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BOTANY AND ZOOLOGY

DIVERGENT TRAJECTORIES OF GENOME EVOLUTION: THE IMPACT OF CENTROMERE DRIVE ON SHAPING GENOME ARCHITECTURE, SPECIES RICHNESS AND COMPETITIVENESS OF FERNS AND ANGIOSPERMS

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Unlike their "younger sisters," the extant flowering plants, ferns generally exhibit lower species richness and reduced dominance across biomes. Unraveling the genomic factors behind these disparities has long been hampered by limited fern data. We assembled the largest genomic trait dataset (genome size, GC content, chromosome size, and number) for 1,100 species (~10%) of ferns, and integrated it with an equally comprehensive angiosperm dataset. Our comparative analyses reveal that the stability and lower evolutionary rate of chromosome size in ferns may be underpinned by the absence of centromere drive, a meiotic mechanism present in angiosperms but not in ferns, as a key factor limiting genomic flexibility in ferns. Additionally, we show that larger fern genomes nearly entirely explain their larger stomatal size (newly estimated for 361 ferns), a critical trait affecting competitiveness via water use efficiency and photosynthesis. We predict that if centromere drive operated in both groups, fern species richness would be only five times lower than angiosperms, rather than the current 30-fold disparity. Furthermore, the similar breakpoint (~10 Gb/1C) in the unimodal relationship between genome size and GC content suggests a causal link between repeatome fossilization and declining genomic GC content, highlighting shared evolutionary constraints in species with large genomes in the two most diverse lineages of vascular plants.

GENOME SIZE OF BULBOUS MONOCOTS IN THE NORTHERN CRADLE OF THEIR DIVERSITY: PATTERNS, GAPS AND CHALLENGES

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Bulbous monocots, characterised by their showy flowers, constitute a prominent component of Mediterranean and other European and Western Asian floras. These plants are highly valued in horticulture, where they are frequently utilised as ornamentals. The high degree of karyological variability resulting from polyploidy and dysploidy in many genera contributes to the high diversity observed within this group. Given the morphological simplicity of monocots, their taxonomy and identification requires futher

knowledge on their cytology. In this regard, genome size can be used as an independent trait for identification across numerous groups, offering insights into their evolutionary origins. Furthermore, the spectrum of genome size variation present within monocots, ranging from small to very large genomes, makes them a compelling subject for the study of genome size evolution. It is noteworthy that numerous species and genera of bulbous monocots present considerable challenges in terms of the methodologies employed in flow cytometry analysis. Consequently, there is a lack of information regarding genome size in many species, despite the popularity of these plants. The focus of this presentation is on the genome size of bulbous species from Asparagales and Liliales orders in Europe, Mediterranean region and West Asia. The presentation will comprise a review of the extant literature on genome size, with particular reference to the database of published and unpublished genome size data. Emphasis will be placed on the evolutionary patterns of genome size variation in bulbous monocots, identification of gaps in existing knowledge, and methodological tips for the analysis of more challenging genera.

USING FLOW CYTOMETRY TO EXPLORE THE BORAGINACEAE FAMILY? CHALLENGE ACCEPTED.

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Flow cytometry (FCM) is widely used to estimate plant nuclear DNA content (GS) in various plant groups, but the Boraginaceae family still remains understudied. This family presents methodological challenges due to its hairy indumentum and various chemical compounds that complicate nuclear isolation, requiring modifications in the isolation buffer, adjustments in the technique, or the use of alternative plant tissues (e.g. roots or filaments). As analyses often suffer from lower quality, multiple measurements per plant are essential to ensure accurate results. Consequently, only a limited number of studies have utilized FCM. From a karyological and cytological perspective, Boraginaceae family exhibits considerable variation in chromosome number (incl. B chromosomes), a high frequency of polyploidy, a range of chromosomal aberrations (aneuploidy, dysploidy), the occurrence of endopolyploidy, as well as hybridization. FCM could therefore serve as a valuable and effective tool.
In our laboratory, we have successfully optimised and applied the protocol for accurate FCM measurements in various members of the Boraginaceae family (Fig. 1). The main goal is to determine the extent of GS variability and to investigate the evolutionary mechanisms influencing this variation. To date, GS estimates have been obtained for 145 taxa (698 accessions) – about 50% of the genera – revealing an 85.5-fold variation, one of the largest recorded within the Lamiidae. The results particularly highlight the role of polyploidy and the transposable elements proliferation. Additionally, knowledge of GS has also proved beneficial from a practical field botany perspective, aiding in the identification of several

problematic and cryptic taxa (e.g. *Buglossoides arvensis* group, *Pulmonaria mollis* group, *Myosotis pallustris* group).

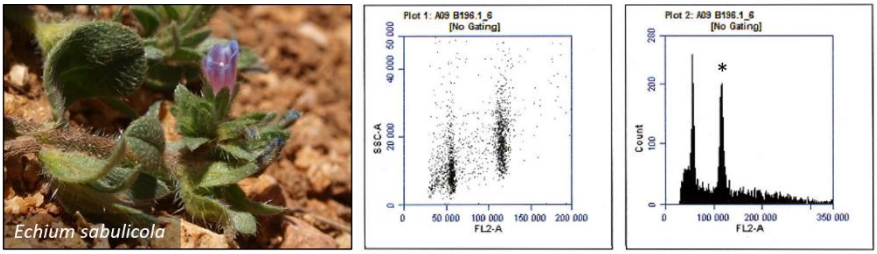

Fig. 1. Example of FCM histogram and side scatter profile (without removal debris by gating to improve the quality) of *Echium sabulicola* (plant from Majorca, using a root tissue for sample preparation) and *Glycine max* 'Polanka' (internal reference standard, marked with *). Nuclei were isolated in LB01 isolation buffer supplemented with PVP-40 (20 mg/ml) and stained with PI. The sample was measured using a BD Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, USA) equipped with a blue laser (488 nm, 20 mW).

ENDOPOLYPLOIDY IS NEGATIVELY CORRELATED WITH GENOME SIZE IN KARYOLOGICALLY UNIFORM TOMATOES

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Endopolyploidization is the genome multiplication that occurs in a coordinated manner as part of ontogenesis at the level of the somatic cell lineage, leading to the formation of tissues and organs with a mosaic pattern of cell distribution with a non-replicated and replicated genome. Flow cytometry became the most influential technique in quantitative evaluation of endopolyploidy and has been successfully applied to show, that the frequency of cells with different ploidy levels is specific for each species, organs and tissues, and has been documented to vary accross phylogenetically differentiated plant groups. Several studies have confirmed a negative trend in the relationship between baseline genome size and endopolyploidy levels. However, by far, groups of plants were studied, where a considerable range of variation for both parameters has been recorded. The studies included diploid-polyploid isogenic groups, cytotypically differentiated species within a genus, or a group of heterogeneous species differentiated from each other cytotypically, but also by the basic number of chromosomes. Here we demonstrate the considerable 1.0914-fold genome size difference (1.870 - 2.041 pg/2C) in 21 cultivars of

tomatoes, *Solanum lycopersicum* (19 cultivars) and *S. pimpinellifolium* (two cultivars). The genome of *S. lycopersicum* and *S. pimpinellifolium* is composed of 24 chromosomes uniformly. Despite these small genome size differences, we demonstrated a statistically supported negative relationship between genome size and the average ploidy level of endopolyploid cells in leaf tissues. The credibility of this relationship is enhanced by the identical result obtained by analyzing two differently grown groups of plants, young with a height of <20 cm and adult (flowering) with a height of ~200 cm, which differed in the overall level of endopolyploidy but only in leaves. In the other organs examined, stem, root and corolla, we did not observe this relationship. The results of the study of endopolyploidy in tomato leaves demonstrate that plants differ in the level of endoreduplication even in the case of a small difference in the genome size independent of the ploidy level difference.

METHODOLOGICAL ASPECTS OF USING FLOW CYTOMETRY TO SAMPLE PLOIDY LEVELS OVER THE WHOLE FLORA OF MULTIPLE SITES

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Ploidy level detection is a common application of flow cytometry in botany. However, most studies focus on related species, use a single protocol, and the number of samples rarely exceeds a few thousands. We studied the distribution of ploidy levels along an elevation gradient on 100 mountains in the Alps, which resulted in over 45,000 samples from about 1,150 species collected during two summer seasons. Due to the enormous number of samples, standard measurement of fresh material was not feasible. Instead, the collected material was immediately dried in silica gel and analysed within several months to two years. We opted for DAPI staining owing to the histogram quality. Due to diversity of species and their secondary metabolites, we had to optimize several sample preparation protocols. We used relaxed quality criteria compared to the fresh material; however, 90% of samples had the sample peak CV < 6.6%. Quite surprisingly, we were able to analyse 97% of species, including the notoriously difficult groups such as *Boraginaceae* and *Rosaceae*, and in as much as 85% of species a universal protocol with Otto buffers was applicable. Acceptable analyses could not be obtained for most *Crassulaceae*, achlorophyllous genera (e.g. *Neottia*, *Monotropa*, *Orobanche*), species with high mucilage content (e.g. *Helianthemum*), and a few others (e.g. *Rubus*). Distributions of the relative genome sizes (RGS) within each species were examined and cytotypes identified. Outliers from the expected Gaussian distribution within each cytotype were checked for identification errors or technical issues. After corrections, most cytotypes exhibited reasonably low RGS variation, with 90% of the cytotypes having a coefficient of variation of the RGS below 5%. Cytotypes with higher variation were generally those technically

challenging or known to involve aneuploids or hybridization. Ploidy levels were assigned to cytotypes based on their RGS values and published chromosome counts. In total, we recorded 17% of ploidy-variable species in our dataset. Despite some limitations, our results demonstrate that flow cytometry can be effectively used for ploidy level screening from dried material across a diversity of plant species, often using a single universal protocol.

Poster: **Endopolyploidy in natural populations of diploid plants and their polyploid relatives**

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In plant tissues, individual cells may undergo repeated genome duplications without cell division – a process known as endopolyploidy (also referred to as endoreduplication or the endocycle). This results in a mixture of cells with different ploidy levels within the same organism, effectively forming a "ploidy chimera". Despite its widespread occurrence across most angiosperms, endopolyploidy remains poorly understood, with most insights derived from model species under laboratory conditions.

My study investigates natural variation in endopolyploidy across a broad phylogenetic spectrum of wild plant species, addressing the following questions: (i) How does endopolyploidy vary among individuals under natural conditions? (ii) How does it differ between plant organs (leaf petiole vs. lamina)? (iii) How is it affected by organ age? These patterns are compared across 42 diploid-polyploid species pairs differing in monoploid genome size.

My results indicate that endopolyploidy shows limited variation among individuals sampled within a single population, even across contrasting microhabitats, suggesting a partial genetic determination of its level. Among closely related taxa, endopolyploidy tends to be higher in diploids and in species with smaller genomes, although the overall level appears to be quite genus-specific. Endopolyploidy is also highly tissue-specific, generally higher in the petiole than in the leaf lamina, though the degree of difference between tissues is again genus-specific. I also confirmed that endopolyploidy levels depend on tissue age and provide practical recommendations for reliable and meaningful tissue sampling in future endopolyploidy studies.

FLOW CYTOMETRIC KARYOTYPING OF WHEAT-*AEGILOPS* ADDITION LINES FACILITATES DISSECTION OF *AE. BIUNCIALIS* AND *AE. GENICULATA* GENOMES INTO INDIVIDUAL CHROMOSOMES

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Wheat breeding for adaptation to changing climates and enhanced resistance to diseases and pests is constrained by a narrow gene pool resulting from domestication and long-term human selection. Annual goatgrasses (*Aegilops* spp.), particularly those with M and U genomes, offer valuable genetic diversity to address these limitations. To accelerate the development of wheat-alien introgression lines, precise knowledge of *Aegilops* chromosome sequences is essential. However, the complexity of *Aegilops* genomes necessitates innovative approaches, such as flow cytometric sorting of individual chromosomes for targeted sequencing.

This study advances chromosome genomics in allotetraploid *Ae. biuncialis* and *Ae. geniculata* by isolating their M and U genome chromosomes. Using FITC-labeled GAA oligonucleotide probes and bivariate flow karyotyping, we successfully discriminated and sorted specific chromosomes. For *Ae. biuncialis*, nine chromosome populations were identified (Figure 1), with high-purity sorting achieved for $7M^b$ and $1U^b$. In *Ae. geniculata*, fourteen populations were resolved (Figure 2), enabling the isolation of nine chromosomes ($1M^g$, $3M^g$, $5M^g$, $6M^g$, $7M^g$, $1U^g$, $3U^g$, $6U^g$, and $7U^g$). To further improve sorting efficiency, we analyzed wheat-*Ae. biuncialis* partial addition lines and a complete set of wheat-*Ae. geniculata* addition lines, which facilitated a clear separation of GAA-rich *Aegilops* chromosomes from GAA-poor wheat chromosomes (Figure 3). Alien chromosomes from addition lines were sorted at purities of 74.5–96.6% (*Ae. biuncialis*) (Figure 4) and 87.8–97.7% (*Ae. geniculata*) (Figure 5).

Comparative analysis of flow karyotypes between the two *Aegilops* species revealed distinct chromosomal patterns. The chromosome-specific genomic resources generated in this study will support gene cloning and the development of molecular markers for efficient alien introgression breeding in wheat.

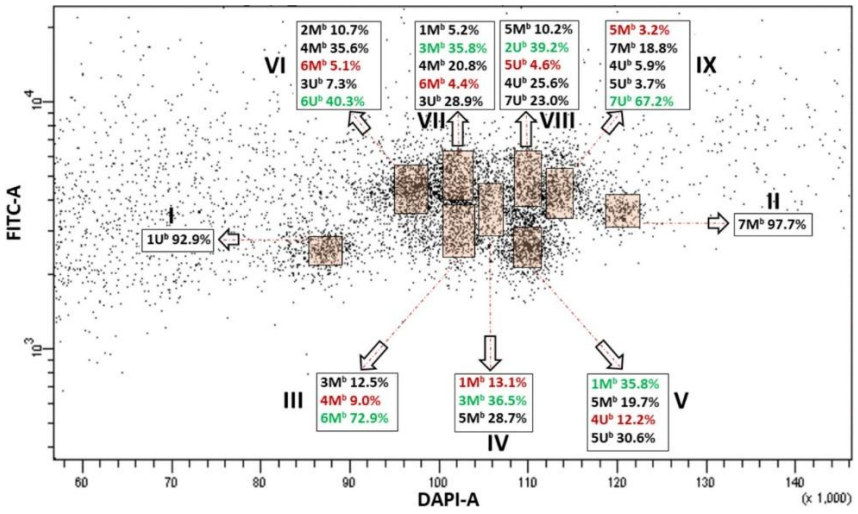

Fig. 1. Bivariate flow karyotype (DAPI-A vs. FITC-A) of *Ae. biuncialis* acc. MvGB382. Flow cytometric analysis of DAPI stained mitotic chromosomes labeled by FISHIS with GAA_7-FITC probe discriminated nine chromosome populations (I–IX) representing the whole genome of the species. Chromosomes in the sort windows (brown) were sorted onto microscope slides and classified by FISH. The red and green numbers within the chromosome content of the sorted populations (black rectangles) refer to the lowest and the highest purities, respectively. x-axis, relative DAPI fluorescence. y-axis; relative GAA_7-FITC fluorescence.

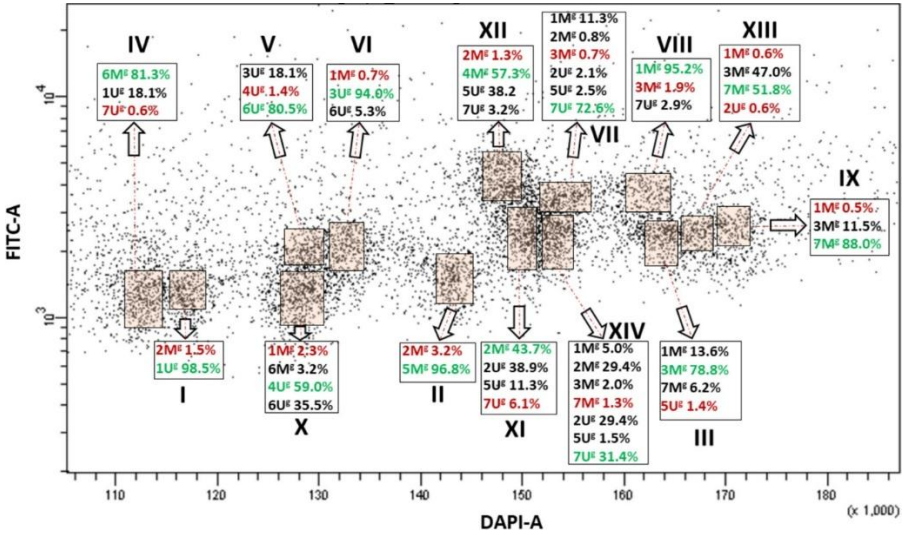

Fig. 2. Bivariate flow karyotype (DAPI-A vs. FITC-A) obtained after FISHIS with the GAA_7 probe on chromosomes isolated from *Ae. geniculata* acc. AE1311/00. The analysis permitted discrimination of fourteen chromosome populations (orange boxes) representing the whole genome of the species. The red and green numbers within a group of chromosomes sorted together refer to the lowest and highest purities, respectively. x-axis, relative DAPI fluorescence; y-axis, relative GAA_7-FITC fluorescence.

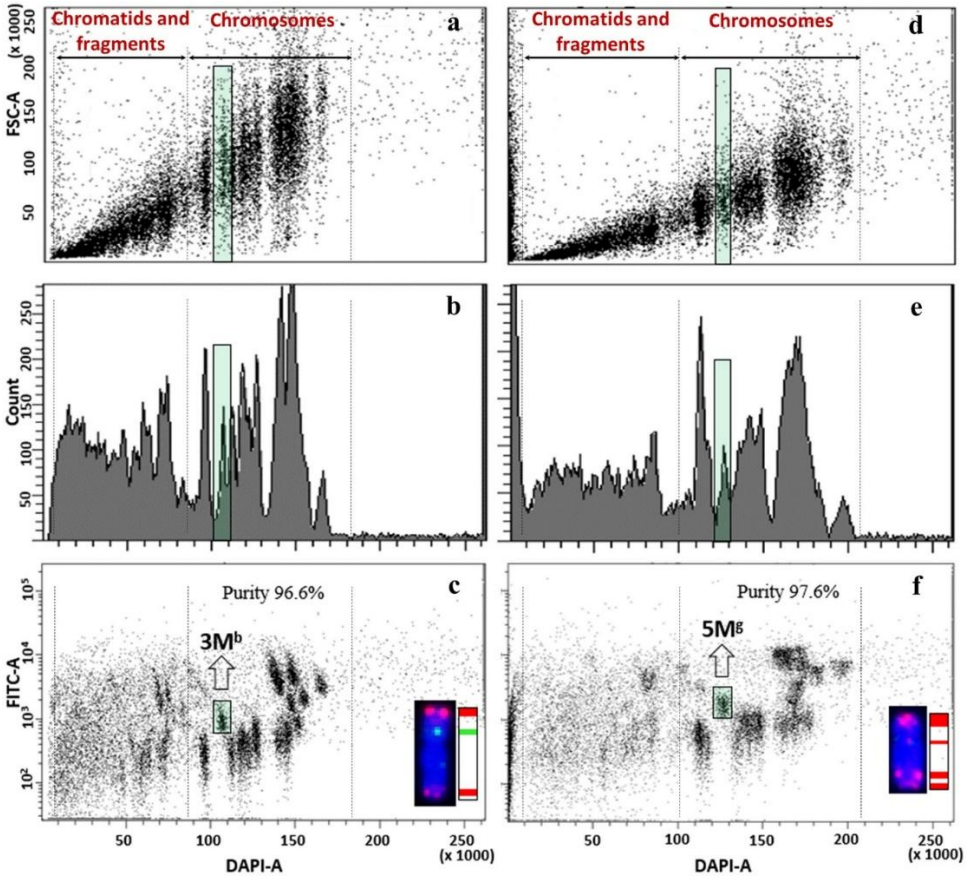

Fig. 3. Flow cytometric analysis and sorting of *Ae. biuncialis* **(a-c)** and *Ae. geniculata* **(d–f)** chromosomes from wheat disomic addition lines. **(a, d)**. bivariate flow karyotypes DAPI (x-axis) vs. FSC (y-axis), **(b, e)** monovariate flow karyotypes DAPI (x-axis) showing count (y-axis), **(c, f)** bivariate flow karyotypes DAPI (x-axis) vs. FITC (y-axis) obtained after FISHIS with $(GAA)_7$ on chromosomes isolated from wheat Mv9kr1-*Ae. biuncialis* acc. MvGB642 **(c)** and wheat CS-*Ae. geniculata* acc. TA2899 **(f)** addition lines. The analysis permitted discrimination and sorting of the tetraploid *Aegilops* chromosomes (green boxes) in the genetic background of bread wheat **(c, f)**. The chromosomes (inset) were sorted at high purity and identified by FISH on microscope slides with probes for *Afa* family repeat (red), 45S rDNA (orange) and *pSc*119.2 repeat (green). Chromosomes were counterstained with DAPI (blue).

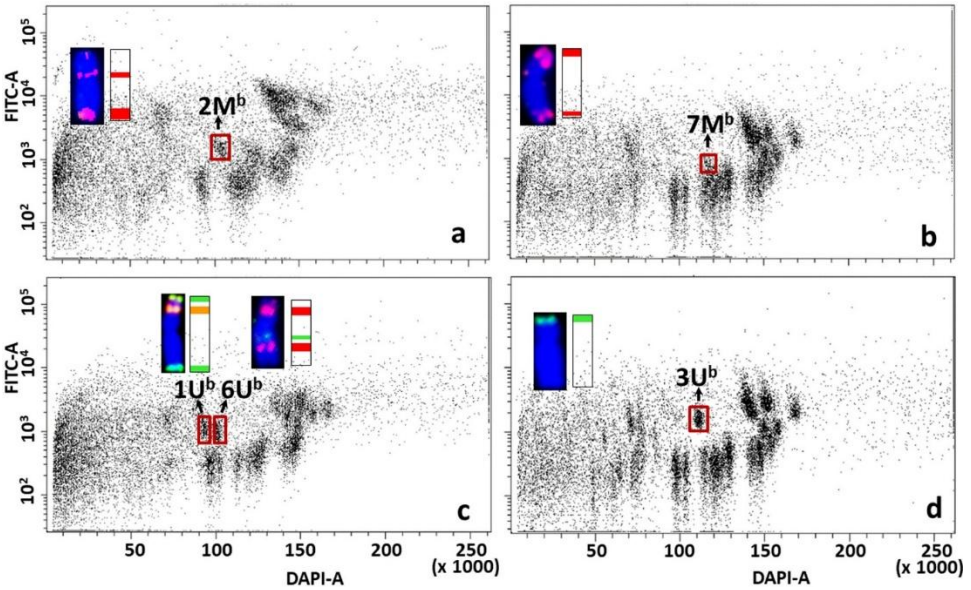

Fig. 4. Bivariate flow karyotypes DAPI vs. FITC obtained after FISHIS with $(GAA)_7$ on chromosomes isolated from wheat Mv9kr1-*Ae. biuncialis* acc. MvGB642 addition lines. The analysis permitted discrimination of chromosomes from tetraploid *Ae. biuncialis* (brown boxes) in the genetic background of bread wheat **(a–d)**. The chromosomes (inset) were sorted in high purity and identified by FISH on microscope slides with probes for *Afa* family repeat (red), 45S rDNA (orange) and *pSc119*.2 repeat (green). Chromosomes were counterstained with DAPI (blue). x-axis, relative DAPI fluorescence; y-axis, relative $(GAA)_7$-FITC fluorescence.

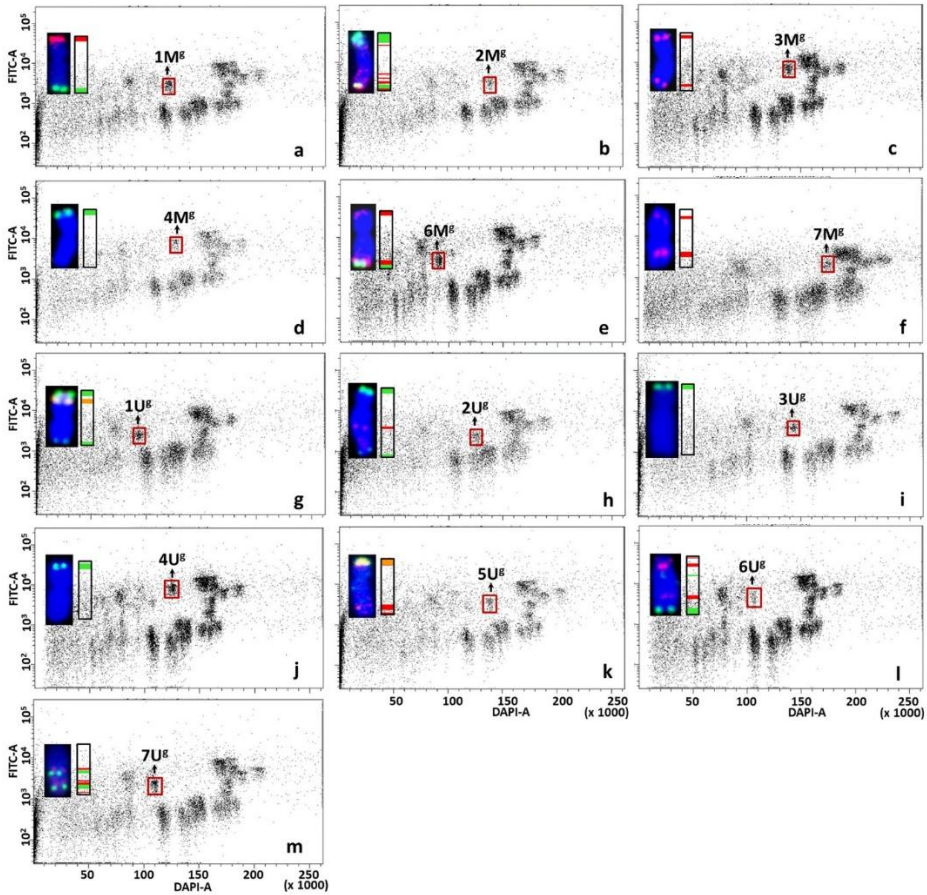

Fig. 5. Bivariate flow karyotypes DAPI vs. FITC obtained after FISHIS with GAA_7 on chromosomes isolated from wheat CS-*Ae. geniculata* acc. TA2899 addition lines. The analysis permitted discrimination of the whole chromosome set from tetraploid *Ae. geniculata* (brown boxes) in the genetic background of bread wheat **(a–m)**. The chromosomes (inset) were sorted in high purity and identified by FISH on microscope slides with probes for *Afa* family repeat (red), 45S rDNA (orange) and *pSc119*.2 repeat (green). Chromosomes were counterstained with DAPI (blue). x-axis, relative DAPI fluorescence; y-axis, relative $(GAA)_7$-FITC fluorescence.

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ADVANTAGES OF ENDOPOLYPLOIDY OVER POLYPLOIDY

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[*]*Presenter*

Endopolyploidy, i.e. multiplication of the genome in differentiated cells without consequent cell division, is common in flowering plants, where tissues and bodies of many species are predominantly formed by endopolyploid cells. Endopolyploidy has very similar effects to whole-organism polyploidy, playing an important role in plant evolution and being associated with several positive effects on overall plant fitness and adaptability. Still, the fact that endopolyploidy can occur only locally in a plant body (such as in response to critical environmental stimuli) must provide some advantages over the rigid whole-organism polyploidy, where genome duplication in all cells likely comes with certain structural, developmental, and/or physiological drawbacks. These advantages, however, remain mostly speculative, as the flexible nature of endopolyploidy makes its accurate quantification by flow cytometry challenging, complicating reliable comparisons across different plants and species.

Within our grant project, we aim to standardize flow cytometry methods for endopolyploidy quantification, study endopolyploidy in natural populations of diploids and their polyploid relatives, and conduct observational and manipulative experiments to detect its potential advantages. In this talk, I will summarize the current results of the project, which are presented in detail on the posters of students working on specific subtopics. The summary will include: (i) the standardization of sampling methods enabling comparisons of endopolyploidy across different plants and species; (ii) variation in endopolyploidy within natural populations of diploids and their polyploid relatives; (iii) results of a manipulative climate chamber experiment testing the possible advantage of endopolyploidy over polyploidy under cold conditions; and (iv) a test of the potential advantage of endopolyploidy in allowing cost-effective body construction while maintaining small seed size in small ruderal annual plants. I will also provide an outlook on future work in this area.

QUALITY CONTROL OF CULTURED OVINE MSCS: CELL VIABILITY, CELL CYCLE, ALDH STEMNESS AND PLURIPOTENCY

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Mesenchymal stem cells (MSCs) are unique stem cell type, which is broadly applied in regenerative medicine due to its immunomodulatory abilities. Besides clinical

applications, animal-derived MSCs are also studied in veterinary medicine. However, the isolated MSCs, independently of their origin, must meet high quality criteria in terms of viability, genomic stability, stem cell attributes etc. prior to their further use. The aim of this study was to analyse the viability and apoptosis, cell cycle and expression of aldehyde dehydrogenase (ALDH) and pluripotency markers (OCT4 and SOX2) in cultured ovine MSCs (oMSCs). Briefly, oMSCs isolated from bone marrow of three young lambs (Native Wallachian sheep breed) using plastic adherence method and cultured until passage 3 were harvested and divided into following analysis. For cell viability and apoptosis, oMSCs were incubated with Annexin V-FITC Apoptosis Detection Kit according to producer's manual. Propidium iodide was added to incubated samples immediately prior the flow cytometry analyses. ALDH activity was assessed using the ALDEFLUOR™ kit according to producer's manual. oMSCs for cell cycle analysis were firstly fixed with 75% ethanol for 30 min. Then, samples were treated with 50 mM NaCitrate buffer supplemented with 0.1 mg/ml of RNAse A overnight at 4 °C. Afterwards, samples were stained with 50 mM NaCitrate buffer supplemented with propidium iodide at final concentration 4 µg/ml and immediately analysed using flow cytometry. At last, oMSCs were pre-fixed using an IC Fixation Buffer and permeabilized with 0.1% Triton X-100. Then, samples were single stained with monoclonal antibodies against OCT4 (40/Oct-3) conjugated with AF647 and unconjugated SOX2 (rabbit IgG) overnight at 4 °C. As secondary antibody for SOX2, goat anti rabbit AF647 conjugated antibody was used. At least 10,000 events were acquired for each sample and analysis type using FACSCalibur flow cytometer and assessed using FlowJo Software (BD Biosciences). Obtained data were statistically evaluated using GraphPad Software. Results are expressed as mean ± SD. The analysed samples exhibited a very high viability (95.9 ± 0.3%) and no apoptosis rate (0.4 ± 0.1%). Moreover, the oMSCs showed stable cell cycle with majority of cells in G1 phase (91.6 ± 0.4%), 5.5 ± 0.1% cells in S phase and 3.6 ± 0.3% cells in G2 phase. All samples highly expressed ALDH (81.2 ± 22.6%) and pluripotency markers OCT4 and SOX2 (98.6 ± 0.5% and 99.2 ± 0.3%, respectively). In conclusion, cultured ovine MSCs exhibited high quality, making them suitable for further applications.

This work was supported by the grants: APVV-23-0141, VEGA 1/0011/23 and KEGA 024SPU-4/2023.

FLOW CYTOMETRY ELUCIDATES COMPLEX EVOLUTION OF THE GENUS DACTYLORHIZA

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Hybridization, respectively allopolyploidization is important and often studied evolutionary mechanism in the family *Orchidaceae*. Nevertheless, direct evidence of hybridization and polyploidization is usually missing. Therefore, we applied the combination of in vitro cultivation of progeny (protocorms) and flow cytometry (DAPI

staining) to allopolyploidizating aggregates, genus *Dactylorhiza*. The aim of this project was to determine frequency of homoploid and heteroploid hybrids (including backcrossing) and unreduced gametes of basic and hybridogenous species of the genus *Dactylorhiza* in vivo and also in the framework of experimental hybridization via flow cytometry of protocorms.
In contrast to hitherto published phylogenies, we found that both directions of hybridization are fully fertile, both at the homoploid and heteroploid levels. Hybrid progeny had intermediate genome size, surprisingly the F1 hybrids are sterile. Potential of further polyploidization was indicated by the production of unreduced gametes.
Our data have potential to increase the effectiveness of *Dactylorhiza* species conservation in nature (including ex situ cultivation). Finally, the unique combination of methods may point to a new way of research in the family *Orchidaceae*.


UNCOVERING GENOME SIZE VARIATION IN BAT LILIES (GENUS *TACCA*, TACCACEAE)

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Genome size is a fundamental biological trait influencing plant physiology, ecology, and evolution. Despite the extensive botanical plant diversity in tropical ecosystems, genome size data remain scarce for most lineages in these regions, especially for monocots. Flow cytometry offers a quick and informative method for estimating nuclear DNA content, yet it remains underutilized in tropical botany due to limited resources and access to advanced scientific facilities. In this study we examine genome size variation in the the monocot genus *Tacca* (Taccaceae) with particular attention to species occuring in Borneo and Peninsular Malaysia. We aimed to explore its implications for phylogeny, cytogeography, and evolutionary patterns. We measured nuclear DNA content in multiple species and populations using flow cytometry with propidium iodide and internal standardization. Preliminary results showed unexpectedly high genome size variability between species, while intraspecific variation was usually low or almost absent. In several cases, genome

size values correlate with known phylogenetic clades, suggesting that knowledge of genome size data can also aid in clade-level identification. Notably, rapid genome upsizing was observed in two distinct *Tacca* lineages, contrasting with the relatively small genome sizes found in their respective sister clades. This pattern indicates that significant shifts in genome size have occurred within the genus and may reflect key evolutionary events. Additionally, one individual displayed elevated genome size value indicative of possible polyploidy. These findings represent the first comprehensive genome size dataset for *Tacca* in Southeast Asia and underscore the value of cytogenetic approaches in resolving taxonomic and evolutionary questions in tropical plant groups.

ENDOPOLYPLOIDY IN ANNUAL RUDERAL PLANTS: A SOLUTION HOW TO BUILD CELL OF LARGE BIG CELLS WHILE KEEPING SMALL SEED SIZE?

Anna Hrádková

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Endopolyploidy (genome duplication without subsequent cell division) is common in plants and has similar effects to whole-organism polyploidy, such as producing larger cells that are generally cheaper in terms of construction costs. However, unlike regular polyploidy, endopolyploidy can be spatially and temporally regulated, allowing plants to increase ploidy levels in specific tissues or developmental stages while maintaining diploid cells elsewhere. This flexibility could, in theory, allow plants to benefit from the advantages of polyploidy (e.g., cheap and rapid growth) in some tissues while avoiding the disadvantages of large cells in others, such as in seeds where small size may be critical for effective dispersal.

To test this hypothesis, I studied small annual ruderal species, where endopolyploidy might promote fast and low-cost growth through large endopolyploid cells, while maintaining small seed size for effective dispersal. I selected approximately 20 species pairs from endopolyploid-rich families (Brassicaceae, Caryophyllaceae, Polygonaceae, Ranunculaceae), consisting of small-seeded annual ruderals and their congeners with larger seeds. I then measured endopolyploidy in the roots of germinating seeds and in various tissues of 2–3-leaf stage seedlings.

Preliminary results do not clearly support the theoretical prediction. They suggest that small seed size may instead be achieved by alternative developmental strategies, such as reduced cell number, and/or that endopolyploidy may not be essential for the development of all studied annual ruderal species.

THE FLOW CYTOMETRY AS A VALUABLE TOOL IN THE STUDY OF *PULMONARIA* (BORAGINACEAE)

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The genus *Pulmonaria* (Boraginaceae) represents one of the outstanding models to study dramatic changes in basic chromosome numbers and remarkable karyological variability. The large variation in chromosome numbers (2n = 14, 16, 18, 20, 22, 24, 26, 28, 30 and 38) indicates, that the evolution of the *Pulmonaria* species has been accompanied by numerous large chromosome rearrangements due to polyploidization and hybridization events. This phenomenon may indicate the presence of dysploid reductions, which could play a crucial role in the diversification of *Pulmonaria*. Traditionally, about 30 taxa (i.e. 16 species and 14 subspecies) are recognised on the basis of morphology, geography and specific chromosome numbers. However, many taxa exhibit high levels of morphological variability, leading to a complex and often unresolved taxonomy. For some cryptic groups, chromosome number and geographic distribution are the only reliable distinguishing characteristics.

Despite the high karyological variability observed within *Pulmonaria*, there have been no complex analyses of genome size (GS) variation. The presented flow cytometric (FCM) analyses (615 accessions/160 populations) represent the most accurate and comprehensive estimates of the GS and genomic GC content in *Pulmonaria* available to date. FCM has proven to be a valuable tool for studying *Pulmonaria*, with GS useful for distinguishing between critical taxa or identifying hybrids. Conversely, almost no variation in genomic GC content has been detected. Notably, several taxa consist of more than one cytotype (e.g. *P. carnica*, *P. dacica*) and mixed populations of different cytotypes occur in some species (e.g. *P. mollis*, *P. stiriaca*). Further research focusing on specific subgroups, combined with cytological and molecular analyses, is essential to fully elucidate the karyological evolution within *Pulmonaria*.

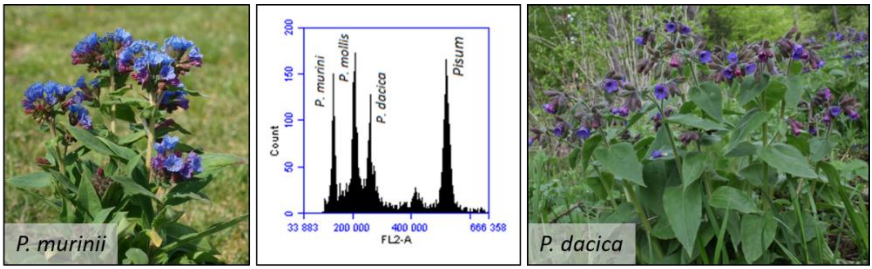

Fig. 1. FCM histogram of a bulk sample of *P. murinii* (Hungary; left), *P. mollis* (Czech Republic) and *P. dacica* (Romania; right) together with the internal reference standard (*Pisum sativum* 'Ctirad'). Nuclei were isolated in LB01 isolation buffer supplemented with

PVP-40 (20 mg/ml) and stained with PI. The sample was measured using a BD Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, USA) equipped with a blue laser (488 nm, 20 mW).

CROSS DIRECTION MATTERS: GENOME SIZE VARIATION IN HYBRIDIZATION BETWEEN DIPLOID LINEAGES OF *FICARIA*

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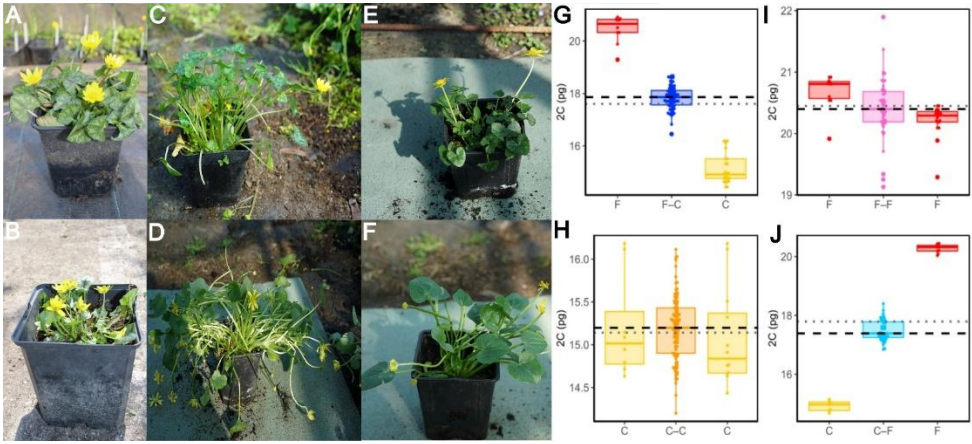
Flow cytometry has greatly increased the number of homoploid hybrids detected, suggesting that the frequency of homoploid hybridization has been underestimated in the past. The genus *Ficaria* presents a suitable model for studying homoploid speciation and reproductive isolation, as hybridization and weak reproductive isolation have been commonly reported for this genus. The most widespread, predominantly tetraploid phylogeographic taxon (lineage) *F. verna* (V) is thought to have arisen from hybridization between two parapatric diploid taxa (lineages), *F. calthifolia* (C) and *F. fertilis* (F), followed by polyploidization.

Based on this, we conducted controlled intra-lineage and pairwise reciprocal inter-lineage crosses between the above diploids to evaluate reproductive compatibility. We assessed genome size (GS), morphology, and fitness in the resulting progeny. Parental lineages apparently differed in GS, and GSs of intra-lineage progeny closely matched those of their respective parents. In contrast, progeny from inter-lineage crosses displayed intermediate GSs, but with notable asymmetry depending on the direction of the cross. Specifically, hybrids from F (seed parent) × C (pollen parent) crosses had higher GS than those from the reciprocal C × F crosses, indicating that GS is influenced not only by parentage but also by cross direction.

No polyploid progeny were detected. Furthermore, the direction of the cross affected reproductive success and hybrid fitness: when C was the seed parent, seed set, germination, and fertility were lower, despite hybrids being taller with larger flowers than reciprocal counterparts. These findings indicate that asymmetric postzygotic barriers, likely due to cytonuclear incompatibilities, strongly influence hybrid success and fertility. Cross direction thus plays a critical role in successful introgression and may shape evolutionary trajectories in *Ficaria*.

Parental lineages (*F. fertilis* (F); *F. calthifolia* (C)) and F_1 progeny from intra- (C–C; F–F) /inter-lineage (C (seed)–F (pollen); F (seed)–C (pollen)) crosses. (A) F; (B) C; (C) F–C; (D) C–F; (E) F–F; (F) C–C. Boxplots of GS of parents and F_1 progeny from (G) F–C; (H) C–C; (I) F–F; (J) C–F crosses. Lines in boxplots = mean GS (dashed = estimated, dotted = expected), dots = individual GSs.



REPRODUCTION MODES IN HEMISEXUAL DOGROSES ACCURATELY DETERMINED WITH FLOW CYTOMETRY

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Representatives of the genus *Rosa* L. are important horticultural plants with international production, markets and uses. Many modern rose cultivars are complex interspecific hybrids and their origin is in naturally occurring species of roses, which are characterized by complex cytogenetic properties due to the variability of ploidy levels between species and basic hybrids, and their reproductive biological properties. These primarily include the occurrence of unbalanced asymmetric (canina) meiosis, in which two sets of bivalent-forming chromosomes undergo segregation and subsequent biparental inheritance, while two to four other sets of univalent-forming chromosomes are inherited only through the maternal lineage. Asymmetric meiosis leads to the formation of a polyploid megaspore and subsequently a female gamete, i.e. 3x, 4x or 5x depending on the ploidy level of the species (4x, 5x and 6x) and the monoploid microspore and sperm cells (1x each time). The peculiarities of asymmetric meiosis raise questions to what extent this process is stable at individual ploidy levels and in individual species. In this work, we considered the models of asymmetric meiosis in three species of roses from the *Rosa* sect. *Caninae*, *R. glauca* (4x),

R. canina (5x), and *R. agrestis* (6x), and we reconstructed the origin of their seeds and thus determined the reproductive methods of these roses applying flow cytometry seed screen. For a correct interpretation of the results, we similarly analyzed the reproductive origin of another seeds from sexually reproducing species *R. multiflora* (2x), *R. palustris* (2x), and *R. rugosa* (2x), *R. gallica* (4x), and *R. pendulina* (4x), We proved that the embryo and endosperm in more than 93% of rose seeds from *Rosa* sect. *Caninae* originated in embryo sacs reduced by asymmetric meiosis, consistent with chromosome inheritance models. Asymmetric meiosis appears to be a highly stable process in these roses. We also demonstrate how the knowledge of flow cytometrically inferred reproduction modes of roses may be advantageous in parentage inference of some spontaneous *Rosa* hybrids. The present work also points out the efficiency and accuracy of the screening method using flow cytometry.

ADVANTAGE OF ENDOPOLYPLOIDY OVER POLYPLOIDY UNDER COLD

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Endopolyploidy (genome duplication without subsequent cell division) is common in many plant species, where endopolyploid cells can sometimes constitute a large portion of their whole body. However, the functional significance of endopolyploidy and its potential advantages for plant development remain poorly understood, especially in comparison to whole-organism polyploidy, which produces similar cellular and tissue-level outcomes. In this study, I explore the hypothesis that endopolyploidy may be more beneficial than polyploidy under cold conditions, where cell division is often constrained and growth may rely more heavily on cell expansion (i.e. by endopolyploidy).
To test this idea, I measured endopolyploidy (in leaf lamina, petiole, main root and lateral roots) and biomass (aboveground and belowground) in ten diploid–polyploid species pairs. Plants were cultivated for several months under standard (22/18 °C, day/night) and cold (12/8 °C) conditions in a climate chamber. As expected, diploids generally exhibited higher levels of endopolyploidy than their polyploid relatives, although the total number of genome copies per cell remained lower in diploids. Cold conditions tended to increase endopolyploidy in leaf lamina and main root, but this response was broadly similar in diploids and polyploids. Biomass analysis further revealed no clear advantage of endopolyploidy over polyploidy for plant growth under cold conditions.

THE EVOLUTIONARY INTERPLAY BETWEEN GENOMIC TRAITS AND GAMETOPHYTE LONGEVITY, SPORE MORPHOLOGY, AND SPOROPHYTE GROWTH FORMS IN FERNS

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Ferns offer a complementary system to angiosperms for understanding environmental influences on genome architecture because their distinctive morphology, free-living gametophyte, and reproductive strategy link genomic traits to functional traits such as gametophyte longevity, sporophyte growth form, and spore morphology. Analyzing 1,100 species (~10 % of known ferns), we related genome size, chromosome size and number, and genomic GC content to gametophyte longevity (short- vs long-lived), sporophyte habits and habitats, and spore type (trilete vs monolete) with phylogenetic comparative methods. Short-lived gametophytes were associated with markedly smaller genomes and chromosomes than long-lived ones, whereas aquatic and tree ferns exhibited pronounced genome downsizing analogous to hydrophytic and arborescent angiosperms, likely reflecting cell-size constraints and selection for rapid growth. By contrast, epiphytic ferns possessed larger genomes and lower GC content, diverging from the genome reduction typical of epiphytic angiosperms and hinting at unique pressures on their free-living gametophytes. Spore morphology correlated with GC content, as trilete spores showed higher GC content than monolete spores, a difference that may relate to desiccation sensitivity. Overall, gametophyte longevity appears to constrain fern genome expansion much as generation time does in angiosperms; convergent downsizing in tree and aquatic forms underscores shared selective forces across vascular plants, whereas the enlarged genomes of epiphytic ferns reveal a fundamental divergence between the clades, and the link between spore type and GC content opens new avenues for exploring the genomic bases of life-history traits in ferns.

HOW DO APPLE TREES REPRODUCE? FLOW CYTOMETRY AS A TOOL TO INVESTIGATE NOT ONLY APOMIXIS IN THE GENUS MALUS.

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Apomixis has the potential for revolutionary applications in agriculture. However, it is rarely studied directly on commercial plants. Therefore, my project focuses on mapping reproductive strategies within the most cultivated temperate fruit, the genus *Malus*. Apomixis and variability in ploidy levels have already been noted here in the past. Today, using flow cytometry, we aim to characterize not only the frequency of apomixis and ploidy variability, but also the contributions of non-reduced gametes to embryo and endosperm formation and reproductive differences between developmental lineages. We compared taxonomic and cultural variability among three groups: wild species, old and local cultivars, and commercially grown *M. domestica* cultivars. Methodologically, flow cytometry (ploidy and seed reproduction analysis) is mainly used, and microsatellite analysis (fingerprinting of part of the cytometrically analysed seeds) is also used to a minor extent. The cytotypic composition and reproductive strategies of each of the above groups are then compared.

Our results confirm, that some taxa within genus *Malus* reproduce via facultative apomixis (both pseudogamy and autonomous apomixis). Moreover even cultivars of *Malus domestica* are capable of apomictic seed formation. These results may significantly contribute to breeding programs of *Malus domestica*.

OPTIMIZED ATAC-SEQ LIBRARY PREPARATION FROM FLOW-SORTED MAIZE POLLEN NUCLEI HARBORING ACCESSORY B CHROMOSOMES

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Pollen grains play a central role in plant reproduction, delivering the male gametophyte to fertilize egg cells. In angiosperms, pollen typically contains one vegetative nucleus and two sperm nuclei. However, small size, rigid cell wall, and cytoplasmic content of pollen grains pose challenges for isolating nuclei, which complicates genomic analyses. Yet, purified nuclei free from organellar and extranuclear DNA contamination are essential for high-resolution downstream applications such as ATAC-seq. In this study, we optimized nuclei isolation and ATAC-seq library preparation from maize (*Zea mays*) pollen obtained from lines with different genetic backgrounds, focusing on lines carrying B chromosomes — supernumerary, nonessential chromosomes that exhibit non-Mendelian inheritance via mechanism termed chromosome drive. In grasses (*Poaceae*), B chromosomes undergo nondisjunction during the second pollen mitosis, resulting in their preferential transmission through sperm cells. While typically not beneficial to the host genome, B chromosome accumulation can negatively impact fertility. We used flow cytometry to isolate nuclei from B73 line with and without 2B chromosomes, Mo17 line, and their hybrid Mo17xB73 (MB), also with and without 2B chromosomes. This approach allowed us to examine chromatin dynamics in maize pollen and to explore the genomic impact of selfish genetic elements like B chromosomes. Our results contribute to a deeper understanding of nuclear organization in plant gametes and lay the groundwork for improved epigenomic profiling in reproductive tissues.

PATTERNS OF GENOME SIZE VARIATION IN THE CYTOLOGICALLY NEGLECTED GENUS *CURCULIGO* (HYPOXIDACEAE)

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Genome size is an important cytological trait. However, the data are geographically unbalanced, with the tropical regions being largely neglected, especially within monocots. With the exception of Orchidales and Zingiberales, where numerous studies have been conducted in recent years, the Hypoxidaceae family (Asparagales) remains cytologically unexplored, despite being widely represented in Old World tropics. Therefore, we focused

on uncovering genome size variation in the genus *Curculigo*, which has its primary centre of diversity and endemism within tropical Southeast Asia.
Our main aims were to evaluate genome size variation in the genus *Curculigo*, to assess whether it correlates with its morphology and can therefore be used as a potential taxonomic marker.
In total, over 200 individuals from 130 populations from various locations across Borneo, Peninsular Malaysia and Singapore were analyzed, with additional material from Indochina, Sumatra, the Philippines, and the Seychelles. Several populations were subjected to morphological analysis. The nuclear DNA content (genome size, 2C) was measured using flow cytometry with propidium iodide (PI) and internal standardization. Various plant organs were tested for sample preparation, showing that they are interchangeable.
In general, genome size reflects evolution in *Curculigo*. The most basal clade including Seychellean taxa possesses the smallest genome size, i.e. 2.08–2.10 pg in *C. rhizophylla* and 2.68–2.73 pg in *C. seychellensis*. On the other hand, phylogenetically the most derived group from western Malesia tends to have larger genomes varying from 3.10 to 4.34 pg. A majority of individuals are presumably diploids, but polyploidy was also detected. The highest genome sizes, both on diploid and polyploid level, were detected in *C. orchioides* group from Thailand. Morphotypes within the *C. latifolia* complex form two main morphologically distinct groups, which differ in their genome size as well. Our study is the first comprehensive genome size survey in the genus *Curculigo*.

ANALYSIS OF THE OVINE MSCS PHENOTYPE IN A COMPREHENSIVE MANNER

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Mesenchymal stem cells (MSCs) possess a unique phenotype, which is commonly used to characterise such cells. In general, human MSCs express CD73, CD90 and CD105, which can be observed also in other animal species. Besides these markers, several other surface markers have been noticed to be expressed by MSCs. Recently, we successfully explored a broader phenotype of rabbit MSCs derived from different biological sources. However, the ovine MSCs (oMSCs) have also been reported to exhibited different MSCs markers depending on the published study. The aim of this study was to analyse the ovine MSCs phenotype by a multicolour panel of antibodies against 23 surface antigens. Briefly, oMSCs isolated from bone marrow of three young lambs (Native Wallachian sheep breed) using plastic adherence method and cultured until passage 3 were stained by following anti-ovine antibodies: CD9 APC (HI9a), CD14 PE (TUK4), CD31 FITC (CO.3E1D4), CD13 (rabbit IgG), CD34 (EQ-8D11-C1), MHCI (Bu8), Stro-1 APC (STRO-1), CD29 FITC (MEM-101A), CD54 AF647 (117G12.02), CD44 FITC (25.32), CD45 PE (1.11.32), CD49f AF647 (GoH3), CD73 PE

(TY/23), CD90 FITC (5E10), CD105 APC (MJ7/18), CD106 (rabbit IgG), MSCA-1 APC (W8B2), W4A5 PE (W4A5), CD146 PE (P1H12), CD166 APC (3A6), CD271 AF647 (C40-1457), MHCII FITC (37.68) and SSEA-4 PE (MC-813-70). Appropriate secondary antibodies were used to stain unconjugated primary antibodies, such as goat anti-rabbit IgG FITC, rat anti-mouse IgG2a PE and rat anti-mouse IgG1 APC. FMO controls were used to set accurate gating of positive signals. To exclude dead cells from the analysis, 7-AAD fluorescent dye was used. At least 15,000 events were acquired for each sample using FACSCalibur flow cytometer and assessed using FlowJo Software (BD Biosciences). Obtained data were statistically evaluated using GraphPad Software. Results are expressed as mean ± SD. The analysed oMSCs highly expressed majority of tested MSCs markers as follows: CD9 (86.4 ± 7.4%), MHCI (89.7 ± 15.8%), STRO-1 (92.1 ± 5.7%), CD29 (99.8 ± 0.1%), CD54 (100.0 ± 0.0%), CD44 (96.5 ± 4.3%), CD49f (75.3 ± 3.0%), CD73 (99.7 ± 0.2%), CD90 (81.3 ± 0.9%), CD105 (100.0 ± 0.0%), W4A5 (98.7 ± 0.4%), CD146 (52.9 ± 3.4%), CD166 (100.0 ± 0.0%) and CD271 (99.6 ± 0.2%). On the other hand, few MSCs markers were dimly expressed in oMSCs: CD106 (8.3 ± 6.0%), MSCA-1 (11.0 ± 2.1%) and SSEA-4 (4.0 ± 2.4%). However, the hematopoietic and endothelial markers CD14, CD31, CD13, CD34, CD45 and MHCII (<3.5%) were negative in analysed oMSCs. In conclusion, a presented flow-cytometric panel is useful for a broader phenotyping of ovine MSCs.

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CLINICAL AND TRANSLATIONAL CYTOMETRY

PROTON RADIOTHERAPY AND LYMPHOCYTE DYNAMICS IN HEAD AND NECK CANCER

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Proton radiotherapy (PRT) is a modern and effective approach to cancer treatment. However, it remains associated with adverse effects such as radiotherapy-induced lymphopenia (RIL). Head and neck (HN) cancer patients are particularly susceptible to RIL due to the high blood flow through the irradiated area. In this study, we investigated peripheral blood lymphocyte changes in HN cancer patients undergoing PRT to characterize treatment-induced lymphocyte dynamics, identify potential predictors of RIL, and explore possible combinations with immunotherapy. Therefore, peripheral blood was collected at multiple timepoints (before, during, at the end, and one month after the therapy), and various flow cytometry analyses were performed on freshly isolated PBMCs. Most patients in our cohort experienced RIL. Our data revealed lymphocyte exhaustion associated with the upregulation of relevant immune targets and transient decline in proliferative capacity during the treatment. Moreover, the presence of specific phenotypes before the PRT significantly correlated with the severity of RIL at the end of therapy, suggesting potential biomarkers for risk stratification. These findings provide insights into PRT-induced changes in peripheral blood lymphocytes and have important implications for predicting RIL severity. Identification of high-risk patients might enable modifications in PRT treatment strategies. Ultimately, integrating these findings with PRT-immunotherapy approaches could lead to optimal treatment outcomes.

LONG-TERM IMPACT OF PEDIATRIC SEPSIS ON IMMUNE FUNCTION: IMMUNOPHENOTYPIC AND METABOLIC SHIFTS IN MYELOID CELLS.

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Sepsis is a heterogeneous syndrome with dynamic progression, yearly affecting 50 million people worldwide, where around 40% of patients are children under 5-year-old. Survivors frequently suffer with immunosuppression, which can persist many months after sepsis onset and impede the patient's full recovery.
The exposure of myeloid cells to environmental stimuli such as invading pathogens or pathogen-originated ligands is followed by their activation leading to a metabolic switch associated with phenotypic changes. The metabolic switch is necessary to obtain sufficient energy and intermediate metabolites to execute the defending processes against invading stimuli. Detailed characterization of immune cells' immunometabolic status and phenotypic alterations together with patient clinical status represent possible approach how to depict the sepsis dynamics, especially with emphasis to the long-term consequences of sepsis in pediatric patients.
Using state-of-the-art techniques – immunophenotypization and single-cell metabolic analysis 'SCENITH' we depicted specific sepsis-induced alterations in myeloid cells from blood of pediatric patients, collected within three time points. We found changes in monocytic subsets frequency and altered expression of HLA-DR, CD86, or CD36 together with specific cytokine pattern. We also revealed the metabolic alterations across all innate immune cells. All markers were correlated to the severity of sepsis (pSOFA score) to obtain comprehensive information associated also with the clinical picture of patients. We also evaluated the decline of quality of life of these patients using self-reporting SF-36 questionnaire.
In summary, we showed a sepsis-induced changes in immunophenotype and immunometabolic profile of myeloid cells of pediatric patients within three timepoints with special emphasis to long-term immunosuppression ~ 6 months after sepsis onset indicating persisting changes in myeloid cell functionality. By further integration of all obtained data by machine learning we will evaluate the potential risk of severe complications' development and design timely treatment strategy to facilitate the patient full recovery.

LYMPHOCYTE POPULATIONS AND SUBPOPULATIONS IN THE CLINICAL IMMUNOLOGY LABORATORY - THEIR USE IN PRACTICE AND RELATION TO DIGITALIZATION IN HEALTHCARE

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Flow cytometry became a common part of investigative procedures in clinical immunology in the 1990s. During this period, the background in our laboratories has changed significantly and we have moved from two-color fluorescence to multicolor. Most

laboratories are now able to analyze multi-color panels targeting specific cell populations. Many laboratories have automated this part of their operation as well.
In clinical laboratories, the EU Regulation 2017/746 of the European Parliament and of the Council on in vitro diagnostic medical devices - the so-called IVD-R Directive - has had a major impact on the use of flow cytometry, emphasizing the use of validated and certified procedures in the diagnostic process for the manufacturer or the laboratory itself. Developing technical capabilities often conflict with validation constraints in clinical practice and in many cases inhibit the diversity of investigations.
Further pressure is being put on the laboratory due to the planned digitisation of medical records and the associated clear definition of methods. In this respect, the National Laboratory Item Code (NČLP) has been in operation since 1997 and is continuously updated to meet the new needs of laboratory medicine. In immunology laboratories, its use in the field of flow cytometry is limited to basic lymphocyte populations that have a clear definition of CD molecule expression. Many of the derived and other subpopulations that are examined in laboratories are not unambiguously defined in terms of the expression of features or the definition of expression units. There is also variability in the results of individual laboratories in the expression of the relative numbers of each subpopulation relative to the parent population. This raises the problem of creating universal codes that could be used in digitisation. This talk will analyze the core and other leukocyte populations that most clinical laboratories offer for their applicants. The NČLP code system and the need to standardize results to ensure compatibility between laboratories and digital medical records will be discussed.

PREPARATION OF SINGLE-CELL SUSPENSIONS FROM SOLID TISSUES FOR RELIABLE FLOW CYTOMETRY ANALYSIS

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Abstract:
While flow cytometry has long been a gold standard for single-cell analysis in blood samples, the growing interest in applying this technique to solid tissues presents unique challenges. In our talk, we will provide an overview of our experience optimizing protocols for the preparation of single-cell suspensions from various solid tissues. Key steps—including mechanical dissociation, enzymatic digestion, filtration, and timing—require careful adjustment to ensure high cell viability, yield, and reproducibility of downstream

analysis. Over the past ten years, we have fine-tuned these steps using both primary tumor samples from patients and xenografts, as well as mouse organs containing infiltrated cancer cells. Additionally, we have developed a workflow for isolating circulating tumor cells (CTCs) from patient blood, establishing CTC-derived xenografts, and preparing xenograft-derived cells for flow cytometry. We will discuss critical considerations, common pitfalls, and practical solutions to improve the robustness of single-cell suspensions from solid tissues, aiming to support others in achieving reliable multiparametric flow cytometry data.

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NOVEL EUROFLOW QUALITY ASSESSMENT ON B-CELL PRECURSOR ACUTE LYMPHOBLASTIC LEUKEMIA MINIMAL RESIDUAL DISEASE. PROGRAM DEVELOPMENT AND FOUR ROUNDS OF IMPLEMENTATION.

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Introduction. Minimal residual disease (MRD) is one of the strongest prognostic factors in pediatric and adult patients with B-cell precursor acute lymphoblastic leukemia (BCP-ALL). The EuroFlow consortium developed a BCP-ALL MRD panel for flow cytometry, enabling highly sensitive MRD measurement in virtually all BCP-ALL patients (Theunissen et al., 2017).

Aims. To develop a quality assessment (QA) scheme for the measurement and interpretation of the EuroFlow BCP-ALL MRD panel.

Methods. We developed a scheme with two components: I) a wet part to assess the performance of the locally performed BCP-ALL MRD panel staining on healthy peripheral blood (PB) samples and II) a dry part to evaluate the analysis and data interpretation of centrally provided BCP-ALL MRD files. We included only tube 1 in both parts of the program.

To establish quality parameters for the wet part, 60 healthy PB samples of tube 1 of the BCP-ALL MRD panel were collected in 11 EuroFlow laboratories. For each parameter (fluorescent n=8, scatter characteristics n=2), we defined 1-2 target populations (in total n=5) for evaluation. For the dry part, we gathered 15 BCP-ALL MRD files of BCP-ALL patients on treatment. Each file was analyzed independently by 3 experts from EuroFlow laboratories.

Results. The median median CV of the 11 evaluated parameters for wet part was 38% (range 17-278%). The analysis of 8 files for dry part showed concordant MRD results with no technical errors.

After two beta rounds, we opened the program to all participants and have run four rounds (2023-2024). The number of participants increased up to 30 in 2024. Results of wet part (1 value per center) and MRD events identification (0-3 values per center) are shown in Figure 1. All parameters are evaluated and discussed in the Summary of the QA round.

Conclusions. We successfully developed and launched a novel QA program for EuroFlow BCP-ALL MRD panel measurement. To date, four rounds were completed. We provide participants with feedback on EuroFlow BCP-ALL MRD panel measurement, data analysis and interpretation, aiming to standardize and improve MRD diagnostics.

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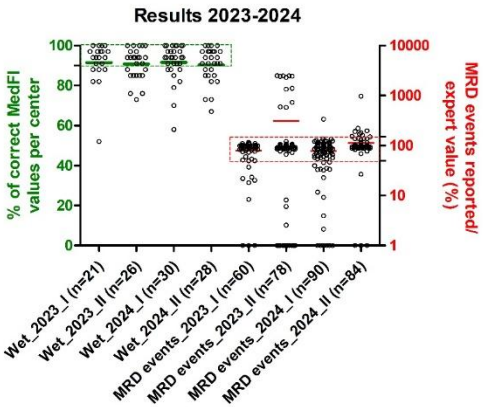

NČLP AND ITS USE IN FLOW CYTOMETRY OF HAEMATOOCOLOGICAL EXAMINATIONS

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Flow cytometry plays a vital role in the diagnosis and monitoring of hemato-oncological diseases. In the context of laboratory standardization, the National Laboratory Item Code (NČLP) plays a key role in providing a uniform description of laboratory tests across specialties. The importance of the NČLP codebook is increasing with the impending computerisation of healthcare, where uniform coding of tests is needed for sharing laboratory data. Although the entries for flow cytometry are the responsibility of clinical immunology, in practice these entries are often used by haemato-oncologists. The current design of the NČLP in the field of flow cytometry does not fully meet the needs of haemato-oncology diagnostics, and does not sufficiently reflect the diversity of materials examined, different methodological approaches, specific antibody combinations and different gating strategies. As a result of these limitations, the current NČLP flow cytometry codebook is not sufficiently usable for most haemato-oncology-oriented departments and is therefore often replaced by items from local codebooks. However, this complicates data unification, sharing of results between sites and electronic communication. This presentation will present the current status of the NČLP in the field of flow cytometry, highlight the difficulties associated with its use in haemato-oncology and open the discussion on the possibilities of further development of the codebook in the context of interdisciplinary collaboration.

INTERPRETATIVE VALIDATION OF FLOW CYTOMETRY IN INTEGRATIVE DIAGNOSTIC HAEMATOONCOLOGY

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Introduction: Analytical and diagnostic validation of in-house monoclonal antibody panels in flow cytometry (FCM) is mandatory within the requirements of the European IVDR directive. To validate their clinical function, SUKL recommends the determination of diagnostic sensitivity and specificity, positive and negative predictive values (PPV and NPV, respectively) and expected values in the population.
Methods: The retrospective cohort included 400 cases of routine multidisciplinary bone marrow (BM) testing. The cohort included 30% reactive haematopoiesis, 15% MDN, 14% multiple myeloma, 13% mature B/T-lymphocytic neoplasms, 8% MPN, 6% AL, 3% CMML and 11% other diagnostic categories. The final diagnosis was established by integrating cytomorphology, FCM, histology and genetic analysis and a review by a multidisciplinary expert panel. The interpretative value of FCM was assessed in relation to the final diagnosis and categorised as diagnostic concordance or discordance, the terms of which were specified for each diagnostic category and panel.
Results: The diagnostic concordance of FCM results with the final diagnosis was 91%. Relevant diagnostic discordance was observed in 8% of cases (number of clonal plasma cells up to 10% in multiple myeloma – 3%, absence of plasma cell or B-cell clone in MGUS – 2%, absence of blast excess in MDS-IB1 and MDS-IB2 – 1%, absence of B-cell clone in mature B-lymphocytic neoplasms – 1%, detection of blast excess in MDS-LB-MLD – 0.5%, myeloblast count below 20% in AML – 0.5%). Diagnostic sensitivity/specificity: 91/95%; PPV 91%, NPV 93%.
Conclusions: The detailed method of performing the interpretative validation of in-house panels in FCM is questionable, especially with regard to the validation standard used. The study presents a practical procedure for the diagnostic validation of FCM in haemato-oncology based on the comparison of the interpretative value of the BM diagnostic panel result with the final diagnosis of the case established by multidisciplinary assessment. The corrective arrangements in the evaluated laboratory aimed at increasing the diagnostic value of FCM in haemato-oncology are based on the results of the study and focus on the pre-analytical phase of the examination process, in particular the preparation of BM samples.

Diagnostická validace

Diskordantní případy (relevantní 8%, irelevantní 1%)

Diagnóza	Počet případů	Důvod diskordance
Mnohočetný myelom	13	Počet klonálních PC pod 10%
MGUS	7	Neprokázán plazmocytární ani B-lymfocytární klon
MDS-IB1 a IB2	4	Neprokázán exces blastů
MDS-LB-MLD	1	Prokázán exces blastů
Akutní myeloidní leukémie	2	Počet myeloblastů pod 20%
Akutní B-lymfoblastová leukémie	1	Alternativní interpretace nálezu
Zralá B-lymfocytární neoplázie	3	Neprokázán B-lymfocytární klon
Zralá B-/T-lymfocytární neoplázie	2	Obtížná diagnóza vzácného lymfomu
Systémová mastocytóza	2	Neprovedeno testování na mastocyty
Reaktivní hemopoéza	1	Podezření na zralou B-lymfocytární neoplázii

PERIPHERAL BLOOD IMMUNE PROFILING REFLECTS ESOPHAGEAL INFLAMMATION IN PEDIATRIC PATIENTS SUFFERING FROM EOSINOPHILIC ESOPHAGITIS

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Eosinophilic esophagitis (EoE) is a chronic, immune-mediated inflammatory disease of the esophagus marked by local eosinophil infiltration and esophageal dysfunction. Current diagnostic and monitoring approaches rely on invasive esophageal biopsies, which provide limited insight into the systemic activation and behaviour of immune cells. With advances in cytometric technologies, there is a growing interest in identifying peripheral blood biomarkers that could serve as non-invasive indicators of disease activity.
This study aimed to evaluate peripheral blood immune profiles that could serve as non-invasive biomarkers of disease activity in EoE. Peripheral blood samples and esophageal biopsies were collected from EoE patients and non-EoE controls. Multiparametric flow

cytometry was used to analyse key immune subsets and markers, including eosinophil-associated (CD193, EMR-1), activation (CD11c, CD44, CD69), and epithelial-associated (EpCAM, CD24, CD47). Additionally, immunofluorescence staining of biopsy sections was performed to localise cell populations within esophageal mucosal tissue.
EoE patients exhibited distinct immune activation profiles in both biopsy samples and peripheral blood compared to controls. Flow cytometric analysis of peripheral blood showed elevated expression of activation markers CD69 and CD44 on circulating T-cells and eosinophils in EoE patients. Dissociated esophageal biopsies demonstrated an increased proportion of $CD45^+$ immune cells, with notable enrichment of $CD193^+$ eosinophils in EoE tissue. These findings were corroborated by immunofluorescent imaging of biopsy sections, which confirmed dense infiltration of eosinophils in the esophageal mucosa, highlighting the robust local immune response.
Flow cytometry profiling of peripheral blood reveals immune activation signatures that mirror esophageal inflammation in EoE. These results highlight the potential of peripheral blood-based biomarkers to support non-invasive disease monitoring, potentially reducing reliance on repeated biopsies.

MONITORING OF CAR T CELLS AND NON-CAR T IMMUNE SUBSETS WITH STANDARDIZED 8-COLOR FLOW CYTOMETRY PANEL UTILIZING DRY REAGENTS

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Background: Chimeric antigen receptor (CAR) T cells represent a groundbreaking therapeutic option for both multiple myeloma (MM) and B-cell malignancies. Monitoring CAR T cell expansion might play a role in the prediction of treatment efficacy and the severity of side effects.

Aims/Methods: An 8-color flow cytometry panel (CD3, CD4, CD8, CD19, CD20, CD45, CD16+CD56, CAR) utilizing dry reagents and drop-in CAR conjugate, was used to assess levels of CAR T cells and other immune subsets. Peripheral blood (PB) samples (n = 73) from 12 MM patients in total were assessed as preliminary dataset at various timepoints starting at day 0 (D0)/day of infusion or D2, with resampling at D7, D10, D14, D21, D28 and M1-M12 each month.
Results: For the preliminary dataset, median number of assessed CD45+ leukocytes was 164 x 10^3 with median limit of detection 0.0122%. The presence of CAR T cells was first detectable at D7 (median count/µl PB, min-max: 9, 0.55-2152) with maximum expansion at D10 (966, 222-11924). From D14 (937, 219-2800), the CAR T cell counts gradually dropped to detectable levels in 3/5 patients at M1-2 and 1/4 patients at M2-3 (Figure 1). The CD4/CD8 ratio significantly decreased over time from D7 to D28 in the CAR T cell compartment (median percentage in CAR T fraction: CD4+, D7 = 74.20% vs. D28 = 54.08%, p = 0.0079; CD8+, D7 = 17.50% vs. D28 = 44.89%, p = 0.0016). A similar shift in the CD4/CD8 ratio was also observed in non-CAR T cells between D0 and M5-6. B cells were completely depleted from D7 to D28 with their first considerable reappearance in circulation at M1-2 in 3/5 patients. The monocyte percentage in the leukocyte fraction significantly increased from D2 to D7 (median: D2 = 2.15% vs. D7 = 25.60%, p = 0.0028). NK cell levels in the lymphocyte fraction were not significantly affected by the therapy in any of the timepoints.
Summary: These preliminary data, obtained through a multicenter collaboration, describe the expansion and dynamics of CAR-T cells as well as non-CAR T cell subsets. A larger dataset involving over 100 patients treated with both anti-BCMA and anti-CD19 CAR-T cells is currently under investigation and is planned to be presented at the conference.

SELECTIVE DEPLETION OF B-CELL LINEAGE SUBSETS DURING TREATMENT WITH ANTI-BCMA VS ANTI-GPRC5D BISPECIFIC ANTIBODIES (BSABS) UNDERLIES DIFFERENT RISK OF INFECTIONS IN PATIENTS WITH MULTIPLE MYELOMA (MM)

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Background:
Anti-BCMA and –GPRC5D bsAbs are effective and approved therapies in relapsed/refractory MM. However, infections pose a significant challenge to their use, particularly in combination with other drugs. Increasing evidence suggests that BCMA bsAbs are associated with higher risk of infections compared to GPRC5D. One of the reasons may be on-target off-tumor toxicity corresponding with different expression pattern of these antigens throughout the B cell lineage.

Aim:
To investigate underlying mechanisms responsible for higher infection rates with anti-BCMA bsAbs.

Methods:
This multi-center study included 75 RRMM patients treated with BCMA (teclistamab, elranatamab; n=28) or GPRC5D (talquetamab; n=47) bsAbs at 4 European centers (Athens, Pamplona, Hradec Kralove and Ostrava). The bone marrow (BM) immune composition was analyzed before (n=70) and during treatment (n=40) using next generation flow cytometry. Additional immunophenotyping was performed to characterize in greater depth, pro-B, pre-B1, large and small pre-B2 and immature subsets within the B cell compartment (n=26). Surface BCMA expression on B cell subsets was evaluated using spectral flow cytometry (n=7). Bulk and single cell RNA sequencing (scRNA-seq) data was used to investigate expression of *BCMA* and *GPRC5D* in B cell subsets (n=11). The effect of anti-BCMA bsAbs was further investigated in MIcγ1 immunocompetent mice that express BCMA in MM plasma cells (PCs) but not in mature B cells or B cell precursors.

Results:
Anti-BCMA bsAbs were associated with higher infection rate (82% vs. 53%; p=0.012), more profound hypogammaglobulinemia and more frequent use of IVIG replacement (74% vs. 32%; p=0.001) compared to GPRC5D. BM immune profiling revealed no significant differences between both groups at baseline. By contrast, during treatment, significant depletion of mature B cells and normal PCs was observed in BCMA group (both $p<0.001$).

Bulk and scRNA-seq uncovered distinct patterns of *BCMA* and *GPRC5D* expression throughout the B-cell lineage. *BCMA* was expressed on mature B cells and, unexpectedly, on B cell precursors. Furthermore, the highest BCMA expression (after PCs) was found in small pre-B2 cells, which was confirmed with multidimensional flow cytometry. By contrast, *GPRC5D* expression was limited to PCs and, unlike *BCMA*, significantly lower levels were observed in normal PCs vs tumor cells.
Deep and longitudinal immunophenotyping unveiled that anti-BCMA bsAbs depleted immature B cells and small pre-B2 cells, which resulted in the accumulation of pre-B1 cells. In addition, depletion of mature B cells and normal PCs persisted throughout treatment and only the interruption of anti-BCMA bsAbs therapy allowed the regeneration of B cell compartment. Using the MIcγ1 experimental model as a negative control since scRNA-seq data displayed negative *BCMA* expression in both mature and precursor B cells, we confirmed no depletion of any B-cell subset after treatment with anti-BCMA bsAbs.
Conclusion:
Anti-BCMA bsAbs are associated with higher risk of infections and more profound and persistent hypogammaglobulinemia. Distinct patterns of BCMA and GPRC5D expression throughout the B cell lineage explain different on-target off-tumor effects, which leads to the depletion of mature B cells, normal PCs and subset of B cell precursors with anti-BCMA bsAbs. These findings may help the management of bsAbs treated patients such as individualized use of IVIG prophylaxis.

THE EFFECT OF BCG VACCINATION AND LATENT INFECTIONS ON CLINICAL PROGRESSION OF SEPSIS AND COVID-19

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Objectives: Several years after the COVID-19 pandemic, the role of trained immunity in COVID-19 remains controversial, and questions regarding the long-term effects of COVID-19 on immune cells remain unresolved. We investigated the roles of Bacillus Calmette–Guérin (BCG) vaccination and latent infections in the progression of COVID-19 and sepsis.

Methods: We conducted a prospective analysis of 97 individuals recovering from mild-to-critical COVID-19 and 64 sepsis patients. Immune cell frequencies, expression of functional markers, and plasma titres of anti-*Toxoplasma gondii*/cytomegalovirus/BCG antibodies were assessed and their impact on disease severity and outcomes were determined. To examine monocyte responses to secondary challenge, monocytes isolated from COVID-19 convalescent patients, BCG vaccinated and unvaccinated volunteers were stimulated with SARS-CoV-2 and LPS.
Results: Post COVID-19 patients showed immune dysregulation regardless of disease severity characterized mainly by altered expression of activation and functional markers in myeloid (CD39, CD64, CD85d, CD11b) and lymphoid cells (CD39, CD57, TIGIT). Strikingly, post-critical COVID-19 patients showed elevated expression of CD57 in $CD8^+$ T cells compared to other severity groups. Additionally, a higher frequency of CMV and *T. gondii* seropositive- alongside a lower frequency of BCG seropositive- patients were associated with severe and critical COVID-19. However, the monocyte response to stimulation was unaffected by the severity of COVID-19.
Conclusion: These findings highlight the long-term alterations of immune cells in post-COVID-19 patients emphasizing the substantial impact of COVID-19 on immune function. However, our data showed no relationship between previous BCG vaccination and protection against SARS-CoV-2 infection.

CLONALITY ASSESSMENT OF SELECTED LYMPHOCYTE SUBPOPULATIONS – TECHNICAL ASPECTS OF ANALYSIS BY FLOW CYTOMETRY

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Background: Clonality assessment of lymphocytes is essential in the diagnostic of selected hematological malignancies. The most frequently used analysis is the surface detection of light immunoglobulin chains kappa and lambda (s□□□) of B lymphocytes (Bly) or their cytoplasmic detection (c□□□) in plasma cells (PC). The availability of MoAbs against the TCR (TRBC1 and TRBC2) also allows the identification of clonal T lymphocytes (Tly). Optimimalization of the settings including antibody (Ab) selection is necessary for proper flow cytometric analysis.

Aim: Detection, unambiguous determination and accurate enumeration of clonal T and B cells including their differentiated forms.

Materials and methods: Clonality was determined in peripheral blood and/or bone marrow of 27 patients using selected Ab combinations: CD4+CD20/CD45/CD8+s□/CD56+s□/CD5+CD34/CD19+TCR□□/CD3/CD38 or CD38/CD45/s□/CD27/CD20/CD19/sIgM/s□ or

CD138/CD45/CD38/CD56/CD27/CD19/c□/c□ or CD4/CD45/CD3/CD56/CD5/TCR□□/TRBC1/CD8.

Results: Pathological pattern of Bly was evident in 6/10 cases with suspect lymphoproliferation ($CD5^{+}$, $CD20^{het}$, $CD19^{het}$, etc.) with evidence of clonality. There was a minor subpopulation of clonal Bly that would not have been detected by conventional analysis in 2/10 cases, and clonal Bly were not identified in 2/10 patients.
PC pathology is detectable in most cases by a specific phenotype ($CD19^{-}CD56^{+}$). Clonal PCs were identified in 5/8 patients, in one patient atypically within $CD19^{+}$ PCs. Comparison of fluorochromes APC-Fire750 vs. APC-H7 was performed for l.
Clonal Tly were identified in 7/9 patients, mainly within the $CD56^{+}$ NKT (LGL) population, with multiple clones present in 4/9 patients. Heterogeneity in CD8 expression was not always associated with the presence of a clone.

Conclusion: The appropriate setting of analytical procedures leading to the identification of a clonal population is necessary not only in the diagnostics of hematological malignancies, but also for monitoring the status of the disease and detecting minimal residual disease (MRD).

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IL-10-DRIVEN REGULATION OF MONOCYTE FUNCTIONALITY AND METABOLISM IN TRAUMATIC INJURY

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Each year, trauma is responsible for approximately 4.5 million deaths worldwide, either as a direct consequence of injury or by dysregulated immune responses, escalating to SIRS or sepsis. During the early and late immune responses, monocytes play an important regulatory role, including cytokine production.

The aim of the project was to identify prominent cytokines identifying trauma and adjacent recovery. For this purpose, blood from patients admitted to the hospital with

various blunt trauma (TR) was collected at 3 timepoints: TP1 (24h after TR), TP2 (3-5ds after TR), and during hospital release (8-24ds after TR). Cytokine profiling of plasma was performed, followed by correlation analysis to assess the clinical relevance of screened cytokines. In blunt trauma patients, we identified that increased levels of immunosuppressive cytokine IL-10 in the first 24 hours after trauma was negatively linked to the survival score TRISS, presumably due to the induction of immunosuppression. Immunophenotyping of IL-10-treated healthy donor blood was performed, accompanied by SCENITH analysis to evaluate IL-10-specific monocyte phenotype and metabolism on a single-cell level in three major subsets (classical, intermediate, non-classical). Moreover, alterations within the bone marrow environment in an established organoid model were observed upon stimulation with IL-10.

In vitro, we observed that IL-10 has a priming effect on highly metabolically active circulating classical monocytes, by increasing their glucose dependence and upregulating CD64 and CD39 but simultaneously decreasing CD11b and HLA-DR cell surface levels. In contrast, these IL-10-induced changes were diminished by glycolysis but not OXPHOS inhibition, indicating that utilization of glucose is not mandatory but is desired for IL-10-induced functional changes in monocytes.

Exacerbated IL-10 cytokine signaling is a hallmark of immunosuppression. To conclude, glycolysis is a preferential metabolic pathway for steady-state and IL-10-induced functional changes in classical monocytes.

IMMUNOPHENOTYPING OF MACROPHAGE SUBTYPES IN OSTEOARTHRITIC SYNOVIAL MEMBRANE AND INFRAPATELLAR FAT PAD

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Emerging evidence highlights the crucial role of synovial macrophages in pathogenesis of osteoarthritis (OA), however deep characterisation of macrophages and their subpopulations present in synovium is missing. This study aimed to investigate the distribution and phenotypic characteristics of $CD206^-$ and $CD206^+$ macrophage subpopulations in the synovial tissue (ST) and infrapatellar fat pad (IFP) obtained in patients with knee OA. Paired ST/IFP tissues were obtained during total knee arthroplasty from 45 patients with late stages of knee OA. Immune tissue cells were stained with an extensive panel of surface markers and analysed using 14-color flow cytometry. Macrophage subsets were identified as $CD45^+HLA\text{-}DR^+CD1c^-CD14^+CD16^+$ cells. In ST, higher

percentage of $CD45^+HLA\text{-}DR^+$ immune cells were detected compared to the IFP (37.3% in ST *vs*. 22.3% in IFP). The proportion of $CD206^-$ macrophages was higher in ST (mean 50.8%) compared to IFP (39.6%), whereas $CD206^+$ macrophages were more prevalent in IFP tissues (57.6%) than in ST (40.2%). Immunophenotyping revealed 2-fold increase of expression of CD36 both in $CD206^-$ and $CD206^+$ cells in IFP tissues, ~2-fold increase in percentage of $CCR2^+$ cells within both subsets, and reduced expression levels of HLA-DR in $CD206^-$ (MFI 5282 in IFP *vs*. 6062 in ST) and $CD206^+$ (5630 in IFP *vs*. 6415 in ST) macrophages in comparison with ST. The $CD206^-$ macrophage population was positive for CD11c marker in both ST (44%) and IFP (30%). These $HLA\text{-}DR^{high}CD11c^+/CD206^-$ macrophages are characterized by enhanced antigen presentation, elevated cytokine production, and increased phagocytic capacity comparing to $HLA\text{-}DR^{low}CD206^+$ macrophages.
Our study demonstrated the distinct distribution and immunophenotypic characteristics of macrophage subtypes between ST and IFP and highlighted the tissue-specific role of macrophages in controlling inflammation and tissue remodeling in OA.
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THE CAPACITY OF PROBIOTIC STRAIN *ESCHERICHIA COLI* O83:K24:H31 TO LIMIT THE SEVERITY OF COLITIS

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Early postnatal administration of the probiotic strain *Escherichia coli* O83:K24:H31 (EcO83) has been shown to promote neonatal immune maturation, including reduced allergy incidence, through enhanced immunoregulatory pathways such as increased Treg activity and elevated IL-10. These effects suggest preventive and therapeutic potential in inflammatory bowel disease (IBD).
Here, we evaluated the protective and therapeutic effects of EcO83 in murine models of intestinal inflammation and investigated underlying mechanisms. Flow cytometry was used to analyze immune cell subsets in mesenteric lymph nodes and intestinal tissues, with focus on regulatory T cells (Tregs) and type 3 innate lymphoid cells (ILC3s). Gene expression of inflammatory and regulatory markers, as well as tight junction proteins, was measured by quantitative PCR. Colonization and persistence of EcO83 were studied using bioluminescent and fluorescent strains visualized *in vivo*.
Early postnatal EcO83 supplementation markedly reduced colitis severity in adulthood, whereas supplementation initiated later had only minor effects. EcO83-treated mice showed decreased expression of *Il6, Tnfa,* and *Cxcl2*, while tight junction genes (*Tjp, Zo, Cldn, Ocln*) were upregulated, indicating improved barrier function. EcO83 enhanced both the proportion and IL-10–producing capacity of Tregs. Furthermore, IL-22, essential

for antimicrobial peptide secretion and gut homeostasis, was elevated in EcO83-supplemented mice, particularly within ILC3s. *In vivo* bioimaging confirmed persistence and distinct colonization dynamics of EcO83 in neonatal versus adult mice.
These findings demonstrate that EcO83 promotes gut homeostasis by strengthening barrier function, inducing antimicrobial peptides and IL-22, and enhancing Treg responses. EcO83 represents a promising candidate for prevention or adjunct therapy of chronic intestinal inflammation.
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FLOW CYTOMETRY IN THE DIAGNOSIS OF FELINE LYMPHOPROLIFERATIVE DISORDERS: A RETROSPECTIVE EVALUATION (2020–2024)

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Lymphoproliferative disorders represent a significant group of oncological pathologies in cats. Accurate diagnosis is crucial for selecting appropriate treatment. Flow cytometry enables rapid and detailed immunophenotypic characterization of lymphoid cells and is essential in differentiating reactive from neoplastic processes. This retrospective study analyzed 133 samples from 97 cats suspected of lymphoproliferative disorders, examined in the Veterinary Research Institute in Brno (Czech Republic) between 2020 and 2024. Samples included lymph node, bone marrow, spleen, and peripheral blood aspirates. The most frequent finding was B-cell lymphoma with a $CD79^+CD21^+$ phenotype (18 patients), in agreement with the high incidence of B-cell lymphomas in feline populations. In seven cases, this phenotype was present in low percentages (7–27% of cells), potentially indicating early neoplastic transformation. An atypical $CD79^+CD21^-$ phenotype was observed in five patients. Mixed T- and B-cell populations with B-cell dominance were detected in 57 samples, possibly reflecting early lymphoma or reactive hyperplasia. A physiological T/B ratio with T-cell dominance was observed in 38 patients. Rare T-cell lymphomas were found in four cases, and $CD4^+CD8^+$ double-positive populations in five cases, which could suggest thymoma, although T-cell neoplasia cannot be excluded without histopathological confirmation. The study highlights the substantial heterogeneity of lymphoproliferative disorders in cats and underlines the diagnostic value of flow cytometry. It also emphasizes the necessity of complementary histopathological evaluation in ambiguous cases. This study was supported by the Ministry of Agriculture (RO0523).

MUTATIONS IN *IKKG* CAUSING IMMUNE DYSREGULATION - FROM PHENOTYPE TO FUNCTION

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Inborn Errors of Immunity (IEI) is a group of genetically determined rare diseases characterized by mutations in genes regulating various components of the immune system. *IKKG*, also called *NEMO*, is X-linked gene which encodes the regulatory subunit gamma of the IkappaB kinase (IKK) complex and is involved in NF-B signaling. The loss-of-function (LOF) mutations in *NEMO* cause high susceptibility to infections whereas gain-of-function (GOF) mutations in *NEMO* give rise to various autoinflammatory phenotypes. Therefore, it is essential to functionally characterize each new variant in the gene in order to set up a proper treatment.
Here, we show a cohort of patients (n=9) with X-linked variants in *NEMO* gene including one functionally uncharacterized and two yet unreported variants. Using 12-color immunophenotyping we characterized peripheral blood T and B cells and found suppressed differentiation towards mature B cells in *NEMO* LOF, which was in concordance with low serum immunoglobulins. Moreover, monocytes in *NEMO* LOF showed decreased activation and production of IL-1, IL-6, and TNFNF-B signaling has been studied using single-cell phospho-flow cytometry and western blotting, and showed decreased phospho-NF-κB p65 (Ser536) and decreased IB degradation in *NEMO* LOF. All these functional aberrations can result in low response to infection. In addition, the functional consequence of the new *NEMO* variants are determined by detecting NF-B-target genes *TNF*, *PTGS2*, *NLRP3*, *BIRC3*, and *BCL2* at the mRNA level.
Detailed identification of cell phenotypes and functional mechanisms damaged by mutations is essential for proper characterization of each novel gene variant underlying IEI. Flow cytometry techniques have an irreplaceable role in this context.
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PROFOUND T LYMPHOCYTE AND DNA REPAIR DEFECT CHARACTERIZES SCHIMKE IMMUNO-OSSEOUS DYSPLASIA

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Schimke immuno-osseous dysplasia is a rare multisystemic disorder caused by biallelic loss of function of the SMARCAL1 gene that plays a pivotal role in replication fork stabilization and thus DNA repair. Individuals affected from this disease suffer from disproportionate growth failure, steroid resistant nephrotic syndrome leading to renal failure and primary immunodeficiency mediated by T cell lymphopenia. With infectious complications being the leading cause of death in this disease, researching the nature of the immunodeficiency is crucial, particularly as the state is exacerbated by loss of antibodies due to nephrotic syndrome or immunosuppressive treatment. Building on previous findings that identified the loss of IL-7 receptor expression as a possible cause of the immunodeficiency and increased sensitivity to radiation-induced damage, we have employed spectral cytometry and multiplex RNA-sequencing to assess the phenotype and function of T cells ex-vivo and to study changes induced by in-vitro UV irradiation and reaction of cells to the presence of IL-7. Our findings highlight the mature phenotype of T cells with proinflammatory Th1 skew and signs of exhaustion and lack of response to IL-7. UV light irradiation caused a severe increase in the apoptosis of T cells, however the expression of the genes related to immune response and regulation remained surprisingly similar to healthy cells. Due to the disease's rarity, more studies will be necessary for complete understanding of this unique immunodeficiency.

IMMATURE NEUTROPHIL RELEASE AND ENHANCED NETOTIC POTENTIAL IN NMOSD PATIENTS

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Neuromyelitis optica spectrum disorder (NMOSD) is an autoimmune neuroinflammatory disease mediated by AQP4 autoantibodies that target astrocytes. However, the neutrophil response in NMOSD and its relationship to disease activity remain poorly understood. We aimed to characterize peripheral neutrophil subpopulations and their functional properties in NMOSD patients across different disease stages.
Peripheral blood samples were collected from patients with active NMOSD (n = 9), stable NMOSD (n = 15), and healthy donors (n = 23) during routine check-ups or acute relapses.

Neutrophil phenotypes were analyzed by flow cytometry using markers CD14, CD15, CD16, CD62L, CD64, CD10, CD11b, CD35, CD71, and CD101. Functional assessment included NET formation by time-lapse microscopy and phagocytic capacity measured via NADPH oxidase activity using flow cytometry. Complement components and IL-40 were measured in serum using multiplex ELISA (MicroVue, Quidel) and standard ELISA, respectively.

Active NMOSD patients exhibited increased granulocyte proportions and the presence of immature neutrophil subpopulations. Flow cytometry revealed an expansion of $CD62L^{bright}CD16^{dim}$ (banded) neutrophils alongside a reduction of $CD62L^{dim}CD16^{bright}$ (segmented) neutrophils, indicating mobilization of immature cells from bone marrow reserves. Elevated CD11b expression further supported neutrophil activation. Functional analyses showed enhanced NET formation both spontaneously and after LPS stimulation, while phagocytic capacity remained unchanged. IL-40 levels were elevated in active disease and positively correlated with neutrophil activation markers. Complement analysis showed consumption of C1q and C3 in both NMOSD groups, with increased factor B in stable patients, suggesting ongoing alternative pathway of complement activation and persistent immune dysregulation.

Our findings reveal a distinct neutrophil phenotype in NMOSD, marked by the mobilization of immature neutrophils, enhanced NETotic potential, elevated IL-40 levels, and dysregulated complement activation. These altered neutrophil responses may contribute to NMOSD pathogenesis and represent potential biomarkers for evaluation of disease activity

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MAPPING OF CD8 T CELL SUBSETS AND CYTOKINE PROFILES UNDERLIES SELF-REPORTED HEALTH STATUS FOLLOWING COVID-19

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Infection with SARS-CoV-2 can have several clinical manifestations and affect multiple organs, including the immune system. There is growing concern regarding possible long-term consequences of COVID-19, termed as post-acute sequelae of COVID-19 (PASC). Several studies demonstrated cognitive impairments in PASC individuals and further associates these impairments with inflammatory processes. Nowadays, a chronic low-grade inflammation linked with aging (inflammaging) is closely related to age-associated diseases including neurodegenerative disorders and several senescence-associated markers have been suggested to affect COVID-19 severity. Thus, dysfunctional immune response pointing towards immunosenescence may be linked to post-acute neurological and cognitive sequelae of COVID-19.
We enrolled 40 individuals with self-reported health issues following COVID-19 (PASC) and 39 individuals who self-reported to be fully recovered from COVID-19 (REC). The study groups were further divided into two timepoints (TP) based on the time since the acute phase of COVID-19, approx. 1 year after COVID-19 (TP1) and 2 years after COVID-19 (TP2). Plasma profiling, whole blood immunophenotyping, and bulk RNA sequencing of CD8 T cell subsets have been performed.
Plasma profiling identified IL-6 and CXCL8 to be increased in PASC TP2 compared to REC TP2. Interestingly, CXCL10 and CRP showed significant increase, while IL-17A and IL-12p70 showed significant decrease with age in PASC TP2 indicative of age-associated changes triggered in PASC individuals. Immunophenotyping and clustering analysis of CD8 T cells show dynamic changes in PASC compared to REC. Importantly, changes in frequency and gene expression signatures of naïve and terminal-effector memory cells re-expressing CD45RA were identified in PASC individuals.
This study reveals changes in CD8 T cell subsets and cytokines that are essential for effective immune response long-term after acute COVID-19 infection. These factors of immunopathogenesis may help to identify the neuro-immune connections accompanying PASC.

CASE OF MISSING CHAINS OR LIMITATIONS OF INTRACYTOPLASMIC LIGHT CHAIN TESTING IN MULTIPLE MYELOMA WITH EXCESSIVE SERUM FREE LIGHT CHAIN LEVELS

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Introduction:Flow cytometry is a bioanalytical method that allows the simultaneous evaluation of various characteristics of individual cells. It plays an important role in the diagnosis, prognosis and measurement of minimal residual disease in monoclonal gammopathies/ multiple myeloma. In all cases, determination of the clonality of the light

chains of plasma immunoglobulins is essential. Standardly, this examination is performed by intracellular staining, which is preceded by a multistep washing process to remove free light chains. However, at abnormally high concentrations of free chains, washing may not be sufficient. We present an atypical laboratory finding in a patient (M, 1963) with multiple myeloma/plasma cell leukemia.
Methods: Determination of plasmocytes in peripheral blood and bone marrow was o performed using BD FACS CANTO II flow cytometer. We identified 77.1% of pathological plasmocytes with the immunophenotype CD38-/CD138+/CD19-/CD56-/CD28-/CD33-/CD117-/CD45-/cy lambda + in the bone marrow sample. The lambda light chain was clearly expressed by intracytoplasmic staining. Examination of circulating plasma cells in peripheral blood revealed 73.1% pathological plasma cells (absolute number 22,999.9 x 10*9/L) with an immunophenotype consistent with the bone marrow findings, but cytoplasmic lambda light chain expression was repeatedly negative. Concurrent biochemical examination revealed an extremely high level of free lambda light chains - 9217 mg/L. We hypothesized that this high free light chain abundance might bind the anti-lambda antibody used. We modified the standardized workflow in terms of adding four washes of the sample with phosphate-buffered saline (PBS) before permeabilization and intracytoplasmic labeling. This process resulted in the removal of interacting free light chains from the sample and clear positivity for intracytoplasmic lambda light chain on the plasmatocytes examined.
Conclusion:In the case of biochemical findings of significantly elevated titer of free light chains, we recommend modifying the procedure with the addition of two to four washes with PBS .This procedure we routinely use for peripheral blood and bone marrow samples with free light chain levels above 500 mg/l.
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CYTOMETRIC DETECTION OF C3D+ IMMATURE ERYTHROCYTES IN A PNH PATIENT TREATED WITH A C5 INHIBITOR

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Introduction: Paroxysmal Nocturnal Hemoglobinuria (PNH) is a rare life-threatening disease caused by a mutation in the PIG-A gene where patients are unable to synthesize glycosyl phosphatidylinositol (GPI) anchor. Treatment of PNH represents the first use of anti-complement therapeutic monoclonal antibodies. However, the wider usage of the terminal pathway inhibition in clinical practice revealed cases of extravascular hemolysis caused by activity of the alternative pathway mediated by the C3 component in patients treated with a C5 inhibitor. We hereby present detection of C3d-related extravascular hemolysis in patient receiving anti-C5 complement monoclonal antibody treatment.

Methods: Immature red blood cells in peripheral blood samples of PNH patients treated with C5 inhibitors were labeled with anti-CD235a FITC (Dako), Clone JC159, anti-CD59 R-PE (Life Technologies), Clone MEM-43, anti-CD71 Pacific Blue (EXBIO Praha a.s.), Clone MEM-75 and anti-C3d APC (EXBIO Praha a.s.), Clone BGRL11 antibodies. Samples were measured on a BD FACS Canto II flow cytometer. The acquisition was a minimum of 1,000,000 singlet events in the erythrocyte gate (CD235 positive events).
Results: C3d positive subpopulation of immature red blood cells was detected in the peripheral blood sample of one PNH patient with insufficient response to C5 inhibitor. C3d positive immature red blood cells were not detected in the control population of PNH patients without C5 inhibitor therapy.
Conclusion: Therapeutic monoclonal antibodies prevent cleavage of C5 into CD5a and C5b, leading to downstream inhibition of the terminal pathway. Pharmacological inhibition of C5 replaces the function of missing CD59, however, concomitant loss of CD55 expression results in C3 activation on PNH-positive erythrocytes. In the environment of anti-C5 inhibitors, C3b is expressed on the membrane of pathological erythrocytes, but it is quickly converted to the inactive form C3d, which lacks enzymatic activity within C3/C5 convertases. Loss of control over C3 convertase activity leads to C3 opsonization of PNH erythrocytes and C3-mediated extravascular hemolysis, which reduces the benefits of anti-C5 therapy. We assume that the degree of C3 opsonization of PNH-positive erythrocytes and thus the degree of adverse effects of anti-C5 therapy, shows significant interindividual variability depending on the polymorphism of complement regulator genes. It may be important that all patients treated with C5 inhibitors should be investigated for activation of the C3 pathway. Flow cytometry allows quick and accurate detection and quantification the C3d component in patients on C5 inhibitor therapy and is able to select patients who could benefit from additional pharmacological C3 inhibition.
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DETECTION OF MELANOMA IN CEREBRO-SPINAL FLUID BY FLOW CYTOMETRY – A CASE REPORT

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Melanoma is a malignant tumor originating from melanocytes, accounting for approximately 1.7% of all diagnosed cancers. It has a high metastatic potential, with 12–20% of metastases involving the brain.
We present the case of a 16-year-old male diagnosed with disseminated melanoma, with metastatic lesions identified in the central nervous system (CNS), liver, and kidneys. Due

to the presence of neurological symptoms, including recurrent headaches and dizziness, episodes of loss of consciousness, and visual disturbances, he was referred for neurological evaluation.
As part of the diagnostic process, a cerebrospinal fluid (CSF) sample was obtained and sent for immunophenotypic analysis. To assess the cell populations present in the CSF, the Lymphoid Screening Tube (LST) developed by the EuroFlow consortium was used. This revealed a population of cells with CD45-, CD3-, CD19-, CD56 dim+ immunophenotype.
To further investigate the extramedullary origin of the cells, the Solid Tumor Orientation Tube (STOT) was applied. The analyzed cell population showed expression of cyCD3/CD271-positivity on the same channel, as designed in STOT. In the next step, an extended panel was performed, including the following markers: CD2, CD99, CD5, CD7, CD45RA, CD44, CD117, CD34, CD8, CD4, and CD10. The results of this staining revealed the expression of CD271, CD117, and CD44, which together with initially shown expression of CD56 and CD45-negativity, indicated melanoma as a potential diagnosis. Further imaging and diagnostic procedures identified a lesion within the leptomeninges. Histopathological examination of the biopsy confirmed the diagnosis of melanoma.
This observation proves high utility of flow cytometry in detection of cells not only of lymphoid or myeloid origin, but also other cell types including mesenchymal, embryonal or melanin-producing neural crest-derived cells.

ANALYSIS OF AML CELLS *IN VITRO*: SENSITIVITY TO CK1 INHIBITION VS. GENETIC BACKGROUND

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Acute myeloid leukaemia (AML) is a heterogeneous malignancy characterized by diverse genetic background, high relapse rate, and poor prognosis. Inhibition or degradation of casein kinase 1 α (CK1α) represents a promising therapeutical approach for AML patients, currently being tested in a clinical trial (BMS-986397 degrader, patients with R/R AML and HR-MDS; NCT04951778).

We treated 48 AML bone marrow samples *in vitro* with an in-house developed CK1 inhibitor (range 0.03-100 uM). Cell viability was measured using the WST-8 assay, and inhibitor efficacy was calculated as the half-maximal inhibitory concentration (IC50). The IC50 values were compared with the mutation status of 25 prognostically relevant genes from a next-generation sequencing (NGS) panel. A lasso regression model, combined with k-fold cross-validation, was employed to identify relevant and redundant variables. The model used genetic background, patient age, and sex as input variables, with IC50 values as the outcome variable.

The final lasso model identified genes associated with both higher and lower responses to CK1 inhibition. Genes associated with higher response (lower IC50) included *FLT3-ITD, IDH1, IDH2*, and *NPM1*. Genes associated with lower response (higher IC50) included *EZH2, FLT3-TKD, PTPN11, TP53*, and *TET2*. The IC50 values predicted by the lasso model correlated significantly with the measured IC50 values (Spearman correlation, $R = 0.69$, $p < 0.001$).

Our findings underscore the diversity of treatment responses observed *in vitro*, reflecting the inherent heterogeneity of AML. While further validation *in vivo* and with larger datasets is necessary, these results emphasize the importance of tailoring therapeutic strategies to enhance patient outcomes.

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HEMODILUTION CALCULATOR: A TOOL FOR BONE MARROW ASPIRATE QUALITY ASSESSMENT

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Acute leukemia diagnostics relies heavily on the quality of bone marrow aspirates (BMA). Hemodilution, the contamination of BMA by peripheral blood (PB), is a common issue that can lead to misinterpretation of samples. Purity of BMA unaffected by chemotherapy or malignancy can be estimated by the presence of bone marrow (BM) specific cells, such as nucleated erythrocyte precursors. However, during chemotherapy, these populations may be depleted or prematurely released into PB, leading to a false interpretation of hemodilution.

We propose an alternative approach for estimating hemodilution based on quantitative differences between BMA and PB. Using equations describing the relationship between

the degree of hemodilution and BMA, PB, and true BM population fractions, we apply logical constraints to define possible hemodilution intervals. We developed an interactive Shiny web application that compares up to five cell subsets between BMA and PB, calculates maximum hemodilution for each population, and predicts real BM percentages while considering experimental and analytical variability.
The calculator is available online at https://breakpoints.vm.cesnet.cz/calculator/ . We tested the tool using 266 paired BMA and PB samples from B-precursor acute lymphoblastic leukemia (BCP-ALL) patients taken on 15th day of chemotherapy. Using an identical flow cytometry tube to compare PB/BMA populations, we found that the maximum BM hemodilution was within one log (<90 %) in 78 % of the BCP-ALL samples. We are continuing to refine the tool by identifying consistent cell subsets that reliably differ between PB and BMA, which will improve the accuracy of hemodilution estimations. This tool provides a quantitative and objective approach to assessing BM hemodilution, addressing a critical challenge in leukemia monitoring. By reducing misclassification of samples, it has the potential to improve minimal residual disease evaluations. While the tool provides an interval estimate, which could lead to overinterpretation of upper-range values, we are working on integrating interpretative guidelines to make the tool more accessible for routine use in laboratories analyzing BM specimens.

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BRONCHOALVEOLAR LAVAGE CELL COMPOSITION DETECTED BY FLOW CYTOMETRY

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Background: Bronchoalveolar lavage (BAL) has become a safe and valuable technique, which is widely used to diagnose patients with interstitial and granulomatous lung diseases. It is also used to exclude inflammatory and infectious diseases, including the detection of neoplasia. Immunophenotyping of BAL samples by flow cytometry (FC) allows discrimination of different leukocytes subpopulations and detection of possible pathological cells of epithelial and/or hematology origin where the number of detected antigens depends on the technical capabilities of the laboratory.
Aim: Flow cytometry analysis of BALs to cover maximum of detected surface markers together with enumeration of selected cell populations.
Material and Methods: BALs were filtered, concentrated by mild centrifugation and incubated with CD4/CD15/CD45/CD235a/CD14/CD20/CD3/CD56/CD326/CD8. Red cells lysis was done if needed by NH_4Cl. Peripheral blood was acquired in parallel with BALs and

TBNK lymphocytes only were analysed. Analysis was done by 3-laser BD FACSCanto II (BD Biosciences) with BALs reanalysis used Infinicyt (Cytognos/BD Biosciences) software.
Results: BALs delivered to the laboratory had variable cellularity and leukocyte representation. Monocytes/macrophages dominated in normal samples. Neutrophilia corresponding to an ongoing bacterial infection was detected, while lymphocytosis with a predominance of the $CD3^+CD4^+$ Tly was found in patients suffered from sarcoidosis. Clonal Bly cells were found in some patients with relapsed B lymphoproliferation. Increased number of $CD326^+$ epithelial cells could indicate the presence of tumour cells. The use of a mix of 2 markers within one fluorescence channel significantly expanded the possibilities of detecting different subpopulations within a single analysis, which is an advantage for samples low cellularity.
Conclusion: Analysis of BALs using FC is a rapid and complex approach which allows for the unambiguous identification of underrepresented subpopulations of leukocytes and non-hematopoietic cells in a single tube.
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EVALUATION OF NEW ANTI-TDT CLONE AND FLUOROCHROMES

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Detection of nuclear terminal deoxynucleotidyl transferase (TdT) is an essential component of the diagnostic workup for hematological malignancies by flow cytometry. Available well-performing clones are limited, and their fluorochrome conjugates are restricted to FITC only.
We evaluated a new commercially available clone, TDT-DAK, conjugated to iFluor488, iFluor700, and APC. Since reagent performance can be influenced by the choice of fixation and permeabilization buffer, we evaluated Fix&Perm (Nordic MUbio, Susteren, The Netherlands) and IntraStain (Dako-Agilent, Glostrup, Denmark) buffers.
We analyzed 14 bone marrow samples (8 diagnostic ALL samples and 6 follow-up samples without malignancy as controls).
We observed similar staining patterns and percentages of positive nuclear TdT events across this dataset, confirming that TDT-DAK iFluor488 performs comparably to HT-6 FITC. iFluor488 and APC demonstrated comparable performance, whereas iFluor700 may underestimate TdT expression in challenging samples with weak positivity.

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PHASE I CLINICAL TRIAL OF CD19 CHIMERIC ANTIGEN RECEPTOR T CELLS WITH AN ENHANCED TSCM IMMUNOPHENOTYPE GENERATED VIA THE PIGGYBAC TRANSPOSON SYSTEM FOR THE TREATMENT OF REFRACTORY B-CELL MALIGNANCIES: PRELIMINARY RESULTS

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The therapeutic efficacy of CAR-T cells is influenced by their immunophenotype, with less-differentiated T stem-cell memory (Tscm) subsets supporting prolonged persistence. We developed a non-viral manufacturing platform using piggyBac transposon DNA electroporation of non-activated T cells, followed by IL-21–mediated expansion to enrich Tscm CAR-T cells. The resulting product, UHKT CAR19, incorporates a novel CD19-specific scFv and 4-1BB-ζ signaling domain.

A phase I trial (NCT05054257) was launched to assess the safety and efficacy of autologous UHKT CAR19 in adults with relapsed/refractory B-ALL or B-NHL ineligible for commercial CAR-T therapy (n=14). Patients receive fludarabine (25 mg/m²/day ×5) and cyclophosphamide (500 mg/m²/day ×2) followed by a single UHKT CAR19 infusion in a dose-escalation design. For comparison, a tisa-cel–treated cohort (n=15) was analyzed for product immunophenotype, in vivo expansion, and preliminary clinical responses.

Preclinical studies showed that UHKT CAR19 T cells exerted cytotoxicity against $CD19^+$ tumor lines, inhibited tumor growth, and were well tolerated in NSG mice. Targeted locus amplification revealed random genomic integration without site preference. Compared with tisa-cel, UHKT CAR19 contained higher proportions of Tscm cells in both $CD4^+$ and $CD8^+$ subsets ($CD45RA^+CD62L^+$: 35% vs. 6%, p= 0,0008; 53% vs. 12%, p= 0,0006; $CD27^+CD28^+$: 77% vs. 40%, p=0.002; 74% vs. 17%, p<0.0001) and fewer $PD\text{-}1^+$ cells (11% vs. 71%, p<0.0001; 7% vs. 32%, p=0.004; UHKT n=15, tisa-cel n=15). In the clinical study, 14 patients received UHKT CAR19 at doses up to 1×10^6/kg. Expansion was detectable in 13/14 patients, including 4 high, 2 mid, and 7 low expanders, with kinetics comparable to tisa-cel even at the lowest dose (0.1×10^6/kg). No grade >3 non-hematologic toxicities occurred; CRS was limited to two cases (grade 1–2). One heavily pretreated patient developed prolonged grade 4 cytopenia from secondary graft failure. Of 14 patients, 6 achieved complete and 2 partial responses.

UHKT CAR19 infusion was safe and induced clinical responses in 8 of 14 patients. The platform enables efficient point-of-care manufacturing of Tscm-enriched CAR-T cells. Enrollment is ongoing in the high-dose cohort.

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FLOW CYTOMETRY-BASED DETECTION OF LOW-GRADE PERIPROSTHETIC INFECTION IN SYNOVIAL FLUID

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Periprosthetic joint infection (PJI) is a serious and rare complication of total joint arthroplasty (TJA). Diagnosis of PJI is a challenging process, mainly due to the presence of implant wear particles and a changed immune microenvironment. Biomarkers for routine use in PJI are still an unmet need in the clinical scenario, especially biomarkers that do not miss low-grade infection and do not result in false-positive tests due to overdiagnosis.
Synovial fluid (SF)-derived immune cells from 44 TJA patients (microbiologically positive PJI, N=16; low-grade PJI (LG-PJI), N=8; TJA with osteolysis/aseptic loosening (OL/AL), N=20) were analysed by 14-colour flow cytometry. A spectrum of surface activation markers and chemokine receptors was evaluated though sequential gating strategy. Soluble infection biomarkers were measured by ELISA. Multivariate bioinformatical approach was utilized to stratify the patients.
LG-PJI patients showed low SF cellularity but high percentage and activation ($CD11b^+$) of neutrophils (NEUs), similarly as observed in PJI patients. On the other hand, levels of SF CRP, defensins and percentage and activation ($HLA\text{-}DR^+$) of NK cells were higher in PJI and lower in OL/AL groups. Similar pattern was observed in LG-PJIs chemokine receptor representation on NEUs, namely percentage of $CCR2^+$, $CCR7^+$, $CXCR4^+$ NEUs and MFIs of CCR5, CXCR1, CXCR2 that were found higher in PJI and lower in OL/AL control groups. Only percentage of $CXCR2^-CXCR4^-$ NEUs, a population of constitutively mobilized NEUs as shown in animal models, was higher in LG-PJI than in control groups. Constructed patient similarity network revealed that 11 markers are needed to separate PJI and LG-PJI from aseptic conditions.
This study revealed that phenotype of SF-derived immune cells reflects the joint microenvironment, and that cellular content of immune cells can be used to distinguish PJI and LG-PJI, but multimodal approach needs to be utilised.

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NEW METHODS & DATA ANALYSIS

INNOVATION AND PRACTICAL POTENTIAL OF □H2AX DETECTION BY FLOW CYTOMETRY

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Detection of the phosphorylated form of nuclear histone H2AX, called □H2AX, is a sensitive indicator of absorbed dose of irradiation that is standardly analysed by fluorescence microscopy on peripheral blood lymphocytes. This analysis is both procedural and time-consuming (days). In contrast, an innovative method for back-estimation of absorbed dose using □H2AX detection by flow cytometry offers a rapid and reliable solution for the biodosimetric analysis of large numbers of potentially exposed individuals following a nuclear accident (or nuclear attack) in a short time (hours). An important advantage of the method is the linear dependence for the dose range 0-10 Gy, which is important for predicting the severity of acute radiation sickness. It is the only available method that can distinguish whether an individual has been exposed to homogeneous whole-body radiation or partial exposure.
However, the disadvantage of this method is that the median intensity fluorescence (MFI) depends not only on the fluorochrome used, but also on the nature of the fluorochrome:antibody binding or the batch of antibody itself. The novel approach of standardising the method by carefully adjusting the optical system of the cytometer, combined with the use of anti-gH2AX-PE antibody calibrated with BD Quantibrite™ PE Beads, allows rapid and accurate analysis, eliminating the influence of physical parameters that affect the MFI value. The new approach offers great potential for the application of this method to internationally established standard biodosimetry procedures.

TVIBLINDI ALGORITHM IDENTIFIES BRANCHING DEVELOPMENTAL TRAJECTORIES OF HUMAN B CELL DEVELOPMENT AND DESCRIBES ABNORMALITIES IN RAG-1 AND WAS PATIENTS

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Detailed knowledge of the human B-cell development is crucial for proper interpretation of inborn errors of immunity and for malignant diseases. It is of interest to understand the kinetics of protein expression changes during development, but also to properly interpret the major and possibly alternative developmental trajectories. We have investigated human samples from healthy individuals with the aim to describe all B-cell developmental trajectories. We validated a 30-parameter mass cytometry panel and demonstrated the utility of "*vaevictis*" visualization of B-cell developmental stages. We used trajectory inference tool "*tviblindi*" to exhaustively describe all trajectories leading to all developmental ends discovered in the data. Focusing on Natural Effector B cells, we demonstrated the dynamics of expression of nuclear factors (PAX-5, TdT, Ki-67, Bcl-2), cytokine and chemokine receptors (CD127, CXCR4, CXCR5) in relation to the canonical B-cell developmental stage markers. We observed branching of the memory development, where follicular memory formation was marked by CD73 expression. Lastly, we performed analysis of two example cases of abnormal B-cell development caused by mutations in RAG-1 and WAS gene in patients with primary immunodeficiency.
In conclusion, we developed, validated and presented a comprehensive set of tools for investigation of B-cell development in the bone marrow compartment.

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DYSREGULATED NEUTROPHIL MOBILITY AND GRANULOPOIESIS AS A LONG-TERM SIGNATURE OF POST-ACUTE SEQUELAE OF COVID

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Post-acute sequelae of COVID (PASC) is associated with long-lasting impairments including cognitive problems and immune dysregulation. Innate immune cells are critical in acute COVID responses, but it remains unclear whether functional dysregulation following the disease may drive PASC-related neuroinflammation and cognitive dysfunction. Here we address differences of innate immune cell phenotypes between PASC patients and patients fully recovered from COVID. We aim to find possible links between prolonged immune dysregulation with neuroinflammation and PASC -related cognitive impairment. We enrolled 38 individuals with self-reported cognitive decline symptoms after COVID development (C19-PASC), and 37 volunteers with self-reported full COVID recovery (C19-Rec) at 1.5 year (TP1) and >1.5 year (TP2) after COVID development. Peripheral whole blood samples were analyzed using flow cytometry and ELISA. Clustering analysis, correlation, regression, propensity matching, and sparse partial least squares discriminant analysis (sPLSDA) integrated the flow cytometry, soluble factors, and patient demographic clinical data (namely age, sex, BMI and Newcastle questionnaire scoring). We identified neutrophils (defined as $CD45^{+}CD3^{-}CD66b^{+/dim}CD16^{+/dim}$) as the most affected population in C19-PASC in TP1. Clustering analysis showed functionally distinct neutrophil subsets, including two $CXCR4^{+}$TREM-$1^{+}CCR2^{+}$ immune suppressive neutrophil subsets, annotated as polymorphonuclear myeloid -derived suppressor cells (PMN-MDSCs). These subsets were reduced in C19-PASC in TP1, suggesting a unique long-term PASC related immune signature. Granulopoiesis and mobility -related chemokine CXCL12, as well as CCL2 and soluble TNF receptor 2 (sTNFR2) were decreased in the C19-PASC group in TP1. At the same time, sTNFR1 and granulopoiesis regulator IL-3 were increased in C19-PASC in TP1 and TP2 respectively. These results suggested low-grade inflammation and dysregulated granulopoiesis. Regression analysis and sPLSDA identified the most characteristic soluble factors and immune signatures for PASC patients for each time point. Our findings pinpoint that dysregulated neutrophil mobility and granulopoiesis are associated with PASC development and aid the understanding and management of PASC.

CLUSTERING VS. GATING IN HIGH-DIMENSIONAL CYTOMETRY: PRACTICAL EVALUATION USING A 22-COLOR MOUSE PANEL

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To facilitate high-throughput immunophenotyping of murine models, we optimized a 22-color panel for splenocyte analysis, consolidating two standard IMPC panels into a single assay compatible with conventional and spectral cytometers equipped with five lasers. This panel enables robust identification of major lymphoid and myeloid populations and their subsets, making it suitable for profiling both steady-state and challenged immune systems.
In this talk, we demonstrate the use of multiple unsupervised clustering tools available as plugins in FlowJo—such as tSNE, UMAP, FlowSOM, and Phenograph—to analyze high-dimensional data acquired from this panel. We evaluate the performance, usability, and biological interpretability of these approaches, comparing them to traditional manual gating.
Additionally, we briefly introduce external platforms including OMIQ and custom R-based workflows, discussing their integration potential and added value for more advanced or large-scale studies. This comparative analysis opens discussion for transitioning from manual gating toward more automated, data-driven cytometry pipelines, helping researchers leverage the full potential of high-dimensional panels for immunological discovery.

HOW WE USE TCR/BCR SEQUENCING DATA TO TRACK THE FATE OF INDIVIDUAL LYMPHOCYTES

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Our laboratory has long-standing experience with single-cell transcriptomics combined with T-cell or B-cell antigen receptor (TCR/BCR) sequencing to study mouse and human lymphocytes. The amino acid sequence of the antigen receptor plays a central role in determining lymphocyte fate. However, our capacity to extract meaningful biological insights from TCR/BCR data remains limited.
I will present our integrative approach to leveraging TCR/BCR sequencing in diverse clinical, preclinical, and experimental settings. These include quantifying clonal expansion and prior antigen exposure across lymphocyte subsets, identifying unconventional T cells,

determining the clonality of atypical B cells in patients with common variable immunodeficiency, and using TCR sequences to trace clonal families.
Finally, I will briefly discuss emerging efforts to predict T-cell antigen specificity from sequence data — a promising yet challenging direction.

IMMUNOMMAP: INSIGHTS INTO IMMUNE AND GENOMIC ALTERATIONS UNDERLYING THE RESISTANCE TO BISPECIFIC ANTIBODIES IN MULTIPLE MYELOMA BY HIGH DIMENSIONAL FULL SPECTRUM CYTOMETRY AND EXOME SEQUENCING

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Background: Bispecific antibodies (bsAbs) targeting CD3 x BCMA (Taclistamab [Tecli], Elranatamab [Elra]), GPRC5D (Talquetamab [Talque]), and FCRH5 (Cevostamab [Cevo]) represent an effective therapeutic option for patients with relapsed/refractory multiple myeloma (RRMM). However, both mutations in target molecules and immune cell exhaustion may affect treatment outcome.
Methods/aims: A 40-parameter panel for 5-laser Cytek Aurora was designed to assess lymphoid subsets and exhaustion status in cancer patients, featuring low complexity and max. 85% overlap. Fresh peripheral blood samples (n=56) from 10 RRMM patients treated with Tecli and Elra were assessed at baseline (D+0) and longitudinally at D+7, D+14, D+30, and at the day of progressive disease (PD). Exome sequencing was performed on sorted bone marrow aberrant plasma cells using the Twist Comprehensive Exome Panel. Samples were collected at diagnosis (n=2), relapse pre-bsAb (n=4), and paired relapse post-bsAb (n=2) to analyze CNVs and SNVs in patients treated with Cevo, Talque, and Tecli.
Results: Within the cytometry cohort, 4/10 patients experienced PD within 3 months after therapy initiation. The PD group exhibited a trend for persistently lower lymphocyte percentages throughout the first month with highest difference at D0 (median noPD/PD: 23.9%/2.5%; p = 0.09). This was primarily due to a significantly reduced proportion of T cells in the lymphocyte fraction (median noPD/PD: D0 84.1%/68.7%, D7 89.7%/61%, D14

87.7%/50.7%, M1 89.1%/48.9%; p<0.03) persisting until progression (median PD: 46.3%). Several distinctive subsets were identified by unsupervised clustering (flowSOM + UMAP) to be enriched or reduced in the PD group longitudinally, including major NK cell cluster expressing inhibitory KIR2DL receptor (CD16+CD56+CD45RA+KIR2DL+) which was significantly enriched in PD patients from D0 (median noPD/PD: 2.4%/10.2%), through D30 (2.5%/17.1%; p<0.03).
In the genomic cohort, 3/6 patients experienced PD. Multiple GPRC5D mutations were detected in one Talque-treated patient, including a W237 likely disrupting the receptor's C-terminus and Talque binding at D239.

Conclusion: Our study describes bsAbs resistance in RRMM via high-dimensional cytometry and paired genomic profiling, associating early T cell depletion and mutations in target molecule with PD.

THE JOURNEY FROM VASCULATURE TO (MICRO)FLUIDIC-BASED *IN VITRO* MODELS

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Cardiovascular diseases (CVD) remain the leading causes of mortality and morbidity worldwide, often arising from an imbalanced interplay among blood flow, vascular endothelial cells, and inflammatory stimuli. Over time, this imbalance can lead to life-threatening conditions such as atherosclerosis, myocardial infarction, and stroke.
Our group focuses on studying CVD using *in vitro* vasculature models. We have employed 3D, 2.5D, and 2D models—ranging from patient-specific constructs to simplified cell-based systems—fabricated via lithography, 3D printing, and polymer casting.
These models have been extensively used to study thrombolysis, revealing how clot structure and stiffness influence thrombolytic resistance and offering insights for future therapeutic strategies. Furthermore, a fluidic model of the middle cerebral artery demonstrated that collateral circulation accelerates vessel recanalization during ischemic stroke without affecting thrombolysis.
Additionally, our models, combined with particle velocimetry, support computational fluid dynamics studies of cerebral aneurysms. Recently, we developed a dimensional reduction

approach to replicate hemodynamic conditions at aneurysm rupture sites and to establish cell-based aneurysm models.

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SPATIOTEMPORAL CHANGES OF HETEROTYPIC HUMAN PANCREATIC DUCTAL ADENOCARCINOMA SPHEROIDS *IN VITRO*

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Introduction: Pancreatic ductal adenocarcinoma (PDAC), the most prevalent malignancy of the pancreas, is characterized by desmoplastic tumor-associated stroma that contributes to the resistance to chemotherapy and immunotherapy and tumor dissemination. Cancer-associated fibroblasts are the most abundant cell type in the PDAC stroma and closely interact at physical and biochemical levels with cancer cells during the tumor progression. 3D heterotypic multicellular spheroids are the *in vitro* models used for investigation of cancer-stromal cell interactions. How the genetic alterations of cancer cells and their epithelial or mesenchymal-like subtype influence the spheroid formation during their interactions with stomal cells is still to be investigated.
Material and methods: PDAC patient-derived stromal cells were outgrown from the resected tumors. They were used together with three different PDAC cell lines, Capan-2 (epithelial morphology), PANC-1 and MIA PaCa-2 (mesenchymal-like phenotype), for formation of heterotypic spheroids. Cancer and stromal cells were seeded in different ratios in multi-well agarose molds while keeping the total number of cells constant. We analyzed the morphological changes of spheroids during prolonged culture and spheroid parameters such as area and circularity.
Results and conclusions: Our results indicate that the epithelial cancer cells (Capan-2) form smaller, more compact spheroids while the mesenchymal-like cancer cells (PANC-1

and MIA PaCa-2) have loosened cellular interactions. Increasing the number of PDAC patient-derived stromal cells, while decreasing the number of PANC-1 and MIA PaCa-2 cells in heterotypic spheroids, led to formation of more compact spheroids and subsequently decrease of spheroid area. Our model shows the cancer cell line-specific interactions with stromal compartment. Utilization of PDAC patient-derived stromal cells increases the variability that corresponds to diversity of PDAC patients observed in clinical practice. Thus, this model has potential to serve for assessment of effects of therapeutic agents on cancer-stromal cell interactions within the complex 3D tumor microenvironment.

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ENHANCING THE EFFICACY OF MDM2 INHIBITORS THROUGH COMBINATION THERAPY

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Molecular targeted anticancer therapies aim for superior efficacy with reduced adverse effects compared to conventional chemotherapy. While strategies targeting oncogenes have been successfully implemented in clinical practice, approaches aimed at reactivating tumor suppressors face significant challenges. The p53 tumor suppressor, activated by highly specific MDM2 inhibitors, represents a promising yet underutilized therapeutic target, as these inhibitors have not been successfully translated into standard clinical treatments. Recent research, however, indicates that the antitumor potential of MDM2 inhibitors can be dramatically enhanced through strategic combination with other therapeutic agents.

Through a comprehensive analysis of the literature, we have identified and characterized compounds that synergize with MDM2 inhibitors and mapped cellular pathways sensitive to p53 activation. Our findings reveal two principal categories of synergistic compounds: those that modulate the p53 network across multiple regulatory levels (including DNA accessibility, transcription, translation, and protein stability), and those that target the same cellular processes as p53 (such as apoptosis, cell cycle regulation, DNA repair mechanisms, metabolic pathways, immune system modulation, and growth signal inhibition). These complementary approaches provide multiple intervention points that collectively amplify the tumor-suppressive effects of MDM2 inhibition.

Our review demonstrates that combination therapeutic strategies offer a promising approach for effectively utilizing MDM2 inhibitors in targeted cancer treatment. By simultaneously activating both the p53 network and other vulnerabilities defined by a specific cancer type, these combinations may overcome the current limitations of p53-based monotherapies and potentially establish new avenues for precision oncology.

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SIMULTANEOUS, HIGH-THROUGHPUT DETECTION OF RNA AND PROTEIN BIOMARKERS USING THE ORIONTM IMAGING SYSTEM AND HCR GOLD FOR COMPREHENSIVE ANALYSIS OF TUMOR SAMPLES IN CLINICAL TRIALS

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Understanding the tumor microenvironment (TME) during cancer progression and treatment has been aided by multiomic analysis of tissues collected at initial patient screening and after treatment. Cyclic staining methods have recently demonstrated multiomic capabilities on a single tissue, but with drawbacks that can include tissue degradation and processing times up to several days or weeks. The Orion™ imaging system overcomes these limitations through single round staining and scanning of whole tissue samples of up to 18 biomarkers simultaneously. When paired with Molecular Instruments' HCR Gold RNA-FISH, a novel product featuring a truly protease-free workflow with next-generation amplification technology, transcriptomic and proteomic multiplex immunofluorescence (mIF) analysis can be carried out in parallel on a single tissue section in a straightforward, high-throughput approach to provide comprehensive insight into tumor biology and immune response.

APPLICATION OF CYTOMETRY IN TOXICOLOGICAL TESTING IN VITRO: TRANSLATING RESEARCH INTO PRACTICE VIA VALIDATION WITHIN THE OECD TEST GUIDELINES PROGRAM

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Toxicological testing using validated methods according to ISO standards or OECD Test Guidelines (OECD TG) is a key field for cytometry applications. The OECD TG system comprises internationally recognized validated tests accepted by all OECD countries, enabling global regulatory acceptance, especially for chemical safety testing needed for safety data sheets, registration and regulatory compliance. New methods are proposed by universities, chemical/ pharma companies and research institutes, with formal submissions coordinated by national OECD TG coordinators. Multiple OECD countries engage in validation projects, often funded by international grants, including EU programs. Outputs include validated test methods, adverse outcome pathways (AOPs), integrated testing strategies (IATAs), and OECD guideline documents. Validated methods can be licensed and transferred worldwide under Material Transfer Agreements (MTAs). Development teams frequently patent and commercialize technologies such as transfected cell lines.

Our team applies promising validated in vitro and in silico toxicological methods from the OECD TG portfolio for custom testing, method guidance, research and state surveillance. We perform tests under Good Laboratory Practice (GLP) or accredited systems (ISO 17025). A recent example is the h-CLAT method for skin sensitization testing in vitro (OECD TG 442E). The h-CLAT test supports REACH submissions and regulatory compliance under CLP, Biocidal, Plant Protection, and Cosmetics Regulations in accordance with the UN Globally Harmonized System (GHS).

In h-CLAT, changes in cell surface markers CD54 and CD86 on THP-1 human monocytic leukemia cells are measured by flow cytometry after 24-hour exposure to test substances. Cytotoxicity is concurrently assessed to ensure marker upregulation occurs at non-cytotoxic levels. Relative fluorescence intensity compared to controls is used to classify substances as sensitizers or non-sensitizers. Proficiency testing confirmed our lab's accurate classification of reference chemicals. The test is fully accepted for regulatory discrimination of skin sensitizers and non-sensitizers and also provides useful screening in early product development stages.

REGULATION OF THE P53 NETWORK ON TRANSLATOME LEVEL

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The transcription factor p53, widely recognized as the "guardian of the genome," is encoded by TP53, one of the most frequently mutated genes in human cancers. As a critical tumor suppressor, p53 transactivates hundreds of genes involved in various cellular programs that maintain genomic stability. During stress responses, p53 orchestrates multiple pathways including cell cycle arrest, DNA repair, and apoptosis, functioning as a

pivotal regulator of cellular homeostasis. While the p53 network operates through several regulatory mechanisms involving multiple negative feedback loops, its primary mode of action involves the transactivation of target genes resulting in elevated mRNA levels. Approximately 90% of p53-induced targets are protein-coding genes, highlighting the significance of mRNA translation as a key regulatory step between p53 network induction and cellular phenotype. Our recent findings demonstrate that activation of the integrated stress response (ISR), characterized by inhibitory phosphorylation of the eukaryotic translation initiation factor eIF2α, leads to preferential translation of pro-apoptotic p53 target genes and subsequent cell death induction. To further investigate this phenomenon, we employed Ribosome profiling (Ribo-seq) to characterize the regulatory impact of ISR on the p53 network and cell fate determination. Our results reveal significant disparities between the p53 transcriptome and translatome upon ISR induction through eIF2α inhibition. These findings suggest that elucidating the impact of mRNA translation on p53 activation outcomes may enhance the efficiency of targeted anti-cancer therapies based on p53 activation.

CELLULAR RESPONSE TO PATIENT SPECIFIC OSCILLATORY SHEAR STRESS CONDITIONS IN MICROFLUIDIC ANEURYSM MODELS

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Intracranial aneurysms are vascular abnormalities with a high risk of rupture, leading to hemorrhagic stroke. Hemodynamic forces, particularly shear stress, regulate endothelial behavior and contribute to aneurysm development. Understanding cellular responses to different flow conditions is essential for uncovering mechanisms of aneurysm progression.

We used microfluidic devices from IBIDI to investigate endothelial responses under patient-specific oscillatory shear stress conditions derived from aneurysm data. Flow parameters were calculated through dimensionality reduction of aneurysmal hemodynamics in collaboration with researchers from the Faculty of Mathematics, Physics and Informatics (MATFYZ). Two experimental setups were established with different levels of specific shear stress: 20.9 dyn/cm² (Setup 1) and 1.77 dyn/cm² (Setup 2). In each setup, two different flow conditions were tested: unidirectional and oscillatory flow, compared

to static control. Human umbilical vein endothelial cells (HUVECs) were used as the model system. Cell behavior was evaluated by image analysis (orientation and elongation), and gene expression changes were assessed by PCR.

In Setup 1, unidirectional flow induced pronounced cell elongation and orientation with the flow direction, whereas oscillatory flow did not result in such cellular responses. In Setup 2, differences between unidirectional and oscillatory flow conditions were less evident. PCR analysis focused on inflammatory markers (VCAM, SELE) and mechanosensitive markers (eNOS, ASS1, KLF2, KLF4, CYP1B1, EDN1). In both setups, oscillatory flow did not significantly induce an inflammatory response but led to alterations in mechanosensitive gene expression.

In conclusion, our findings demonstrate that specific shear stress conditions significantly influence endothelial cell behavior. Particularly, oscillatory flow alters endothelial homeostasis, which could contribute to endothelial dysfunction and aneurysm progression.

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ASCITES-DERIVED ORGANOIDS AS PREDICTIVE MODELS FOR PERSONALIZED TREATMENT OF OVARIAN CANCER

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Ovarian cancer (OC) is an aggressive malignancy that frequently disseminates into the peritoneal cavity, where it promotes the accumulation of ascitic fluid. This fluid harbors tumor spheroids—three-dimensional clusters of cancer cells capable of surviving hostile conditions and initiating metastasis. Standard treatment for advanced OC includes cytoreductive surgery followed by systemic chemotherapy targeting residual

micrometastases. Therefore, spheroids can serve as a valuable source for transforming into organoids (also known as tumoroids) that maintain the tumor's genetic and phenotypic characteristics and can be used to predict treatment response.

In this study, we explore the potential of ascites-derived organoids as patient-specific models to predict therapeutic response. Tumor spheroids are isolated from ascitic fluid collected during cytoreductive surgery, then embedded in BME Cultrex (Matrigel) and cultured in organoid-supporting media. These organoids are treated with standard-of-care drugs—such as carboplatin, paclitaxel, and PARP inhibitors—and their viability assessed via WST assay. To evaluate their predictive accuracy, we correlate in vitro drug response with the actual clinical outcomes of the patients.
Chemotherapy, that comes after the cytoreductive surgery, usually leads to initial response. However, up to 80% of patients experience recurrence due to the development of treatment resistance, and a quarter of patients are inherently resistant even before the therapy. Sensitivity to platinum derivatives is a key prognostic factor, but its assessment is retrospective, based solely on the response to therapy and time to recurrence.
This organoid-based approach offers a promising avenue for timely, personalized therapy selection. If validated, this strategy could revolutionize treatment planning in OC, improving outcomes by tailoring therapies to the individual tumor's response profile.

INTEGRATIVE CYTOMETRY AND SINGLE-CELL TRANSCRIPTOMICS FOR PROFILING RARE NEURAL POPULATIONS

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Single-cell RNA sequencing (scRNA-seq) is a transformative tool in neuroscience that enables the dissection of cellular heterogeneity and developmental trajectories within the brain. While droplet-based scRNA-seq methods are widely used, their requirement for large cell numbers poses challenges in capturing rare or fragile neural populations. To address this, we implemented a plate-based scRNA-seq approach that combines precise single-cell isolation with nanoliter-scale reagent dispensing, facilitating cost-effective, full-length RNA-seq library preparation.
To extend our single-cell datasets with additional phenotypic information, we integrated fluorescence-activated cell sorting (FACS) with index sorting into our workflow, allowing for the capture of rare and delicate cell populations. This integration links cytometric data such as surface marker expression with transcriptomic profiles, providing a multidimensional view of cellular identity. Additionally, we incorporated another accurate and flexible technology for single cell isolation - image-based sorting using the CellenONE system. These complementary strategies have proven effective across multiple brain-focused collaborative projects, enhancing our ability to profile rare neural populations.

The integration of cytometry, index sorting, image-based selection, and full-length scRNA-seq offers a robust framework for profiling of rare neural populations. This multidimensional approach overcomes common bottlenecks in single-cell neuroscience research, providing deeper insights into cellular diversity and function.

AUTOMATING ANTIBODY COCKTAIL PREPARATION AND SAMPLE STAINING WITH THE C-FREE PLUTO LT SYSTEM

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The preparation of antibody cocktails for flow cytometry is a time-intensive and variable process, particularly for surface or intracellular staining workflows that require precise reagent handling and consistency. The C-FREE Pluto system introduces an automated solution designed to streamline this critical step, minimizing human error, reducing reagent waste, and enhancing reproducibility. Pluto LT workstation increases the ability to automate staining and washing as well as master-mix preparation, giving the user experience of an “all in one” sample prep solution.

QUANTITATIVE CELL–CELL INTERACTION ASSAY USING IMAGING FLOW CYTOMETRY

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Cell-cell interactions are essential for numerous biological processes, such as tissue development, immune responses, and oncogenesis. Due to their vital function in modulating cellular behavior, they are examined through a range of experimental methodologies. In this context, we introduce a quantitative assay designed to investigate cell–cell interactions utilizing imaging flow cytometry with the BD FACSDiscover™ S8 CellView™ imaging system.

Utilizing T-47D wild-type and desmoglein-2 knockout (KO) cells, we established and refined conditions to ensure consistent aggregate formation while reducing user-dependent variability. Desmogleins, which are desmosomal cadherins, are vital for epithelial cohesion and tissue integrity, thus serving as an ideal model to investigate altered cell-cell interactions. Validation of the assay across multiple repetitions demonstrated a robust capacity to distinguish between single cells and aggregates through imaging-derived parameters. Moreover, the assay was successfully integrated with fluorescent antibody staining (Trop-2), confirming its compatibility with multiparametric analysis.

Together, these results establish a robust, quantitative, and reproducible platform for studying cell-cell interactions and their regulation by adhesion molecules, with potential applications in cancer research and beyond.

Acknowledgment: This work was supported by grants from the Czech Science Foundation (grant no. 24-11793S and 23-06472S).
Spectral flow cytometry generates high-dimensional single-cell data, enabling deep phenotypic profiling and complex cellular population analysis. Given the large volume of data produced by high-throughput techniques, unsupervised analysis is highly desirable; however, it presents challenges in data interpretability and biological insight extraction. Even with exemplary preprocessing and QC, algorithm selection and tuning can lead to significantly different results, impacting reproducibility and the identification of biologically relevant populations. Moreover, due to the extensive data generated by modern spectral flow cytometry, performance becomes a crucial consideration. Downsampling to as low as 10% is a common practice to accommodate computationally intensive community detection algorithms, which often yield superior results. However, this approach can hinder the detection of rare but biologically significant cell populations. Here we examine commonly used approaches, their respective advantages and limitations, and possible optimization strategies for managing large datasets without compromising clustering quality or resolution.

HIGHLY ACCURATE PROFILING OF LEUKOCYTE COMPOSITION FROM BULK PERIPHERAL BLOOD WITH TARGETED DIGITAL CYTOMETRY

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Background:
Cell profiling methods such as flow/mass cytometry and single-cell RNA-sequencing are powerful tools for quantifying immune composition from healthy and neoplastic tissues. However, only a modest number of markers can be interrogated by the former and the latter remains cost prohibitive for large-scale analysis. Here we demonstrate robust, accurate, and reproducible enumeration of immune cell subsets from 36 whole blood samples using SureSelect XT HS2 RNA sequencing combined with CiberMed's iSortTM digital cytometry solution. Enrichment of genes in the LM22 signature matrix, a well-established collection of reference profiles for deconvolving 22 human immune subsets, was achieved with two new targeted sequencing panels—Agilent SureSelect CD CiberMed Heme and Agilent SureSelect CD CiberMed Heme + HiRes. Both panels were assessed for their ability to profile leukocyte subsets with CiberMed's iSortTM Fractions software, an optimized and standardized version of CIBERSORTx with novel enhancements.

Methods:
Whole blood samples were freshly collected from 36 healthy donors and split into two fractions—one was immediately processed for complete blood count (CBC) and flow cytometric enumeration of major leukocyte populations; the other was stored in PAXgene Blood RNA tubes for subsequent RNA sequencing. A Sysmex system was employed for CBC quantification and a Becton Dickinson 6-color TBNK MultiTest in vitro diagnostic (IVD) assay was employed for enumerating B cells, CD8 T cells, CD4 T cells, NK cells. These data were used as ground truth to assess iSortTM deconvolution performance from targeted and whole-transcriptome bulk RNA sequencing data. RNA-seq expression values were used as input to iSortTM Fractions to deconvolve 22 immune subsets in each sample.

Results:
Across 7 major populations, cell type fractions determined by iSortTM Fractions were highly concordant with ground truth fractions determined by clinical grade standards ($r \geq 0.96$) and exhibited strong reproducibility across technical replicates ($r \geq 0.98$). Furthermore, targeted enrichment using the SureSelect CD CiberMed Heme panel reduced the sequencing requirement by nearly 50-fold compared to whole-transcriptome sequencing, while also improving accuracy.

Conclusion:
These new panels for digital cytometry are being released through the Agilent Community Design program to enable focused, reliable, and high-throughput analysis of cell type composition from peripheral blood samples.

EXPERIMENTAL CYTOMETRY

FAM83H/SACK1H SHAPES THYMIC STROMAL ARCHITECTURE AND POSTNATAL LYMPHOPOIESIS VIA CK1 INTERACTION

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Family of Sequence Similarity 83H (FAM83H/ SACK1H) is primarily expressed in epithelial cells, where it associates with CK1 and keratins to regulate cytoskeletal organization, cell proliferation, and vesicular trafficking. Truncating mutations in FAM83H are the primary cause of autosomal dominant hypocalcified amelogenesis imperfecta (AI). Notably, two AI patients from a Czech family with a confirmed FAM83H mutation also developed juvenile rheumatoid arthritis.

To investigate the role of FAM83H in immune system homeostasis, we generated two mouse models: Fam83h-deficient mice (Fam83hem2(IMPC)Ccpcz, Fam83h-/-), and mice lacking a part of the N-terminal CK1-binding domain (Fam83hΔ87/Δ87). Both strains exhibit nearly identical phenotypes, underscoring the essential role of the CK1-binding domain in FAM83H function.

Consistent with other Fam83h-deficient models, these mice are subviable, smaller in size, and display a sparse, scruffy coat, scaly skin, weakness, and hypoactivity. Importantly, both strains exhibit impaired lymphoid cell production during early postnatal development. While fetal hematopoiesis remains intact, B cell and NK cell development in the bone marrow is partially blocked at the pro- and pre-B cell stages and the immature NK cell stage in the absence of FAM83H. In the thymus, Fam83h is expressed by thymic epithelial cells (TECs), and its deficiency in stromal cells results in a severe impairment of DN3 T cell expansion, ultimately leading to insufficient T cell production.

Fam83h-deficient cortical TECs (cTECs) show elevated expression of circadian rhythm genes and reduced expression of the TEC master regulator Foxn1, along with its multiple downstream targets. This suggests a role for FAM83H and CK1 in cTEC maturation.

In summary, FAM83H, together with CK1, is essential for organizing the keratin cytoskeleton in thymic epithelial cells, thereby maintaining thymic stromal architecture and supporting normal T cell development.

CD MOLECULES NOMENCLATURE 2025: ANTIBODY VALIDATION AND EXPRESSION PROFILING OF IMMUNE SYSTEM G PROTEIN-COUPLED RECEPTORS (GPCRS)

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Monoclonal antibodies (mAbs) targeting cell-surface molecules are indispensable tools in biomedical research, diagnostics, and biotechnology. They are increasingly recognized for their critical roles in the treatment of malignancies and autoimmune diseases. Over the past four decades, the CD nomenclature system, established by the Human Leukocyte Differentiation Antigens (HLDA) Workshops and endorsed by the International Union of Immunological Societies (IUIS), has provided a standardized framework for naming mAbs and their target molecules.
G protein-coupled receptors (GPCRs), althrough beeing the largest family of cell-surface receptors, are underrepresented in the availability of well-validated mAbs suitable for flow cytometry.
At the Eleventh HLDA Workshop (HLDA11), 104 mAbs targeting GPCRs were evaluated for their reactivity with 27 peripheral blood leukocytes subsets obtained from healthy donors (n=4), cell lines (n=9), and transfected COS cell line using conventional or spectral flow cytometry. These studies facilitated the validation of the mAbs and characterization of their expression profiles.
As a result, fourteen new CD designations were assigned for GPCR cell-surface molecules expressed on immune cells: CD198 (CCR8), CD199 (CCR9), CD372 (CCR10), CD373 (CX3CR1), CD374 (XCR1), CD375 (GPR15), CD376 (GPR26), CD377 (SSTR3), CD378 (C3AR1), CDw379 (FPR2), CD380 (LTB4R), CDw381 (GPR138), CDw382 (F2RL1) and CD383 (P2RX7). We present the newly assigned CD nomenclature for GPCRs and provide detailed quantitative expression profiles across various subsets of innate and adaptive leukocytes. In addition, we provdie validation data for these mAbs and discuss the therapeutic implications of these expression profiles in targeting immune-mediated diseases and cancer. In conclusion, the HLDA workshops and the CD classification system continue to

evolve, maintaining their relevance and value in the face of ongoing advancements in imunology.
This work was supported by projects - NU23J-03-00026, UNCE/24/MED/003, LX22NPO5102. The reagents were kindly provided by Exbio (Vestec, Czech Republic).

LPS TRIGGERS ER STRESS–MEDIATED HYBRID EMT IN HUMAN EXPANDABLE LUNG EPITHELIAL CELLS

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Lipopolysaccharide (LPS), a bacterial endotoxin, is known to trigger inflammation in lung tissue, yet its direct effects on the lung epithelium remain insufficiently understood. In this study, we utilized expandable lung epithelial cells (ELEPs) derived from human embryonic stem cells (Kotasova et al., 2022) to investigate the response to LPS exposure. LPS treatment was shown to induce features of hybrid epithelial-to-mesenchymal transition (EMT), including retention of E-cadherin within the endoplasmic reticulum (ER), activation of the unfolded protein response (UPR), and increased intracellular accumulation of β-catenin. Although cell viability remained unaffected, the ELEPs exhibited enhanced migratory capacity. ER stress induced by tunicamycin produced similar effects, while treatment with the chemical chaperone TUDCA partially reversed these phenotypes by restoring E-cadherin membrane localization and reducing UPR markers. Additionally, 3D spheroid assays revealed structural disruption upon LPS treatment and reduced growth with tunicamycin, both of which were partially reversed by TUDCA, further supporting the observed phenotypic changes. These findings were demonstrated through MTT assay, western blotting, qRT-PCR, immunofluorescence microscopy, morphometric analysis, and cell migration analysis. Our results reveal that LPS promotes a non-lethal but transformative stress response in lung epithelial progenitors, highlight the role of ER stress in promoting migration, and position ELEPs as a relevant non-cancerous model to study inflammation-driven epithelial remodeling.

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Reference:

Kotasova H. et al. Tissue Eng Regen Med, 2022, 19(5):1033-1050. doi: 10.1007/s13770-022-00458-0
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SYSTEMIC ADMINISTRATION OF HYALURONIC ACID: PHARMACOKINETICS AND THERAPEUTIC POTENTIAL IN RHEUMATOID ARTHRITIS

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Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease characterized by joint inflammation and cartilage breakdown. Hyaluronan (HA) is a glycosaminoglycan, composed of D-glucuronic acid and N-acetyl-D-glucosamine, which is an important part of extracellular matrix present in high concentration in cartilage and synovial fluid where it is responsible for proper mechanical function of the joint. HA or its derivatives is currently administered intra-articularly in the RA treatment for improvement of mechanics of the joints, however, intravenous administration of hyaluronan is emerging as an alternative particularly because intra-articular injections can be challenging for clinicians when targeting small or swollen joints.

Adjuvant induced arthritis mouse model was used to study pharmacokinetics and therapeutical effect of intravenously administered middle-Mw HA. Our unique 13C-, biotin- and fluorescently labelled HA was preferentially accumulated in inflamed joint tissues while distribution in other organs remained similar to healthy controls. With repeated administrations, the significant attenuation of clinical arthritis symptoms such as swelling and redness, decrease in levels of RANKL, iNOS, COMP and prostaglandin E2 was observed. Moreover, HA treatment prevented dextran-FITC penetration into inflamed limbs suggesting reduced vascular permeability at the site of inflammation. These findings advocate for a potential shift towards systemic HA administration as a novel and effective treatment strategy in the treatment of RA.

Animal experiments were approved by the institutional Animal Care and Use Committee (protocol no.92/2024).

CHANGES IN BONE MARROW NICHE DURING SEPSIS INVESTIGATED USING IPSC-DERIVED BONE MARROW ORGANOIDS.

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Sepsis remains a major global health concern, profoundly disrupting hematopoiesis and bone marrow (BM) function. While current experimental models have offered valuable insights into BM responses under systemic stress, they often fail to fully capture the complexity of human BM dynamics.. Human induced pluripotent stem cells derived bone marrow organoids (BMOs) perfectly reconstruct the BM microenvironment, including critical hematopoietic elements, providing a novel platform to study sepsis-driven changes.

In this study, we aimed to explore the pathophysiological effects of sepsis on bone marrow using human BMOs as a model system.

To gain a comprehensive understanding of cellular interactions and stress responses within the bone marrow microenvironment, we modeled sepsis in BMOs by exposing them to endotoxins and a pro-inflammatory cytokine storm. Confocal microscopy was employed to visualize cellular interactions, while flow cytometry enabled profiling of immune cell populations and their activation status. Additionally, ELISA was used to quantify cytokine secretion, providing a functional readout of the inflammatory response of the BM.

Exposure to pro-inflammatory cytokines and bacterial endotoxins induced hallmark features of septic response, including elevated IL-1β secretion, endothelial activation, and enhanced phagocytosis. Using flow cytometry, we characterized shifts in hematopoietic lineage commitment, revealing a pronounced bias toward myelopoiesis over lymphopoiesis, indicative of emergency granulopoiesis. Furthermore, we were able to analyze immune cell activation and phenotypic changes on a single cell level within complex BMO structure.

Our findings support the use of BMOs combined with flow cytometry as a powerful platform for investigating BM immunopathology during sepsis and pose a useful solution for future possible applications, including preclinical drug screening and personalized medicine.

RESPIRATORY SYNCYTIAL VIRUS PREFUSION F3 VACCINE IN LUNG TRANSPLANT RECIPIENTS ELICITS CD4+ T CELL RESPONSE IN ALL VACCINEES

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Respiratory syncytial virus (RSV) causes seasonal acute respiratory illness significantly impacting vulnerable groups, including lung transplant recipients (LTRs), who are at increased risk of hospitalization, acute rejection, and allograft dysfunction. The immunogenicity of the novel RSV Prefusion F3 (RSVPreF3-AS01, Arexvy, GlaxoSmithKline) vaccine in immunocompromised patients remains largely unknown. In this study, we assessed both antibody using and cellular immune responses two months after a single dose of the RSVPreF3-AS01 vaccine in 30 LTRs aged 60 years or older, who were at least six months post-transplant. The antibody response was assessed using enzyme-linked immuno sorbent assay for detection of serum anti RSV-F IgG specific antibodies, and the CD4+ T-cell response was measured by flow cytometry intracellular cytokine secretion assay. Our findings show that all vaccinees exhibited a CD4+ T-cell response two months post-vaccination, while only 40% demonstrated an antibody response. These results suggest that some patients may derive clinical benefit from the vaccine through cellular immunity, even without an antibody response. Furthermore, the vaccine was well tolerated in this vulnerable population, with no major safety concerns observed.

UNDERSTANDING THE ROLE OF DESMOGLEIN 2 IN PRESERVING THE EPITHELIAL PHENOTYPE OF BREAST CANCER CELLS

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Desmoglein 2 (DSG2) is a surface protein that plays a physiological role in desmosomal cell-cell adhesion and in maintaining epithelial tissue integrity. In cancer, the role of DSG2 remains controversial, as it has been reported to exhibit both pro-tumorigenic and tumor-suppressive effects. Given that the connection between DSG2 and the metastatic cascade in cancer is underexplored, we focused on its association with epithelial-mesenchymal transition (EMT) traits in breast cancer cells.
CRISPR-Cas9 knockout (KO) cells were validated using western blotting, immunofluorescence, and sequencing of gRNA targets to confirm the loss of DSG2 in the T-47D cell line. DSG2 KO cells were tested for their attachment to ECM matrix components utilizing the ECM Select® Array Kit Ultra-36 (Advanced Biomatrix). Multicolor spectral flow cytometry was used to detect the epithelial-mesenchymal transition (EMT) surface profile of the DSG2 KO cells, assessing markers such as EpCAM, Trop2, CD9, CD29, CD49c, GD2, and ITGB5.
To evaluate the impact of DSG2 loss in vitro, we established a T-47D DSG2 CRISPR knockout model and successfully confirmed the deletion of DSG2. We investigated the capacity of DSG2 knockout cells to adhere to various extracellular matrix (ECM) proteins and noted a general reduction in cell adhesion to most individual ECM components. The measurement of an in-house developed and validated EMT surface panel indicated the upregulation of multiple mesenchymal markers in DSG2 knockout cells and the downregulation of epithelial markers.

Our study demonstrates that in the T-47D breast cancer cell model, the loss of DSG2 results in a reduced capacity to adhere to various ECM components. These alterations are also evident in the overall mesenchymal-like cell surface profile of DSG2 KO cells. We conclude that DSG2 in the tested model is associated with EMT and may play a role in suppressing the metastatic process.

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T-MAPS: NOVEL SURFACE MARKERS FOR MEASURABLE RESIDUAL DISEASE MONITORING IN PEDIATRIC T-CELL ACUTE LYMPHOBLASTIC LEUKEMIA

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Pediatric T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive malignancy. Despite improved prognosis at diagnosis over recent decades, relapse still occurs in approximately 20 % of patients and is associated with poor outcome. The level of measurable residual disease (MRD) on day 15, assessed by flow cytometry, serves as a key prognostic marker. However, accurate MRD evaluation is often complicated by phenotypic overlap between malignant and non-malignant T cells. A particularly challenging subtype is early T-cell precursor (ETP) ALL which exhibits an immature T-cell phenotype and aberrant myeloid/stem cell marker expression.

Conventional and full-spectrum cytometry were used to assess the expression of surface markers (n=307) in pediatric T-ALL cases (n=47). These expression profiles were compared to healthy donor peripheral blood (HDPB, n=15) T cells, as well as to developmental stages of T cells in non-malignant thymus (n=3) and bone marrow (n=3).

First, we manually identified aberrantly expressed markers of T-ALL cells (at least a 2-fold difference in expression compared to HDPB T cells). The number of the markers ranged from 26 to 78 across samples. In total, 11 CD markers were aberrant in at least 75% of T-ALL samples. Notably, the expression level of CD26, CD38, CD52 and CD98 did not mirror any physiological developmental stage of T cells, indicating their specifity to T-ALL ($p<0.01$, Mann-Whitney test). In ETP-ALL (n=12), manual analysis identified 10 additional CD markers aberrant in over 80 % of samples. In parallel, principal component analysis revealed 8 CD markers that distinguished ETP-ALL cells from HDPB T cells. Of these, 4 markers (CD28, CD27, CD200, CD166) were identified by both manual and unsupervised analysis. The remaining 4 markers were either overexpressed in 8/12 ETP-ALL cases (CD123) or considered negative in all samples based on manual analysis.

In conclusion, we identified 11 CD markers potentially suitable for MRD monitoring in at least 75 % of pediatric T-ALL samples. Moreover, we characterized the ETP-ALL group by the aberrant expression of 4 additional CD markers. Selected markers are prospectively tested on day 15 to assess their treatment stability and potential utility. This work was supported by GAUK 318225. PE antibodies were provided by Exbio (CZ) and Biolegend (USA).

SKYRIN ACTION IN COLORECTAL CANCER CELL LINES

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Skyrin (SKR) is a natural anthraquinone with anticancer potential. In our previous study, SKR upregulated death receptor DR5, an apoptosis inducer in cancer cells, and exhibited some cytostatic and cytotoxic properties in colorectal cancer (CRC) cell lines HCT 116 and HT-29, with the effects being more pronounced in HCT 116 cells. Our aim was to explain these differences by focusing on the roles of protein p53 and ABC transporter protein BCRP.

In addition to HCT 116 (wt p53, low BCRP expression) and HT-29 cells (mutant p53, high BCRP expression), we included p53-null HCT 116-derived cell line. First, we focused on the mechanism of SKR-induced DR5 upregulation. Elevated protein expression of DR5 and its transcription factor CHOP was detected in both HCT 116 cell lines, but not in HT-29 cells. This suggests that DR5 induction is p53-independent and most likely mediated by CHOP. CHOP is known to be induced by oxidative stress, which is also one of the proposed mechanisms of SKR action in cancer cells. Therefore, we investigated the impact of SKR on intracellular accumulation of reactive oxygen species (ROS). To our surprise, ROS levels decreased in all three CRC cell lines following SKR treatment, suggesting its antioxidant properties. Lowered ROS levels may be the result of mitochondrial depolarization. SKR slightly, but significantly increased the number of HT-29 cells with dissipated mitochondrial membrane potential.

Secondly, we investigated the relationship between SKR and BCRP activity. Since SKR has a similar structure to hypericin, a BCRP substrate, we analyzed the impact of BCRP inhibitor Ko143 on metabolic activity (MA) of HT-29 cells and their ability to accumulate SKR. While the MA decreased in Ko143-pretreated group, intracellular SKR content was not affected. However, in another experimental model, leukemic HL-60 cells with low BCRP level and their BCRP-overexpressing derivative cBCRP, we observed marked increase in SKR content in Ko143-pretreated cBCRP cells, whereas no such effect was detected in the parental cell line.

In sum, our results indicate that SKR is a potential BCRP substrate and its activity is in some respects independent of p53.

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RADIOPROTECTION EFFECT OF VITAMIN C

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The relevance of radioprotective research has grown significantly in light of the deteriorating global security environment and the increasing likelihood of exposure of both military personnel and civilian populations to weapons of mass destruction. Among these, nuclear weapons are the most devastating, resulting in large-scale human casualties and material damage. Radioprotective agents act via diverse biological mechanisms, but their common function is mitigating ionizing radiation's adverse effects. This contributes to enhanced operational capability, reduced mortality, and improved quality of life for those affected.
In this study, the radioprotective potential of the antioxidant vitamin C was evaluated in combination with amifostine. Female BALB/c mice were assigned to eleven experimental groups: C (control), A (amifostine), AI (amifostine + irradiation), AS (amifostine + stress), ASI (amifostine + stress + irradiation), I (irradiation), S (stress), VC (vitamin C), VCI (vitamin C + irradiation), VCS (vitamin C + stress), and VCSI (vitamin C + stress + irradiation). Relative changes in T and B lymphocyte populations were assessed via flow cytometry at the following time points: days 0, 1, 2, 7, 14, 21, and 30. This research was supported by the Ministry of Defence of the Czech Republic "Long Term Organization Development Plan 1011"— Healthcare Challenges of WMD II of the Military Medical Faculty, Hradec Kralove, University of Defence, Czech Republic (project no DZRO-FVZ22-ZHN II).

TUMOR HYPOXIA AND NONPHOTOACTIVATED HYPERICIN – TWO FACTORS AFFECTING METASTASIS IN LUNG ADENOCARCINOMA CELLS

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Tumor hypoxia represents a critical factor that greatly affects the process of metastasis in solid tumors. Hypoxia-inducible transcription factor, HIF-1α, can directly regulate the expression of genes involved in the metastasis process, such as *SNAI1*. The presence of HIF-1α in tumors may indicate a worse patient prognosis. Lung cancer patients are known to have higher rates of metastasis, and therefore metastasis-directed therapy could improve survival outcomes in these patients.
Since anticancer research is once again leaning towards using natural substances with the least possible toxic effects on the body, hypericin, a secondary metabolite of *Hypericum* genus, appears to have the necessary effects to suppress metastasis. Firstly, hypericin is

predominantly accumulated in tumor tissue [1]. Secondly, hypericin reduces the protein levels of a key hypoxic transcription factors, HIF-1α and HIF-2α, in A549 lung adenocarcinoma cell line. In the current study, the protein level of Snai1 was also decreased after hypericin treatment and this result was more pronounced under hypoxia. Hypericin successfully reduces HIF-1α levels due to a reduction in intracellular pH and subsequent increase in cathepsin B activity [2]. To verify whether hypericin affects Snai1 protein levels through HIF-1α degradation, we applied a cathepsin B inhibitor, which in the presence of hypericin prevented the decrease of HIF-1α and Snai1 levels. The wound-healing assay confirmed these results as the migration ability of the entire A549 population was decreased by hypericin in hypoxia. On the other hand, hypericin in hypoxia not only reduced the side-population (SP) sorted from A549 cell line, but also significantly stimulated SP cells migration.
Hypericin application in the treatment of hypoxic tumors could potentially augment success rate of metastasis-directed therapy, however, at the same time, hypericin in hypoxia may have a selective and adverse effect on a small subpopulation of cancer cells representing cancer stem cells.

This work was supported by the Scientific Grant Agency of the Ministry of Education of the Slovak Republic under the contract No. VEGA 1/0003/23.

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HYALURONIC ACID NANOPARTICLES AS A POTENTIAL RADIOPROTECTIVE AGENT

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Radioprotective agents operate through various biological mechanisms, aiming to reduce the adverse effects of ionizing radiation and improve the quality of life in exposed individuals. In the past three decades, significant advances have been made in developing and applying nanomaterials—particularly nanoparticles (NPs)—across numerous research disciplines and clinical settings. The potential impact of this research on the field of radiobiology is significant, offering hope for improved treatments and outcomes. Due to their wide-ranging potential in diagnostics, therapy, and disease prevention, the implementation of NPs in medicine represents one of the most notable scientific breakthroughs of recent years. Given their demonstrated beneficial effects in treating various pathological conditions, including oncological, neurodegenerative, and intravascular diseases, it is understandable that research interest has also expanded into radioprotection, where initial promising results have already been reported.

The study aims to evaluate the radioprotective potential of hyaluronic acid nanoparticles (HANPs). The experiments were conducted on human and mouse T and B lymphocytes, both healthy and cancer-derived cell lines from primary and commercial origins. The primary outcome measure was cellular viability and molecular and cellular reactions following exposure to a 60Co gamma source of ionizing radiation.
This work was supported by the Ministry of Education, Youth and Sports of the Czech Republic (Specific Research project no. SV/VLF202401) and the Ministry of Defence of the Czech Republic "Long Term Organization Development Plan 1011"— Healthcare Challenges of WMD II of the Military Medical Faculty Hradec Kralove, University of Defence, Czech Republic (project no. DZRO-FVZ22-ZHN II).

KDM5 IN CHEMORESISTANCE OF NEUROBLASTOMA (NBL) CELLS.

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A significant challenge in the treatment of cancer is chemoresistance. Drug resistance is caused by various mechanisms: inhibition of cell death, altered expression of drug transporters, changes in drug metabolism, alterations in drug targets and increasing DNA repair. Histone methylation plays an important role in regulation of expression, its dysregulation has been observed in various cancers and is associated with chemoresistance. Histone methylation is mediated by histone methyltransferases- add methyl groups to arginine and lysine residues, and lysine demethylase (KDM)- remove them. A lot of information has been published about the relationship of individual KDM to the chemoresistance of cancer. The NBL lines and their derived CDDP and DOXO resistant lines were screened for KDM5DA-D mRNA. It has been demonstrated that there is a loss of KDM5D expression and a reduction of KDM5B expression in chemoresistant NBL lines (in KDM5D with genotype XY, the gene is at Y) to both CDDP and DOXO. Incubation with cytostatic reduced both KDM5B and D expression in sensitive cells at both mRNA and protein levels. Knock down of KDM5B as well as D reduced H3K4 acetylation (flow cytometry), demonstrating that KDM5B and D function cannot be fully replaced by other KDMs. KDM5B and KDM5D silencing increase proliferation (xCELLigence) and migration (wound healing assay, xCELLigence), and modulate cell cycle (flow cytometry) in resistant cell lines. KDM5B and D knock-down increases sensitivity of resistant cells to CDDP, whereas artificial expression of KDM5D induces resistance to CDDP. Finally, we examined the expression of the gene *CUL4A*. Sensitive cell lines showed lower expression of *CUL4A* than resistant ones and its expression increased after silencing of KDM5D or CDDP treatment in sensitive but not in resistant cells, which do not express KDM5D. It suggests that CDDP affect CUL4A expression *via* KDM5D. Low KDM5D and high CUL4A expression correlate with poor survival in male NBL patients (public database analysis). We

hypothesize that KDM5D and KDM5B may serve as marker of chemoresistance, and CUL4A inhibition could be used as a therapy for NBL, after further studies.
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APPLICATION OF THE CRISPR/CAS9 METHOD IN THE PREPARATION OF MPO-DEFICIENT HEMATOPOIETIC PROGENITOR CELLS

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Myeloperoxidase (MPO)-ANCA glomerulonephritis is a rare autoimmune disease that is characterized by a loss of immune tolerance, leading to the production of anti-neutrophil cytoplasmic antibodies (ANCA) targeting the neutrophil-derived protein MPO. Affected individuals experience severe inflammation of small blood vessels, resulting in impaired kidney function that can progress to kidney failure. Current treatment typically involves a combination of glucocorticoids with other immunosuppressive agents (e.g. methotrexate, rituximab or cyclophosphamide).[1] One very potential treatment option is the use of CRISPR/Cas9 in the preparation of cMPOko.
The primary objective of our study was to prepare MPO KO using the CRISPR/Cas9 method. To optimize electroporation and gRNA parameters for the most efficient transfection, the 32D cell line was initially used. The transfection efficiency on the 32D cell pool ranged from 65-89% for each setup, which was determined by Sanger sequencing. To verify successful MPO KO, an MPO KO clone was selected by sorting the transfected clones and subsequently verified at the protein level by Western blot and MPO activity assays for the absence and non-function of MPO. After successful preparation of MPO KO on 32D cells, this approach was transferred to lineage-negative (Lin-) cells isolated from mouse (CD45.1 strain) bone marrow. Flow cytometry was used to analyze cell populations before and after isolation. After further optimization, MPO-deficient Lin-cells were successfully generated and validated at the DNA level. These transfected cells were prepared for subsequent transplantation into an ANCA-mouse (CD45.2 strain) model.
These results show preliminary data for novel potential therapy of MPO-ANCA glomerulonephritis with use of CRISPR/Cas9 method to make MPO deficient neutrophil in effected individuals with aim to reduce pathological immune response.
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INHIBITION OF LACTOSYLCERAMIDE SYNTHASES B4GALT5 OR B4GALT6 ENHANCES OXALIPLATIN-INDUCED CELL DEATH IN COLON CANCER CELLS

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Glycosphingolipids (GSLs) are an important group of bioactive lipids that serve as both structural components of membranes or signaling molecules involved in regulation of numerous biological processes including cell growth and death. They have been found to be deregulated in colon cancer cells, with a potential role in modulation of disease progression. Lactosylceramide (LacCer) is a key intermediate in GSL metabolism and an important precursor of more complex GSLs. We evaluated the impact of inhibition of LacCer synthesis (using selective targeting of specific LacCer synthases, B4GALT5 or B4GALT6 by CRISPR/Cas9 gene knockout) on control of human colon adenocarcinoma cell death. The efficacy of the downregulation/inhibition of selected enzymes was verified by decrease in respective mRNA (RT-qPCR) and protein (Western blotting) levels, and reduced levels of LacCers and several more complex GSLs (LC-MS/MS) in LacCer synthase-deficient cells. We found that downregulation of B4GALT5 or B4GALT6 enhanced the DLD-1 colon cancer cell sensitivity to the cytotoxic effects of oxaliplatin, a drug frequently used in colorectal cancer treatment. This was demonstrated by a general decrease in cell viability (resazurin assay), an enhanced apoptosis/cell death (annexin V/PI assay, flow cytometry), caspase-8, -9, -3 activation, cleavage of caspase substrates (specific antibodies, flow cytometry or Western blotting) or stimulation of other cell death-related markers. Our findings support the functional roles of LacCer synthases in the regulation of chemosensitivity of colon cancer cells. [This study is supported by the Czech Ministry of Health, grant no. NU21-03-00421.]

CIRCULATING TUMOR CELLS-DERIVED XENOGRAFT AS A MODEL OF CASTRATION-RESISTANT PROSTATE CANCER

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Prostate cancer remains one of the most common cancers in men worldwide. There is a need for models that will make it easier to study the metastatic stage of this disease. The present study describes a unique model of advanced-stage prostate cancer derived from circulating tumor cells from a patient with metastatic castration-resistant prostate cancer (mCRPC). A detailed immunohistochemical analysis of the developed CTCs-derived xenograft (CDX_IBP_02) and matched needle biopsy from the primary tumor revealed strong expression of the neuroendocrine marker synaptophysin, simultaneously with the expression of luminal markers (AR, PSA, NKX 3.1), indicating that the developed xenograft represents a rare type of amphicrine prostate cancer. To investigate the phenotype of the established xenograft, a multiparametric panel of selected surface markers was examined using spectral flow cytometry. Whole-exome sequencing and proteomic analyses have been carried out to further characterize this model. Finally, we verified the sensitivity of our model to surgical castration or to the AR inhibitor enzalutamide *in vivo*. In addition, given the fact that the xenograft is positive for the surface marker Trop2, the effect of an antibody-drug conjugate, sacituzumab govitecan, was examined on tumor growth *in vivo*. In conclusion, our CTC-derived xenograft from a patient with mCRPC represents a unique model of advanced prostate cancer, which can be used for further studies of the metastatic stage of the disease.
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POTENTIAL OF TARGETING NLRP3 INFLAMMASOME IN NON-SMALL CELL LUNG CANCER: INSIGHTS FROM 2D AND 3D *IN VITRO* MODELS

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The NLRP3 inflammasome plays a central role in promoting inflammation and has recently been implicated in the progression of various cancers. Non-small cell lung cancer (NSCLC) is characterized by a highly inflammatory tumor microenvironment (TME), with *NLRP3* gene alterations present in more than 15% of cases. Additionally, chronic inflammation in NSCLC is associated with poor clinical outcomes, yet the precise role of NLRP3 remains insufficiently explored.

In this study, we aimed to investigate the impact of NLRP3 targeting on tumor cell behavior using both 2D and 3D *in vitro* models of NSCLC, with a particular focus on cell viability, spheroid formation, and TME modulation, especially in relation to immune cells. The experiments were conducted on the A549 NSCLC cell line, which overexpresses NLRP3, and where we confirmed chronic activation of the NLRP3 inflammasome. The spheroids were formed in ultra-low attachment plates, and NLRP3 was targeted through two complementary approaches: gene silencing via siRNA transfection and pharmacological activation of NLRP3 inflammasome using the small-molecule inhibitor MCC950. To comprehensively assess the effects of NLRP3 targeting, we employed flow cytometry, RNA sequencing, immunostaining, and microscopy. Additionally, we developed an *in vitro* protocol to study the impact of NLRP3 on immune cell infiltration into spheroids.

Our data demonstrate that NLRP3 is essential for long-term cell survival of A549 cells, and NLRP3 expression and activation promote the expression of immune checkpoint molecules (PD-L1, PD-L2) and epithelial-mesenchymal transition (EMT) markers (CXCR4). These findings suggest that NLRP3 contributes to NSCLC progression by supporting immune evasion, EMT, and cancer cell survival. Targeting NLRP3 may thus offer a promising new strategy for improving NSCLC treatment outcomes.

PRENATAL EXPOSURE TO ENVIRONMENTAL CHEMICALS AND THE IMPACT ON IMMUNITY IN EARLY CHILDHOOD

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Poly- and perfluoroalkyl substances (PFAS) are environmental chemicals linked with various adverse health outcomes, the most significant of which is immunomodulation. Exposure to PFAS begins during prenatal development, when the immune system undergoes rapid development and is especially vulnerable to environmental chemicals. Even subtle disturbances in immune cell differentiation or maturation during this period may have lifelong implications.
The project explores the impact of maternal PFAS exposure on newborns' immunity by examining immune cell subpopulations in the umbilical cord blood. Analysis of maternal exposure and isolation of cord blood mononuclear cells (CB-MNCs) from newborns was performed in a subgroup of mother-child pairs from CELSPAC: TNG cohort (Brno, Czech Republic, n=600+ pairs).
Immunophenotyping is conducted in two panels with shared backbone markers, using BD FACSymphony A1. Panels focus on T and B cell subpopulations, but available markers and clustering tools allow for identifying over 25 distinct populations.
The project aims to determine the impact of maternal exposure to PFAS (as well as chemical mixtures) on the immune profiles of newborns. Further, the project will investigate children later in life to link prenatal exposure, immune cell profiles, and immune-mediated diseases.

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CAN PERORAL ADMINISTRATION OF HIGH-MOLECULAR WEIGHT HYALURONAN AND PECTIN AFFECT T CELL SUBSETS IN MICE?

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Hyaluronic acid (HA) is a nonsulfated glycosaminoglycan composed of repeating disaccharide units: glucuronic acid and N-acetylglucosamine. It is an important hydrophilic component of extracellular matrix with multiple roles. High-molecular weight HA (HMW HA) is thought to have anti-inflammatory and anti-fibrotic properties. Due to its beneficial properties, biodegradability, and biocompatibility, HMW HA is included in medical devices and joint dietary supplements. Oral administration of HMW HA has been shown to reduce intestinal inflammation in mouse models of chemically/pathogen-induced colitis, linked to alteration in gut microbiota. Moreover, it was observed that imbalance of microbiome affects immune system, especially T cells, which are key players of adaptive immunity.
The aim of this experiment was to detect T cells subsets after peroral administration of HMW HA in drinking water to healthy mice for two weeks in comparison to another polysaccharide, pectin. Samples were prepared from lymph nodes, stained using a 12-colour panel and analysed on spectral cytometer BD FACSDiscover S8 Cell Sorter. The basic T cells subsets, including helper ($CD3^+$, $CD4^+$), cytotoxic ($CD3^+$, $CD8^+$), regulatory ($CD3^+$, $CD4^+$, $GITR^+$, $CD25^+$), and natural killer T cells ($CD3^+$, $CD161^+$) were analysed together with further specific identification of T cell maturation defined by CD62l and CD44 expression: naïve ($CD62l^{high}$, $CD44^{low}$), central memory ($CD62l^{high}$, $CD44^{high}$) and effector memory ($CD62l^{low}$, $CD44^{high}$) T cells. Additionally, KLRG1 expression was used to discriminate short-lived effector cells ($KLRG1^+$) from memory precursor effector cells ($KLRG1^-$).
Obtained data show that percentage of helper, cytotoxic and regulatory T cells is not changed, however the presence of natural killer T cells is significantly decreased after HA and pectin administration. In conclusion, peroral administration of HMW HA and pectin induces changes in composition of T cell subsets.

MUTUAL EPITOPE INTERFERENCE BETWEEN ANTI-CD3 AND ANTI-TCR ANTIBODIES IN FLOW CYTOMETRIC ANALYSIS

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CD3 is an integral component of the T cell receptor (TCR) complex, physically associated with both α/β and γ/δ TCR heterodimers. In Exbio, we investigated the epitope accessibility of anti-TCRα/β, anti-TCR Cbeta1, anti-TCR Cbeta2 and anti-TCRγ/δ antibodies following preincubation with anti-CD3 antibodies. Using multicolor flow cytometry on human peripheral whole blood, we demonstrate that anti-CD3 binding sterically hinders subsequent recognition of TCR-specific epitopes by their respective antibodies. This mutual interference has important implications for the design and interpretation of immunophenotyping panels, particularly when aiming to quantify minor T cell subsets or inspecting TCR Cbeta clonality. Our findings highlight the necessity of optimizing staining protocols and antibody sequences to avoid competitive blocking in multiparametric cytometry.

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

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Tumor necrosis factor-stimulated gene 6 (TSG-6) is a multifunctional protein primarily secreted at inflammatory sites, where it has been found to play a crucial role in immune regulation. While TSG-6 has been shown to influence immune responses at both cellular and cytokine levels, its effects on the adaptive immune system, particularly on T helper (Th) cells and their activation mechanisms, remain largely unexplored. We show that TSG-6 modulates Th cell activation and spontaneous differentiation, driving distinct immune responses. We observed the promotion of both regulatory T cells and inflammation-associated subsets. Notably, TSG-6 did not significantly alter T cell migration. With that, we propose that hyaluronan (HA) may be the main mediator of the interaction between TSG-6 and T cells. Gene expression, complemented by confocal microscopy, showed endogenous production of HA by T cells, albeit in low amount, and confirmed its surface expression, respectively. Finally, effects of HA degradation and presence of other potential ligands, such as CD44 receptor, on described effects are underway. Together, these findings highlight the potential of TSG-6 as a therapeutic agent in Th cell-mediated pathological processes, offering new immunomodulatory strategies.

THERAPEUTIC POTENTIAL OF NANOBODIES TARGETING THE TUMOR ANTIGEN CAIX

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Cancer cells face insufficient oxygen after reaching a certain tumor size, leading to the upregulation of genes controlled by hypoxia-inducible factors to promote survival in low-oxygen conditions. These include genes encoding glycolysis enzymes, angiogenesis-promoting proteins, and pH-regulating proteins. Carbonic anhydrase IX (CAIX) is a hypoxia-regulated transmembrane protein that maintains optimal intracellular pH by hydrating carbon dioxide, leading to tumor microenvironment acidification.
There is growing evidence that combinational immunotherapy could enhance therapeutic efficacy and improve patient clinical outcomes. Antibody-dependent cytotoxicity (ADCC) utilizes the response of innate immune cells, such as NK cells, to provide antitumor cytotoxicity triggered by the interaction of the antibody's Fc with the immune cell's Fc receptor.

Nanobodies are widely investigated as therapeutics due to their many advantages, such as small size and higher tissue penetration. Therefore, we aimed to study the therapeutic potential of four CAIX-specific nanobodies fused to human Fc.
Using immunofluorescence staining, we confirmed the specific binding of these nanobodies fused to Fc to hypoxia-induced tumor antigen CAIX. Additionally, we detected a significant increase of CAIX-specific nanobodies bound to cancer cells exposed to hypoxia compared to normoxic cells using flow cytometry analysis. Crucially, our pilot experiment showed the potential ability of our nanobodies to increase the cytotoxicity of primary peripheral blood mononuclear cells (PBMCs) towards cancer cells compared to the cytotoxic effect of PBMCs without nanobodies, suggesting the role of ADCC in boosting therapeutic efficacy. In the future, we will confirm the observed impact of nanobodies fused to Fc on NK cell-mediated cytotoxicity with NK cell lines.
Our findings indicate the potential of our CAIX-specific nanobodies as tools for targeted immunotherapy via the ADCC mechanism.

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REGULATION OF PROLIFERATION DURING CARDIOMYOGENESIS

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Cardiomyogenesis, the formation of myocardium during embryonic development, involves intensive proliferation of cardiomyocytes and their progenitors. In contrast, the adult myocardium has minimal regenerative capacity, making it unable to effectively repair damage after a heart attack. In principle, pluripotent stem cells represent an unlimited source of new cardiomyocytes, but in practice, generating a therapeutically relevant number of cardiomyocytes is time-consuming and inefficient. To improve cardiomyocyte yield, we aim to identify factors that would promote proliferation along the cardiomyogenic lineage. As a first step, we need to analyze the inherent dynamics of proliferation during cardiomyocyte differentiation.
We analyzed the frequency and absolute number of cardiomyocytes and their progenitors. Encouragingly, the results from our *in vitro* model closely matched data from *in vivo* studies. We also examined cell cycle dynamics and found that cardiac progenitors proliferate most at the beginning of differentiation, interestingly even more than other cells in the population. And as differentiation progresses, cardiomyocyte proliferation decreases. Our analysis of publicly available scRNA-seq data suggests that this decrease may result from a shift in regulation of proliferation during specification of cardiomyocytes into atrial or ventricular subtypes. Ventricular cardiomyocytes maintain Cyclin D2

expression and remain capable of proliferation, while atrial cardiomyocytes preferentially express Cyclin D1, which in cardiomyocytes is linked to hypertrophic growth.
In conclusion, our findings point to a narrow window early in differentiation when cardiomyocyte proliferation is at its peak and most responsive to regulation. Targeting this period could enhance cardiomyocyte yield and improve the efficiency of cardiomyocyte production for research and therapeutic applications.

BIOCOMPATIBILITY OF CMC-PEG HYDROGELS AND THEIR USE AS DRUG VECTORS

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Hydrogels based on natural compounds are popular in biomedicine as they are naturally biocompatible, and economically and ecologically viable. Besides their use as scaffolds in the regenerative medicine, they could be also used as vectors for deliverance of different agents. Well known is their use as a wound dressing bearing antibiotics and healing growth factors (Zhu et al. 2024), however, our aim is to prepare hydrogels that could serve as vectors of chemotherapeutic drugs. The idea is to mix the sol version of hydrogel with the drug and crosslinking agent, inject it in the vicinity of tumour, where it solidifies and slowly releases the drug. We used carboxymethyl cellulose (CMC) hydrogel cross-linked with polyethylene glycol (PEG) in different concentration. Fibroblast and pancreatic carcinoma cells had the best proliferation in the 8 % CMC-PEG hydrogel, however, it was the most viscous one. We expected the gel to harden by addition of calcium ions and that it will then float in the DMEM media. However, this never happend, so we needed to use the cell crown inserts with PET membrane, in which we put the hydrogel with mixed campthotecin (CPT). CPT is the chemotherapeutic inducer of apoptosis via inhibition of topoisomerase I (Venditto and Simanek 2010). During 3-days-long experiment we observed decrease in cell viability caused by CTP released from hydrogel. Never the less, the toxicity of released CPT was lower than the original dose added to the cells directly, so we can assume that the release of CPT from CMC-PEG hydrogels is slow and continuous and thus it is favourable for its potential use as the “on site” chemotherapeutic agent.

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GUT MICROBIOTA DETERMINES THE BIOAVAILABILITY AND METABOLIC FATE OF ORALLY ADMINISTERED HYALURONAN

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Hyaluronan (HA), a polysaccharide used widely as a dietary supplement for its anti-inflammatory properties and benefits on skin and joint health, demonstrates unclear bioavailability and metabolic fate when orally administered. We employed stable isotope (13C)-labelled HA coupled with LC-MS and MALDI-MS imaging analyses in conventional and germ-free mice models to unravel its intestinal absorption and systemic metabolism. Our results reveal a significant role of gut microbiota, particularly Bacteroides species, in cleaving HA into unsaturated oligosaccharides (<3 kDa), essential for intestinal absorption. These fragments exhibited limited systemic bioavailability (~0.2%), predominantly as unsaturated disaccharide. High-resolution size exclusion chromatography documented progressive MW reduction across the gastrointestinal tract, microbiota-dependent, culminating in complete bacterial metabolism to short-chain fatty acids (SCFAs). Imaging analyses confirmed low-MW oligosaccharides' intestinal epithelial penetration but failed to show absorption of intact high-MW HA. Metabolomic profiling revealed substantial bacterial conversion to SCFAs, which may mediate HA's beneficial systemic effects rather than direct transport to distal tissues. Collectively, our cytometric and single-cell analytical approaches elucidate the microbiota-driven metabolism and systemic bioavailability of HA, providing insights into its therapeutic mechanisms and potential optimization strategies.

PHENOTYPIC PROFILING OF T CELL SUBSETS IN PHILADELPHIA-NEGATIVE MYELOPROLIFERATIVE NEOPLASMS

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Background: In Philadelphia-negative myeloproliferative neoplasms (Ph^- MPN), $CD4^+$ and $CD8^+$ T cells show chronic activation, exhaustion, and shifts toward Th2, Th17, and regulatory subsets, fostering immune suppression. These alterations, together with changes in memory and terminally differentiated populations, may influence disease course and therapy response. Integrating immune profiling with JAK2 allele burden monitoring could improve understanding of treatment effects and identify novel immunomodulatory targets.

Aim: To analyze CD4+ and CD8+ T cell subpopulations in Ph^- MPN patients, assess their correlation with clinical parameters, and compare them to healthy controls (HC).
Methods: Peripheral blood from Ph^- MPN patients (n = 84) and HC (n = 50) was stained with CD45RA-PB, CD279-BV480, CD161-BV605, CD3-FITC, HLA-DR-PerCP, CD197-PE, CD25-PE-DL594, CD196-PC7, CD4-APC, CD127-APC-AF700, and CD8-APC-AF750, and analyzed on a four-laser flow cytometer (Omnicyte, Cytognos). Data were processed in FlowJo (BD). Patients were stratified by therapy (interferon-α, ruxolitinib, other, untreated), mutation status (JAK2, CALR), and clinical parameters. JAK2V617F allele burden was quantified from granulocyte DNA using qPCR. Statistical analysis was performed in R.
Results: Compared to HC, Ph^- MPN patients showed broad disturbances in $CD4^+$ and $CD8^+$ T cell compartments, with evidence of skewed differentiation, altered activation, and impaired memory formation. These immune perturbations were largely consistent across $JAK2^+$ and $CALR^+$ patients. Current therapies only partially normalized T cell profiles: interpheron-alpha tended to increase regulatory subsets, while ruxolitinib affected differentiated effector populations. Clinical features such as splenomegaly or JAK2 allele burden had little impact on T cell composition.

Conclusions: High-dimensional cytometric analysis revealed marked T cell dysregulation in Ph^- MPN, only partially modulated by therapy. These results support T cell subsets as potential biomarkers for monitoring immune imbalance and therapeutic response.

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EFFECT OF SELECTED CYTOKINES ON ENDOMETRIAL RECEPTIVITY

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Despite many years of research, the percentage of successfully implanted embryos during *in vitro* fertilisation (IVF) process remains low, indicating a significant number of patients with non-receptive endometrium. Although tests for receptivity are used in clinical practice, the underlying mechanism of endometrium non-receptivity is still not clearly understood. Consequently, no targeted therapies are currently available. The receptivity of the endometrial epithelium is regulated by different factors including hormones and immune cells` secretome. Thus, modulation of the receptivity by selected cytokines was investigated.
First, we re-analysed transcriptomic data from GEO database to confirm the expression of selected cytokine receptors in RL95-2 cell line, as representative of endometrial epithelium. Next, we used Jar cell line as trophoblast model in a functional receptivity assay to test effect of selected cytokines on RL95-2 receptivity. Adhesion rate of Jar cells (stained with Calcein AM) was assessed by fluorescence microscopy. Finally, we analysed the effect of selected cytokines on gene expression (RT-PCR), protein expression (flow cytometry) and localisation of integrin αvβ3 and E-cadherin as a key receptivity markers.
We confirmed expression of IFNGR1, IFNGR2, TNFR1, IL-6R and IL-1R1 receptors in the RL95-2 cell line. Corresponding cytokines demonstrated effect in modulating of endometrial receptivity. IL-6 and IL-1β increased RL95-2 cell receptivity, while IFN-γ decreased it. TNF-α enhanced receptivity at low concentrations but reduced it at higher doses. These effects correlated with *ITGB3* expression. IL-6 and IL-1β had minimal impact on E-cadherin, whereas TNF-α and IFN-γ reduced *CDH1* expression and disrupted its membrane localization.
These results highlight the importance of immunological factors in modulation of endometrial receptivity. Understanding the mechanisms and effect of immune cells-derived cytokines in this process may offer novel therapeutic approaches with the aim of improving IVF success rates.

OVERVIEW OF SOME MARKERS IN CELL LINES OF PATIENTS WITH GLIOBLASTOMA MULTIFORME

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Glioblastoma (GB) is the most common and aggressive primary brain tumor in adults with with a very poor prognosis. Conventional treatment focuses on temporary removal of the tumor but is often followed by recurrence.
A partial aim of this study was to prepare a comprehensive portfolio of primary cell cultures from patients and characterize their selected surface markers for the study of new drugs. New drugs will behave quite differently on primary patient cell lines than immortalized commercial cell lines. The multifaceted behavior of patient cells will already incorporate the difficulties of newly introduced drugs in vitro and will help to select only truly potentially effective agents. Another aim was to determine whether galectins 3, 8 and 9 can be used as prognostic markers.
We present an overview of the primary patient lines and a few selected markers such as p53, TRA-1-60, A2B5, MGMT, the aforementioned galectins and other markers generally associated with glioblastomas. Most of our samples have low expression of p53, TRA-1-60 and A2B5. However, some markers behave quite erratically in our samples and have expression levels ranging from virtually 0 to 100%, such as MGMT and VEGFR and PDGFRα and PDGFRb. As far as galectins are concerned, only galectin 8 appears promising so far, with the best coming directly frompatient blood. However, these results are not yet as pronounced in patient cell cultures.

GADOLINIUM DOPED CARBON DOTS AS A THERANOSTIC AGENT OF NEURODEGENERATIVE DISEASES

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Carbon-related dots (CDs) are a family of highly studied nanomaterials, which show excellent photo- and chemical stability, good biocompatibility, and high water solubility. They have gained tremendous attention as they could be used as drug delivery systems in therapy of many diseases. Using CDs as theranostics agents links their therapeutic potential and their ability to be visualized for diagnostic purposes (Ray et al. 2021). In this work, we will focus on Alzheimer´s disease, which is accompanied by accumulation of amyloid fibres. We tested gadolinium-doped carbon dots (Gd-CDs) that emit the most in violet and ultraviolet spectrum. Gadolinium is mostly used as a contrast agent in magnetic resonance imaging (MRI) (Caravan 2008). Its binding to the CDs core makes it a versatile agent for both fluorescence imaging and MRI applications. Recently, we have discovered that CDs have the ability to prevent the building of amyloid fibres and they usually have

good penetrability through the ABC transporters´ rich blood-brain barrier (Gomez Perez et al. 2024). Herein, we proved that Gd-CDs are really not substrates of major ABC transporters, so their crossing of the barrier should be not hindered. We also confirmed the ability of Gd-CDs to penetrate the mouse glioma cell line GL261 via endocytosis and their perinuclear co-localisation with mitochondria. Importantly, by Thioflavin T assay we found out that the Gd-CDs can inhibit the polymerisation of amyloid fibres. To sum up, in this work we characterized the new potentially powerful theranostic agent for Alzheimer's disease.

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ASSESSMENT OF HUMAN SPERM PATHOLOGY BY FLOW CYTOMETRY USING A PANEL OF SIALIC ACID BINDING LECTINS

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Mammalian sperm glycocalyx, composed of glycoproteins and glycolipids, plays a critical role in several reproduction-related processes. Lectins, which detect specific glycan variations (isomers, linkages, terminal modifications), serve as valuable tools for studying this structure. In this study, we used fluorescent (FITC) sialic acid binding lectins (SALs) in flow cytometry to profile human sperm glycocalyx alterations in the context of sperm pathology, focusing on differential sialic acid linkage recognition. SALs (WGA, SNA I and MAL I) were used in sperm samples from 7 normospermic (N), 11 teratospermic (T), 6 asthenoteratospermic (AT) and 9 oligoasthenoteratospermic (OAT) ejaculates, with simultaneous staining by propidium iodide (PI) determing sperm viability. Samples were analysed by FACS Canto II cytometer (BD Biosciences), the data were processed using Floreda.io (free web-based software). Non-sperm events were excluded from the analysis based on FSC/SSC gating, and two-dimensional plots of FITC versus PI fluorescence events were drawn. Individual subpopulations were divided by gates (viable; non-viable: pre-

moribund, moribund, dead) and quadrants (positive and negative events) and statistically evaluated using GraphPad Software. Significantly higher percentages of SNA I-positive spermatozoa were observed in all non-viable subpopulations compared to viable spermatozoa mainly in N ($p \leq 0.01$) and T ($p \leq 0.001$) ejaculates. Similarly, a significantly higher abundance of MAL I positivity was also detected in non-viable compared to viable spermatozoa in all ejaculates (N: $p \leq 0.0001$, T: $p \leq 0.0001$; AT: $p \leq 0.01$; OAT: $p \leq 0.01$). In the case of WGA in non-viable sperm subpopulations, the percentage of positive sperm remains unchanged in all groups tested (N, T, AT, OAT). SNA I and MAL I positivity, probably related to a disrupted sperm plasma membrane, create a prerequisite for their use in flow cytometric assessment of ejaculate quality.

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50 SHADES OF FLUORESCENCE TO FOLLOW IMMUNE RESPONSE (SAFFIR) WITH SPECTRAL FLOW CYTOMETRY.

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Whooping cough persists as an endemic disease (40million cases and 300000 child death per year worldwide) even in vaccinated populations. This disease is caused by the gram(-) bacterium *Bordetella pertussis*. This bacteria expresses multiple virulence factors acting in concert to facilitate its adherence, survival and proliferation in human respiratory tract. More, it has been shown, in a mouse model, that CD4+ Trm are generated during the time course of infection1. Our objectives is to decipher the cellular mechanisms underlying the "cross-talk" between antigen presenting cells and T cells that allow the differentiation, maintenance and function of Trm at the tissue level. To do this aim we developped a 48 markers panel to follow the behavior of several immune populations in the mean time in the same tube. Mice were infected with a wild strain of *Bordetella pertussis* (Tahoma I). 15 days later they were sacrificed. After intra-cardiac perfusion with PBS, collected lungs were digested with a mix of Collagenase IV/DNAse I for 30 minn at 37C. Lung cells were seeded at 3x106 cells/well and stained in PBS, 2mM EDTA, 0,5% BSA, 40% brilliant violet stain buffer(BD Biosciences) and 10% Fc block at 37oC for 45mn. Cells washed 3 times before staining in PBS + live/dead marker (Zombie NIR) for 15mn. After 3 washes cells were resuspended in PBS for analysis on Spectral Cytometer Aurora (Cytek Biosciences). Data analysis were performed using OMIQ.

THE ROLE OF ENDOPLASMIC RETICULUM STRESS IN IMMUNE SURVEILLANCE AND ITS RELEVANCE FOR CANCER IMMUNOTHERAPY

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Ovarian cancer (OC) remains the most deadly gynaecological malignancy, with a limited five-year survival rate of less than 30% in advanced disease. Immunotherapy offers promising potential, but clinical responses are often hindered by immune evasion and resistance mechanisms. Crosstalk between immune (IC) and cancer cells (CCs) in the tumor immune microenvironment (TIME) promotes immunosuppression. Disruption of endoplasmic reticulum (ER) homeostasis leads to chronic ER stress (ERS), which promotes tumor characteristics in CCs and impairs IC function. Stressed CCs can also transmit ERS to nearby non-tumor cells—termed transmissible ER stress (TERS)—a recently described mechanism contributing to cancer progression. Therefore, a deeper understanding of the tumor immune microenvironment (TIME) is crucial for improving treatment outcomes.

In our study, we primarily focused on TERS mechanisms. Peripheral blood mononuclear cells (PBMCs) exposed to this TERS conditions showed reduced viability and altered effector profiles, as confirmed on gene expression as well as protein levels. Co-treatment with the ER stress inhibitor TUDCA significantly restored PBMC function, suggesting that TERS is a modifiable barrier to immune surveillance in OC. In parallel, we investigated the role of TUSC3, a gene implicated in ER homeostasis and tumor suppression in OC context. Our findings indicate a link between TUSC3 expression, immune evasion, and therapy resistance. Moreover, mass spectrometry-based techniques (MALDI-TOF MS) may support its use as a predictive biomarker for patient stratification and treatment response.

Targeting ER stress pathways and understanding TUSC3-related mechanisms could enhance immunotherapeutic strategies and support personalized oncology.

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PURIFICATION OF GLIA FROM FRESH AND FIXED MOUSE BRAIN USING THE WOLF G2 CELL SORTER

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The brain is a heterogenous organ composed of different cell populations. Some of these cells are the neuroglia that include astrocytes, oligodendrocytes, and microglia. Neuroglia are known to have important functions in the brain such as modulation of homeostatic functions, myelination, nerve signal propagation, and responses to neural injuries1. The disruption in any one of these key functions is of importance in research because that can help explain mechanisms underlying a number of developmental and neurodegenerative diseases. Microglia typically account for less than 10% of the target cells in brain samples. Oligodendrocytes make up a target population of about 20% or higher, and astrocytes can make up between 17 to 61% of target populations3,4. Cell sorting can assist in these glial studies and enhance purification of individual cell types from diverse cellular populations, as found in the brain. In this work, the WOLF G2 Cell Sorter was used to sort astrocytes, microglia, and oligodendrocytes from fresh mouse tissue. In addition, a second sort was performed using fixed mouse brain due to the fact that researchers may not have access to fresh tissue or may have to flash-freeze the brain before the tissue can be processed.

IMMUNOMETABOLIC CHANGES IN AML CELL LINES SPHEROIDS AS A MODEL OF LEUKEMIC MICROENVIRONMENT

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The leukemic bone marrow (BM) niche supports immune evasion and therapy resistance in myeloid leukemia. To more accurately replicate this microenvironment in vitro, we use 3D spheroid cultures that integrate mesenchymal stromal cells (MSCs) and leukemia cell lines MOLM13 or THP1. This model exhibits several characteristics resembling the tumor microenvironment, providing an experimental advantage to traditional suspension cultures.
To compare the differences in the immunometabolic states of leukemic cells cultured in either suspension or 3D spheroids, we utilized high-dimensional metabolic flow cytometry techniques, including MetFlow and SCENITH. Our comparative analysis revealed significant metabolic reprogramming in the cells assembled into spheroids. Specifically, expression of glycolytic markers such as GLUT1, HK1, and LDH was significantly

upregulated in spheroids, indicating enhanced glycolytic activity. Additionally, ACAC (lipid metabolism), TKT (pentose phosphate pathway), and PRDX2 (oxidative stress response) were differentially regulated, suggesting broader metabolic adaptation of leukemic cells to the niche-like environment.
Our results underscore the critical impact of the microenvironment on leukemic cell metabolism and emphasize the importance of combining 3D in vitro models with advanced analytical cytometry techniques to identify new therapeutic vulnerabilities.
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SURFACE PROTEIN PROFILING OF CHILDHOOD B-CELL PRECURSOR ACUTE LYMPHOBLASTIC LEUKEMIA USING SPECTRAL FLOW CYTOMETRY

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Introduction:
B-cell precursor leukemia is the most common childhood cancer, with an overall survival rate of over 90% due to stratification, response monitoring, and advanced supportive care. Adopting approaches for minimal residual disease (MRD) monitoring for patients treated with targeted therapies is needed.

Methods:
In this study, using full-spectrum cytometry, we evaluated CDmaps (Kužlkova´ et al. Front Immuno 2022) - the expression of surface markers - (n=305) on BCP ALL cells obtained from thawed samples at diagnosis (n=15) or relapse (n=3). In this pilot phase, the following genotypes were included: DUX4r (6), ETV6::RUNX1 (11), BCR::ABL (1). One sample of CD19 negative relapse was analyzed (ETV6::RUNX1). We barcoded samples with anti-HLA-I antibodies for high-throughput analysis and used backbone antibodies to differentiate blast cells from non-malignant B cells. The marker expression was evaluated as a percentage of positivity and quantified as antibody bound per cell using the BD Quantitation kit.

Results:
We compared marker expression in CDmaps with fresh material from diagnosis or relapse using conventional flow cytometry (34 out of 305). No significant differences were found between fresh and thawed samples. We confirmed a specific expression signature in DUX4r (CD371pos, CD10 heterogeneous, CD34 bright). DUX4r cases showed higher expression of CD146 ($p < 0.003$), CD49c ($p < 0.02$), and CD66b ($p < 0.02$). In a CD19-negative relapse patient, we identified the expression of other B cell markers - CD22, CD24, CD72, and CD81.

Conclusion:
While barcoding works in other projects, BCP-ALL presented some challenges. Despite this, high-throughput flow cytometry is effective for protein profiling and MRD marker identification in childhood leukemias, though caution is needed for BCP-ALL. Promising markers will be further validated.

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POLYCYCLIC AROMATIC HYDROCARBONS (PAHS) ACTING AS AHR LIGANDS MAY ALTER GLUCOSE PRODUCTION AND/OR TRANSPORT IN HUMAN HEPATOCYTE-LIKE HEPARG CELLS

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Polycyclic aromatic hydrocarbons (PAHs) are widely distributed environmental pollutants that have been primarily studied as genotoxic and carcinogenic toxicants. However, many PAHs are also potent AhR ligands that can interfere with signaling pathways controlling immune and endocrine signaling. Using a set of PAHs representing both weak and strong AhR ligands among PAHs, we studied their effects on metabolism, in particular metabolism of glucose in differentiated hepatocyte-like HepaRG cells, prepared as 2D and 3D cultured variants. We found that exposure to benzo[*k*]fluoranthene (BkF) a potent AhR agonist, significantly decreased glucose release into the glucose-free medium in differentiated HepaRG cells at 2D cultivation conditions. Simultaneously, mRNA levels of phosphoenolpyruvate carboxykinase 1 (PCK1), rate-limiting enzyme in both gluconeogenesis and glyceroneogenesis, were strongly reduced upon BkF exposure. At the

same time, BkF reduced mRNA levels of SLC2A2/GLUT2 (a major glucose transporter in liver cells) and SLC2A9/GLUT9 (which acts as a transporter of uric acid, glucose and fructose). Using both 2D and 3D (Hepoid®) variants of cultivation of HepaRG cells, we evaluated import of fluorescent 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxyglucose (2-NBDG) using fluorescence microscopy and flow cytometry. The results suggested that incubation of HepaRG cells significantly reduced glucose import. Collectively, these results suggest that exposure of liver cells to BkF and other potent AhR ligands among PAHs may impair their ability to produce and/or transport glucose. These findings suggest the need to study more deeply the relationship between PAHs (and their metabolites), AhR activation and impaired intracellular glucose metabolism and transport in liver cells. [Funded by the Czech Science Foundation, project no. 24-10086S.]

GENOME DOUBLING AND BEYOND: ENDOREDUPLICATION DYNAMICS IN POLYPLOID PLANTS UNDER STRESS

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Endoreduplication, the repeated duplication of the genome without subsequent cell division, plays a major role in plant development and its higher levels are often associated with stress response. In *Arabidopsis arenosa*, a marked decrease in endoreduplication has been observed in multiple naturally occurring autotetraploid populations. However, it remains unclear whether this is a direct consequence of whole genome duplication (WGD) or of subsequent evolutionary processes. To disentangle these effects, we compared natural diploids, natural established tetraploids, and synthetic neotetraploids generated from a diploid population via oryzalin treatment. Plants were grown under control (high water) and stress (low water) conditions to assess environmental effects.
We found significant differences among cytotypes. Diploids and neotetraploids showed nearly identical endoreduplication indices – calculated as the average number of endocycles *per* cell – while natural tetraploids had significantly reduced values. As a result, average DNA content in leaf tissue was higher in neotetraploids than in both diploids and natural tetraploids. This indicates that WGD alone does not reduce endoreduplication; rather, the decrease observed in natural tetraploids likely results from post-WGD evolution. In contrast, neither water availability nor its interaction with ploidy had a significant effect on endoreduplication. Thus, endoreduplication levels appear stable under drought stress across all cytotypes.
Our study suggests that reduced endoreduplication in natural *A. arenosa* tetraploids reflects evolutionary changes following WGD, not WGD itself. While often linked to stress responses, endoreduplication in this case remains unaltered by water availability. Whether this shift represents adaptation or a neutral consequence of genome duplication remains to be clarified.

RADIATION-INDUCED FIBROTIC DAMAGE, EPITHELIAL-MESENCHYMAL PLASTICITY AND HYALURONIC ACID NANOPARTICLES

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Ionizing radiation-induced fibrotic damage is a form of chronic tissue injury characterized by scarring and thickening of otherwise healthy tissue, a condition known as fibrosis. The severity of radiation-induced organ fibrosis (RIOF) is influenced by several factors, including the radiation dose, exposure regimen, and an individual's genetic susceptibility. The radiosensitivity of various organs differs based on their specific characteristics. Depending on the affected organ, RIOF typically presents clinically between 4 to 12 months after radiation exposure and may continue to progress over several years. However, the underlying molecular and cellular processes that drive fibrosis are activated within the first few days after exposure.

A key mechanism driving fibrosis, not only in response to irradiation, is the differentiation of normal fibroblasts into pathological myofibroblasts through a process known as epithelial-mesenchymal transition (EMT), more recently described as epithelial-mesenchymal plasticity (EMP). This process is accompanied by an increase in the production of pro-fibrotic cytokines, particularly transforming growth factor-β (TGF-β). During EMT, epithelial cells lose their polarity and intercellular junctions, undergo shape changes, increase extracellular matrix production, lose dependence on the basement membrane, become more motile, and acquire resistance to apoptosis. The completion of EMT is marked by degradation of the basement membrane and the emergence of migratory cells.

Treating radiation-induced fibrosis remains a significant challenge. Although several therapeutic strategies exist, their effectiveness is often limited by the high toxicity of the agents involved. A promising new approach involves the use of nanoparticles, such as hyaluronic acid nanoparticles (HANPs), which have shown beneficial effects in mitigating fibrosis. Our research focuses on targeting RIOF and EMP using HANPs. Preliminary results demonstrate that HANPs influence cellular behavior in both human and mouse cell lines after irradiation, particularly during the active phase of EMP. HANPs play a significant role in regulating cell behavior during morphogenesis and tissue repair processes associated with inflammation, injury, and healing, making them a promising tool for treating RIOF.

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

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Acute myeloid leukemia (AML) is a malignant hematological disorder characterized by the aberrant expansion of myeloid progenitor cells in the bone marrow and peripheral blood. The properties of the AML microenvironment define the ability of the immune system to control AML. In this environment, the phenotype of immune and leukemia cells is shaped by cell-to-cell interactions and by the production of soluble proteins, including cytokines and soluble receptors.

In this work, we combine the phenotyping of immune and leukemia cells using a 36-fluorescent-parameter spectral flow cytometry panel with the measurement of soluble plasma protein levels using the cytometry-based LEGENDPlex assays. We present data obtained from peripheral blood samples of 99 patients at diagnosis and, if available, remission together with 29 healthy donors.

We detected altered phenotype of immune effector cells in the AML microenvironment. Downregulation of activating receptors NKG2D and DNAM-1 impairs capability of immune cells to eliminate AML cells. Activation of immune cells may be further inhibited by interaction of strongly upregulated inhibitory receptor Tim3 on immune cells with its ligand Galectin-9 elevated in plasma. Moreover, we observed potential inhibition of co-stimulatory signaling facilitated by soluble forms of activating receptors such as sCD27 and sCD25. These soluble receptors may compete for ligand with their membrane-bounded counterparts and interfere with co-stimulatory signaling. We detected alterations in the cytokine profile of patients demonstrated by increased levels of IL-6 and IFN-α2.

In conclusion, we described the immune profile of AML patients. We detected soluble receptors as players potentially regulating co-stimulatory signaling in the AML microenvironment. The capabilities of immune cells are further reduced by the downregulation of activating receptors NKG2D and DNAM-1.

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THE EFFECTS OF EXOGENOUS HYALURONIC ACID ON PRE-ADIPOCYTES

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The main functions of adipose tissue are thermal insulation and energy storage. However adipose tissue can also react to inflammatory processes occurring in the body. During systemic inflammation adipose tissue usually reacts by elevating lipolysis resulting in size-reduction of the tissue. Recently a few studies observed expansion of adipose tissue in reaction to inflammation calling it reactive adipogenesis.
Hyaluronic acid (HA) is a glycosaminoglycan formed from repeating units of the D-glucuronic acid and N-acetyl-D-glucosamine disaccharide. Depending on the size of HA polymer it is categorised into high (H), middle (M) or low (L) molecular weight (MW) HA. HMW-HA is produced by various cells as part of extracellular matrix where it mainly maintains tissue hydration and provides mechanical support. Under inflammation HMW-HA is degraded into LMW-HA fragments. Several studies propose that LMW-HA has pro-inflammatory properties or that it serves as a signal molecule. Degradation of extracellular matrix by hyaluronidases results in slower differentiation of pre-adipocytes into adipocytes.
The aim of our study was to evaluate the effects of exogenous HA of various molecular weight on differentiation and metabolism of pre-adipocytes. We exposed 3T3-L1 cell line undergoing differentiation to HMW-, LMW-HA as well as HA oligo- and disaccharides. We observed lower expression of *adipoq*, a marker of adipocyte differentiation, after exposure to HA disaccharides. Contrary to studies using hyaluronidases we did not see changes in primary cilia dynamics compared to untreated cells. Exogenous HA is incorporated into the glucose metabolism shown by utilising ^{13}C-labeled HA. It is used to produce uridine diphosphate N-acetylglucosamine that could be used for synthesis of new HA or for the post-translational modification O-linked GlcNAcylation.
The currently obtained data suggest involvement of HA in the cell metabolic processes and adipocytes functions.

MAPPING YOLK SAC-DERIVED HEMATOPOIETIC LINEAGES: A HEMATO-ENDOTHELIAL PERSPECTIVE

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During embryogenesis, blood cell production is sustained by three independent hematopoietic waves. The first two originate in the yolk sac (YS), while the third arises intra-embryonically and is driven by hematopoietic stem cells (HSCs), which later establish lifelong hematopoiesis. However, HSCs are dispensable prenatally, highlighting the importance of YS-derived progenitors for early development and potential prenatal therapies.
Our study aims to map YS-derived hematopoietic lineages, investigate their origins, and assess their contribution to myeloid cell production. Using single-cell RNA sequencing (scRNA-Seq), we identified a common hemato-endothelial ancestor for YS-derived waves. We show that the first wave generates cells with megakaryocyte-erythrocyte (MkE) potential, while the second wave, driven by erythro-myeloid progenitors (EMPs), gives rise to both MkE progenitors and the first myeloid cells. To further characterize EMP-derived blood production, we identified differentially expressed genes and searched for potential surface marker candidates. Notably, we found complement receptor CD88 to be a marker of the early EMP subset, which could enhance flow cytometry-based identification. Additionally, we are developing a novel lineage-tracing model to distinguish between hematopoietic waves across different niches.
Our findings suggest that YS-derived waves share a hemato-endothelial ancestor and that EMPs are responsible for the first myeloid cells in embryogenesis. Ongoing *in vivo* validation of this model will provide deeper insights into early hematopoietic development and its clinical implications.

CELL-BASED ASSAYS FOR EARLY DETECTION OF IMMUNOGENICITY RISK FOR PROTEIN AND PEPTIDE THERAPEUTICS

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Immunogenicity refers to the ability of protein- or peptide-based therapeutics to provoke unwanted immune responses. Such responses can lead to reduced drug efficacy, hypersensitivity reactions, or the formation of neutralizing antibodies. Assessing immunogenicity risk is therefore essential during drug development to ensure safety and therapeutic performance.
The immune reaction to biologics typically involves antigen-presenting cells such as dendritic cells and macrophages, which internalize and process the therapeutic compound, presenting derived peptides to T lymphocytes. Upon recognition of foreign epitopes, CD4+ T cells become activated, orchestrating a broader immune response, including cytokine production and antibody formation by B cells.
In our laboratory, we have employed a range of *in vitro* immune cell-based assays to assess the immunogenic potential of biologics. The Monocyte Activation Test (MAT) is routinely used to evaluate pyrogenicity and innate immune activation. To analyze T-cell responses, we perform proliferation assays using flow cytometry as well as cytokine secretion profiling with the Fluorospot technique, which enables sensitive detection of IFN-γ, IL-2, and other cytokines. These validated methods are available in our lab and support early-stage screening of therapeutic candidates for immunogenic risk.
Understanding and applying these assays early in development can help predict immunogenicity risks, reduce late-stage failures, and support the creation of safer biotherapeutics.

CYTOMETRY APPLICATIONS IN BIODOSIMETRY SCREENING

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This study aimed to evaluate selected peripheral blood lymphocyte subsets as in vitro biodosimetric markers of ionizing radiation exposure (3–20 Gy) and to identify a suitable time window for detecting radiation-induced apoptosis. Additionally, we sought to establish a rapid, practical method for biodosimetric screening using peripheral blood mononuclear cells (PBMCs). Human peripheral blood samples were irradiated with 60Co gamma rays and incubated for defined intervals. Multicolor surface immunophenotyping and flow cytometry were used to assess lymphocyte subset distributions. Early apoptotic

changes were detected through decreased forward scatter and Annexin V binding, indicating phosphatidylserine translocation. The radiosensitivity of specific lymphocyte subsets was quantified using the IVNIR (Irradiated Versus Non-Irradiated Ratio), comparing their representation in irradiated versus control samples. Conventional markers such as CD4 and CD8 proved suboptimal due to the heterogeneity of these populations, which include cells at various differentiation stages with differing radiosensitivities.

In contrast, well-defined subsets—$CD8^+$ natural killer (NK) cells, $CD21^-$ B-cells, and $CD27^+$ B-cells—exhibited consistent and dose-dependent responses, indicating their suitability for biodosimetric purposes in humans. In pigs, however, where NK cells predominantly express CD8, this marker does not identify radiosensitive subpopulations as in humans. Interestingly, $CD8^+$ NK cells in pig blood increased after irradiation, potentially due to mobilization or radioresistant precursor maturation. Despite differing kinetics, porcine $CD8^+$ NK cells may also serve as biodosimetric indicators.

Conclusion: Three lymphocyte subsets—$CD8^+$ NK cells, $CD21^-$ B-cells, and $CD27^+$ B-cells—demonstrated promising biodosimetric potential in humans, underscoring the importance of subset specificity and interspecies variability in biodosimetry.

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OPTIMALIZATION OF MONOCYTE-LIKE CELL LINE THP-1 DIFFERENTIATION FOR A QUICK BIOMATERIAL IMMUNOCOMPATIBILITY TESTING

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Immunocompatibility testing is the second step applied to materials that have passed the first step of cytokinetic parameter testing. The most popular approach is the use of monocytes isolated from the blood of a healthy donor. Monocytes usually differentiate into basal state M0 macrophages after the addition of macrophage colony stimulating factor (M-CSF) and are then transported to grow on the tested surfaces for 2-3 days (Brown et al., 2012). Their response is polarization to either a pro-inflammatory (M1) or pro-regenerative (M2) phenotype, which is assessed by qRT-PCR for the expression of specific cytokines or surface markers. As blood monocyte isolation is laborious and time-consuming, there is a tendency to use monocytic cell lines derived from leukaemia patients. We used the THP-1 cell line in our first attempt to test the immunocompatibility of biomaterials functionalised by plasma treatment (Buchtelová et al., 2024). We repeated the procedure used for blood monocytes, only replacing M-CSF with 10 ng/ml PMA - incubation lasted 2 days, followed by 3 days of rest. Although we have obtained reliable

results showing that our material reduces the pro-inflammatory response, we continue to optimise the test procedure. Recently, we aimed to shorten the differentiation process. Higher doses of PMA trigger the differentiation process sufficiently to overcome the genetic blockade and THP-1 cells could be seeded directly on the tested surfaces (Toda et al., 2024; Zhu et al., 2017). The results of this method are similar to those obtained previously, although the concentration of PMA plays a crucial role. The assay is then reduced to more than half the time previously required and avoids the most difficult part of harvesting M0 macrophages from standard culture plastic.

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UTILIZING BIOID TO IDENTIFY INTERACTION PARTNERS OF HASPIN KINASE

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Haspin is an atypical Ser/Thr kinase that is critical during mitosis. It has been specifically shown to regulate chromosome alignment and ensure the timely segregation of sister chromatids. Although its well-characterized functions are limited to mitosis, Haspin is present throughout the entire cell cycle. This observation raises the possibility that Haspin may have additional roles in other cell cycle phases. Understanding these potential roles requires investigating the Haspin interactome, which encompasses the network of protein-protein interactions involving Haspin.

To analyze the interactome of Haspin, we employed the BioID method. This proximity-dependent biotinylation technique labels potential interaction partners based on their spatial proximity to the protein of interest. BioID was executed in HeLa cells, followed by mass spectrometry analysis to identify biotin-tagged proteins. Subsequent bioinformatics analyses were conducted to determine the putative interaction partners and evaluate their association with various cell cycle stages.
We identified 127 proteins as potential interaction partners of Haspin kinase through mass spectrometry. Some proteins, such as Pds5b, were previously recognized as Haspin interaction partners. However, most identified proteins had no known association with Haspin, suggesting novel functional links. Furthermore, a subset of these proteins exhibited variations in their interaction patterns across different cell cycle stages, indicating that Haspin's interactome may be dynamically regulated throughout the cell cycle. These findings imply that Haspin might have broader cellular roles beyond mitosis, possibly influencing other cell cycle phases. In the next step, we refined our approach by employing the Fucci2 reporter system, which allowed cell sorting based on specific cell cycle phases before proteomic analysis. This strategy facilitated the elucidation of the dynamic changes in Haspin's interactome and its broader biological significance.
Our study offers insights into the interactome of Haspin kinase and its potential role in cell cycle regulation beyond mitosis. The discovery of new interaction partners emphasizes untapped facets of Haspin's function.
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CHARACTERIZATION OF THE CD2$^-$CD8^{low} ΓΔ T CELL SUBPOPULATION IN PIGS: BIOLOGICAL ENTITY OR METHODOLOGICAL ARTIFACT?

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Porcine γδ T cells have traditionally been classified into three main subpopulations based on CD2 and CD8 expression (CD2$^+$CD8$^+$, CD2$^+$CD8$^-$, and CD2$^-$CD8$^-$). Recently, a CD2$^-$CD8$^{low/dim}$ subpopulation has been mentioned, raising the question of whether this is a true biological subset or a methodological artifact resulting from the increased sensitivity of modern flow cytometry. Notably, the proportion of CD2$^-$CD8^{low} cells increases with age, especially in the first two weeks of life, and reaches the highest values in older sows. Furthermore, only the CD2$^-$CD8^{low} cells, but not the CD2$^-$CD8$^-$ cells, are transferred into the colostrum. Functional analyses revealed differences in cytokine production between

these subpopulations. Different clones of anti-CD8 antibodies can be used to detect the $CD2^{-}CD8^{low}$ population, and the frequency of positive cells is influenced by staining protocols, cell concentration, and sample washing procedures. However, it cannot be excluded that the detected signal corresponds to a molecule other than CD8. Our findings highlight the need for caution in the interpretation of such data, but also suggest a potential biological relevance of this population that warrants further investigation. This study was supported by the Ministry of Agriculture (RO0523).

TGF-B DECREASES NK CELL MOBILITY AND CYTOTOXIC EFFICACY IN COMPLEX *IN VITRO* MODELS OF THE LEUKEMIA MICROENVIRONMENT

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Background: Natural killer (NK) cell-based therapies are a promising approach to prevent relapse in acute myeloid leukemia (AML), leveraging NK cells' antigen-independent cytotoxicity. However, AML-induced NK cell dysfunction – driven by immunosuppressive signals in the bone marrow niche (BMN), including TGF-β – remains a major hurdle. Here, we assessed the effects of TGF-β on NK cell phenotype and function using transcriptomics, 2D co-cultures, and 3D MSC-leukemia spheroid models. **Results:** RNA-seq analysis revealed TGF-β–mediated downregulation of genes essential for cytotoxicity (*GZMB*, *PRF1*, *IFNG*), adhesion, and migration. Functionally, TGF-β exposure impaired NK-mediated killing of K562, THP-1, MOLM-13, and U937 cells in a time-dependent and donor-variable manner. Notably, even short-term TGF-β exposure – mimicking in vivo conditions – disrupted NK cell function and migratory potential. In 3D spheroid models, TGF-β reduced NK cell infiltration into leukemic-MSC spheroids, particularly in THP-1, MOLM-13, and U937 constructs. Transcriptomic data confirmed altered expression of migration-related genes linked to extravasation and chemoattraction. **Conclusions:** These results

suggest TGF-β impairs not only cytotoxicity but also NK cell mobility within the AML microenvironment. Our study underscores the need to assess both functional and migratory NK cell properties in cytometric assays. Incorporating this knowledge into in vitro expansion protocols may enhance therapeutic efficacy in AML by selecting donor NK cells capable of overcoming BMN-associated immunosuppression.

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TIME DEPEDENT MACROPHAGE ACTIVATION IN THE INITIAL PHASE OF TUMORIGENESIS IN ANIMAL MODEL

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Background

The concept of immunoediting emerged in 2003 defining the principles of interaction between tumors and the immune system. Equilibrium shift between the two is decisive for tumor progression or regression. Progression to clinically detectable tumor is considered the escape phase of immunoediting. In many types of tumours, macrophages constitute the most numerous cells in the tumor microenvironment after tumour cells and their effect on the process of tumor growth is dependent on activation status. In addition to main pathways M1 and M2, many studies described various different populations of macrophages, each with a unique phenotype.

Methods

In a rat model of early tumorigenesis, we injected BP6-TU2 fibrosarcoma cell line into the peritonteal cavity of Wistar rats. The control group was injected with RPMI-1640 cell medium with no oncocells. Lavage samples were processed with flow cytometry where we monitored the representation of 4 cell pupulations in one and two weeks interval after application with monoclonal antibodies CD68, CD80, CD163, CD197, CD206.

Results

We calculated the ratio of each population phenotype and estimated the fluctuation timeline
($CD68^+CD80^+CD197^+$; $CD68^+CD206^+CD163^+$; $CD68^+CD80^+CD197^-$; $CD68^+CD206^+CD163^-$) in the peritoneal cavity. We detected singificant change in the amount of each subtype in comparison to the control group, confirming the tumor induction effect on macrophage polarization starting from 1 week post injection. We also observed a significant change in the M1/M2 ratio in the first and second week in comparison with the control group.

Conclusion
Our early tumorigenesis experiment model depicts changes in the tumor microenvironment corresponding to the pre-clinical phase of tumor progression, i.e. equilibirum phase. Understanding such dynamics will aid in the understanding of the pathophysiological mecahnisms of tumorigenesis and are key for the optimalization of timing of immunotherapy intervention.

Key words
macrophage, tumor microenvironment, imunoediting, M1 and M2 phenotype

EFFECT OF TUMOR-CONDITIONED MEDIUM ON NEUTROPHIL HETEROGENEITY

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All components of the tumor microenvironment (TME), including hypoxia and immune cells, are involved in tumor progression and interact with each other. The therapy itself also has a major impact. The effects of radiotherapy are strongly dependent on the availability of oxygen and the potential for the generation of oxygen radicals, which subsequently damage the DNA of tumour cells. In this study, we focus on changes in the phenotype and functional properties of neutrophils (NEU) exposed to tumor-conditioned medium (TCM) from head and neck cancer cell lines (FaDu, 2A3 and Detroit-562) exposed to hypoxia and γ-radiation.
To assess the effect of hypoxia and ionizing radiation on HNSCC tumor cells, the cell lines were cultured in hypoxic and normoxic conditions for 3 weeks and irradiated once a week with a dose of 6 Gy (3 times in total). Subsequently, changes in proliferation and cytokine production (especially IL-8, MIF, Serpin) were observed. TCM prepared in this way was used to affect NEU isolated from healthy donors. The results showed that TCM increased the viability of NEUs from 20% (unaffected NEUs) to 80% for Detroit TCM. This increase correlated with the concentration of IL-8 in TCM, but no significant change in viability was observed using the IL-8 neutralizing antibody. Furthermore, an increase in ROS production, a slight increase in CD11b and a decrease in CD62L surface markers were observed. TCM also affected the NEU phenotype, with TRAIL-R2 decreased, TRAIL-R3 and CD170 increased, and PD-L1 was not altered. In addition, TCM were able increased NEU migration and VEGF production.
On the basis of these results, we can conclude that hypoxia and γ-radiation affect both the tumor cells themselves and the TME and thus affect the function and phenotype of the NEU and thus tumor progression.

This study was supported by the project of Ministry of Education, Youth and Sports (LUC23033) “Effects of radiation and hypoxia on head and neck cancer cells: influence on neutrophil heterogeneity”

MODIFIED TRYPTAMINE COMPOUNDS ABROGATE INFLAMMATORY CUES IN AHR DEPENDENT MANNER IN INTESTINAL EPITHELIAL CELL MODEL

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The mucosal intestinal epithelial barrier (IEB) shields our body from harmful agents within gut lumen. To accomplish this, the plethora of cells that constitute it expresses the ligand-regulated aryl hydrocarbon receptor (AhR) that has an active role in cell proliferation, migration, immune respond and inflammation through its activation by tryptophan-derived metabolites. Dysregulation of AhR has been associated with physiological disorders, including inflammatory related diseases and cancers. Thus, AhR could be a target for therapeutic modulation of the immune response in IEB. Here, using a human colon cancer-derived cell model, Caco-2 cells, we studied the effects of chemically modified tryptamines targeting AhR and assessed their anti-inflammatory and IEB protective activities. After characterization of AhR activation and anti-inflammatory potential of a set of modified tryptamine derivatives, as model compounds, we established AhR deficient Caco-2 cell line (AhR KO) variant. We observed that loss of AhR impaired IEB, while simultaneously promoting pro-inflammatory effects of a cocktail of inflammatory cytokines. Then, we evaluated the anti-inflammatory properties of selected tryptamine derivative 3a in a co-culture model employing Caco-2 cells (either wild-type or Caco-2 AhR KO variant) and mucus-producing HT29-MTX cells. Our data so far indicate that tryptamine and its derivatives, such as 2-(1-methyl-1 H-indol-3-yl)ethanamine may inhibit inflammatory responses in Caco-2 WT cells, partly in AhR-dependent manner. Nevertheless, our data also suggests that 2-(1-methyl-1 H-indol-3-yl)ethanamine may also reduce the production of some pro-inflammatory cytokines/chemokines and inflammatory damage in AhR KO cells via yet unidentified signaling pathway. Future work should identify the mechanisms responsible for this. [This work was supported by the project No. 22-00355S of the Czech Science foundation.]

MISMATCH AIRE MUTATIONS AND *C. ALBICANS* RESPONSE

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Involvement of AIRE in immune response to *C. albicans* is well documented, as patients with a disfunctional AIRE protein suffer from autoimmune polyendocrinopathy syndrome type 1 (APS-1), with chronic mucocutaneous candidiasis (caused by *C. albicans*) as the most common symptom. We previously showed that the peripheral function of the AIRE in RORγt+ eTACs is crucial for mounting an effective T cell response to *C. albicans*. In APS-1, various patient groups with distinct AIRE mutations exist, however, only patients with the AIRE Y85C mutation manifest autoimmune symptoms, yet their susceptibility to CMC is notably lower compared to those with other mutations. To examine the role of specific Aire mutations in the immune response to *C. albicans*, we used Aire Y86C mice, which mirror the patient mutation AIRE Y85C, and challenged them with *C. albicans* via gastrointestinal colonization. We observed similar numbers of Candida-specific CD4 T cells in Peyer's Patches and other lymphatic organs in Y86C mutants and WT animals. To characterize RORγt+ eTACs in Y86C animals, we developed a strategy for their isolation and identification. We conducted analysis of published and in house generated scRNA seq datasets of WT mice and found extracellular markers for characterization of 3 different RORγt+ eTACs subpopulations. Based on our scRNA seq analysis and in vivo experiments, we hypothesize these eTACs populations corresponds to different maturation stages. Our data suggest that the Y86C mutation preserves AIRE's ability to induce a Candida-specific immune response, indicating that it is an ideal mouse model to dissect the mechanistic role of AIRE in the immune periphery.

EVALUATION OF MITOCHONDRIAL, ER AND OXIDATIVE STRESS ENDPOINTS INDUCED BY POLYCYCLIC AROMATIC HYDROCARBONS IN HUMAN HEPATOCYTE-LIKE CELLS

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Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental contaminants forming a wide range of genotoxic and non-genotoxic metabolites in cells exposed to these toxicants. Understanding the nature of stress induced by exposure to PAHs and/or their metabolites is crucial for characterization of molecular mechanism underlying their impact on target cells, and whole organism. Our previous results have indicated that exposure to selected PAHs that activate the aryl hydrocarbon receptor (AhR) and the AhR-dependent formation of PAH metabolites in human hepatocyte-like HepaRG cells leads to induction of the stress markers, such as GDF15 and to increased expression of ATF3 and GADD34, genes associated with general cellular stress responses. However, a majority of classical endoplasmic reticulum stress markers are not significantly altered, suggesting the activation of alternative pathways leading to induction of ATF3, GADD43 or GDF-15. These observations led us to further investigate stress-related endpoints, focusing on mitochondrial function and oxidative stress. Our preliminary data indicate that PAHs may alter levels of glutathione (GSH) and GHS/GSSG ration, and induce expression of some oxidative stress gene targets, as well as they can modulate mitochondrial metabolism when applied at sufficiently high concentrations. To assess mitochondrial integrity, mitochondrial membrane potential (MMP) was measured using TMRE staining and flow cytometric detection. A slight, concentration-dependent increase in MMP was observed after PAH exposure, which nevertheless seemed to arise from production of fluorescent PAH metabolites. To address this challenge, we are now testing the use of spectral flow cytometry to obtain a better discrimination between fluorescence signal of PAH metabolites and that of specific probes detecting MMP, as well as formation of reactive oxygen species (ROS). Multiple approaches are currently being optimized to evaluate ROS production, using a set of probes including hydroethidine, 2',7'-dichlorodihydrofluorescein diacetate, MitoSOX™ Mitochondrial Superoxide Indicators, and CellROX™ Deep Red Reagent. [This work is supported by the project No. 24-10086S of the Czech Science foundation.]

CHALLENGES IN FLOW CYTOMETRIC ANALYSIS OF B CELL POPULATIONS CO-CULTURED WITH STROMAL FEEDER CELLS

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The co-culture of human B cells with stromal HS-5 feeder cells stably expressing CD40L, in the presence of interleukins IL-4 and IL-21, represents an *in vitro* model designed to support B cell survival, activation, proliferation, and differentiation. This model was

initially developed to stimulate the proliferation of chronic lymphocytic leukemia cells *in vitro* and mimics interactions with follicular T helper cells within the lymph node microenvironment.

Although HS-5 cells themselves do not directly interfere with flow cytometric analysis, we observed several changes in B cell fluorescence and phenotype arising from the co-culture that introduced substantial challenges to the analysis. Notably, we detected increased levels of autofluorescence, particularly in channels excited by the violet laser, which can interfere with the detection of commonly used fluorochromes and complicate standard gating strategies. Furthermore, we observed altered expression patterns of surface markers, especially CD27, which is frequently used to distinguish memory B cells and plasmablasts. These shifts appear to result from activation and differentiation processes triggered by the co-culture conditions.

Our findings highlight the importance of careful panel optimization, the inclusion of appropriate controls (e.g., freshly isolated B cells, fluorescence-minus-one controls), and awareness of culture-induced phenotypic variability when analyzing B cell populations by flow cytometry in complex co-culture systems.

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IMAGE CYTOMETRY

QUANTITATIVE ANALYSIS OF B-CELL MIGRATION: FROM PHENOTYPE TO MOLECULAR MECHANISMS

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B-cells represent an under-studied immune cell type, especially regarding their cell migration and polarity properties. This is despite the fact that migration is an important aspect of B-cell maturation including but not limited to germinal center reaction. Migration and polarity therefore have high relevance to B-cell immune functions and pathologies, including B-cell-derived malignancies. Therapeutic agents targeting haematological cancers are often shown to negatively affect cell migration. The molecular mechanism of these effects is however rarely investigated in detail. Here, using chronic lymphocytic leukemia cell line, we demonstrate the combined use of live-cell migration and proteomic analyses to identify downstream mechanisms controlling CLL cell migration, which are disrupted following inhibition of casein kinase 1 and compare them to well established cytoskeleton-targeting drugs. Our results should contribute to better understanding of the intracellular effects of the anti-cancer treatments and their more effective use.

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QUANTITATIVE AI-BASED DNA FIBER (QAID) WORKFLOW USING SCANR HIGH-CONTENT IMAGING SYSTEM

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Replication stress (RS) is a prominent source of genome instability and human diseases, including developmental defects, premature aging, and cancer. Understanding the molecular mechanism of RS has great potential for early diagnosis and development of

effective strategies for human diseases characterized by unstable genomes. One of the most powerful methods to study DNA replication dynamics and its alterations at the single molecule resolution is the DNA fiber assay. However, this method relies exclusively on manual image acquisition and analysis, which can affect the interpretation and reproducibility of the collected data. To address this, we developed an automated workflow for the acquisition and analysis of DNA fibers using the scanR high-content imaging system and TruAI deep learning module. Our quantitative AI-based DNA fiber (qAID) workflow enables imaging and real-time multiparameter analysis of thousands of DNA fibers within several dozen minutes. The multiparameter analysis classifies fibers into five standard classes and measures properties such as fiber length and symmetry of two-colour labelling. qAID multiparameter analysis is complemented by visual inspection of individual DNA fibers using unbiased image galleries. In summary, qAID workflow provides a fast and effective examination of replication dynamics and its alterations at the single-molecule resolution. At the same time, it also opens new avenues in understanding the molecular mechanism of replication stress.

SPHINGOSINE 1-PHOSPHATE RECEPTOR-1 CONTROLS T CELL MECHANOSENSITIVITY.

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Lymphocytes constantly navigate through crowded lymphoid tissues in search of their cognate antigen. Traditionally, this process has been attributed to guidance by chemotactic compounds such as chemokines or bioactive lipids. However, what is the contribution of environmental mechanical cues to lymphocyte motility remains unknown. To address this, we have adopted a PDMS-based confiner system that mimics the physical constraints of lymphoid tissues in vitro. Using this platform, we show that T cells exhibit spontaneous motility driven solely by mechanical confinement, independent of chemotactic signals. Surprisingly, this motility relies on G protein αi and phospholipase C signaling, implicating the role of a G protein-coupled receptor. Indeed, we have identified sphingosine 1-phosphate receptor-1 (S1PR1) as a key receptor controlling T cell responses to mechanical stimuli. S1PR1 deletion restricts T cell motility under confinement, and S1PR1 natural and synthetic modulators adjust the migration speed by regulating S1PR1 surface abundance. These findings present a novel layer of regulation of lymphocyte migration and reveal an unexpected function of mechanosensitive GPCRs in cell motility.

COMPARATIVE MIGRATION ANALYSIS OF HEALTHY HUMAN B CELL POPULATIONS

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Cell migration is an essential feature of the immune system. Among others, it is vital for the development and proper functioning of B cells. However, B cell migration has received relatively little attention so far. B cells are a leukocyte population that experiences clonal expansion in chronic lymphocytic leukaemia (CLL). Cell migration and trafficking have been previously shown to play an important role in CLL cell expansion and survival (Davids *et* Burger, 2012), therefore further investigation of cell migration and the differences between healthy lymphocytes and CLL cells holds promise for development of novel therapeutic strategies targeting CLL. Moreover, as the cellular precursor of CLL cells has not been identified yet and migration is one of the processes dysregulated in CLL, studying the migratory properties of different healthy B cell populations may provide new insights into the origin of CLL.

In this study, we have tested the migratory potential of B cells isolated from peripheral blood (PB) of healthy donors and CLL patients using the transwell assay and the under-agarose assay. Following the transwell assay, transmigrated cells were stained with a panel for identification of B cell subpopulations and analysed by flow cytometry. The under-agarose assay provided additional information, such as the speed or persistence of migration.

Using these two approaches, we analysed the migratory properties of naive B cells and memory B cells from healthy donors, and the patient CLL cells. Additionally, we tested the effect of casein kinase 1 inhibition (CK1i) on healthy primary cell migration, as the CK1i been shown to interfere with the migration of multiple CLL cell lines and CLL cells from Eμ-TCL1 mouse model (Čada *et al*., 2022, Janovská *et al.*, 2018). Our results show that naive B cells from PB migrate less than memory B cells, and that CK1i disrupts B cell migration in this *in vitro* model.

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VERLO: A NOVEL IMAGING CELL SORTER FOR ENHANCED ANALYSIS OF TUMOR INFILTRATION BY IMMUNE CELLS

Adonary Munoz, Micayla George, Rea Dabelic, PhD

Nanocellect Biomedical, Inc, San Diego, California

Cancer ranks as the second leading cause of death in the US. Central to cancer progression is the tumor microenvironment (TME), which significantly influences the disease's spread. Immune cell infiltration into the TME is crucial for effective immunotherapy. Traditional infiltration assessment methods, including microscopy and flow cytometry, have limitations such as low throughput and lack of imaging. Addressing these issues, we present VERLOTM, an image-guided cell sorter combining low-pressure microfluidics with dual-laser scanning to enhance cell analysis. We utilized VERLO to examine T cells infiltrating breast cancer spheroids, which simulate tumors. VERLO's application enabled us to preserve T cells-tumor interactions, facilitating the exploration of factors that may augment immune infiltration. This research underscores VERLO's utility in studying immune cell dynamics within the TME, advancing immunotherapy optimization for cancer treatment. Our findings indicate that VERLO can significantly contribute to understanding and improving immune cell-mediated cancer therapies.

REVOLUTIONIZING STEM CELL SELECTION: A LABEL-FREE APPROACH USING THE VERLO IMAGE-GUIDED CELL SORTER

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Nanocellect Biomedical, Inc, San Diego, California

In the evolving landscape of stem cell research, the ability to distinguish between undifferentiated and differentiated cells is paramount for advancing both basic science and therapeutic applications. The traditional reliance on fluorescent markers for cell sorting, although effective, introduces complexity and may affect cell viability and functionality. Our recent study leverages the VERLO Image-Guided Cell Sorter's capabilities to address this challenge in a label-free manner, revolutionizing the process of stem cell selection. Utilizing imaging features and advanced analytics, the VERLO cell sorter enables the precise identification and separation of undifferentiated from differentiated human pluripotent stem cells without the need for fluorescent labels. This approach preserves cell integrity and eliminates the potential impacts of labeling on cell physiology. Our results demonstrate a significant advancement in stem cell research methodologies, offering a more efficient, reliable, and non-invasive alternative for cell sorting. We successfully applied this label-free sorting technique to isolate pure populations of undifferentiated stem cells, achieving high efficiency and viability. This method not only enhances the potential for scalable stem cell applications but also opens

new avenues for research into cellular development, disease modeling, and regenerative medicine. The utility of the VERLO Image-Guided Cell Sorter in this context underscores its value as a critical tool for the next generation of stem cell research.

FANCJ AND RTEL1 FACILITATE PRE-REPLICATION COMPLEX DISASSEMBLY FOLLOWING REPLISOME COLLISION

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Eukaryotic DNA replication begins with origin licensing, during which inactive MCM2-7 complexes are loaded onto replication origins, leading to the formation of pre-replication complexes (pre-RCs). Despite excess MCMs loaded on chromatin, only a small subset is converted into active replicative CDC45-MCM2-7-GINS1-4 (CMG) helicases during origin firing. Although excessive pre-RCs are beneficial for error-free DNA replication, their removal needs to be precisely and timely regulated. Nevertheless, the mechanisms by which the incoming replication fork interacts with pre-RCs remain largely uncharacterized. Here, using *Xenopus laevis* egg extracts and human cell models, we show that pre-RCs are frequently displaced from DNA upon collision with replication forks, a process facilitated by the helicases FANCJ and RTEL1. Notably, simultaneous depletion of both helicases results in frequent replication stalling events, the accumulation of pre-RCs on chromatin, and the induction of DNA damage. Our findings highlight the critical roles of FANCJ and RTEL1 in promoting pre-RC removal, ensuring efficient replication fork progression, and maintaining genome stability.

IDENTIFIYING EXTRACELLULAR VESCICLES BINDING AND INTERNALIZATION IN BLOOD CELL SUBSETS BY IMAGING FLOW CYTOMETRY

Haley Pugsley

Cytek Biosciences inc. Freemont, California

Extracellular vesicles (EVs) are cell-derived, membrane-bound small particles less than a micron in size. EVs are potentially valuable biomarkers and therapeutic agents in many disease environments, including cancer, autoimmunity, and neurodegenerative disorders. Due to their small size and heterogeneity, EVs can be difficult to characterize.1 The single

vesicle flow cytometry vFC assay is a method developed with rigor and reproducibility capable of single EV measurements. Our results demonstrate the sensitivity and resolution of the Aurora spectral flow cytometer. Using single vesicle flow cytometry and an Aurora spectral flow cytometer, we were able to detect vesicles and their surface cargo.

QUANTIFICATION OF KEY DIAGNOSTIC BONE MARROW CELLS USING AUTOMATED DIGITAL CYTOMORPHOLOGY

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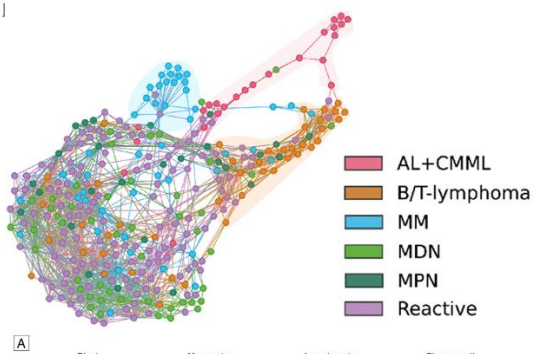
Introduction: Correct quantification of key bone marrow (BM) cells (blasts, monocytes, lymphocytes, and plasma cells) through cytology, flow cytometry, histology, and immunohistochemistry is crucial for diagnosis. Automated digital morphology (ADM) using AI is an innovative approach in this field.
Methods: In 328 BM smears, the quantification of BM cells using ADM (Morphogo system) was compared with expert optical microscopy in six diagnostic groups: myelodysplastic neoplasms (15%), multiple myeloma (MM, 14%), mature B/T-cell neoplasms (13%), acute leukaemia (AL) and chronic myelomonocytic leukaemia (CMML, 9%), myeloproliferative neoplasms (8%), and reactive haemopoiesis (41%). The expert/ADM differences were quantified for all cell types and the patient similarity network was constructed. Numerical differences in the representation of key BM cells were displayed in the network. The Matthews correlation coefficient (MCC) and odds ratio (OR) values were calculated for the key cells.
Results: Three separate subgroups of three diagnostic groups were identified: AL+CMML, B/T-lymphoma, and MM (Fig. 1). The largest differences in blast counts occurred in AL+CMML, monocyte counts in AL+CMML, lymphocyte counts in B/T-lymphoma, and plasma cell counts in MM groups (Fig. 1A, 1B; red dots). MCC and OR calculated for the key attributes in the diagnostic groups (AL+CMML, B/T-lymphoma, and MM) confirmed the findings from the network (Fig. 1C). The major reason for these differences is the automatic selection of the adaptive area for 100× immersion lens analysis by ADM, which is more representative and reliable than region selection in conventional light microscopy. ADM reduces the variability associated with user bias in region and cell identification by analysing a significantly larger number of slide areas and cells.
Conclusion: The largest numerical differences in the representation of blasts were observed in AL+CMML, monocytes in AL+CMML, lymphocytes in B/T-lymphoma, and plasma cells in MM groups. This raises a major question regarding the accuracy of

quantifying key diagnostic cells as well as a provocative question about the absolute correctness of expert opinion. The method used in this study has enormous potential to drive diagnostic transformation by reducing subjectivity and variability in BM assessments.



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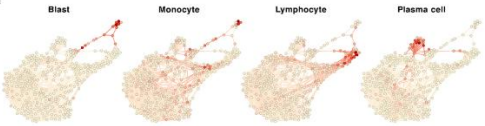

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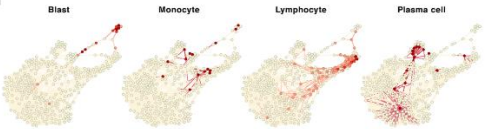

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Dg. group	Attribute	MCC	Odds ratio	95 % CI	P-value
AL+CMML	Blast	0.578	84.9	21.1 - 341.6	< 0.001
AL+CMML	Monocyte	0.219	7.8	2.4 - 24.9	< 0.001
B/T-lymphoma	Lymphocyte	0.274	5.4	2.6 - 11.0	< 0.001
MM	Plasma cell	0.356	18.0	5.4 - 60.5	< 0.001

CRITICAL MISCLASSIFICATIONS INFLUENCE THE RELIABILITY OF AUTOMATED DIGITAL BONE MARROW CYTOMORPHOLOGY

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Introduction: The correct classification of individual bone marrow (BM) cells in closely related or similar morphological categories can be challenging. The clinical viability of

automated digital cytomorphology (ADM) has not been fully explored as for the occurrence of critical BM cell confusions.

Methods: In 328 diagnostic BM smears, BM cell recognition using ADM (Morphogo system) was compared with expert optical microscopy in six diagnostic groups: MDN (15%), multiple myeloma (MM, 14%), mature B/T-cell neoplasms (13%), acute leukaemia and CMML (9%), MPN (8%) and reactive haemopoiesis (41%). To maintain clinical consistency, the percentage of correctly classified cells in individual patients was assessed. A detailed analysis was conducted for critical misclassification cases with clinical consistency of <80%. The patient similarity network (PSN) revealed differences between conventional and ADM myelograms.

Results: The relevant clinical consistency was 97.1%, with 5.5% of critical misclassifications, caused by a failure to recognise atypical neoplastic lymphocyte/blast, myeloblast/lymphocyte, lymphoblast/lymphocyte, immature plasma cell/blast and monoblast+promonocyte/promyelocyte. (Fig. 1) Atypical cytomorphology of misclassified cells was observed in all cases. Patients with critical misclassifications were only seen in the diagnostic categories AL+CMML, MM and B/T-lymphoma, not in the MPN, MDN and reactive groups. Using PSN, the majority of patients with low values of clinical consistency and all critical misclassifications occurred in three separate subgroups of AL+CMML (4/17), MM (5/46) and B/T-lymphoma (9/46) groups.

Conclusion: This study identified the most frequently misclassified cell types by ADM with potential critical impacts on BM diagnosis. These included atypical lymphocytes resembling blasts, small lymphoblasts and myeloblasts resembling lymphocytes, granular monoblasts resembling promyelocytes, dysplastic monocytic elements, plasmablasts, and immature plasma cells (candidate cells for future AI training). Although unfavourable outcomes are fully mitigated by expert supervision, we consider them to be a restriction of ADM's absolute reliability.

Diagnosis	Cell	Expert classification	ADM classification
Splenic MZL		Atypical lymphocyte	Blast, NOC
Splenic MZL		Atypical lymphocyte	Blast, NOC
MCL, blastoid variant		Atypical lymphocyte	Blast, NOC
HCL		Atypical lymphocyte	Monocyte
MCL		Atypical lymphocyte	Blast, NOC
HCL		Atypical lymphocyte	Blast, NOC
MZL		Atypical lymphocyte	Blast, NOC
CLL, pleomorphic variant		Atypical lymphocyte	Blast, NOC

Diagnosis	Cell	Expert classification	ADM classification
T-ALL		Lymphoblast	Lymphocyte
AML		Myeloblast	Lymphocyte
AML		Monoblast	Promyelocyte
MM		Plasma cell	Lymphocyte
MM		Plasmablast	Blast, NOC
MM		Plasmablast	Blast, NOC
MM		Immature plasma cell	Blast, NOC
MM		Immature plasma cell	Monocyte

SINGLE-CELL GENOMICS

BRIDGING TWO WORLDS: MERGING FLOW CYTOMETRY WITH SINGLE-CELL RNA-SEQUENCING

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Flow cytometry and single-cell RNA sequencing (scRNA-seq) are powerful and complementary tools for exploring the complexity of cellular populations. However, directly linking flow cytometric data with gene expression at the single-cell level remains challenging, especially for cell populations that are readily accesible by flow cytometry but difficult to assay by transcriptomics. Here, we present a workflow that integrates fluorescence-activated cell sorting (FACS) and index sorting with Smart-seq3xpress (SS3x), an advanced, cost-efficient, and sensitive plate-based single-cell RNA sequencing method. This combined approach takes advantage of the high-throughput precision of FACS to isolate specific cell populations and pairs this with in-depth gene expression profiling using SS3x. By directly connecting cytometric features to transcriptomic data, our workflow enables the identification and characterization of cell groups previously difficult to annotate solely based on cytometric information. This strategy allows a precise match between cell phenotype and gene expression, providing valuable insights into cell diversity and functional states.

Our results demonstrate that combining flow cytometry with single-cell transcriptomics effectively bridges the gap between these two methods. This integrated SS3x-FACS workflow offers cytometrists an accessible and powerful tool for a more comprehensive exploration of cellular identity and function, directly guided by cytometric data.

COMPOSITION AND FITNESS OF T AND NK CELLS IN EXTRAMEDULLARY MYELOMA TUMOR MICROENVIRONMENT

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Background:
Extramedullary disease (EMD) is an aggressive manifestation of multiple myeloma (MM), when clonal plasma cells (PCs) become independent on the bone marrow (BM) microenvironment and invade distant tissues and organs. The incidence of EMD is increasing and is associated with drug resistance and poor prognosis. The efficacy of modern immunotherapy is highly dependent on the patient's own immune system. However, there is limited knowledge about the composition of the EMD microenvironment and the fitness of its effector immune cells.

Aims:
To investigate the composition and fitness of T and NK cells in extramedullary tumor and corresponding BM microenvironment

Methods:
Biopsy of EMD soft tissue tumor was performed in relapsed/refractory MM patients and samples were processed immediately after surgery (Jelinek, Leukemia, 2024). Single-cell RNAseq was performed using Chromium GEM Single Cell 3' reagent kit v3.1 (10x Genomics) and cell suspensions from 7 EMD tumors, 5 BM from time of EMD relapse (EMD_BM, 4 paired with EMD tumors), and 5 unrelated RRMM_BM without EMD. The computational pipeline combined scanpy python framework and R packages SoupX for ambient RNA filtering and scDblFinder for doublet removal. Cytotoxicity and dysfunctional score of T cells were based on Li et al., 2019, Cell. For flow cytometry (FC) assessment, four 8-color panels were used consisting of CD3, CD4, CD8, CD16, CD27, CD45, CD45RA, CD56, CD57 as backbones (and other activation or inhibitory molecules) for dissection of T and NK cell subsets in EMD (N=6) and EMD_BM (N=5).

Results:
Single-cell RNA-seq analysis yielded a median of 3,489, 1,409, and 4,611 cells for EMD, EMD_BM, and RRMM_BM samples, respectively. The median proportion of PCs was 92% for EMD and 36% for RRMM_BM samples. Interestingly, the median number of PCs in EMD_BM samples was only 0.2%. The median proportion of cells in the T/NK cluster was 7% for EMD, 18% for EMD_BM, and 20% for RRMM_BM.

Semi-automated cell type annotation using Celltypist, the Leiden clustering algorithm, and literature review resulted in the identification of 7 distinct T cell and 2 NK cell clusters. We observed a substantially lower number of CD4+ T cells in EMD compared to unrelated RRMM_BM (median 2% vs 31% of T cells; $p = 0.02$) and EMD_BM (12%; $p = 0.05$). Importantly, this finding was validated by FC (median 10.7% vs 31.6% for EMD vs EMD_BM; $p = 0.05$). The remaining six T cell clusters were all CD8+ with markedly lower estimated cytotoxicity (e.g. FAM65B, IL7R, PLEK, etc.) and dysfunction (e.g. LAG3, TIM3, PD1, TIGIT, etc.) scores in EMD compared to EMD_BM or RRMM_BM. This trend was attributed to different composition of T cells between the groups. EMD had the highest proportion of cells in clusters with low cytotoxicity and high dysfunction, including CD8_proliferating_MKI67 (median 14% vs 2% and 0.2%; $p = 0.03$ and 0.02 for EMD_BM and RRMM_BM), CD8_exhausted-like_Tox (3% vs 0% and 0%; $p = 0.09$ and 0.13), and in the cluster specific almost exclusively for EMD that we annotated as CD8_MTRNR2L12 (19% vs 2% and 1%; $p = 0.05$ and 0.05). Additionally, using FC we found an increased percentage of T cells positive for exhaustion marker PD-1 (39.4% vs 15.9%; $p = 0.03$) in

EMD compared to EMD_BM and higher proportion of central memory CD8+ T cells (61.9% vs 31.9% of CD8+ T cells).
Analysis of NK cell compartment revealed significantly higher proportion of CD16- NK cells (median 70.3% vs. 7.5% and 6.8%; p=0.007 and 0.003) compared to CD16+ cytotoxic NK cells (29.7% vs. 92.5% and 93.2%), a well-known phenomenon in solid oncology (Rebuffet et al. 2024, Nature Immunology). This finding was further confirmed by FC (37.9% vs 7.6% of total NK cells; p = 0.016). Importantly, CD16- NK cells from EMD exhibited significantly higher expression of inhibitory receptor NKG2A compared to EMD_BM (p < 0.001).
Conclusion:
In this study, we revealed that majority of T cells in EMD tumor microenvironment are CD8+ T cells with impaired cytotoxicity and increased exhaustion compared to BM CD8+ T cells. For the first time, we demonstrated that NK cells infiltrating EMD tumors are represented dominantly by CD16- NK cells (in contrast to BM) resembling the situation in solid cancers. Moreover, EMD CD16- NK cells have significantly higher expression of NKG2A checkpoint compared to BM CD16- NK cells suggesting potential therapeutic opportunity with NKG2A inhibitors in EMD MM patients.

ROR-1+ B CELLS FROM PERIPHERAL BLOOD COMPRISE ATYPICAL B CELL POPULATION THAT POTENTIALLY REPRESENTS PRECURSORS OF THE MALIGNANT CLONE IN CHRONIC LYMPHOCYTIC LEUKAEMIA

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Chronic lymphocytic leukaemia (CLL) is a haematological malignancy of unknown origin, that with its growing incidence remains among primary health issues in adult population. Wnt signalling was shown to play an important role in CLL. In fact, receptor for Wnt5a, ROR-1, is even in such heterogeneous disease as CLL among uniform diagnostic markers. Our previous study showed that not only ROR-1 but many of its downstream molecules were upregulated in CLL, whereas their expression was undetectable in healthy donor (HD) B cells. In combination with evidence about only 1-5% of mature B cells in HDs being ROR-1+, this raised questions on the characteristics of ROR-1+ B cells and whether these cells represent/include the CLL cell of origin.

To address this, we used several single cell-based methods to characterize B cell subsets collected from PB of young and elderly HDs, CLL patients and donors with CLL-preceding condition – monoclonal B lymphocytosis (MBL). Firstly, we were able to show (using spectral flow cytometry) that ROR-1+ B cells are a heterogeneous population of cells that cluster into most of the normal B cell subsets and are enriched in transitional and unswitched memory B cell subsets. Secondly, 30-marker CyTOF panel uncovered that the ROR-1+ B cells include a rare population of atypical B cells that display several anergic traits (absence of CD21 and CD81, upregulation of T-bet). Importantly, this population was also identified as potential precursor to CLL cells using topological data analysis tool for trajectory inference. Finally, we were able to characterize the atypical B cell population using single-cell RNA sequencing which provided us by more information about ongoing intracellular processes in these cells, that were assessed in context of developing CLL.

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MANIPULATING EFFECTOR AND MEMORY FATES IN CD8+ T CELLS: A NOVEL TPAM TRANSITIONAL STATE AND THE ROLE OF TGF-B

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Upon activation, naive CD8+ T cells differentiate into short-lived effector cells (SLECs), which provide immediate protection against infections, and long-lived memory cells, which retain immunological information long after pathogen clearance. Despite the crucial roles of these subsets in developing effective anti-cancer therapies and vaccines, the molecular mechanisms governing **SLEC vs. memory cell commitment** remain unclear.
To address this gap, we built a comprehensive single-cell multiomics atlas of CD8+ T cells, profiling them across multiple steady-state and infection conditions, including various pathogens and time points. Alongside conventional types of CD8+ T cells, this approach revealed a novel transient state in effector differentiation—dependent on the polyamine biosynthesis pathway and hence named "**Tpam**".
To study Tpam cells in vivo, we developed an **inducible OT-I TCR mouse model** (TCR-switch), in which T cells initially develop with a polyclonal TCR repertoire but, upon induction, switch to express an OVA-reactive TCR. Using this system, we demonstrated that Tpam is required for the emergence of SLECs in vivo.
From our atlas, we also uncovered key regulatory factors of effector vs. memory cell differentiation, including **Bach2, Myc, Odc1, Satb1,** and **Zbtb20**, whose CRISPR-mediated knockouts significantly skewed T-cell fate. Moreover, single-cell RNA and ATAC multiome sequencing of the earliest T-cell divisions during infection revealed a pivotal requirement

for **TGF-β signaling** in regulating Tpam and effector cell development. Disruption of TGF-β receptor subunits or downstream signaling components led to an enhanced effector phenotype and improved SLEC survival. Finally, spatial transcriptomics demonstrated Tpam state emergence in tissue, dependent on interactions with cDC1 dendritic cells.
Collectively, our findings indicate that effector vs. memory fate is determined remarkably early after T-cell activation, involves a newly defined Tpam transitional state, and can be manipulated through targeted genetic interventions—offering new avenues to optimize T cell–based immunotherapies.

REGENERATION-INITIATING CELLS IDENTIFIED IN *XENOPUS* TAIL REGROWTH: INSIGHTS INTO TUMOR PLASTICITY AND MICROENVIRONMENTAL REMODELING

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Tissue regeneration and tumorigenesis share several fundamental biological processes, including cellular plasticity, niche remodeling, and signaling dynamics. In our recent study, we applied an integrative transcriptomic approach—combining bulk, single-cell, and spatial RNA sequencing—to dissect the molecular events underlying tail regeneration in *Xenopus laevis*. We identified a previously uncharacterized population of regeneration-initiating cells (RICs) that emerge early after amputation and orchestrate the regenerative process.
Functional assays revealed that RICs play a key role in extracellular matrix (ECM) remodeling and modulate multiple developmental signaling pathways. These cells also exhibited remarkable transcriptional plasticity and dynamic interactions with surrounding cells. Notably, several gene expression signatures in RICs resemble those found in aggressive, invasive tumors, suggesting a conserved program that governs both regenerative and malignant tissue remodeling.
By characterizing RICs, we provide new molecular targets and mechanistic insights that may inform therapeutic strategies in cancer biology. The *Xenopus* tail system thus serves not only as a regeneration model but also as a valuable comparative framework for understanding tumor initiation, progression, and resistance.

LINEAGE SWITCHING IN PEDIATRIC ACUTE LYMPHOBLASTIC LEUKEMIA RESOLVED BY SINGLE-CELL MULTI-OMICS

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Certain subtypes of pediatric acute lymphoblastic leukemia (ALL) are prone to a myeloid lineage switch (swALL), most often displaying a monocytic phenotype. This switch typically occurs during early therapy, though it may be detectable at diagnosis (Dx). In both scenarios, it complicates diagnostics and response assessment. Single-cell multi-omics offers high-resolution insights, but dynamical processes such as lineage switching require tailored analytical approaches.

We investigated lineage switching in five pediatric ALL patients carrying DUX4r, PAX5 P80R, or ZNF384r rearrangements using the 10x Genomics Single Cell 5' v2 platform. This allowed simultaneous profiling of RNA expression, surface proteins, and BCR rearrangements from bone marrow or peripheral blood at Dx and early therapy (day 5, 8, or 15 of a BFM-type protocol).

We applied state-of-the-art computational methods to analyze transcriptomic topology, cell density, and RNA splicing patterns to identify switching subpopulations. Intermediate populations connecting B- and myeloid-lineage blasts were observed in four of five samples, supporting a model of direct transdifferentiation. In the PAX5 P80R case, an abrupt switch to a myeloid phenotype was evident on day 5, with a transcriptomically distinct intermediate population already present at Dx. In ZNF384r, lineage instability was detectable at Dx, with a subpopulation expressing myeloid features yet carrying a clonal immunoglobulin heavy chain rearrangement on a DNA level (determined by bulk sequencing of sorted blasts). DUX4r patients showed gradual switching with residual B-blast populations.

In all cases, intermediate cells showed B-blast surface markers but expressed myeloid-associated genes (e.g., CSF2RA, CEBPD, CD86, CD68, CYBB, S100A4, TYROBP, SRGN). Trajectory inference placed these cells prior to the emergence of monocytic blasts, with transcriptomic changes preceding immunophenotypic shifts.
These results support a transdifferentiation model of swALL and emphasize the importance of integrated single-cell approaches in resolving early phenotypic instability.

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INTEGRATIVE CELL IDENTIFICATION REVEALS HUMAN RECENT THYMIC EMIGRANTS ACROSS MULTIOMIC PLATFORMS

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Aging leads to a progressive decline in immune function, with CD8 T cells particularly affected. This includes altered subpopulation distributions, reduced TCR repertoire diversity, and functional impairments—contributing to heightened vulnerability to infections and chronic inflammation in older adults. However, a consistent and precise definition of human T cell subsets—including recent thymic emigrants (RTEs)—remains lacking across both transcriptomic and cytometric methodologies.

Using a multiomic systems immunology approach, we identified RTEs based on a shared transcriptional program across CD4 and CD8 T cells, characterized by high expression of SOX4, IKZF2, and TOX transcription factors. We then validated high surface expression of CD38 as a reliable marker for both CD8 and CD4 RTEs. This definition enabled consistent identification of RTEs across single-cell RNA/ATAC-sequencing and spectral flow cytometry. Leveraging this integrative definition, we tracked RTEs and mature T cell subsets in a cohort of 158 individuals using high-dimensional spectral flow cytometry, revealing age-associated changes in their frequency, cytokine production profiles, and transcriptional state.

By anchoring transcriptional identity to accessible surface markers, we provide a robust and reproducible strategy for identifying and tracking RTEs across multiomic platforms. This integrative definition enables biologically grounded, cross-platform monitoring of thymic output over time and across individuals—supporting improved immune profiling in aging and disease.

COMBINING VIZGEN'S MERSCOPE® SPATIAL TRANSCRIPTOMICS AND INSITUPLEX® SPATIAL PROTEOMICS PROFILING TO UNRAVEL THE COMPLEXITIES OF THE TUMOR-IMMUNE MICROENVIRONMENT

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Despite remarkable results for many patients, cancer therapies have still a poor response rate. Biomarkers used to select patients for treatment are few and only single-biomarker assays are clinically approved. Here we aim to enable a comprehensive understanding of the molecular, morphological and functional landscape of tumors using a multi-omics approach **combining spatial transcriptomics and proteomics** assays. Vizgen's MERSCOPE® Ultra platform allows for high-resolution, spatially resolved analysis of up to 1000 genes, enabling the study of molecular and cellular signatures associated with the Tumor-immune MicroEnvironment (TiME), while Ultivue's InSituPlex® (ISP) spatial proteomics assay allows for pathology-grade high-throughput detection of tumor and immune cell interactions, functions and spatial distribution. We demonstrate the ability to orthogonally validate findings and expand on the biomarker-driven protein data with pathway-level data from the transcriptomic data. The integration of gene expression profiling enabling screening of a broad range of targets, and multiple immunofluorescence assays for target validation, allows for a holistic understanding of spatial and molecular insights of the TiME. These complementary technologies can be used in parallel for biomarker discovery and immuno-oncology pathways interrogation

ALTERATIONS IN ENDOMETRIAL IMMUNE CELL COMPOSITION IN RECURRENT IMPLANTATION FAILURE: A REANALYSIS OF SINGLE-CELL RNA-SEQ DATASETS

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The endometrium, the lining of the uterus, plays a pivotal role in embryo implantation during *in vitro* fertilization (IVF). Endometrial non-receptivity is a major contributor to recurrent implantation failure (RIF) and remains a key barrier to successful assisted reproduction. A well-balanced immune milieu within the endometrium is essential, as

resident leukocytes are the principal source of cytokines that orchestrate epithelial functions required for implantation. Together with steroid hormones, these cytokines regulate the expression of adhesion molecules (e.g., integrin αVβ3, E-cadherin) and extracellular matrix (ECM) components in the luminal epithelium; perturbations in immune cell composition can therefore disrupt cytokine networks, impair adhesion-molecule and ECM expression, and compromise endometrial receptivity.
Here, we reanalyzed publicly available single-cell RNA-sequencing datasets (GEO: GSE250130, GSE243550, GSE183837) to compare endometrial cellular landscapes between fertile controls and RIF patients. Unbiased clustering identified discrete epithelial (glandular, luminal, ciliated), endothelial, stromal, and immune populations. In RIF specimens, we observed a marked decrease in uterine natural killer cells and regulatory T cells, accompanied by an increased proportion of pro-inflammatory macrophages and cytotoxic T cells. These shifts correlate with the downregulation of key receptivity markers—integrin αVβ3 and E-cadherin—in epithelial cells.
Our findings underscore the critical importance of immune–epithelial crosstalk in establishing endometrial receptivity and suggest that targeted modulation of specific leukocyte subsets may represent a novel therapeutic strategy to enhance IVF success rates.

INTRATUMORAL HETEROGENEITY OF P53 AND ATF4 ACTIVITY IN COLON CARCINOMAS

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Transcription factor p53 is the most frequently altered tumor suppressor in human cancers. In response to various stress signals, p53 activates a range of genes that mediate its tumor-suppressive functions. This anti-cancer network, controlled by a single protein, presents a major opportunity for targeted therapies. However, inhibitors of its primary negative regulator, MDM2, induce effective apoptosis in only a limited number of cancer types. Recently, we demonstrated that combined inhibition of MDM2 and eIF2α acts synergistically to induce a strong apoptotic response *in vitro*. In contrast, *in vivo* treatment led only to arrested tumor growth and insufficient levels of apoptosis. We hypothesize, that limited efficiency of the combined treatment is driven by resistant subpopulations of tumor cells. To test our hypothesis, we employed spatial transcriptomics in colorectal cancer samples and analyzed available single-cell RNA sequencing data of colorectal tumors. Importantly, we observed a remarkable correlation between activities of p53 and an eIF2α downstream target ATF4 across subpopulations of colorectal tumor cells. Our

results suggest, that both pathways may be muted by shared resistance mechanisms. Therefore, we further characterized both sensitive and non-responding populations to propose strategies for sensitizing tumors to therapies based on MDM2 and eIF2α inhibition. Altogether, our findings pave the way towards more efficacious targeted cancer treatment.

DEVELOPMENT OF T CELLS IN PATIENTS WITH CONGENITAL AND ACQUIRED THYMIC DEFECT

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The clinical effect of post-natal thymectomy remains a matter of debate, but laboratory findings of low thymic output are prominent in the early years of life of patients who undergo this procedure. Reducing thymic tissue is often necessary when performing cardiac surgery on infants, where it presents an obstacle and bleeding risk when accessing the heart. As the thymus is active during the prenatal period and before the thymectomy, there is significant difference between individuals whose thymic function is reduced due to thymectomy and patients with congenital defects leading to impaired thymopoiesis.
We have assessed the T cell function and phenotype in these patients with focus on differentiation, activation and exhaustion and transcriptomic profile, as well as cytokine profile in the serum of patients with impaired thymic function. We also monitor allergic, autoimmune and infectious complications in the Czech cohort of patients with thymic defects. Our preliminary data show that patients with congenital defects of the thymus tend to have more severe state of immunodysregulation than the patients that undergone thymectomy, who despite similar laboratory findings have minimal clinical problems.
The variance in severity makes researching and managing thymic defects complex and novel approaches and treatments should be considered for these children to increase thymopoiesis and the homeostasis of the immune system in the context of lymphopenia and increased exhaustion and proinflammatory state caused by the thymic defect.

SMALL PARTICLES, EVS

MACROPHAGE EVS: MEDIATORS OF INFLAMMATION

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Macrophages (Mfs) are essential players in innate immunity, contributing to pathogen defense, inflammation, and tissue repair. They are also significant producers of extracellular vesicles (Mf-EVs), whose functional properties remain insufficiently understood. This study hypothesized that bacterial stimulation of maternal Mfs could modulate the production, composition, cargo, and functionality of Mf-EVs.

To investigate this, Mf-EVs were isolated by ultracentrifugation from the conditioned medium of RAW 264.7 Mfs, both untreated and treated for 24 hours with lipopolysaccharide and bacterial lysates derived from *Lacticaseibacillus rhamnosus* CCM7091. Characterization of the Mf-EVs followed MISEV 2023 guidelines and employed techniques such as Cryo-EM, NTA, and WB to detect typical EV markers. The expression of pro-inflammatory cargo, including inducible nitric oxide synthase (iNOS), in Mf-EVs was analyzed. Lipid profiles of both Mf-EVs and their parental cells were determined using liquid chromatography-mass spectrometry lipidomic analysis. Functional properties of Mf-EVs were assessed in RAW 264.7 and MS-1 cell lines using confocal microscopy, flow cytometry, ELISA, and WB.

The results confirmed the presence of bilayer membrane-enclosed Mf-EVs within the size range characteristic of small EVs. Mf-EVs from stimulated Mfs expressed typical EV markers, including TSG101, CD63, and CD81, while the negative EV marker ApoE was absent. Notably, bacterial stimulation of Mfs increased iNOS expression in their EVs and induced changes in their lipid profile. Furthermore, these Mf-EVs exhibited pronounced pro-inflammatory properties.

Our findings demonstrate that bacterial stimulation significantly modulates the composition and cargo of Mf-EVs, underscoring their role as carriers of pro-inflammatory signals. This highlights the potential of Mf-EVs to influence inflammatory responses and emphasizes their relevance in therapeutic research.

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ABSOLUTE QUANTIFICATION OF NANOPARTICLES BY OPTICAL MICROSCOPY AND DEEP LEARNING: A SIMPLE YET POWERFUL TOOL FOR NANOPARTICLE ANALYSIS

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Understanding the biological interactions of nanoparticles – whether engineered or naturally occurring – requires robust and reliable methods for their quantification. However, standard techniques often fall short in providing absolute particle counts, especially for sub-100 nm particles at biologically relevant concentrations. Here, we present a microscopy-based counting assay that enables absolute quantification of nanoparticles in macroscopic sample volumes.

This method, termed Evaporated Volume Analysis (EVA) [1], involves dropping the sample dispersion (~2.5 µL) on a glass substrate, evaporating the entire droplet volume, and imaging the dried residue using conventional optical microscopy. By combining large-area image stitching and deep learning-based particle recognition, EVA allows reliable counting of hundreds of thousands of nanoparticles from a single droplet. We validated the method using photon-upconversion nanoparticles (size ~60 nm) [1,2], and extended it to fluorescent polystyrene beads and plasmonic silver nanoparticles, demonstrating broad applicability across particle types and imaging modalities.

Importantly, EVA offers straightforward uncertainty modeling, making it well-suited for standardization and inter-laboratory comparisons (we achieved a relative standard uncertainty of 3% in estimating the nanoparticle concentration). The method is compatible with aqueous buffers, biological matrices such as diluted plasma and fruit juice, and organic solvents such as chloroform. By requiring only basic lab infrastructure, EVA holds promise for nanoparticle quantification in biological research.

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Reference: [1] Hlaváček et al., 2025, *Analytical Chemistry*, 97 (5), pp. 2588 - 2592, DOI: 10.1021/acs.analchem.4c05555. [2] Hlaváček et al., 2022, *Nature Protocols*, 17 (4), pp. 1028 - 1072, DOI: 10.1038/s41596-021-00670-7.

GETTING TO KNOW EXTRACELLULAR VESICLES: BETWEEN PROMISE AND PITFALLS

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Extracellular vesicles (EVs) are no longer seen as mere cellular debris. These small, membrane-bound particles, released by cells, carry critical molecular cargo—proteins, RNAs, lipids, and metabolites—that can influence recipient cells both locally and systemically. EVs have become central to understanding cellular communication, disease mechanisms, and the development of novel diagnostic and therapeutic strategies. However, as interest in EVs grows, so does the complexity of working with them. The promise of EVs is vast—offering potential breakthroughs in biomarkers, targeted therapies, and drug delivery systems. Yet, the path forward is fraught with challenges. Inconsistent methodologies, difficulties in isolation, and issues with characterization have made EV research both exciting and frustrating. The ability to isolate high-quality EVs from diverse biological sources remains a major challenge, as sample preparation and isolation techniques are often inconsistent, leading to enormous variability in downstream analysis. Characterizing and quantifying these particles, particularly at the single-vesicle level, requires precise, standardized methods—a task that continues to evolve. Additionally, tracking EVs *in vivo* to understand their biodistribution and cellular uptake adds another layer of complexity. While new technologies, such as microfluidics, advanced imaging, and AI, offer promising solutions, many technical challenges persist.
This talk will serve as an accessible primer for researchers from other fields, emphasizing the groundbreaking potential of EVs while addressing the hurdles that persist. From technical limitations to ongoing debates about nomenclature and function, we will explore why understanding EVs is more critical than ever. Attendees will leave with a clearer perspective on the current state of the field, ready to engage with the complexities and opportunities that EV research and their translation into clinics presents. Join us to uncover the big stories behind these remarkably small structures.

DEVELOPEMENT OF A UNIVERSAL CHEMICALLY-DEFINED MEDIUM FOR THE ANALYSIS OF „STRESSED“ BACTERIAL EVS IN THE CONTEXT OF UNDERNUTRITION

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Undernutrition represents a significant global health challenge, impacting diverse populations including vulnerable groups such as the elderly and chronically ill. Its influence on the gut microbiome is complex, causing both quantitative and qualitative shifts that may alter inter-bacterial communication and host interactions. Recent insights suggest that bacteria under nutritional stress produce extracellular vesicles (bEVs), which could serve as universal messengers modulating the gut environment and potentially impacting host health and disease.
This project aims to elucidate the role of undernutrition-induced stress on bacterial EV production, focusing on key nutritional deficiencies such as iron, vitamin A, and excess bile

salts. To achieve this, we are developing a universal chemically-defined medium (CDM) optimized for the reproducible culture of representative commensal strains under stressed and standard conditions. Using CDM we can easily change its composition and analyse bacterial growth and the resulting bEVs without the need of analysing medium-derived EVs. The CDM enables systematic comparison of bEVs, including proteomic and transcriptomic profiling of bEVs pairs to identify functional cargo involved in microbiome-host interactions. Moreover, we investigate the biophysical properties, cargo composition, and inter-bacterial as well as host cell transfer capabilities of these EVs.
This research aims to establish a standardized platform for the production and analysis of bEVs, fostering a deeper understanding of their role in malnutrition and gut health. Ultimately, these insights may pave the way for novel biomarkers or therapeutic strategies targeting microbiome-mediated pathways in undernutrition and related digestive pathologies.
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LIPIDOMIC PROFILING OF DU145 TETRASPANIN DEFICIENT CELLS AND EXTRACELLULAR VESICLES

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Tetraspanins are integral membrane proteins involved in cancer cell proliferation, progression, adhesion, and chemoresistance. They organize tetraspanin-enriched microdomains by interacting with partner molecules, influencing extracellular vesicles (EVs) formation. EVs are lipid-rich particles released by cells into extracellular space, that mediate intercellular communication.
This study investigates the impact of three key EV-associated tetraspanins—CD9, CD81, and CD63—on lipid composition in DU145 prostate cancer cells and EVs. Using CRISPR-Cas9, we generated knockout (KO) lines for each tetraspanin and conducted targeted HPLC-MS/MS analysis to quantify sphingolipids (SLs), glycosphingolipids (GSLs), globosides (Gbs), and phospholipids (PLs). Our results indicate that tetraspanins

influence lipid metabolism. Notably, GSLs are upregulated in all KO lines, particularly in CD9-deficient cells. Among phospholipids, phosphatidylcholine—a key determinant of membrane structure—shows differential regulation: increased in CD81 KO cells and decreased in CD9 and CD63 KO lines. Comparative analysis of intracellular and EV lipid profiles further reveals EV-specific lipid alterations, highlighting the role of tetraspanins in lipid sorting and EV cargo selection.

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PRECISE AND EFFICIENT EXTRACELLULAR VESCICLES PURITY DETECTION WITH EXOPLORER NANO FLOW CYTOMETER

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VDO Biotech, Suzhou, China

The Exoplorer Nano-Flow Cytometer redefines nanoparticle analysis as the all-in-one solution for revealing nanoscale secrets with groundbreaking precision. Combining superior sensitivity, resolution, and throughput, this instrument delivers comprehensive insights across the full 5–3000 nm spectrum, enabling simultaneous size distribution mapping, bead-free absolute counting, and multi-parameter biochemical profiling. In this study we underline the fact that reliable measurements of extracellular vescicles (EV) concentrations are essential for biomarker research. Moreover, different instruments measures different sample concentrations. Using ExoPlorer nano flow cytometer we have been able to accurately measure EVs concentration and size.

SEPARATION OF BLOOD-DERIVED EXTRACELLULAR VESICLES USING DROPLET-BASED SPECTRAL SORTING

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Extracellular vesicles (EVs) are cell-derived, membrane-bound, small particles measuring less than a micron in size, that demonstrate potential as valuable biomarkers and therapeutic agents in various disease environments. Due to their small size and heterogeneity, EVs can be difficult to characterize; however, single vesicle flow cytometry is a proven method to detect and analyze EVs. Growing interest exists for using high-speed,

droplet-based sorting to isolate specific EV populations for further characterization in both functional assays and to explore their use in therapeutics. The work described herein outlines a proof-of-concept assay that demonstrates the ability to effectively enrich specific EV populations using a Cytek Aurora CS, droplet-based Spectral Cell Sorter.

BACTERIAL STIMULATION ALTERS LIPID COMPOSITION OF MACROPHAGE-DERIVED EXTRACELLULAR VESICLES: IMPLICATIONS FOR IMMUNE MODULATION

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Macrophages (Mfs) are key innate immune cells involved in pathogen defense, inflammation, and tissue repair. Their extracellular vesicles (Mf-EVs) play important roles as important mediators in various diseases. We focus on the effects of dysregulated microbiota on host cells during intestinal inflammation, where immune cells play a crucial role. While most studies examine protein cargo, the lipid profile of Mf-EVs remains underexplored.

Mf-EVs were isolated by gradient ultracentrifugation from untreated RAW 264.7 macrophages and those treated for 24 hours with bacterial lysate (BL) from *Lacticaseibacillus rhamnosus* CCM7091. EVs were characterized using Cryo-EM, NTA, and western blotting following MISEV 2023 guidelines. Lipidomic profiles of both Mf-EVs and their maternal cells were analyzed using LC-MS and evaluated through bioinformatic tools. The immuno-boosting effect of BL on RAW 264.7 macrophages was confirmed by increased pro-inflammatory markers production. Cryo-EM and NTA confirmed that Mf-EVs possessed a bilayer membrane and fell within the size range of small EVs. Typical EV markers (TSG101, CD63, CD81) were detected, while the negative EV marker was absent. Lipidomic analysis revealed significant differences in the lipid composition between BL-EVs and those from untreated cells, as well as between their respective maternal cells.

Our study highlights a crucial gap in current research, focusing on the lipid composition of Mf-EVs under inflammatory conditions. Bacterial stimulation of Mfs alters the lipid profile of both maternal cells and their EVs, indicating that the activation state of Mfs may influence lipid distribution within EVs. The functional implications of these lipid changes warrant further investigation.

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INTERACTION OF MACROPHAGE-DERIVED AND BACTERIAL EXTRACELLULAR VESICLES WITH TARGET CELLS

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Extracellular vesicles (EVs) are nano-sized, membrane-bound particles released by both eukaryotic and prokaryotic cells, playing a pivotal role in intercellular communication by transporting molecular cargo. Among these, macrophages - key player in the innate immune system - are crucial for pathogen defense, inflammation and tissue homeostasis. The EVs they release help regulate immune response, highlighting their functional significance. Similarly, bacteria release bacterial extracellular vesicles (bEVs) which are increasingly recognized for their roles in modulating host-pathogen interactions and affecting inflammation-connected processes. Given the critical importance of EV interactions with target cells in determining their functional effects, precise monitoring of these interactions is essential in EV research.

This study aims to visualize the interaction of representative G+ bEVs and macrophage EVs (Mf-EVs) with the target cell such as RAW 264.7 cells, bone marrow-derived macrophages (BMDMs) and the MS-1 cell line. Mf-EVs were isolated from RAW 264.7 conditioned media by ultracentrifugation and purified via sucrose cushion. bEVs were isolated from cultures such as *Lacticaseibacillus rhamnosus* CCM7091 and *Staphylococcus aureus* 3.496 using a similar workflow. EV characterization followed MISEV 2023 guidelines using Cryo-EM, NTA, and WB. Mf-EVs expressed EV markers TSG101, CD63 and CD81, while negative EV marker (ApoE) was absent. bEVs were positive for lipoteichoic acid (LTA), verifying G+

bacterial origin. Both types of EVs were labeled with CFSE to visualize their uptake. Interactions with target cells were analyzed using Leica TCS SP8 SMD which is a part of IBP confocal microscopy, standard fluorescence microscopy and BD FACSDiscover™ S8 flow cytometer with digital microscopy. Results showed that Mf-EVs and bEVs interact with target cells, providing a basis for future studies of its mechanism. Further research will be needed to uncover specific interaction partners.

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INSIGHT INTO THE PROPERTIES OF CANDIDA ALBICANS EXTRACELLULAR VESICLES WITH A FOCUS ON ACTIVE MOLECULES IMPORTANT FOR HOST-PATHOGEN INTERACTION

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Candida albicans, a dimorphic fungus, is a regular component of the normal human microbiota. Nonetheless, *C. albicans* belongs among the major opportunistic fungal pathogens, causing superficial cutaneous-mucosa (oral and vaginal) and deep-seated life threatening disseminated infections. Anti-*C.albicans* immunity focuses upon surface antigens which are involved in the adherence to the target host cell, mainly structural antigen variation, in avoidance of phagocytosis and in the suppression of host immune response itself. In order to understand host-fungus interactions it is essential to study the fungal components which elicit respectively modulate the host immune responses and the expression and production of these components during *Candida* growth and morphogenesis. In addition to various well-known mechanisms contributing to *Candida* virulence, the microbial cells produce also extracellular vesicles (EVs) carrying a diversified biologically active cargo. The discovery of EVs was a breakthrough in the field of secretion as it provided a new mechanism for the release of components into the extracellular milieu. Most of the molecular mechanisms underlying EVs release and cargo loading into the *Candida* EVs are still unknown. EVs could carry bioactive substances, mainly proteins, nucleic acids, lipids, and carbohydrates. They represent number of immunogens, virulence factors and regulators that are involved in intercellular communication, biofilm matrix production, and these components could potentially impact disease outcomes by interacting with the host. It was described, that yeast comprised a larger population of bigger EVs and hyphal EVs were smaller, but with much higher protein diversity and contained a greater number of virulence-related proteins. The aim of our present study was the isolation and characterization of EVs produced by *C.albicans* obtained after cultivation at different condition (media, temperatures) with a particular focus on

proteomic analysis and the flow cytometric determination of proteins exposed at the surface of EVs that come into immediate and direct contact with host cells.

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ROLE OF SERUM EXTRACELLULAR VESICLES AS MEDIATORS OF INCREASED SYSTEMIC INFLAMMATION IN PATIENTS WITH SLEEP APNOEA

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Obstructive sleep apnoea (OSA) is the most common sleep-related breathing disorder. It affects 2-4% of adults, with men being almost twice as often diagnosed as women. The World Health Organization estimates that more than 80% of OSA cases are undiagnosed. This disorder is clinically characterised by repetitive periods of reduced or absent airflow, caused by transient upper airway collapse during sleep. Moderate to severe OSA is mostly treated with continuous positive airway pressure (CPAP) therapy, a medical device delivering a constant flow of air through a mask. In non-obese individuals, surgical treatment may be successful. However, if untreated, OSA is associated with an increased risk of cardiovascular disease and metabolic disorders. A chronic systemic inflammation seems to be important interlink between OSA and its adverse impacts. There is increasing evidence of the effectiveness of CPAP treatment in reducing overall mortality and cardiovascular risk.

Extracellular vesicles (EVs) are small particles, typically about 100 nm in size, surrounded by a lipid bilayer that cannot replicate themselves. They are present in blood serum and known as potent mediators of systemic inflammation in various diseases such as rheumatoid arthritis. In this work, we investigate the potential of EVs isolated from the blood serum of OSA patients and healthy controls to induce activation and polarization of macrophages using *in vitro* culture of macrophages differentiated from THP-1 monocytes. We designed and tested a flow-cytometry multicolor protocol for immunophenotyping of monocytes and macrophages after their direct co-culture with EVs obtained from OSA patients for 24-48 hrs. EVs were further analysed for cytokine expression and the presence of surface inflammasome. Our preliminary data suggest that EVs derived from OSA patients may influence inflammatory responses occurring along the monocyte-macrophage axis and support their role as mediators of systemic inflammation in untreated OSA patients.

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MOLECULAR INSIGHTS INTO THE IMMUNE MODULATION BY MEMBRANE VESICLES OF *LACTICASEIBACILLUS RHAMNOSUS* CCM7091

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Membrane vesicles (MVs) produced by probiotic bacteria *Lacticaseibacillus rhamnosus* CCM7091 hold significant potential in modulating immune responses. However, their precise molecular effects on immune cells are not fully understood. Here, we aimed to isolate and comprehensively characterize *L. rhamnosus*-derived MVs and examine their immunomodulatory properties, focusing on the molecular basis of the interaction. Understanding these interactions could provide critical insights into host-microbe communication and support the development of new therapeutic strategies for immune modulation.

The MVs were isolated from the late-stationary growth phase of the maternal culture by multiple centrifugation, filtration, and ultracentrifugation steps, followed by purification on a sucrose cushion. Subsequently, the MVs were characterized (according to MISEV2023 guidelines) by NTA, Cryo-EM, and Western blotting. To assess their functional capacity, we evaluated the MVs' effects in vitro on RAW264.7 macrophage cell line. Additionally, to explore the molecular mechanisms underlying *L. rhamnosus* MVs' immunomodulatory effects, we used HEK293 cells overexpressing various pattern recognition receptors. To study the involvement of the inflammasome pathway in the interaction, we used primary murine bone marrow-derived macrophages (BMDMs) and detected the production of Caspase 1, IL-1beta, and IL-18.

Our findings revealed that *L. rhamnosus*-MVs elicited a robust immune response in macrophages, as shown by increased production of TNFα, IL-6, IL-10, and reactive species of nitrogen. At the molecular level, we observed that the *L. rhamnosus*-MVs induce TLR2 signaling pathways. Moreover, the MVs treatment of BMDM showed that the MVs may interfere also with the inflammasome signaling.

In summary, our results indicate that MVs derived from L. rhamnosus CCM7091 activate TLR2 and inflammasome pathways in macrophages, significantly enhancing cytokine and inflammasome-associated responses. These results highlight their potential role as potential modulators of innate immune pathways.

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CHARACTERIZATION AND IMMUNOMODULATORY ROLE OF *STAPHYLOCOCCUS AUREUS*-DERIVED VESICLES IN INFECTIVE ENDOCARDITIS

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Infective endocarditis (IE) is a serious inflammatory condition affecting the inner lining of the heart, particularly the valves, and is predominantly caused by gram-positive bacteria such as *Streptococcus*, *Staphylococcus*, and *Enterococcus*, which are responsible for 80–90% of cases. Recent attention has been focused on bacterial extracellular vesicles (bEVs), membrane particles secreted by all cell types, as potential mediators of virulence and immune modulation.

In this study, bEVs were obtained from a clinical strain *Staphylococcus aureus* 3.496 characterized by whole genome sequencing (employing Oxford Nanopore Technologies). Furthermore, bEVs were isolated using centrifugation, filtration, ultracentrifugation, and sucrose cushion purification. Characterization was performed using nanoparticle tracking analysis (NTA), cryo-electron microscopy (Cryo-EM), FTIR, Western blotting, and protein quantification assays. *In vitro* stimulation of RAW264.7 macrophages with purified bEVs resulted in significant upregulation of proinflammatory mediators production, including TNFα and IL-6 (ELISA), nitric oxide (Griess assay), and inducible nitric oxide synthase expression (iNOS, Western blot). The vesicles, approximately 80 nm in diameter and lipid-bilayered, induced a robust inflammatory response, supporting their role in host immune activation. These findings underscore the potential contribution of *S. aureus*-derived EVs to IE pathogenesis. Ongoing studies aim to characterize the vesicular cargo via proteomic analysis to identify associated virulence factors.

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CONVENTIONAL FLOW CYTOMETRY AS A TOOL FOR EXTRACELLULAR VESICLE CHARACTERIZATION AND INITIAL ASSESSMENT FOR SUBSEQUENT SURFACEOME PROFILING

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Introduction:
Conventional flow cytometry (cFCM), when appropriately optimized and validated, can be a powerful tool for extracellular vesicles (EVs) immunophenotyping and surfaceome profiling. However, it is limited in size resolution (particles larger than 300–500 nm), compared to high-sensitivity instruments like nanoFACS. On the other hand, most laboratories are not equipped with a nanoFACS analyzer; therefore, using a conventional flow cytometer remains beneficial for general EV characterization, particularly for identifying EV-specific markers.

Methods:
EVs were isolated from conditioned media of primary hepatocytes and hepatic stellate cells via a dichotomic size exclusion chromatography (SEC) approach. For general EV characterization, a semi-quantitative immunophenotyping strategy was employed, incorporating size calibration beads, detergent lysis controls, isotype-matched antibodies, and EV-specific surface markers, leveraging the detection capabilities of a conventional flow cytometer. cFCM was additionally utilized to evaluate the binding affinity of a biotinylated functional chemoproteomic reagent toward mildly oxidized and native EVs, serving as a pre-evaluation step for further downstream surfaceome profiling.

Results:
Our results indicate that the lower detection threshold of the flow cytometer was established at approximately 180 nm. The analyzed EV populations were detected within a size range of 180–590 nm and showed surface positivity for EV markers CD9 and CD63, as well as the hepatocyte-specific marker ASGPR1. Additionally, we confirmed the binding

of a biotinylated functional chemoproteomic reagent to EVs, with a notably enhanced binding affinity observed in oxidized EVs compared to their non-oxidized counterparts.

Conclusion:

In conclusion, our results underscore the advantages of conventional flow cytometry, particularly its ability to detect EVs and other particles above 180 nm. However, to gain a more comprehensive understanding of EV populations, we emphasize the importance of complementing cFCM analysis with nanoFACS.

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