolayers and exhibited loss of contact inhibition. Cell lines from urothelial carcinomas remained MHC class I deficient, PD-L1^{dim}, CD54^{neg}, CD80^{neg} and FasL^{neg} which indicates their selective growth advantage during the tumor development although they still retained ability to upregulate MHC class I/PD-L1 expression by exposure to IFN gamma. We proved that Hgf-Cdk4^{R24C} transgene is present in our cell lines. We now aim to assess tumorigenicity of the cell lines in in vivo experiments and plan to isolate DNA/RNA for the upcoming analysis of TMB, dysregulated pathways, copy number alternations or presence of neoantigens. These novel cell lines complement the already well characterized MB49 cell line. MB49 cells express MHC class I at steady-state and respond well to immunotherapy. These new bladder cancer cell lines, also stemming from the C57BL/6 background, with low or no MHC class I expression at steady-state may respond differently to immunotherapy and are thus important contributions to the field of immunotherapy and can be used to further understand the immuno-biology when

NK CELLS IN REPRODUCTIVE IMMUNOLOGY

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Infertility is defined as the inability to achieve pregnancy after one year of regular, unprotected intercourse, spontaneous abortion is defined as a clinically recognized pregnancy loss before the fetus achieves the point of viability out of uterus, i.e. the period since conception until 24th week of gestation.

The aim of this work was to assess selected peripheral blood NK-cells parameters, i.e. activity after JAR-cells stimulation, percentage of NK-cells and absolute count of NK-cells in a group of female patients, and to compare obtained values with control group.

There were 73 female patients in fertile age diagnosed with defined form of reproductive failure in this work, whose blood was taken from October 2018 to March 2019. The subjects in the patient group had not been treated for any possible immunological health condition. All the parameters were investigated by flow cytometry, and the stimulation with JAR-cells and CD69 activation marker was used. There were women in fertile age with history of successful pregnancy in the control group.

We were successful in introduction of the method of measuring peripheral blood NK-cells activity by flow cytometry into laboratory practise. We were not able to demonstrate elevated peripheral blood NK-cells activity in women with reproduction failure compared to the control group. We found out elevated percentage of peripheral blood NK-cells in a group of women with reproductive failure. We also found out a higher number of patients with more than 12% of peripheral blood NK-cells, which is the rate connected to reproductive failure. We were not successful in demonstrating elevated NK-cells absolute counts in the patients group compared to the control group.

The role of NK-cells in immune response in pregnancy is indubitable even though their accurate role has not been thoroughly investigated yet. The accurate comprehension of their roles in reproduction processes remains one of the challenges in the reproductive immunology field.

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

Clare Szybut

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

IMMOPHENOTYPING AND CYTOKINES PROFILE AFTER TOTAL BODY IRRADIATION

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The lungs are complex organ, consisting of the large central airways (the trachea and main steam bronchi), the smaller conducting airways and the alveolar sacs where gas exchange occurs. Biological effects of ionizing radiation (IR) is irreversible damages to various cell types, and may thus have implications for the level of the whole tissue[1]. Radiation induced lung injury can disrupt multiple aspects of normal pulmonary physiology, including efficient gas exchange, optimal matching of perfusion and ventilation, and adequate airflow. Simultaneously, the lungs are slowly proliferating systems and radiation effects occur in delay (months, till years)[2].
The aim of our presentation was to determine the effects of IR in early time intervals: 4, 8 and 24 hours, as well as in later time intervals: 7, 21 and 30 days. This time periods, also known as radiation pneumonitis associated with early response, are the most critical phase for preventing and possible treatment of radiation damages[3].
The chosen model of C57BL/6 mice was whole body irradiated by the dose of 8 Gy. We studied changes in major cell populations in the peripheral blood and in the lung (lymphocytes, monocytes and granulocytes), lymphocyte subpopulations (T, B and NK cells) and the myeloid subtypes (monocytes, granulocytes and macrophages). We had investigated variations in cytokine levels, as possible mediators of lung toxicity by multi-color flow cytometry. Sections of lung tissue were also fixed and processed for histopathological analysis and immunofluorescent staining.
We had observed changes during all days in experiment schedule. The most significant changes were observed at 21 day. Changes included significant infiltration of T lymphocytes ($CD3^+$ $CD4^+CD8^-$) in the lung tissue, increased level of MCP- 1 and obvious histological changes in alveolar – capillary complex.
The results of this study clearly demonstrated a time - dependent response of the whole body damages after gamma irradiation.

LOW MOLECULAR WEIGHT LEUKOCYTE EXTRACT (TRANSFER FACTOR) ACTIVATES PORCINE T-LYMPHOCYTES

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Transfer factor (TF) is low molecular weight (under 10 kDa) leukocyte extract, also known as dialysable leukocyte extract. It has been known for its immunomodulatory capacity for more than 40 years. The term "transfer factor" describes TF's main function, which is the ability to transfer specific cell mediated immunity from immunized donor to non-immune recipient.

Additionally, TF shows many other non-specific immunomodulatory functions including anti-inflammatory and hematopoietic capacity, ability to stimulate immune cells and potentiate immune response. These predetermine TF as a potential immunostimulant in human and also in veterinary medicine. Few nutrition supplements with transfer factor activity are currently available. However, the mechanism of TF non-specific biological activity is known very poorly.

In the present study, different sources of leukocytes from porcine tissues for TF preparation were compared. Blood leukocytes isolated using "buffy coat" separation, mononuclear blood leukocytes isolated by gradient centrifugation and leukocytes from mesenteric lymph nodes were included in the study. Basic molecular characteristics of extract and tested its biological activity were performed. Molecular composition of extracts from different sources was compared using liquid chromatography and present proteins and peptides were identified by mass spectrometry. Very similar results were obtained when different leucocytes types were used for TF preparation. Also biological activity tested as a capacity to stimulate porcine T lymphocytes has been proved for all extracts. Co-cultivation with TF has a significant stimulatory effect on T lymphocyte subpopulations analysed by flow cytometry. The increase of percentage of activated cells was weak in γδT lymphocyte (γδ TCR+) population and both types of CD4+ lymphocytes: T- helper (Th) and double positive CD4+CD8+ (DP). On the contrary cytotoxic T lymphocytes (Tc, CD3+CD8hi) and NK cells (CD3-CD8+) showed strong stimulatory effect after 24 hour co-cultivation with TF.

This work was supported by TAČR GAMA project TG03010038.

OPTIMIZATION OF A METHODOLOGY FOR THE ANALYSIS OF IMMUNE CELLS IN GLIOBLASTOMA MICROENVIRONMENT BY FLOW CYTOMETRY

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Glioblastoma multiforme (GBM) is the most common malignant primary brain tumor with a median survival of only 15 months. Immunosuppression is one of the hallmarks of GBM and insight into the mechanisms through which it is established may facilitate the development of effective immunotherapies. Flow cytometry can be used to study various immune cell subpopulations in the tumor microenvironment, but obtaining a single cell suspension is an important prerequisite. The aim of our study was to establish a suitable protocol to generate single cell suspension of viable cells from GBM tissues.

Using tissue samples from experimental syngeneic mouse tumors, four tumor tissue dissociation methods (manual mechanical dissociation of the tissue, use of a dissociator [Miltenyi Biotec GmbH], both with or without enzymatic digestion) were compared for the preparation of single cell suspension. The quality of the cell suspension was evaluated by determining leukocyte viability, preservation of the studied markers and visual determination of the presence of cell clumps and subcellular debris.

The combination of mechanical and enzymatic dissociation using the dissociator and Tumor Dissociation Kit (Miltenyi Biotec GmbH) yielded immune cells with more than 90% viability as compared to other methods where cell viability did not reach more than 50%. Moreover, this approach did not alter the studied surface markers and produced less cell clumps. Debris removal using Debris Removal Solution (Miltenyi Biotec GmbH) further improved the quality of the single cell suspension.

In summary, an effective method for the preparation of a high quality single cell suspension from GBM tumor tissue and subsequent analysis of immune cell populations in the tumor microenvironment was implemented in our laboratory.

Financial support: Ministry of Health of the Czech Republic, grant No. 15-31379A, project “Center for Tumor Ecology” (reg. n. CZ.02.1.01/0.0/0.0/16_019/0000785), Progres Q28/1LFUK and 2015064 LM EATRIS.

THE EFFECT OF COLD ADAPTATION ON THE IMMUNE SYSTEME

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Maintaining energy homeostasis at reduced temperatures is essential for the survival of the organisms. In this study, we determined the influence of cold stress and cold adaptation on the immune system in a rat model. Various factors participate at the process of thermoregulation, a crucial step involves the binding of noradrenaline (NE) to adrenergic receptors, and in long-term adaptation, thyroid hormones play an important role. Changes induced by the binding of these factors to its receptors lead to the formation of brown adipose tissue, which is necessary for temperature homeostasis, as well as for energy balance and metabolism regulation in rodents. To decipher neuro-immune interaction in the cold, we monitored changes in the presence, activation and soluble products of individual immune cell populations at various time points of cold adaptation and correlated them with the expression of adrenergic receptors and enzymes responsible for the NE activity. Bioactive products of adipocytes undergoing changes during thermogenesis modulate the immune system, which is also influenced by nerve cell signalling. Interconnection of the immune and nervous system seems to be very important in many biological processes. Deciphering basic mechanisms of the influence of cold adaptation on immune cells can therefore explain other clinically relevant topics, such as treatment of obesity or Cardiovascular diseases. This work is supported by grant 1154217 from Grant Agency of Charles University.

EVALUATION OF PROBIOTIC CANDIDATES FOR THE RE-PROGRAMMING OF MACROPHAGE SUBSET-DRIVEN PRO-INFLAMMATORY RESPONSES: REBUILDING THE GUT ECOSYSTEM VIA FAECAL MICROBIAL TRANSPLANTATION IN INFLAMMATORY BOWEL DISEASE (IBD)

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IBD is a global issue in the 21st century, associated with increased level of pro-inflammatory Mϕ 1 which leads to a chronic inflammatory response. Most of the current therapies for IBD focus on the management of the inflammation by using immune modulators. Changes in bacteria composition and number are now recognized as a key element in gut inflammatory processes suggesting microbiome-based therapies as an attractive approach. Lactobacilli are known to be efficient modulators of intestinal inflammation due to their effect on the M1/M2 switch, which is desirable in initial acute phase of IBD. The extremely high success rates achieved with faecal microbiota transplantation (FMT) in the treatment of diarrhoea have catalysed the attention of researchers for its potential applications in IBD treatment. However, donor selection is a big challenge, because the major concern of the FMT is the possibility to spread viral or bacterial infections. To overcome this problem, targeted modulation of autochthonous microflora by probiotics could represent safer therapeutic approach of IBD patients.

The aim of this work was to evaluate the reprogramming potential of selected *Lactobacillus* strains utilising Mϕ subsets *in vitro* model.

The human monocytic cell line THP-1 was differentiated into M1 and M2 macrophages. Phagocytic properties were measured by culturing Mϕ subsets with CFSE-loaded bacteria *L. reuteri* (LR) and *L. plantarum* (LP) and analysed by flow cytometry (BD FACSVerse, software BD FACSuite). Cytokine profile (IL1β, TNFα) was investigated upon Mϕ culture with Lactobacilli and quantified by sandwich ELISA. DPPH radical scavenging and antioxidant activities (AA) were detected by spectrophotometry.

The phagocytic response was generally greater for M2 macrophages. The greatest phagocytosis was observed immediately after 1 hour of co-culture with LR while with LP after 12 hours. Both strains decreased cytokine release after LPS treatment. Tested strains showed good AA, specifically 26% inhibition rate of LA peroxidation for LR and 32% for LP. DPPH assay also indicated strong AA of used strains (almost 90%). Our results proved that tested strains can be promising candidates for targeted modulation of autochthonous microflora of IBD patients.

The work was supported by APVV-16-0176, VVGS-PF2019-1064, VVGS-2019-1073.

CYTOMETRY METHODS FOR CELL BIOLOGY

SPECTRAL CYTOMETRY ADVANCES IN HIGH DIMENSIONAL DATA ACQUISITION – THE POSSIBILITIES OF A 24 COLOUR PANEL ON A 3 LASER PLATFORM USING THE CYTEK AURORA

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Recent advances in flow cytometer design and the development of polymer dyes have resulted in a dramatic increase in the ability to construct and perform multicolour panels with flow. However, limitations in antibody conjugates to appropriate spectrally distinct dyes still hinder the expansion of multicolour panels in research. Spectral flow cytometry with the Aurora presents the ability to utilise highly overlapping dyes that conventional flow cytometers are unable to use increasing the need to run high complexity assays on more expansive platforms with more laser excitation possibilities.
We demonstrate the development of an assay aimed at identifying the main circulating cell subsets in whole blood including 24 different markers on a 3 laser Aurora Spectral Cytometer. 53 commercially available fluorochromes were characterized in terms of spectrum signature, brightness and spread using an Aurora full spectrum cytometer equipped with 3 lasers (405, 488 and 635 nm). 24 fluorochromes that could be used in combination on the 3 laser Aurora were selected. Following the principles of panel design, a theoretical panel was created, evaluated and optimised on bead and cell controls to identify optimal controls and sensitivity for best panel performance in high dimensional flow cytometry.
The efficacy of complex multicolour panels utilising commercially available fluorophores has been readily demonstrated by choosing dyes with unique spectral characteristics that can be deconvoluted through spectral unmixing. Spectral flow cytometry offers an affordable approach to answering complex research questions without the use of more costly reagents for less well used laser lines.

HAEMATOLOGY

Peripheral blood of CLL patients is repopulated with complement resistant malignant cells one day after rituximab infusion: possible implications for subsequent chemotherapy

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Monoclonal antibody rituximab (RTX) has been used for 20 years, however, it remains unclear how it functions in vivo. It is known that RTX acts in vivo rapidly by CDC (complement dependent cytotoxicity). We studied prospective in vivo response of 44 chronic lymphocytic leukemia (CLL) patients treated with RTX. Peripheral blood was collected immediately before RTX infusion (BT), immediately after the end of infusion (AT) and on the next day before any chemotherapy has been administered (D+1). CLL samples in the cohort had an average CLL cell count of 151 $x10^9$/l prior to RTX infusion, and had 84 $x10^9$/l CLL cell count at the AT time point. Individual responses to RTX were highly heterogeneous with CLL cell count reduction ranging from 8% to 88%. Only 17 patients (39%) experienced reduced or stable count of CLL cells on D+1. Surprisingly, average CLL cell count increased to 115 x 10^9/l on D+1, and 18% of patients had even the same or higher count of CLL cells than before the start of the treatment. While we have detected only a small population of membrane attack complex-positive (MAC^+) B cells in samples before treatment (mean=0.5%), we found on average ~10% of viable B cells to be positive for MAC in the samples collected after RTX infusion (AT). These viable MAC^+ B cells represent CLL cells fully resistant to CDC. Interestingly, patients with increased B cell count on D+1 had relatively higher % of viable MAC^+ B cells in AT samples compared to patients with stable CLL cell count on D+1 (13% versus 6%, p=0,014). The observed phenomenon is probably caused by complement consumption during CDC in vivo. Patients with stable count of CLL cells on D+1 were found to have higher complement capacity in D+1 plasma than others (70% vs. 50%; p=0,025), most likely due to lower density of complement inhibitor CD59 on their CLL cells (p=0,019). Interestingly, "re-population" of peripheral blood with CLL cells 20 hrs. after rituximab administration transformed into a clinical impact as patients with increased lymphocytosis on D+1 had longer progression-free survival (p=0,034). This can be potentially caused by higher sensitivity of CLL cells to chemotherapy outside of immune niches.

Supported by grant 16-32743A provided by the Ministry of Health of Czech Republic.

DEVELOPMENT OF MULTIPARAMETER FLOW CYTOMETRY COMBINATION FOR MYELOID MATURATION

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Myeloid maturation in bone marrow (BM) is a complex process involving granulocytic, monocytic, erythroid and megakaryocytic lineage development. Recent advancement of flow cytometry (FC) enables users to combine more parameters simultaneously and to improve knowledge about normal maturation patterns. Building up FC combinations with >4 fluorochromes brings up challenges with a need of profound testing. Our aim was to introduce a new 10-color FC combination for myeloid maturation applicable also in a terrain of myeloid malignancy.
We performed testing of antibody combinations from different vendors (BD Biosciences, Beckman Coulter, Exbio, Biolegend, ThermoFisher). Chosen set of markers included: CD117, CD34, CD15, CD45, CD71, CD33, HLA DR, CD38, Syto (cell-permeant nucleic acid stain), with one free position for patient-specific marker. We stained BM samples from pediatric patients without apparent aberrancies in myeloid development. Samples were acquired on BD FACSLyric and Sony SP6800 cytometers and compared to original in-house panel (CD15/CD117/CD7/CD45/CD34/CD71/CD33/Syto41). Data were analyzed using Flow Jo software.
In total we stained and analyzed 34 samples. For our panel (user-specific) needs we found superiority of Syto16 over Syto41, CD15 IgG antibody over IgM antibody, Alexa Fluor 700 fluorochrome over APC-Alexa Fluor 700 fluorochrome and CD117 PE Cy7 conjugate over APC on Sony SP6800 cytometer. Next, we studied influence of overspill from APC tandem dyes on APC conjugated marker staining pattern, differences in CD15 staining patterns on developing promyelocytes (dependent on antibody used) and finally unspecific binding of distinct PE Cy7 conjugates on mature monocytes. Final combination consists of 9 parameters and free position for patient-specific marker. Five of those markers will be prepared in lyophilizate format to reduce possible pipetting errors.
We developed improved 10-parameter combination for analyzing most immature myeloid lineage compartment in BM for routine use. We encountered common challenges in multiparameter FC, such as overspill, unspecific binding, clone and fluorochrome influence on staining patterns. Our study illustrates building up multicolor FC panel and may inspire similar studies.
Supported by NV18-03-00343, NV18-07-00430, UNCE/MED/015

NK CELLS FOR THE PREVENTION AND TREATMENT OF RELAPSE AFTER ALLOGENEIC HAEMATOPOIETIC STEM CELLS TRANSPLANTATION

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NK cells play a crucial role in relapse control and therefore they are great candidate for using them as an advanced therapy medical product after allogeneic haematopoietic stem cells transplantation (allo-HSCT). In our ongoing clinical trial, we apply NK cells for prevention or treatment of relapse in high-risk haematological malignancies, mainly AML. However before clinical application, many preclinical testing had to be done.
During development of NK cells based medical product, we did several testing providing us optimal cell source and the best culture condition. We compared immunophenotype and cytotoxic activity of NK cells from healthy donors and patients after allo-HSCT. There were no significant differences detected except a higher number of CD16 negative NK cells in group of patients (median 45±17%) compared with donors group (median 26±12%). Than we sorted two distinct populations - CD25pos/NKp44neg or CD25neg/NKp44pos – and evaluated their cytotoxic potential and cytokine expression. We detected variabilities in 6 tested analytes. CD25 expressing cells had higher expression of CCL3, TNFβ, GM-CSF, IFN-γ in contrast with NKp44pos cells, where CCL5 and TNFα were elevated. The functional proteins (FasL, perforin, granzyme B) were without any association with expression of CD25/NKp44 and there was no difference in their cytotoxic activity. We also evaluated whether a low dose chemotherapy could increase DAMPS expression and sensitize NK-resistant cell line KG1a to NK cells and whether their cytotoxic potential is dependent on type or on level of inhibitory KIRs. We pre-treated KG1a cells with ara-C or/and applied anti HLA-ABC antibody for blocking KIR-HLA interactions. In co-cultivations experiments, both treatments increased the proportion of dead KG1a cells as compared with untreated cells. We proved the importance of both types of receptors (activation and inhibitory) for cytotoxic potential of NK cells. Increase of DAMPs is more suitable for clinical applications and could be done using standard chemotherapy.
All above mentioned testing led to preparation of standard and robust protocol for NK cells preparation as well as determination of importance of chemotherapy pretreatment before immunotherapy.

The work was funded by the grant of Ministry of Health of the Czech Republic (FNPl, 00669806).

EXTRAMEDULLARY EXPANSION OF MYELOMA PLASMA CELL INTO CNS

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Background: Multiple myeloma (MM) is characterised by a presence of clonal plasma cell (a-PC) which are usually localised in bone marrow (BM) as heterogeneous suspensions, not often as solitary lesion. A BM microenvironment dependency is lost in a subset of patient and a-PCs spread out of BM, probably because of changes in adhesive molecules expression. Circulating myeloma PCs (cPCs) were detected in peripheral blood of all MM. Moreover, primary and/or secondary extramedullary disease (soft tissue and/or bone-related) was revealed in many patients. On the other hand, presence of a-PCs in cerebrospinal fluid (CSF) is relatively rare.
Aim: Detection of a-PCs in CSF and comparison of their phenotype with other compartments.
Patients and Methods: CSF of 15 relapsed MM patients was analysed by polychromatic flow cytometry (FC). Centrifuged samples were immediately incubated with MoAb (mostly CD38/CD138/CD45/CD19/CD56/CD14/CD5/CD27), lysed by NH_4Cl and analysed.
Results and Conclusion: Whole group of analysed CSF has median of leukocytes 231 with range 19-6013. Myeloma a-PC infiltration was detected in 26.7 % (4/15) with range 1.2-70.0% or 22-2985 cells from leukocytes. Only a-PCs were detected, these were always $CD19^-$ and in 50% $CD56^+$. Phenotype profile in CSF, bone marrow, peripheral blood and tumour was similar according to CD19 and CD56. Flow cytometry is a highly efficient method for a-PCs detection. Unfortunately, for these small samples more markers have to be analysed simultaneously to perform diagnostics and to analyse a-PCs as well, so only 8-colour FC is relatively insufficient in term of detail a-PC phenotype assess. All CSF^+ patients were almost refractory to the treatment and they have cPCs in peripheral blood; extramedullary relapse was detected in 2 patients as well. It is evident, that prognosis of patients with CNS infiltration is generally poor.
Supported by Ministry of Health of the Czech Republic, grant nr. 17-30089A and 17-29343A.

DYNAMIC CHANGES OF INHIBITORY KIR RECEPTORS AFTER ALLOGENEIC HSCT

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The number of allogeneic hematopoietic stem cells transplantations (allo-HSCT) increases in Czech Republic. Standard selection of the best donor is driven by HLA genotypes matching and occasionally in some malignancies also by evaluation of KIR (Killer-cell immunoglobulin-like receptor) haplotype. KIRs are NK cells receptors consisting of activating as well as inhibitory group. The basic division classifies them into two haplotypes A and B. Haplotype B is composed from more genes coding for activating KIRs and therefore is connected with lower risk of relapse. But KIR genes are very polymorphic and this polymorphism can influence the strength of inhibitory signalling or even its expression. Therefore, evaluation of expression of KIRs instead of genotyping seems to be closer to real situation.

In our study we followed inhibitory KIRs expression before and after allogeneic allo-HSCT and determined their kinetics and association of their level with the outcome of patients. An expression of inhibitory KIRs (iKIRs) was evaluated on the surface of NK cells from 35 donors and then in transplanted patients – sequentially at 1-2-3 month after HSCT. Blood samples were stained with CD45-Horizon (BD Bioscience, USA), CD3-Pacific Blue, CD16-PerCP, CD56-PeCy7 (all Exbio, Czechia), KIR2DL1-PE, KIR2DL2/2DL3-APC, KIR2DL3-FITC or KIR3DL1/DL2 PE, KIR3DL3-APC, KIR3DL5-FITC (all Miltenyi, Germany). The samples were measured using BD FACSCanto II, analysis were performed in FlowJo software (FlowJo, LLC, USA). For standardization of biological differences, we determined the expression before HSTC as 100% and then we calculated percentage from this value. The KIR-HLA mismatch was evaluated in each donor patient pair. Expression of iKIRs was also evaluated after *ex vivo* activation.

Our study confirmed dynamics of expression of inhibitory KIRs receptors with association of their HLA ligands. We detected opposite behavior of KIRs. KIRs binding HLA-C molecules were strongly downregulated, on the other hand KIRs receptors for HLA-A and B ligands showed rather increase of expression. We did not see any correlation with presence of relapse or changes of KIRs expression after *ex vivo* activation.

The study was funded by the Ministry of Health of the Czech Republic - Czech health research council (project no. 15-30661A).

SINGLE CELL PROFILING OF SIGNALTRANSDUCTION PATHWAYS IN PEDIATRIC T-CELL ACUTE LYMPHOBLASTIC LEUKEMIA

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Acute leukemia is the most common malignancy of childhood. About 15% of pediatric leukemias are derived from T-cells leading to T-cell acute lymphoblastic leukemia (T-ALL). The survival of T-ALL patients is comparable to other acute leukemia patients, however about 30% of T-ALL patients relapse and their survival rates dramatically decrease. Therefore further investigation of the biology and treatment possibilities of T-ALL is urgently needed.
Using single-cell mass cytometry we developed panel of 39 metal-labeled monoclonal antibodies (moAbs) identifying T-ALL blasts and non-malignant T-cells. Using phospho-specific moAbs and moAbs targeting proliferation and apoptosis we detected signal transduction upon in vitro treatment of 17 diagnostic and 5 relapse T-ALL samples with IL-7, Ruxolitinib, BEZ-235, combination of Ruxolitinib and BEZ-235 and Pervanadate.
First, we evaluated quality of the data using previously described targets of stimuli. E.g. pSTAT5 was phosphorylated upon IL-7 stimulation (Wilcoxon test, $p<0.05$). 4E-BP1 (Akt downstream target was dephosphorylated after BEZ-235 treatment (Wilcoxon test, $p<0.01$). We detected strong phosphorylation of pLck in all T-ALL samples after treatment with Pervanadate. In general, in T-ALL blasts we observed a constitutive hyper activation of many down-stream targets (e.g. pErk1/2, p-p38 or pAkt) compared to residual non-malignant T-cells.
Next, we compared specific subgroups of T-ALL patients regarding their prognosis. pRb, a marker of proliferating cells, was hyper activated in subgroup of patients responding to treatment (prednisone good responders) compared to patients responding slowly to treatment (prednisone poor responders, Wilcoxon test $p<0.05$). Additionally, we observed enhanced pRb in patients with low and very low level of minimal residual disease (MRD) compared to patients with high MRD.
In summary, using mass cytometry we phenotypically and functionally characterized pediatric T-ALL samples. We detected constitutively active signal transduction pathways in T-ALL blasts compared to residual non-malignant T-cells. We provide evidence of the feasibility of CyTOF-based protocol in T-ALL biology investigation.
Supported by: PRIMUS/17/MED/11, NPU I nr. UNCE 204012 , NPU I No. LO1604

HIGH QUALITY MULTIPARAMETRIC FLOW CYTOMETRY: SEEING THE FULL PICTURE THROUGH FULL SPECTRUM CYTOMETRY

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Introduction: Full Spectrum Cytometry differs from conventional cytometry in the way that light emitted from the excitation of a particular fluorochrome is collected. Rather than collect this light at only the peak emission wavelength, full spectrum cytometry captures this light across a range of wavelengths from UV to far red (420-830 nm). This strategy makes it possible to approach multicolor flow cytometry in a more flexible way when it comes to fluorochrome choices and enables high dimension (> 20 color) high resolution flow assays with fewer lasers. The aim of this work was to optimize a 24-color panel for immunophenotyping in human whole blood.
Methods: An assay aimed at identifying the main circulating cell subsets in whole blood was designed and included 24 different markers. 53 commercially available fluorochromes were characterized in terms of spectrum signature, brightness and spread using an Aurora full spectrum cytometer equipped with 3 lasers (405, 488 and 635 nm). 24 fluorochromes that could be used in combination on the 3 laser Aurora were selected.
Results: Detailed analysis of the single stained controls revealed that beads were not always optimal controls as the spectral characteristics of these controls sometimes differed from cell controls. Moreover, the analysis of the initial multicolor panel showed good resolution for the great majority of the markers but needed further optimization to better resolve 3 out of the 24 markers. A second panel was designed and showed optimal resolution of all the markers in the panel.
Conclusion(s): Developing a highly multiparametric panel using a full spectrum flow cytometer proved to be a straightforward process that resulted in high resolution data. The possibility to fully assess the spectrum of each dye not only guided fluorochrome selection but was also key for successful full spectrum cytometry panel design.

KIRNOME ANALYSIS FOR IMPROVED TRANSPLANTATION EFFICIENCY

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Killer-cell immunoglobulin-like receptors (KIR) are group of transmembrane proteins on the surface of mostly Natural Killer (NK) cells responsible for either activation or inhibition of NK cell function. Sixteen KIR genes is differentiated with totally 977 alleles, that can be combined into specific haplotypes. KIRs gene polymorphism can affect strength of inhibitory signal or level of surface expression influencing NK cells behaviour. Given the NK cells functions, KIR genotyping became very important in hematopoietic stem cells transplantation where the presence/absence of KIR gene has impact on outcome of transplanted patients Although assessment of KIR haplotype is of great clinical importance, currently it is not typical marker that is investigated before HSCT. We assume that knowledge of donor KIR haplotypes may be used as crucial secondary marker in cases when multiple donors are available based on matching human leukocyte antigen (HLA) haplotype and can improve efficiency of HSCT.

The goal of the study is to characterize KIR haplotypes common in Czech population, explore possibility of detailed KIR haplotyping in clinical practice and, given the role of NK cells in malignant diseases, to link presence of tumour disease to particular KIR haplotypes. Method-wise, complete KIR genome is amplified by long-range PCR using a mixture of 6 primers from isolated DNA, resulting in a population of full-length KIR genes (4-17kbp). Next generation sequencing library is then prepared by modified NEBNext® Ultra™ II FS DNA Library Prep Kit for Illumina (New England Biolabs) and libraries are sequenced in pair-end mode on Illumina Miseq instrument. Data are processed by in-house algorithms and KIR haplotype is assessed by detection of particular alleles.

Currently, proof-of-concept part of the project is completed, and patients and donors are enrolled into the study for full-scale description of KIR haplotypes and the connection between genotype and phenotype is evaluating.

This work is funded by the Ministry of Health of the Czech Republic - Czech health research council (project no. NV18-03-00277).

THE DEVELOPMENT OF ERYTHROCYTES POPULATIONS OF HEREDITARY SPHEROCYTOSIS PATIENT IDENTIFIED BY FLOW CYTOMETRY IN TIME

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Hereditary spherocytosis (HS) is an inborn disease characterized by extravascular hemolysis and spherocytes presence in peripheral blood. The erythrocytes (Ery) membrane defects cause its instability, loosing of membrane material and finally creating a spherocyte. HS is often discovered in child age however; it can be diagnosed during the whole lifetime.

Flow cytometry (FMC) approach is diagnostically specific and sensitive. Eosin-5-maleimide binds on BAND3 protein in Ery membrane and it is detectable by FMC. EMA binding on BAND3 is worse in HS positive samples than healthy control. Thus, the fluorescence is also lower. The result is expressed as a patient vs. healthy control ratio.

The presented case of child patient (*2016) was intercepted after repeated transfusions in 7/2018. Patient's family moved to the Czech Republic in 2017, where she received her first transfusion 3.7.2018. The patient underwent 10 transfusions in total.

First FMC analysis was performed 21 days after transfusion. The doubled Ery population was detected – the patient's own and transfusion-origin with higher EMA fluorescence, which hid the presence of small population of HS Ery. Another three blood samples were sent in the following 5 months. The continual reduction of transfusion-origin Ery was observed as well as the total ratio decrease.

The blood transfusion influence analyses of Ery as in HS patient analysed by FMC may cause a false-negative result. Our analyses proved a continual decrease of transfusion-origin Ery which do not interfere with positive test result after 3 months from transfusion.

Supported by MZ ČR-RVO (FNBr, 65269705) and specific research project MUNI/A/1105/2018.

MICROFLUIDICS

DETECTION OF PHOTON-UPCONVERSION LUMINESCENCE FROM MICRODROPLETS IN MICROFLUIDIC CHIPS

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We report instrumentation for reading photon-upconversion luminescence from water microdroplets in a microfluidic chip, which was not described previously. From the early times of their introduction, droplet-based microfluidics presents a paradigm for high-throughput screening in a broad spectrum of fields from physics to biomedicine. Droplet-based microfluidics uses a two-phase system of aqueous microdroplets (1 pL to 1 µL) embedded in immiscible oil. This system enables to manipulate single droplets at a high-throughput as well as to incubate stable droplets off-chip and reintroduce them into the microfluidic environment for further processing and analysis. UCNPs are lanthanide-doped nanocrystals that can be excited by near-infrared light and emit light of shorter wavelengths, which is very different from the luminescence of organic fluorophores or quantum dots [1,2]. Advantages of UCNPs include multiple and narrow emission bands, negligible autofluorescence and high photostability making UCNPs an ideal luminescent label for use in droplet microfluidics; recent UCNPs are sufficiently bright even for single nanoparticle imaging [3]. We suppose that our results are applicable for single cell experiments: multiparameter titrations, biological and chemical assays, and digital immunochemical detections [2,3].

Acknowledgment

We acknowledge financial support from the Czech Science Foundation (18-03367Y) and the Institutional support RVO: 68081715.

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HIGH-THROUGHPUT CHARACTERIZATION OF HALOALKANE DEHALOGENASES BY USING CAPILLARY MICROFLUIDICS

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Here we present a microfluidic platform for characterization of haloalkane dehalogenases (HLDs) in terms of enzyme activity, substrate specificity and temperature profiles. The experiments are carried out in 1000-fold decreased reaction volume and 100-times reduced time as compared to conventional methods. The platform utilizes a novel route for substrate delivery, based on the partitioning of the substrate between the carrier oil and the reaction aqueous phase of the droplet containing the enzyme. We validated the microfluidic system by measuring the substrate specificity of model enzyme haloalkane dehalogenase LinB towards 27 halogenated substrates and compared the results with data obtained by conventional and robotic measurements. Subsequently, we utilized the microfluidic system for the high-throughput analysis of substrate specificity profiles and temperature profiles of 8 well-known HLDs. The obtained results and microfluidic method performance were critically compared with conventional measurements, showing a high correlation of $R^2 = 0.89$ for activities of LinB and $R^2 = 0.95$ for temperature profiles [1]. The newly developed microfluidic platform has been systematically applied for the high-throughput characterisation of enzyme candidates identified within integrated workflow for gene database mining [2]. The usage of microfluidic methods significantly reduces both sample and time requirements and increases the speed of the novel biocatalysts discovery.

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DETECTION OF CHLOROALKANES BY SURFACE-ENHANCED RAMAN SPECTROSCOPY IN MICROFLUIDIC CHIPS

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Optofluidics, a research discipline combining optics with microfluidics, currently aspires to revolutionize the analysis of biological and chemical samples, e.g., for medicine, pharmacology, or molecular biology. In order to detect low concentrations of analytes in water, we have developed an optofluidic device containing a nanostructured substrate for surface enhanced Raman spectroscopy (SERS). The SERS substrate (nanostructured gold surface) was enclosed in a microfluidic system, which allowed transport and precise mixing of the analyzed fluids, while preventing contamination or abrasion of the highly sensitive substrate. To illustrate its practical use, we employed the device for quantitative detection of persistent environmental pollutant 1,2,3-trichloropropane in water in (sub)millimolar concentrations. The developed sensor allows fast and simple quantification of halogenated compounds and it will contribute towards the environmental monitoring and enzymology experiments with engineered haloalkane dehalogenase enzymes [1].

[1] Pilát *et al*., Sensors 18(10), 3212 (2018)

FABRICATION OF A MICROFLUIDIC CHIP TO STUDY THE BIOLOGY OF VASCULAR ENDOTHELIUM UNDER FLOW CONDITIONS

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Blood vessel inflammation is an initial process preceding the development of cardiovascular diseases. However, the mechanisms behind the onset of inflammation are not yet fully understood. Majority of vascular system research has been conducted using either rodent models or static cell cultures. Both aforementioned approaches are limited in terms of physiological relevance. Microfluidic chips are a good alternative to overcome these limitations. However, commercially available chips usually have rectangular cross-section of the channels, which causes non-physiological behaviour of the cells. To overcome these limitations, we designed a microfluidic chip with circular cross-section of the channels. The chip was made of polydimethylsiloxane (PDMS). PDMS is gas permeable and optically transparent elastomeric material making it suitable for cell culture. Its main limitation is hydrophobicity, which does not favour cell adhesion. To overcome this issue, we screened several surface modifications (silanisation, oxidation, protein coating, etc.). The most potent modification in terms of cell adhesion, cell viability and temporal stability was combination of plasma surface oxidation followed by collagen IV deposition. Channels with surface modified this way were then seeded with murine vascular endothelial cells (cell line MS1). By using flow conditions cultivation, we were able to repeatable get uniform layer of highly viable endothelial cells with morphological features well resembling morphology of endothelium in vessels *in vivo*.

This work was supported by the Czech Science Foundation grant No. 17-24707S, National Program of Sustainability II (MEYS CR) project no. LQ1605 and Internal project of the Institute of Biophysics.

OPTICAL MICROMANIPULATION AND RAMAN SPECTROSCOPY OF CELLS IN MICROFLUIDIC SYSTEMS

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Measuring antibiotics induced changes in E. coli cells via Raman spectroscopy, LTRS and microfluidic systems is useful method for identification pathogens. Optical micromanipulation by a laser beam allows noncontact and noninvasive manipulation of small object, for example bacterial cells. Microfluidic device consists of microchannels and microchambers in transparent polymer and it is used for isolation, observation and cultivation of bacterial cells. Combination of these methods gives an effective tool for observation, manipulation and analysis of microorganisms. E. coli is a microorganism potentially pathogenic for humans and faster detection of its sensitivity to antibiotic treatment would make the whole process of diagnostics and treatment easier. We performed laser tweezer-Raman spectroscopy and conventional Raman spectroscopy of bacterial cells and cells under antibiotic stress and collected Raman spectra and characteristic areas were compared with literature to establish the reliability and usefulness of this method.

DATA ANALYSIS

MULTIDIMENSIONAL DATA ANALYSIS AND BREAST CANCER HETEROGENEITY

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Breast cancer is the most common cancer in women which includes remarkably heterogeneous subtypes, with the relatively poor outcome due to aggressive clinical behaviour and lack of characterized molecular targets for therapy. Both intratumoural heterogeneity (ITH) and heterogeneity in microenvironmental constituents account for patient prognosis and response and resistance to therapies. ITH, characterized by the presence of multiple distinct clones of cancer cells within one tumour, is one of the main obstacles in effective cancer treatment. Cancer stem cell (CSC) concept elucidates how one mutated cell on the origin can give rise to many different cells in the tumour, thus it reflects intra ITH. In the breast, CSCs presumably originate from basal and luminal cells. To shed new light on ITH in breast cancer, we introduced high-throughput, validated antibody-based, flow cytometric screening platform for simultaneous analysis of hundreds of surface antigens in a single run and in up to six cell lines. Such analysis of surface pattern in multiple epithelial cell lines and their mesenchymal counterparts revealed distinct surface changes, accompanying epithelial-to-mesenchymal transition in these models. To prove the clinical relevance of our findings, we further validated heterogeneity in expression of ten most robustly changed antigens in dissociated breast cancer patient samples at a single cell resolution. [1]
Therefore, we established a 12-colour flow cytometric panel for the multiparametric description of breast cancer heterogeneity and plasticity in patient samples. Analysis and visualization of the multidimensional flow cytometric data are challenging, and traditional methods of sequential gating are no more possible to employ for analysis. In this study, we aimed to apply automated clustering methods implemented in the R scripting language. [2, 3] Four luminal type A patient samples of breast cancer were analysed with the specific aim to compare results of algorithms and more importantly to describe inter and intra tumour variability.

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This work was supported by the Ministry of Health of the Czech Republic, grant no. 15-28628A and NV18-08-00245; project no. LQ1605 from the National Program of Sustainability II (MEYS CR) and by project HistoPARK (CZ.1.07/2.3.00/20.0185).

BOTANY

DNA REPLICATION TIMING PROGRAM IN BARLEY (*HORDEUM VULGARE*)

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Nuclear genome is replicated during the S phase of cell cycle under strict rules, which ensure accuracy and completeness of this important process. Replication timing programs, which are predictive of genomic features and activity, have been described in prokaryotes, yeasts and many animal species. In plants, DNA replication was analysed in more detail only in species with relatively small genomes, such as maize and *Arabidopsis*. To provide more insights, we studied the replication machinery in barley (*Hordeum vulgare*), a representative of plants with large genome (1C ~ 5100 Mb) and high proportion of DNA repeats (more than 80%). In order to study DNA replication in both time and space we combined flow sorting of 5-ethynyl-2'-deoxyurdine (EdU) labelled nuclei, 3D acrylamide FISH and Repli-Seq. Nuclei at different stages of the cell cycle (G1, G2, early, middle and late S-phase) were isolated and used for 3D FISH with probes specific to different DNA repeats and rRNA genes. We observed that replication process of different DNA repeats varied in time, e.g. centromeric retrotransposon Cereba was replicated during all stages of S phase and sub-telomeric satellite repeats psc119 was replicated during the early and middle S phase. Difference in replication timing was observed also for rRNA genes, while 5S rRNA genes were replicated during early S phase, majority of 45S rRNA genes were replicated in very late S phase, probably reflecting the presence of large amounts of pseudogenic 45S rDNA units. Genome-wide replication timing program in barley was described based on Repli-Seq data. Genomic regions that replicate predominantly during early, middle and late S-phase were identified. Our results provide the first picture of the complexity of DNA replication in time and nuclear space, reflecting different types of DNA sequences and their role in genome organization and function in barley.

CHARACTERIZATION AND DISSECTING THE COMPLEX NUCLEAR GENOME OF CRESTED WHEATGRASS BY CHROMOSOME FLOW SORTING

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Agropyron species are a potential source of beneficial traits for wheat improvement. Among them, *Agropyron cristatum* comprises a complex of diploid, tetraploid and hexaploid forms with the basic genome P, with some accessions carrying supernumerary B chromosomes (Bs). In this work, we applied flow cytometry to characterize and dissect the complex genome of *A. cristatum* into individual chromosomes to facilitate its analysis. Flow cytometric analysis classified 26 accessions as diploid, 51 accessions as tetraploid and 3 accessions as hexaploid. Mean nuclear 2C DNA content of diploid plants was estimated as 12.99 pg DNA (1C genome size = 6.352 Gbp). Flow karyotypes obtained after the analysis of DAPI-stained mitotic chromosomes of diploid and tetraploid accessions consisted of three peaks, each corresponding to a group of two or three chromosome types. To improve the resolution, bivariate flow karyotyping after fluorescent labeling of $(GAA)_7$ microsatellite was applied and allowed discrimination and sorting of P genome chromosomes from wheat-*A. cristatum* addition lines. Chromosomes 1P - 6P and seven telomeric chromosomes could be sorted at purities ranging from 81.7 to 98.2% in disomics and from 44.8 to 87.3% in telosomics. Chromosome 7P was sorted at purities reaching 50.0 and 39.5% in diploid and tetraploid *A. cristatum,* respectively. In addition to the whole complement chromosomes (A), supernumerary B chromosomes (Bs) could be easily discriminated and sorted from a diploid accession at 95.4% purity. The sorted chromosomes will streamline genome analysis of *A. cristatum*, facilitating gene cloning and development of molecular tools to support alien introgression into wheat.

ACKNOWLEDGEMENT
Supported by GACR Grant 16-16992S • ERDF (No. CZ.02.1.01/0.0/0.0/16_019/0000827) • OTKA K116277 • MarieCurie AEGILWHEAT H2020-MSCA-IF-2016-746253 • Grants award AGL2014-52445-R and RTI2018-093367-B-I00 co-financed by FEDER (EU).

NEW METHODS

PROBIOTIC EFFECT ON MUCOSAL IMUNITY TO CAMPYLOBACTER JEJUNI IN CHICKENS

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Mucosal immunity included cecal IEL and LPL, to *Lactobacillus reuteri* B6/1 in chickens infected with *Campylobacter* jejuni was study by flow cytometry. Day-old chicks (120) were divided into four groups (n=30): control (C), L. reuteri (Lr), *Campylobacter jejuni* (Cj), and combined *L. reuteri*+*Campylobacter* (LrCj). *L. reuteri* was individually per os administrated to chickens during 7 days and *Campylobacter* infection was done at day 4. Direct immunofluorescence and double staining of isolated lymphocytes with mouse anti-chicken antibodies were used in combination CD3PE/IgM FITC, CD4PE/CD8FITC, CD45PE/IgAFITC (Southern Biotech, USA). Samplings were done 4 and 7 days post infection (dpi). Monitoring of mucosal immunity by determination of cecal IEL on 4 dpi indicated overstimulation in both probiotic groups (Lr and LrCj) of CD45, CD3, IgM, and in combine LrCj group of CD8 and IgA. Upregulation of CD3 and CD8 persisted on 7 dpi in both probiotic groups, and of IgM in combine group. Cecal LPL showed activation of CD3 in both probiotic groups and both samplings. Within T cell subpopulations CD4 showed overstimulation 4 dpi and CD8 7 dpi in both probiotic groups. B cells, included IgM and IgA subpopulations, were upregulated in combine LrCj groups of both samplings, 4 and 7 dpi. Our results suggest beneficial effect of *L. reuteri* B6/1 at the beginning of campylobacter infection by involvement both cellular mediated and humoral immunity.
Study was supported by grants APVV-15-0165 and VEGA 1/0112/18.

QUANTITATIVE, LIVE-CELL KINETIC ANALYSIS OF MICROGLIAL FUNCTION AND MORPHOLOGY

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Microglia are the resident immune cells of the central nervous system and play significant roles in the regulation of homeostasis and the management of tissue response to inflammatory or pathological insults. Microglia impact synaptic remodeling and turnover of dendritic spines through the removal of damaged or unnecessary neurons or synapses. Limited tools and invitro model systems exist to enable optimizing, monitoring, and analyzing functional and morphological changes of these cells. We characterize RatPrimary, iPSC derived, and immortalized microglia and present data evaluating the ability of these cells to phagocytose pHrodo®labeled E.coli bioparticles and apoptotic Neuro-2A (N2A)cells using a quantitative, live-cell imaging approach with the IncuCyte®S3 Live-Cell Analysis System for Neuroscience.

HIGH PARAMETER FLOW CYTOMETRY – SETUP AND OPTIMALIZATION

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New generation of fluorescence flow cytometers allows for the measurement of more than 30 fluorescent parameters. However, this adds further complexity in setting up the instrument for optimal performance and designing flow cytometry panels. We have therefore compared different methods for setting optimal PMT voltages with regard to population separation and minimalization of spreading error. The tested methods include the gold standard maximum staining index method compared to the "10^{2}" method, which sets the negative population to 10^{2} on the log scale, and the method using a source of uniform light produced by the LED pulsar QuantiFlash.
Using these methods, we have further evaluated the spectral properties of 54 fluorochromes to identify the best fluorochromes for the development of 30+ fluorescent panels using the BD Symphony A5 flow cytometer.
Supported by OPVVV CZ.02.1.01/0.0/0.0/16_025/0007428

3D BIOPRINTING OF STEM CELLS FOR CREATING TISSUE MODELS

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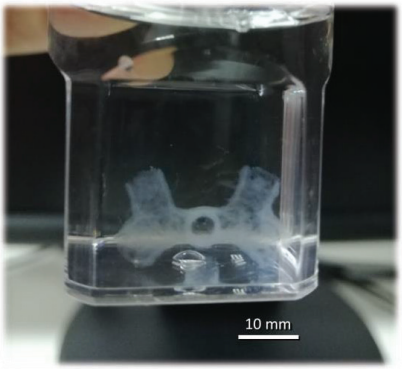
3D cell culture techniques provide deeper insights to cellular behavior in comparison to standard planar cultivation, due to more physiological conditions – e.g. cell-cell and cell-matrix interactions, nutrition/soluble factors accessibility. We utilize 3D bioprinting to create cellular/hydrogel structures of simple structures up to complex 3-dimensional tissue models. For creation of such models, it is necessary to apply suitable materials, cells and design, with respect to architecture and size of mimicked tissue, cell interaction as well as nutrient/waste exchange.
We are printing several biocompatible hydrogels – natural and synthetic. In this work we present results with alginate, which is broadly applied material for 3D bioprinting. Human stem cells printed in alginate hydrogels can be grown for a prolonged period of time, however cells suffer from minimal interaction with environment as alginate lacks bioactive motifs. Therefore we are fine-tuning physio-chemical properties of alginate by immixing extracellular matrix proteins and components with aim to stimulate specific cell response (adhesion, proliferation, differentiation, migration, cell aggregation).
We show that we can print simple and complex tissue models including viable stem cells and alter their behavior during 3D culture in hydrogel by its modifications.

This work was supported by project of Czech Science Foundation GA18-05510S, project from Masaryk University MUNI/A/1565/2018*. Vaclav Chochola is holder of Brno Ph.D. Talent Scholarship – funded by the Brno City Municipality.*



ISOLATION OF STERLET (*ACIPENCER RUTHENUS*) TYPE A SPERMATOGONIA USING FLUORESCENCE-ACTIVATED CELL SORTING

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Sturgeons belong to the order Acipenseriformes, most ancient of actinopterygian fishes. Nowadays 64% of sturgeon species are listed as critically endangered. Moreover, most sturgeon species are large and late maturing, which makes their culturing very costly and time consuming. Therefore, germ cell culture and xenotransplantation of germ cells could be an affordable and a time-efficient method for surrogate production of endangered sturgeons . Germ stem cells (GSCs) are a crucial component in reproduction, with the ability to self-renew as well as to differentiate into other types of germ cells. In fish transplantation system, only undifferentiated germ cells - spermatogonia type A (ASGs), are capable of undergoing gametogenesis after their incorporation into the genital ridges of recipients. Thus, enriched population of ASGs, is expected to increase the transplantation success rate. Besides, the morphology as well as cellular and molecular mechanisms of sturgeon germ cell development have been poorly understood and remain a question of broad interest. Development of new methods for enrichment of different germ cell populations *in vitro* is very essential.

In our study, we established a method for identification and isolation of sturgeon ASGs using fluorescence-activated cell sorting (FACS). Flow cytometry analysis of freshly prepared whole testicular cell suspension showed a few distinguished cell populations formed according to different values of light scatter parameters. FACS of these different cell populations was performed directly on glass slides for further immunocytochemistry to identify germ cells and ASGs. The same cell populations were also single cell sorted into 384-well plates for q-PCR of vasa gene. Results of q-PCR and immunocytochemistry of post sorted cells, as well as immunohistochemistry and histological observation of whole testis sections showed that the cell population in gate P1 on a flow cytometry plot (with high forward scatter (FSC) and high side scatter (SSC) parameter values) contains the highest amount of ASGs – 81.92 ± 2.71% compared to a non-sorted sample – 11.59 ± 1.86%.

We expect that the use of this FACS strategy can improve the production of sturgeons with surrogate broodstock and further study of cellular and molecular mechanisms of sturgeon germ cell development.

MALDI-TOF BIOTYPING IN QUALITY CONTROL OF CLINICALLY RELEVANT CELL TYPES

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Dynamic development of stem cell (SC) biology resulted in protocols driving human pluripotent SC into various clinically relevant cell types. These advances make SC biology a promising field for clinical applications of SC-based products in context of modern regenerative medicine. One of the most important questions related to clinical use of SC-based products is homogeneity of cell populations and its quality control.
Several methods for assessing SC viability and genetic stability have been developed, however, most of them are time consuming with great variations between different facilities, which is a problem for quality testing in clinical settings.
Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) have been adapted for analysis and determination of complex biological samples, including bacteria, yeasts, and mammalian cells.
MALDI-TOF MS analysis requires only simple sample preparation, and in some cases can be automated in combination with Artificial Intelligence-based algorithms.
Much effort has been put into understanding the sources of SC heterogeneity and into improving generation of high-quality pluripotent SC. Previously, we have identified extracellular form of Cripto-1 protein as a potent marker of multipolar mitotic spindle-linked genetic instability in human pluripotent SC.
Herein, we analyzed changes in cell status by intact cell mass spectrometry fingerprinting in differentiating human embryonic stem cells, with respect to Cripto-1 linked alterations. This study describes cell mass spectrometry combined with chemometric and artificial intelligence approaches as a simple quality control tool for determining induced and spontaneous alterations in human pluripotent SC populations and its derivatives in routine cell cultures.
This study was supported by funds from the Ministry of Health of the Czech Republic (grant nr. 18-08-00299) and from the Ministry of education, Youth, and Sports of the Czech Republic (NPUII; LQ1605).

AUTOMATED SCORING OF THE IN VITRO MICRONUCLEUS ASSAY FOR GENETIC TOXICOLOGY TESTING USING IMAGING FLOW CYTOMETRY

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Introduction: The in vitro micronucleus (MN) assay is a well-established test for evaluating genotoxicity and cytotoxicity. Scoring by manual microscopy is laborious and variability between scorers may impact results. Automated microscopy and flow cytometry methods have been developed, but are limited by the inability to visualize the cytoplasm or to confirm legitimacy of potential MN. The Amnis®ImageStream®XMk II imaging flow cytometer can overcome these limitations by combining the speed of flow cytometry with the high resolution imagery of microscopy

Materials and Methods: TK6 cells were exposed to well-known MN-inducing chemicals for 3 hr**.** Following this, cells were transferred to fresh media for an additional 24 hr in the presence of the cytokinesis blocker Cytochalasin B**.** Cells were then enlarged with KCl, fixed with Formalin, and stained with Hoechst**.** High resolution images were captured on the ImageStreamXMk II and a data analysis template was used in the IDEAS®Software package to automatically identify and enumerate mono-, bi-, and polynucleated cells, as well as binucleated cells (BNCs) with MN**.** Genotoxicity was determined by quantifying the rate of MN in BNCs**.** Cytotoxicity was determined by quantifying cell proliferation through enumeration of mono-, bi-, and polynucleated cells

Conclusions: An automated method to perform scoring for the in vitroMN assay using the ImageStreamXMk IIhas been developed. Mono-, bi-, and polynucleated cells as well as MN were automatically identified and scored using masks and mathematical algorithms in IDEAS®. Statistically significant increases in MN frequency were observed for all MN-inducing chemicals tested when compared to solvent controls. ImageStreamXMk II derived MN frequencies and MN fold increases compare well to manual microscopy. The ImageStreamXMk II-basedmethod to perform the in vitroMN assay has the potential to overcome limitations inherent in slide-based microscopy and conventional flow cytometry techniques

TEST OF METALLOPROTEINASES PRODUCTION BY MESENCHYMAL STROMAL CELLS AFTER COMBINATION OF DIFFERENT BIOPHYSICAL STIMULI

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Matrix metalloproteinases (MMPs) constitute a family of enzymes which are implicated in tissue remodeling processe. MMP has strong impact to cell migration and effectivity of cell invasion to specific site of ischemic tissue or pathological site. To investigate the role of biophysical stimuls to MMP production by mesenchymal stormal cells (MSC), the set of experiments wae prepared. Used cells (rat MSC, rabbit MSC) were cultured on three different types of microplates with three different physical microstructure : collagen I, fibronectin + gelatin, planer net of nanofibers prepared from PLC. All variants of plates were prepared for safety stimulation by high inductive magnetice stimuls or hypoxic condition in temperated box for 3 days.

The MMPs/actin expression was detected by Western Blotting and RNA analysis of MMPs changes in cells were measured on all variants of cell samples. Two samples were detected as variant with the significant differenties of MMPs production after biophysical stimul: MSC after high inductive magnetic stimuls and after hypoxic stimul (both on collagen I variant of microplate)

This work was supported by the Ministry of Health of the Czech Republic (NV16-28784A)

SCREENING FOR PRODUCTION OF INTRACELLULAR BENZO[a]PYRENE METABOLITES BY SPECTRAL FLOW CYTOMETRY

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Benzo[a]pyrene (BaP) is one of the most abundant polycyclic aromatic hydrocarbons (PAHs) in polluted air, processed food or tobacco smoke, which is a potent mutagen and carcinogen. BaP is innocuous by itself and it requires its metabolic activation (catalyzed by cytochrome P450 family 1 (CYP1) enzymes), in order to exert its genotoxic effects. The CYP1-dependent metabolism of BaP is regulated by the aryl hydrocarbon receptor (AhR), and it may lead to production of different types of metabolites including hydroxylated intermediates, quinones, dihydrodiols and various conjugated metabolites, with distinct toxicity profiles, including production of dihydrodiol epoxides forming covalent DNA adducts. The metabolism of PAHs, such as BaP, thus plays a pivotal role in their toxic mode of action; however, the analysis of metabolism of PAHs is often time-consuming and expensive. Here, we explored a possibility of developing a method for screening of CYP1-dependent PAH metabolism by detecting the production of fluorescent metabolites of BaP in living cells, using a spectral flow cytometry. We used cell lines with a well-characterized BaP metabolism previously analyzed in our lab (HCT-116 colon cancer cells and MCF-7 breast cancer cells). We employed both wild-type cells and cells with compromised metabolism of BaP (due to disruption of AhR signaling), in order to analyze the spectral differences among cells with distinct BaP metabolism. The data sets, obtained with Sony SP6800 Spectral Analyzer were converted to FCS format and then analyzed with FlowJo. We applied t-Distributed Stochastic Neighbor Embedding (t-SNE) for dimensionality reduction of multiparametric data, in order to identify cell populations with low and high rate of BaP metabolism. Our preliminary results seem to suggest that the analysis of uptake and/or metabolism of BaP in living cells by spectral flow cytometer may have a potential for screening of metabolism of PAHs in cells treated with AhR agonists/antagonists or in cells with genetically-manipulated CYP1 expression/activity. This may help to provide a novel insight into mechanisms of cellular injury caused by PAHs, as well as to allow for screening of potential chemopreventive compounds. [Supported by the Czech Science Foundation, project no. 19-00236S.]

CELL SEPARATION OF CD3+ AND CD19+ CELLS FROM SAMPLES OF LEUKEMIC PATIENTS

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Physical separation of cell subsets from clinical samples is an important preparatory step for multiple methods that analyze the bulk sample without single cell resolution. This is the case for clinical samples of patients with leukemia, where genetic landscape needs to be analyzed separately in the tumor and in the non-malignant tissues. We tested and validated alternative cell separation approach, the releasable antibody based immunoaffinity matrix (FABian cell selection device; IBA Lifesciences, Goettingen, Germany), to separate CD19+ B-cells and CD3+ T-cells. FABian passes whole blood or bone marrow through the Fab-TACS Auto column filled with a Strep-Tactin coated cell-grade agarose matrix. Target cells adhere to the affinity matrix based on the exclusive binding of the Fab-Strep to the target cell. Non-target cells are washed away efficiently. Finally, FABian loads biotin onto the column which causes the elution of the target cells and the Fab-Streps spontaneously self-dissociate from the cell surfaces due to their reduced affinity.

In total 45 FABian separations (42 patients) were performed, CD3+ (n=36) and CD19+ (n=9). The purity of CD3+ separation products was 96.2% to 99.4% (median 98.05%) and the purity of CD19+ was 94.5% to 99.1% (median 98.1%). DNA isolated from the separated cells is mostly used as nonmalignant control in SNP analysis of leukemic specimens. Therefore the contamination of final product with leukemic cells is another important parameter that is checked beside the purity. The maximal contamination with leukemic cells is set up as 2% for SNP analysis. This criterion was always fulfilled. The contamination by leukemic cells was 0% to 1.1% (median 0.03%) in CD3+ products. Most of the CD19+ separations (7/9) were performed with B-cell precursor (BCP) ALL specimens to enrich the leukemic cells proportion. The contaminating leukemic cells assessment is irrelevant here but its overall percentage (after nonmalignant CD19+ cells exclusion) was evaluated. In two T-ALL cases CD19+ cells were separated as nonmalignant controls.

We evaluated Fab based separation and consider it a robust tool for nonmalignant as well as leukemic cell enrichment. This method is suitable, fast and efficient for clinical sample processing. Separated cells are routinely used genetic diagnostics.

Supported by UNCE 204012 and NPU I nr. LO1604.

CTHRC1: A NOVEL PROGNOSTIC MARKER IN CHRONIC LYMPHOCYTIC LEUKAEMIA

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WNT signaling pathway is one of the cornerstones in development of multicellular organisms and its defects have been described in a vast number of malignancies. We have previously described the upregulated expression and activity of WNT/Planar cell polarity pathway (PCP) components in the chronic lymphocytic leukemia (CLL) cells. Our research also pointed to association of the WNT/PCP pathway and poor CLL patient prognosis. The more thoroughly described branch of WNT signaling, β-catenin-dependent, has been also connected to CLL progression by others. Our work however suggested that higher *WNT3* expression (a typical WNT/β-catenin-activating ligand) is associated with better prognosis of CLL patients.
In addition to the clear upregulation of the key WNT/PCP components (ROR1, FZD3/7, PRICKLE1, DVL1-3, CK1Ɛ, WNT5A/B etc.), we have described in our projects a set of atypical WNT/PCP regulators, including COBLL1 protein or the family of amyloid precursor proteins (APPs).
In this study, we describe the role of another WNT/PCP regulator - CTHRC1, which is an extracellular protein, described earlier as a potential switch between β-catenin-dependent and WNT/PCP signaling pathway. Our in vitro analysis partly confirms these findings. Moreover, we were able to demonstrate that high *CTRHC1* expression in primary CLL cells correlates with poor overall survival rate of the patients. This makes *CTRHC1* a potential prognostic marker in CLL and points the role of WNT/PCP activity in CLL biology as well as normal B cell function.

HIGH QUALITY MULTICOLOR FLOW CYTOMETRY - SEEING THE WHOLE PICTURE THROUGH FULL SPECTRUM CYTOMETRY ON THE 5 LASER AURORA

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Flow cytometry is rapidly evolving and with the advent of the development of spectral flow cytometry we see for the first time a realisation of the potential to exceed 28-colour panels with existing commercial dyes.
Cytek Biosciences introduces the first 5 laser spectral flow cytometer with multimode automated microplate sampler (AMS). This high-parameter system offers full spectrum assessment from 5 independent laser excitations simultaneously across 64 detectors offering unparalleled resolution of data from highly overlapping dyes. Spectral unmixing can be performed in real-time from tubes or multi-well plates. We present performance characteristics of this unique instrument along with a variety of examples of multiparametric applications We will also show how this spectral analyser yields superior performance/resolution compared to traditional cytometers.